Medicinal and molecular documentation of some members of Mimosaceae and their microsymbionts

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Declaration

I declare that the thesis entitled "**Medicinal and molecular documentation of some members of Mimosaceae and their microsymbionts**" has been prepared by me under the supervision of Prof. Arnab Sen, Department of Botany, University of North Bengal as supervisor and Dr. Dilip De Sarker, Department of Botany, Raiganj University as co-supervisor. No part of the thesis has formed the basis for the award of any degree or fellowship previously.

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Certificate

We certify that Mr. Manas Ranjan Saha has prepared the thesis entitled "Medicinal and molecular documentation of some members of Mimosaceae and their microsymbionts", for the award of Ph.D. degree of the University of North Bengal, under our guidance. He has carried out the work at the Department of Botany, University of North Bengal and at the Department of Botany, Raiganj University. The results incorporated in this thesis have not been submitted for any other degree elsewhere.

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Abstract

Since time immemorial plants are being served as rich sources of foods and medicines. There are about 54 million of aboriginal people belonging to different ethnic groups in India relies on medicinal plants. Concurrently, extensive survey revealed that the medicinal plants became the backbone of herbal drugs being used over the world wide. More than 3000 plants species from 200 families were identified to have medicinal properties in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Island of India. Out of several medicinal families, Mimosoids contributes a major position in the management of several diseases and/or disorders. Moreover, the medicinal properties of a plant species are attributed to their phytochemical constituents which are belived to make cure from varied ailments. Herein, a comprehensive attempt was intended with selected members of the genera like, *Mimosa*, *Acacia* and *Albizia* of Mimosaceae (Mimosoideae) to explore their ethnopharmacological claims as well as the genetic variation lies within the species.

In-depth antioxidant profiling revealed decent free radical scavenging activity of leaf extracts of *Acacia catechu* (ACL), *A. nilotica* (ANL), *Mimosa pudica* (MPD) ands *M. invisa* (MIN) confirming their potent role against oxidative stress. In brief, the *in-vitro* free radical scavenging assay through DPPH revealed enhanced (P <0.001) antioxidant potentiality of ACL extract (86.30±0.18% at 100 µg/ml) compared to ANL (83.79±0.92 at 100 µg/ml), MPD (82.29±0.85 at 100 µg/ml), MIN extract (80.63±1.25 at 100 µg/ml) and standard ascorbic acid (27.93±1.10 at 100 µg/ml) while ANL extract was found to be comprised of elevated hydroxyl radical ($OH^{\bullet}$) quenching activity in all studied doses (94.98±0.39% at 200 µg/ml) than the others. Decent amount of singlet oxygen ($^1O_2$) scavenging activity was observed in the following order MIN> ACL> MPD > ANL indicating the presence of certain active metabolites which reacted with singlet oxygen, thereby exhibits scavenging activity. Similarly, superoxide radical, NO, peroxynitrite, H_2O_2, HOCl, Iron chelation, lipid peroxidation and total antioxidant activity assay exhibited convincing free radical scavenging activity which could establish ACL, ANL, MPD and MIN as potent anti-oxidative candidates. Elevated amount of phenolic and flavonoid content in those extracts judicially supports this finding since phenolics and flavonoids are the chief contributers of exerting anti-

oxidative chattels.

Based on the ethnopharmacological claims, ACL, MPD and MIN were further evaluated to explore their probable function over cognitive disorders by means of *in-vitro* acetylcholinesterase (AChE) inhibitory assay. ACL was found to show 73.47±0.303% of AChE inhibitory activity at 200µg/ml with lower value of 75.91 ±2.28 µg/ml while ANL, MPD and MIN revealed 68.21±0.47%, 74.40±0.52% and 75.24±0.85% of inhibition at 200µg/ml respectively suggesting potent role of ACL, ANL, MPD and MIN extracts as cholinesterase inhibitors (AChEI) and might be useful as anti-cholinesterase drug against Alzheimer's and Perkinson's. After obtaining remarkable result from *in-vitro* AChE inhibitory assay, effect of MPD and MIN extract was further evaluated on scopolamine-induced learning and memory paradigm in mice. It was observed that the administration of MPD and MIN extracts significantly (P <0.001) attenuated the initial latency (IL) and step therough latency (STL) time period in scopolamine-induced mice. The depleted antioxidant capacity of SOD, catalase and GSH in brain tissues were further ameliorated by the treatment of MPD and MIN extracts indicating potent neurotherapeutic role of MPD and MIN in neutralizing ROS-mediated neuronal damage that could be treated as future drug.

What's more, *Acacia nilotica*, an important medicinal member of Mimosaceae, was found to be used as a traditional anti-diabedic remedy in Bangladesh, Pakistan, Egypt, Nigeria and in Ayurveda as well. However, hardly a comprehensive report was obtained regarding the anti-diabedic efficacy of leaves of *A. nilotica*. Hence, an attempt was made to evaluate the efficiencies of *A. nilotica* leaf (ANL) in ameliorating diabetes and its systemic insult through its antioxidative defense. The hyperglycemic condition in alloxan-induced mice was found to be lowered significantly (P < 0.001) after 20 consecutive days of high-dose (200 mg/kg BW) ANL treatment. The blood glucose level was reduced from 237.2±9.23 mg/dl on day 1 to 74±14.37 mg/dl on day 20 after the treatment of high ANL dosage with a decrease of 68.80%. However, reduced level of insulin (64%) in the diabetic group was not restored upto the normal level, it was significantly (P <0.01) improved 85% by high ANL treatment. It was further observed that the hepatic glycogen level in diabetic group was dramatically deceased by 56% comparison to control group which was improved by 45%, 15% and 30% in glibenclamide, low and high ANL group respectively. Restoration of hepatic injury and dyslipidemia was also evidenced after the treatment of ANL using serum ACP, ALP, AST, ALT and triglyceride test. A significant decrease (P <0.01 - 0.001) in catalase and peroxidase activities were detected in liver, kidney and skeletal muscle, which were subsequently reversed

due to glibenclamide and ANL treatment. A 34%, 30% and 38% lowered peroxidase activity and 48%, 44% and 57% lowered catalase activity was measured in liver, kidney and skeletal muscle. High ANL treartment revealed highest increase (51%, P <0.001) of peroxidase and catalase activity (123%, P <0.001) in case of skeletal muscle suggesting ANL could be beneficial for correcting the hyperglycemia and diabetic complications due to lipid peroxidation and free radical oxidation, thereby holds a hope towards the discovery of new anti-diabetic drug.

Since therapeutic properties of plants are attributed to their phytoconstituents, present study was further focused on the identification of bioactive metabolites in the considered extracts. FT-IR, GC-MS and NMR analysis were employed in this regard. FT-IR analysis of *A. catechu* (ACL) and *A. nilotica* (ANL) leaf extract accounted the presence of several functional groups including alkynes, aldehydes, ketones, aromatics, primary alcohol etc. A total of 41 different bioactive compounds have been identified in ACL by GC-MS analysis while MIN exposed 29 phytometabolites. Subsequently, 34 bioactive metabolites were characterized in MPD whereas MIN showed a total of 40 bioactive compounds through GC-MS. Amongest several compounds, galiic acid, squalene, catechin, epicatechin, isoquercetrin, dopamine, β-sitosterol, campesterol, stigmasterol, pyrogallol, catechol, α-tocopherol, γ-tocopherol etc. were the main active biomolecules identified so far. Of these, few have anti-oxidative properties; others have anti-diabetic activity, anti-cancerous activity and some have anti-neurodegenerative actions which were judicially explained in the present study. Furthermore, galiic acid, squalene, phenols and aromatic compounds were detected through NMR analysis. Interestingly, finding of dopamine (a neurotransmitter) in ACL extract and its probable biosynthetic pathway is the first report that enriched this endeavor to a great extent.

Plethora of evedences suggest that excess amount of ROS (reactive oxygen species) causes necrotic cell death by means of several enzymatic and non-enzymetic pathway. On the contrary, phytochemicals are well-known ROS scavengers as we as exert several remedial therapy. Therefore, an *in-silico* computational approach was undertaken to explore the underlying mechanism of phytochemical action on selected proteins associated with different impairments. To investigate the probable function of β-sitosterol, campesterol and stigmasterol (identified in MPD and MIN extract) in neutralizing oxidative stress, molecular docking was performed with ROS-generating proteins including FAS Ligand protein (FasL; ID- 4MSV), Toll like receptors (TLR; ID- 5AWA) and NADPH oxidase (NOX; ID- 1OEY). Result reveals that the selected compounds

(ligands) bind with those proteins effectively at their active sites signifying their probale role in hindering ROS generation which is evident from the *in-vitro* antioxidant assys. In addition, decent binding pattern was also admired between the same ligands (β-sitosterol, campesterol and stigmasterol; identified in MPD and MIN) and human brain membrane protein (dopamine receptor D3 protein; ID- 3PBL) claiming their strong function in the management of neurodegeneration like Alzheimer's disease, Perkinson's disease etc. A good deal of pharmacokinetic study supports this belief and confirms that MPD and MIN extracts might be treated as future CNS drug. Furthermore, successful binding between selected phytochemicals (α-tocopherol, γ-tocopherol; identified in ANL extract) and Nrf2 protein (5FNQ) demonstrates anti-diabetic signature of those metabolites from ROS-mediated diabetic complications. Hence, present finding could open a new door to understand the roots of several diseases and disorders facilitating new drug discovery.

To investigate the probable cytotoxic effects of extracts, a good deal of experiments were performed. In brief, significant ($P < 0.001$) percentage of erythrocyte membrane protection was detected in all of four extracts while negligible amount of haemolytic effect towards human erythrocytes was observed in ACL (7.79±0.55 %), ANL (6.74±0.43 %), MPD (8.20±0.84 %) and MIN (8.79±0.29 %) extract indicating non-detrimental effect on erythrocytes. Furthermore, MTT cell viability test on splenocyte and macrophage cells revealed no cytotoxic effect on either of splenocyte or macrophage cells during the exposure of highest dose. Therefore, all the extracts could well be treated as consumable bio-safety stuff, making it suitable for the preparation of drugs involved in the treatment of various diseases.

Apart from medicinal aspect, no comprehensive attempt was carried out to explore the genetic variations among the different members of Mimosaceae (Mimosoideae). Hence, an initiative was undertaken to discriminate the genetic variations among 9 important Mimosoids including *Mimosa pudica*, *M. invisa*, *Acacia nilotica*, *A. nilitica* var. *indica*, *A. catechu*, *A. concinna*, *Albizia lebbeck*, *A. chinensis* and *Samanea saman* (*Albizia saman*) through DNA fingerprinting techniques. A good deal of genetic diversity was recorded in the present study. For instance, a total of 330 polymorphic bands were generated during RAPD analysis ranging from 190 bp to 1763 bp with 100% of polymorphism using 23 primers while PCR-RFLP analysis accounted a total of 20 polymorphic bands with 86.96% of polymorphism. It may be presumed that this polymorphism was resulted due to their polyphyletic nature of the different

genera under Mimosoideae. Further DNA barcode analysis by means of matK and TrnL-F clearly reflected that the members of Mimosoideae and Caesalpinioideae are closer than the members from Papilionoideae which validates the traditional classification of Cronquist. Molecular documentation of micro-symbionts (*Rhizobium* strains) isolated from selected Mimosoids of different locations revealed 97.39% of polymorphism expressing its host specific nature. Six partial sequences of different Mimosoids were also submitted to NCBI GenBank making this study more valuable one.

In conclusion, so far my knowledge concerned, the present endeavor is the first hand information to represent a comprehensive therapeutic aptitude of *A. catechu*, *A. nilotica*, *M. pudica* and *M. invisa* exposing potent antioxidant activity, erythrocyte membrane stabilizing activity, cytoprotective activity, anti-cognitive activity, anti-diabetic activity and memory -enhancing activity by means of *in-vitro* and *in-vivo* trials. In addition, chemical characterization and *in-silico* approach provided a noteworthy impression during drugability prediction of ligands. In fact, selected phytocompounds exposed prominent binding pattern with respective proteins justifying their role in neutralizing ROS generation, diabetes, neurodegeneration and memory decline. A good deal of genetic variation was further obtained among the 9 species of Mimosoids and their micro-symbionts using several molecular skills. Apart from these, submission of few partial sequences of different plant species to NCBI GenBank enriched this endeavor as well.

Preface

This Ph.D. thesis is the outcome of my five years of restless journey which was commenced in February 2012 at the Molecular Biology laboratory, Department of Botany, University of North Bengal and Department of Botany, Raiganj University. Since evidenced-based pharmacognostic studies on complementary and herbal medicines had been paid great attention because of some imperative advantages over synthetic drugs, I have decided to evaluate several ethnopharmacological claims of varied medicinal Mimosoids under the title-"*Medicinal and molecular documentation of some members of Mimosaceae and their microsymbionts*". During this tenure, I came in contact with several individuals who helped me a lot to accomplish my research work. Henceforth, it's my pleasure to express thanks to all those personnel who contributed in many ways to the success of this endeavor and made it a memorable experience for me.

First and foremost, I would like to take this opportunity to express my sincere gratitude to my research supervisor, Prof. Arnab Sen, Department of Botany, University of North Bengal for his excellent guidance, valuable suggestions, motivation and constant encouragement throughout my research journey. This endeavor would not have been possible without his unconditional support. I am truly grateful to Sir for making me aware of A to Z of research - "*Anyone who has never made a mistake has never tried anything new*". He pushed me in the right direction whenever he thought I needed it. His unwavering courage and conviction will always inspire me and I hope to continue to work with his noble thoughts. Thank you very much Sir, for everything you made for me.

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Words are short to express my deep sense of gratitude towards my dearest friend cum colleague, Dr. Priyankar Dey. I was lucky to have the chance to work with him, who patiently taught me every method of antioxidant assay, in addition to a number of other laboratory skills, and who worked closely with me in the analysis of GC-MS, diabetic complications etc. that I represented in this thesis. In addition, I gained a lot from his vast bio-chemistry knowledge and scientific curiosity. It is obvious that without his efforts my job would have undoubtedly been more difficult. Eventually, this paragraph is not enough to express my gratitude to him.

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Most of the results described in this endeavor would not have been obtained without a close collaboration with few laboratories. I owe a great deal of appreciation and gratitude to Prof. Bratati De, University of Calcutta, for HPLC and GC-MS analysis as well as for her valuable suggestion and advice; Dr. Sajal Das, Department of Chemistry, University of North Bengal, for NMR analysis. My warm gratitude is due to all the research fellows of those labs also.

Words fail me to express my admiration to my dear lab-mate cum brother, Pallab Kar and sister, Indrani Sarkar. I can see the good shape of my thesis because of their unvarying help and suggestions in formatting the entire thesis. My thanks go in particular to Ayan Roy, Somit Dutta and Arnab Chakraborty with whom I started this work and many rounds of discussions on my research with them helped me a lot. Thank you doesn't seem sufficient to them for their unvarying support, care, encouragement, understanding and valuable

friendship.

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Finally, I would like to convey high regards to my parents, Shri Madhai Chandra Saha and Smt. Jyostna Saha for their sincere encouragement and inspiration throughout my research work and lifting me uphill this phase of life. I doubt that I will ever be able to express my love and respect fully, but I owe them my eternal gratitude. Baba and Maa, I love you a lot. Moreover, I want to dedicate this thesis to them. In addition to this, it's my fortune to gratefully acknowledge the love and support of my sweetest sister ever, Ms. Maitri Saha.

Last but not least , I like to thank all those personnel whose names are not listed here but, knowingly and unknowingly helped me a lot in the successful completion of this endeavor.

Manas Ranjan Saha

[**Manas Ranjan Saha**]

29/5/2017

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Chapter 1
Introduction

"Look deep into nature, and then you will understand everything better"
-Albert Einstein

Man has been dependent on nature for their survival since time immemorial. This dependency led the indigenous people living in harmony with nature to evolve a unique system of medicinal plant practices (Teron and Borthakur, 2014). This new branch of science is known as "*Ethno-botany*". The traditional knowledge of medicinal plants in India has been accumulated in course of many centuries based on several ancient therapeutic systems, including Ayurveda, Unani and Siddha (Lone and Bhardwaj, 2013). According to the survey report of World Health Organization (WHO) (World Health Organization, 2002), 80% people of the developing world use plant remedies for several therapeutic purposes.

India, one of the richest floristic regions of the world, has diverse socioeconomic, ethnic, linguistic and cultural areas. There are about 54 million indigenous people of different ethnic groups colonizing various regions of the country. The aboriginal groups have their own distinctive culture, religious rites, food habit and a rich knowledge of plant utilization (Mahishi *et al.*, 2005; Boro and Sarma, 2013) which pass orally generation to generation. Therefore, the traditional knowledge of medicinal plants and their use in treating several ailments might reasonably be expected in India due to its rich floristic vegetation (Shil *et al.*, 2014). Chandel *et al.* (1996) have reported that nearly 70% of tribal and rural inhabitants of India are to a large extent depended on medicinal plants for their primary healthcare management due to either insufficient or inaccessible or less availability of modern healthcare

system. Virtually, ethnobotanical survey may be regarded as one of the most reliable approaches towards new drug discovery and it is a prerequisite for any developmental planning concerned with the welfare of tribal and their environment (Lokho and Narasimhan, 2013). Nonetheless, in recent times medicinal plants became the backbone of herbal drugs being used over the world wide.

Medicinal plants are also well-documented for their eminent curative properties that prevent alterations in human body due to presence of rich polyphenols (Justesen and Knuthsen, 2001). The polyphenols are the antioxidants with redox properties that act against reactive oxygen species (ROS) and reactive nitrogen species (RNS) including hydroxyl radical ($OH^{\bullet-}$), superoxide anions ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite radicals ($ONOO^-$) etc. In addition, polyphenols play an important role against oxidative stress which is related to the pathogenesis of various important diseases (Finkel and Holbrook, 2000). However, human body has its own inherent antioxidative mechanism by which it exerts several biological functions such as the anti-mutagenic, anti-carcinogenic, anti-aging, anti-immunosuppression and anti-neurodegeneration responses (Gocer and Gulcin, 2011). An imbalance between ROS and the inherent antioxidant capacity of the human body leads to the use of dietary and/or medicinal supplements particularly during the disease attack. This ROS are mainly originated from molecular oxygen as a result of normal cellular metabolism and also from the mitochondrial electron transport chain (ETC). Besides, FasL, a type II membrane protein belonging to the tumor necrosis factor (TNF) family, has also been reported to be associated with ROS generation causing necrotic cell death (Medan *et al.*, 2005; Vercammen *et al.*, 1998) while NADPH oxidase, a membrane bound enzyme complex was also involved to yield different ROS (Han *et al.*, 1998; Pham-Huy *et al.*, 2008). Simultaneously, toll like receptors (TLR) can also induce the ROS production (Marcato *et al.*, 2008) which trigger the signals for cell apoptosis.

A plethora of evidences suggested that herbal plants, vegetables, and fruits own antioxidative compounds such as phenolics, flavonoids, tannins, and proanthocyanidins which alleviate or neutralize the free radicals. Moreover, the intake of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders (Gulcin, 2012). However, several synthetic antioxidants namely, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) etc. available in the market that have been reported to show adverse health effects (Shahidi and Zhong, 2005). Hence, there is an emerging tendency to shift towards naturally occurring antioxidants for the prevention

and treatment of diseases as well as maintenance of human health (Halliwell and Gutteridge, 1981).

Cognitive disorders (CDs), one kind of abnormalities that affect brain's capability to remember and process information (Trivedi, 2006) at late age, could be balanced by the antioxidative defense system. CDs include dementia, amnesia, Alzheimer's disease (AD), Parkinson's disease (PD), trauma, seizures, and other neurodegenerative disorders (NDs) (Chattipakorn *et al.*, 2007). Dysfunction of dopaminergic neurons, cholinergic abnormalities, mitochondrial dysfunction and extensive neuronal loss in brain are the main factors of occurring NDs. In addition, ROS generates continuously in brain leading to a progressive accumulation of cellular damage which is correlated with the onset of AD and PD (Gandhi and Abramov, 2012). AD is believed to be linked to a deficiency in the brain neurotransmitter, acetylcholine (ACh). Inhibition of acetylcholinesterase enzyme (AChE) is a rational pathway for the systemic treatment of AD (Prince *et al.*, 2013). PD, the second most common neurodegenerative disorder after AD, is characterized by resting tremor, bradykinesia, muscular rigidity, and postural imbalance occurring due to progressive death of substantia nigral cells leading to dysfunction of dopaminergic neurons (Chattipakorn *et al*., 2007). A recent survey reflected that about 35.6 million people lived with dementia worldwide in 2010 which is likely to be doubled by 2030 (Prince *et al*., 2013). Hence, neurodegenerative disorder has emerged as a great public health concern, thereby demand intervention to ameliorate oxidative stress. Several approved drugs including donepezil, tacrine, rivastigmine, galanthamine etc., to some extent, alleviate the symptoms of cognitive impairments. However, their chronic use is often associated with exerting side effects (Chattipakorn *et al*., 2007). Herbal formulations on the other hand have been documented effective against several cognitive disorders so far (Mathew and Subramanian, 2014).

Correspondingly, diabetes, a metabolic disorder, is progressively affecting a large number of populations and fatally reducing their quality of life. Utilization of conventional medicines in diabetes management is quite expensive as well as unreasonable to most of the patients. Furthermore, these drugs exert adverse side-effects due to chronic use. Inclusion of herbal remedy into conventional healthcare system may considerably improve the overall healthcare system. The advantage of herbal remedy over modern medicines is that most of the herbal medicines are plant-based and comparatively cheaper, possess fewer or negligible side-effects owing easy acceptability.

Therefore, the study on medicinal plants

and vegetables strongly supports the idea that plant constituents (i.e. phenolics, flavonoids, tannins, proanthocyanidins etc.) along with antioxidant properties are capable of exerting protective effects against oxidative stress, diabetes, cancer, neurodegeneration, aging etc. Approximately 3,000 plants species of more than 200 families were identified to have medicinal properties in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Island of India (Prakasha *et al.*, 2010). Out of several medicinal families, Mimosoids contributes a major position in the management of several diseases and/or disorders (Joy *et al.*, 1998). In addition, recent surveys made by (Saha *et al.*, 2014a; Saha *et al.*, 2014b), exhibited their medicinal importance not only as a remedial for human being but also in the management of domesticated animals and birds.

According to Cronquist (1981), Mimosoids have been usually recognized either as the family Mimosaceae or as the subfamily Mimosoideae within the family Fabaceae (Leguminosae) of the order Fabales. Mimosoideae or Mimosaceae consists of about 80 genera and 3,370 species of trees, shrubs, and lianas found mainly in tropical, subtropical, and warm temperate regions of the world (Luckow *et al.*, 2003). The members of Mimosoideae are chiefly characterized by their valvate aestivation of petals, bi-pinnate leaves, regular flowers grouped into spicate or capitate inflorescence with flat brown pods. Economically, they have been acknowledged as food and fodder (e.g. *Pisum sativum*, *Glycine max* etc.), oil (e.g. *Arachis hypogea*, *Glycene soya* etc.), fruits (*Tamarindus indica*, *Phaseolus coccineus* etc.) and timber (e.g. *Dalbargia sisso*, *Acacia auriculiformis* etc.). Apart from these, most of members of Mimosoideae have been distinguished to comprise several medicinal properties. The genera like *Mimosa*, *Acacia* and *Albizia* under Mimosaceae were extensively reported to have several medicinal properties including anti-diuretic, anti-dysenteric, anti-diarrhoerial, anti-asthmatic, aphrodisiac, analgesic, antidepressant and antioxidant properties (Ahmad *et al.*, 2012; Ali *et al.*, 2012; Anonymous, 2001; Saha *et al.*, 2016). In fact, these species are characteristically used by the ethnic people as a source of polyherbal formulations in the management of several diseases in different parts of India (Ali *et al*., 2012; Anonymous, 2001). The traditional classification of these three genera is as follows:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Fabales
Family : Fabaceae
Sub-family : Mimosoideae/ Mimosaceae
Genus : ***Mimosa*** L.
Acacia Mill
Albizia Durazz

Mimosa, (Mimosoideae; Fabaceae) the large monophyletic genus of flowering plants, consists of >500 species, mainly native to the New World (Simon and Proenca, 2000). It is native to South America and Central America and regarded as an invasive species in Tanzania, South Asia, South East Asia and many Pacific Islands (Shelef, 1984). The species vary in habit from tall trees and shrubs to vines and herbs and they are found in a wide variety of habitats from wet to dry, growing on many different soils. Amongst several species, *M. pudica, M. invisa* (Syn: *M. diplotricha*), *M. pigr*a and *M. tenuiflora* are the most familiar species that have been found to possess several medicinal properties in India (Ahmad *et al*., 2012; Joseph *et al.*, 2013; Joy *et al*., 1998). *M. pudica* is locally known as Lajjaboti (লজ্জাবতী) or touch-me-not whereas *M. invisa* is called as Swet-lajjaboti (শ্বেত লজ্জাবতী) in Bengali. They are usually found alongside the road of entire Bengal. On the other hand, hardly a report was found regarding the molecular documentation of *Mimosa*.

Acacia, one of most economically important genera under Mimosoideae, is consisting of about 980 species of trees and shrubs that can be found in Australia, and in the tropical and subtropical areas of America, Asia, Africa and Europe (Lewis, 2005). Different Australian species, like *A. mangium*, *A. mearnsii* and *A. saligna* are economically important and are widely planted for wood products, tannin, firewood and fodder. Another African species, *A. senegal*, is the chief source of gum arabic while the Afro-Indian species *A. nilotica* and *A. catechu* are planted in India and elsewhere, as a source of wood, khair and stock fodder. Moreover, gum arabic, catechu or kath or khair, tannins from bark, essential oils from flowers and seeds are mainly used in the industry of pharmaceuticals, preservatives, beverages, confectionery, adhesive, dye, perfumes, cosmetics etc. In addition, acacia woods are mostly used for the manufacture of floorings, furniture, toys, jewellery and tools. Besides, acacias are well-known agents of agro-forestry by increasing nitrogen content of the soil through their interaction with symbiotic bacteria. Despite of immense economic worth, many of *Acacia* species show numerous medicinal properties. In India, acacias, most notably *A. nilotica* (বাবলা), *A. catechu* (খয়েড়), *A. concinna* (শিকাকাই), *A. auriculiformis* (আকাশমণি) etc. are being used in the management of several ailments such as conjunctivitis, haemaptysis, catarrh, cough, pruritus, leprosy, leucoderma, skin diseases, helminthiasis, anorexia, diarrhea, dysentery, ulcers and wounds, eczema, haemoptysis, haematemesis, hemorrhages, fever, anemia, and diabetes (Asolkar *et al.*, 1992; Jain, 1994; Khare, 2008). However, there are some controversies over the past decade generated on the scientific use of

the generic name *Acacia* due to its broad systematic position as well as similar kind of characteristics with different species and still it is a confusing one after the XVIII-International Botanical Congress in Melbourne (Smith and Figueiredo, 2011).

Albizia is a large fast-growing genus comprising approximately 150 species that are widely distributed in Asia, Africa, Madagascar, America and Australia. Albizias are basically known for production of fuelwood and timber. However, some species like *A. lebbeck*, *A. amara*, *A. saman*, *A. ferruginea* etc. are some medicinally important species used to treat rheumatism, stomachache, cough, diarrhea, wounds, and as an anthelmintic (Singab *et al.*, 2015).

Evidences suggested that a few preliminary works including antioxidant activity, anti-aging, anti-diarrhea, anti-asthmatic, hypoglycemic, anti-tumour etc. have been done with selective members of *Mimosa*, *Acacia* and *Albizia*. However, no attempt was made to support the ethno-pharmacological claims. Even though, in-depth medicinal properties showing a comparative and comprehensive information of Mimosoids is still missing.

More surprisingly, it has further been observed that there has no sufficient information illustrating the comprehensive genetic variation among the medicinal members of Mimosaceae within the order Fabales. Since the morphological variation between two closed species is difficult to distinguish; an appropriate knowledge of molecular documentation would be a rational way to understand the genetic relationship among different families. Hence, an initiative step was carried out to explore the genetic variations of some medicinal Mimosoids through DNA fingerprinting techniques. Of the various DNA fingerprinting techniques developed for plant research, random amplified polymorphic DNA (RAPD) analysis has become increasingly popular which are being used to evaluate the genetic relationship among species, cultivars and varieties (Williams *et al*., 1990). Besides, restriction fragment length polymorphism (RFLP) analysis is also used to investigate the phylogenetic relationships among species. Subsequently, a new modified molecular technique i.e. DNA barcoding was developed recently to explore the evolution, identification and genetic relatedness of unknown plants and animal species resolving various anomalies in the taxonomic levels by using a short stretch of DNA sequence (Hebert and Gregory, 2005).

Hence, based on the therapeutic appraisal as mentioned above, 9 different medicinal species under Mimosoideae including, *Mimosa pudica* L., *M. invisa* Mart. Ex Colla, *Acacia nilotica* (L.) Delile, *A. nilitica* var. *indica* (Benth.) A.F. Hill., *A. catechu* (L.f.) Willd, *A. concinna* (Willd.)

Fig. 1.1. Map of the study area. The spots (*) represent the places of plant collection sites in the northern parts of Bengal province (consisting of three districts) in West Bengal, India.

DC., *Albizia lebbeck* (L.) Willd., *A. chinensis* (Osbeck) Merr. and *Samanea saman* (Jacq.) Merr. (Syn: *Albizia saman*), found in different territories of West Bengal in India (Fig. 1.1), were employed in the present study. Hence, an effectual initiative was undertaken to explore the varied medicinal properties of these 9 species with the following objectives:

- Selection and collection of medicinal members of the family Mimosaceae from different parts of North Bengal.
- Documentation and analysis of ethnic knowledge of Mimosoids.
- Profiling of medicinal and bio-chemical properties of selected Mimosoid extracts.

    I. Contouring of antioxidant activities and other medicinal properties.

II. Screening of correlation patterns between different antioxidant traits used.

- In-depth study of medicinal properties of selected plant including anti-diabetic activity.
- Evaluation of plant extract against cognitive impairment including behavioral trial on mice.
- High throughout phytochemical analysis using FT-IR, GC-MS and NMR analysis.
- Selection of target compounds for novel drug discovery targeting using *in-silico* approach.
- Determination of cytotoxicity of extracts.
- Isolation, characterization and molecular documentation of selected Mimosoids and its micro-symbionts (*Rhizobium* sp.).

Chapter 2
Review of Literature

"Study the past, if you would define the future."
-Confucius

The recent vogue on plant research has enlightened all over the world and a plethora of evidences have been collected to confirm the immense potentiality of medicinal plants used in traditional healthcare systems. Several medicinal plants have been identified and studied using modern scientific approaches for their phytochemistry. Selected members of Mimosoideae have already been remarked as valuable medicinal plants, which are rigorously used in Ayurvedha and Unani systems of medicine. Previous phytochemical reports on some species of Mimosaceae have already revealed their probable role in medical care.

This chapter, besides representing a general overview, considers selected members of Mimosoideae from the perspectives of Ethnobotany, Parmacological activities, Antioxidant activities, Neuroprotective activity, Phytochemistry, focusing also on Molecular documentation of selected taxa as well as on rhizobial diversity.

2.1. Brief history of Mimosoideae

Mimosoids form a major group within legumes. According to Cronquist (1981), Mimosoids have been usually recognized either as the family Mimosaceae or as the subfamily Mimosoideae of the family Fabaceae (Leguminosae) under the order Fabales. Mimosoideae consist of about 80 genera and 3,370 species of trees, shrubs, and lianas found mainly in tropical, subtropical, and warm temperate regions of the world (Luckow *et al*., 2003). The members of Mimosoideae are usually characterized by their bipinnately compound, alternate leaves; spicate or capitate inflorescence; bisexual flowers, rarely unisexual, actinomorphic, in tight clusters with numerous stamens and legume fruits. Besides, most of the species under Mimosaceae exhibit an associationship with nitrogen fixing bacteria.

2.2. Ethnomedicinal studies

'Ethnomedicine' may be defined as the sum of knowledge of plants, skills and

practices based on the oral theories, beliefs, and experiences curing diseases and disorders by native people belonging to different culture (Tamuli and Saikia, 2004). Several plants are being used to treat different ailments since time immemorial. Utilization of different plant species of different families like, Asteraceae, Poaceae, Malvaceae, Mimosaceae etc. were mentioned in Indian traditional and ayurvedic medical system to alleviate dysentery, inflammation, burning sensation, asthma, leucoderma, leprosy, cholera, vaginal and uterine complaints, bile, bilious fevers, piles, jaundice, leprosy, bronchitis, cold and cough, fatigue, blood diseases etc (Kirtikar and Basu, 2006). Selected members of Mimosaceae, especially, *Mimosa pudica*, *M. hamata*, *Samanea saman*, *Prosopis cineraria*, *Parkia biglandulosa*, *Albizia procera*, *Acacia senegal*, *A. chundra* etc. were reported to be used as ethnomedicine by local people of the Idar-Vadali forest area of Sabarkantha district of India to treat rheumatoid arthritis, fever, headache, piles, fistula, swellings, diarrhea, diabetes, cataract, hydrocele erysipelas, ulcer, leucorrhoea and also used as anti-venom agent in case of scorpion sting (Patel and Jangid, 2013). An ethnomedicinal report from Araku valley Mandalam, Visakhapatnam district, India claimed the effectiveness of Mimosaceae in local healthcare management (Padal and Sathya vathi, 2013). Besides, ethnomedicinal survey in Caprivi region of Namibia (Chinsembu and Hedimbi, 2010) reflected the beneficial effects 6 Mimosoids treating different HIV/AIDS-related opportunistic infections. Saini *et al.* (2008) mentioned the ethnomedicinal value of five acacias (*Acacia nilotica*, *A. tortilis*, *A. senegal*, *A. catechu* and *A. jacquemontii*) in Rajasthan of India which were regularly used to treat asthma, toothache, stomach complaint, skin infections, caugh, leprosy, indigestion and diarrhea. Simultaneously, selected members of Mimosaceae are also used by the indigenous people of Bargarh district of Orissa, India for their local therapeutic purposes (Sen and Behera, 2008). In continuation, Saha *et al*. (2014a) described the use of *M. pudica* (root and leaf decoction) as a remedy of leucorrhea and breast cancer whereas *A. nilotica* was found to have anti-diabetic property (Saha *et al.*, 2014c) in Malda district of West Bengal, India.

2.3. Medicinal properties of selected Mimosoids

Plants have been exploited for the management of diseases for centuries because of their very limited adverse effects. Therefore, their scientific evaluation is a logical way of searching new drugs. Moreover, 80% of world population depends entirely on herbal medicines prepared almost exclusively from plants. Numerous indigenous medicinal plants have been found to be

successfully used to control different ailments, which can counter the high cost and poor availability of the current synthetic drugs for many rural populations in developing countries like India. Consequently, different medicinal members of Mimosoideae have already been evaluated for their various pharmacological activities followed by isolation of active principles. In this section, an overview of different pharmacological activities of selected Mimosoids is summarized below:

2.3.1. Antimicrobial activity

Antimicrobials are such kind of substances that kill microorganisms or inhibit their growth. Numerous plants are being used as antimicrobial agent since the beginning of human civilization. Likewise, plenty of plants under Mimosaceae were studied for their antimicrobial activity. Genest *et al.* (2008) reported a comparative account of antibacterial and antioxidant activities of dichloromethane (DCM) and methanolic extracts of stems of *Mimosa rubicaulis* and *M. pudica* exhibiting considerable encouraging activity against *Bacillus subtilis*, *E. coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In addition, potent antibacterial activity of *M. pudica* was also tested by Balakrishnan *et al.* (2006). The ethanolic leaf extract of *Acacia nilotica* effectively showed highest zone of inhibition at 70mg/ml extract concentration against the bacteria *Campylobacter coli*, isolated from goats (Solomon-Wisdom and Shittu, 2010). Antibacterial activity of *A. concinna* bark was also observed against *P. aerogenosa* and *S. aureus* (Vergeese and Sivaraj, 2012). Furthermore, the antimicrobial activity of the *A. nilotica* against several bacterial strains was also examined by Khan *et al.* (2009) while ethyl acetate extract of *Albizzia lebbeck* leaves confirmed successive antibacterial activity against gram positive and gram negative bacteria (Rahul *et al.*, 2010). Antibacterial activity and antifungal activity of *Samanea saman* leaves were also noticed against *Pseudomonas aeruginosa*, *Fusarium solani* and *Trichophyton longifusus* (Azhar *et al.*, 2009).

2.3.2. Anti-inflammatory activity

Inflammation may be regarded as a complex pathophysiologic process and can be initiated in response to injury involving the accumulation of cells and exudates in the affected tissues (Markiewski and Lambris, 2007). Several plants were being applied therapeutically for many years leading to the production of major anti-inflammatory drugs. Therefore, natural products with anti-inflammatory activity are a chief concern in the present circumstance. Studies with *M. pudica* leaves revealed significant (p<0.05) inhibitory activity in a dose-dependent manner than the standard drugs, indomethacin when it was tested on carrageenan-induced paw oedema and cotton pellet granuloma in rats (Goli *et al.*,

2011). In addition, similar type of experiment with ethanolic leaf extract of *M. pudica* was executed by Mistry *et al.* (2012) and the result was found to be significant against acute and chronic inflammation. Catechin, isolated from *Acacia catechu* revealed the inhibition of Cyclooxygenase (COX) and 5-Lipoxygenase (LOX) enzyme activity showing its anti-inflammatory potentiality (Altavilla *et al.*, 2009). Further, the butanolic fraction of *Acacia pennata* dried leaves exhibited significant protective effects against chemical stimuli (acetic acid and formalin) as well as an inhibitory effect in carrageenin-induced rat paw oedema in the late phase (Dongmo *et al.*, 2005).

2.3.3. Wound healing activity

Wound healing is an immune-mediated obscure process where the skin or other soft tissue repairs itself after injury. The healing process begins with the clotting of bloods as well as a set of biochemical actions takes place to repair the damage (You and Han, 2014). Numerous plants were explored in the management and treatment of wounds over the years as they promote the repair mechanisms in natural way. The methanolic extract of aerial parts and roots of *M. pudica* was found to be a potent candidate revealing greater wound healing activity in Wistar Albino rats compared to the standard drug Gentamicin (Kannan *et al.*, 2009). In addition, Kokane *et al.* (2009) reported noteworthy wound healing activity of ointment containing methanolic and aqueous extract of *M. pudica* root at 2% level in rat models. Concurrently, similar type of result was also found in bark extract of *Albizia lebbeck* (Gupta and Jain, 2010).

2.3.4. Antinociceptive activity

Pain, a subjective symptom, is affected by psychological factors which could be alleviated by different chemical agents by means of central or peripheral mechanisms. Different plant species have been considered as natural pain-relievers in search of new potent drugs. The methanolic extract of *A. catechu* was proved as a relieving agent from pain when tested in acetic acid-induced gastric pain mice models (Rahmatullah *et al.*, 2013). The aqueous extract of *Mimosa pudica* exhibited significant ($p<0.001$) inhibition of writhing response in acetic acid-induced animal model which might be due to the inhibition or reduction of proinflammatory mediators (Karthikeyan and Deepa, 2010). On the other hand, analgesic activity of a Brazilian native plant, *Abarema cochliacarpos* was evaluated in mice model by Silva *et al.* (2009) exhibiting higher activity of bark extracts than that of standard drug used.

2.3.5. Antidiabetic activity

Diabetes, a metabolic disorder critically affecting the population, is rapidly emerging as a major public health challenge in developing countries.

Although, several drugs and interventions are available to manage diabetes, in most of cases these are either expensive or show adverse effects like hypoglycemia. Therefore, there always remains a need to find an effective and safety drug for the treatment of diabetes. Despite of having decreased or nil adverse effects, natural products play a vital role in this regard. For example, the ethanolic leaf extract of *M. pudica* revealed significant decrease of blood glucose level in alloxan induced diabetic Wistar rats as compared to standard, Metformin whereas pet ether extract did not show any significant decrease in serum glucose level up to 7 days of treatment (Sutar *et al.*, 2009). Likewise, the methanolic extract of the pods and tender leaves of *Acacia nilotica* was found to be very beneficial to treat diabetes (Gilani *et al.*, 1999). Besides, significant reduction of blood glucose level was observed in diabetic albino rats at the doses of 250 and 500mg/kg body weight (p<0.001) by the application of ethyl acetate extract of *Acacia catechu* (Ray *et al.*, 2006). The bark extract of *Albizia odoratissima* also significantly (p< 0.01) reduced the levels of serum cholesterol, triglycerides, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, alkaline phosphatase as well as the extract decreased the level of total proteins in alloxan induced diabetic mice suggesting their potent role as antidiabetic agent (Kumar *et al.*, 2011).

2.3.6. Diuretic activity

Diuretics are the drugs which increase the rate of urine flow, sodium excretion and are used to adjust the volume and composition of body fluids. Traditionally, several plants were claimed to be possessed diuretic properties, but lack proper clinical validation. However, a few reports are available regarding the diuretic activity of members of Mimosoideae. Sangma *et al.* (2010) revealed that the aqueous extract of *M. pudica* leaves was significant (at 100mg/kg) showing diuretic activity in normally fed albino rats with increased electrolyte excretion (p<0.01 for urine output, p<0.01 for Cl, p<0.05 for K^+, and p<0.01 for Na^+). The methanolic extract of *Albizia lebbeck* was administered to experimental rats at a dose of 200 and 400mg/kg for diuretic activity and obtained significant increase in the volume of urine and urinary concentration of Na^+, K^+ (Sivakumar *et al.*, 2013).

2.3.7. Antidiarrheal activity

Diarrhea is a frequent digestive chaos caused by enterotoxins of several bacteria. Indigenous systems of Indian therapeutic exhibited several numbers of medicinal plants to be used as antidiarrheal activity. For instance, the crude ethyl acetate extract of *A. catechu* at a dose of 250mg/ kg was found to be highly significant (p<0.001) against diarrhea in albino rats in respect of latent period of onset of diarrhea (Ray *et al.*, 2006) whereas the ethanolic

leaf extract of *M. pudica* inhibited castor oil induced diarrhea and PGE2 induced enteropooling in Wistar albino rats and found 200 and 400mg/kg was significant (p<0.001) dose (Khalid *et al.*, 2011). Besides, the bark powder of *A. nilotica* was found to be a potent inhibitor when castor oil and magnesium sulphate induced diarrhea in Swiss albino mice was studied by Misar *et al.* (2006). Similar type of result was also reported by Besra *et al.* (2002), when they tested the antidiarrhoeal activity of *Albizzia lebbeck* seeds employing conventional rodent models.

2.3.8. Antiulcer activity

Ulcers are the lesions on the surface of skin or a mucous membrane caused by superficial loss of tissue. Ulcer healing is a complex process involving the combination of wound retraction and re-epithelialization. Several medicinal plants within Mimosoideae were mentioned in ayurvedic medicine for their antiulcer properties over the years. For example, Vinothapooshan and Sundar (2010) reported the antiulcer activity of different extracts of *M. pudica* in aspirin, alcohol and pyloric ligation induced models of gastric ulcer in albino rats revealing ulcer damage suppression capability of all extracts (p<0.001). The aqueous extract of *A. catechu* heartwood significantly inhibited the formation of ulcers in the pylorus ligated rat models (Patankar *et al.*, 2011). Similar type of activity was also noticed in hydroethanolic extracts of young seedless pods of *A. nilotica* (Bansal and Goel, 2012).

2.3.9. Hypolipidemic activity

Hypolipidemic drugs are the substances that reduce the level of lipids and lipoproteins in the blood. The plant species namely, *M. pudica* exhibited potent hypolipidemic activity (p<0.05) against atherogenic diet in wistar albino rats by lowering the serum levels (Rajendran and Krishnakumar, 2010).

2.3.10. Hepatoprotective activity

Hepatoprotection is such type of capability to prevent the damage of liver. Natural products have been used traditionally for the prevention and treatment of liver disease. Scientific research on hepatoprotective activity has been supported the claims of the medicinal efficacy of several herbal compounds. Ethanolic (50%) extract of *M. pudica* exhibited significant hepatoprotection when the extract was examined on CCl_4-induced liver damage in Wistar albino rats (Kumar and Kumar, 2010). Additionally, methanolic leaf extract of *M. pudica* was also found to be hepatoprotective (p<0.05) by means of lowering of serum levels (Sohil and Sundaram, 2009). Subsequently, ethanolic extract of *Acacia concinna* pods exhibited significant hepatoprotective effect in $CC1_4$ induced liver damage rat model (Maqdoom, 2016). Further, significant (p<0.001) hepatoprotective activity was observed at a

dose of 250 mg/kg p.o. of ethyl acetate extract of *Acacia catechu* on albino rats after the administration of seven days (Ray *et al*., 2006).

2.3.11. Immunomodulatory activity

An immunomodulatory agent is a drug or inhibitor that may be used as an immunosuppressant or an immunostimulator based on its effect on the immune system. The logical design of novel drugs from traditional formulation offers new prospects in modern healthcare management. Ayurveda, one of the oldest traditional medical systems in India, reveals certain plants which strengthen the host immune system. For instance, the hot water extract and butanol fraction of *Albizzia lebbeck* bark successfully proved immunostimulatory effect against macrophage migration model as well as cell mediated arms of the murine immune system (Barua *et al.*, 2000). The aqueous extract of *Acacia catechu* stimulated the murine neutrophil adhesion and the phagocytic index. Additionally, the extract was found to be helpful in protection against cyclophosphamide induced neutropenia in murine system which was evident through its immunoglobulin production (Ismail and Asad, 2009). The ethanolic extract of *Albizia lebbeck* leaves have been reported to be exhibit strong immunomodulatory effect by increasing the swimming or survival time ($P<0.001$) and also decreased the writhing produced by glacial acetic acid ($P<.001$) employing swim endurance test and acetic acid induced writhing test model (Chaudhary *et al.*, 2012). Further, significant immunological adjuvant activities of saponin extracts from the pods of *A. concinna* was examined by Kukhetpitakwong *et al.* (2006).

2.4. Antioxidant activities of selected Mimosoids

Reactive Oxygen Species (ROS) or free radicals such as hydroxyl radical, singlet oxygen, superoxide anion and hydrogen peroxide play a chief role in the development of various diseases including atherosclerosis, heart disease, ageing, immunosuppression, and others (Young and Woodside, 2001). The free radicals in human body are produced by means of aerobic respiration or from exogenous sources and react with various biological molecules namely proteins, lipids and deoxyribonucleic acids resulting in the imbalance between oxidants and antioxidants. Moreover, oxidative stress or excessive oxidation of cellular substrates result in the formation of type II diabetes, neurodegenerative diseases, or even some types of cancer. The most effective pathway to reduce the action of free radicals causing oxidative stress is antioxidative defense mechanism. Antioxidants may be defined as the compounds inhibiting or delaying the oxidation of other molecules by means of reducing the initiation or propagation of

oxidizing chain reactions.

Medicinal plants play a key role in treating various human ailments due to presence of certain components of therapeutic value. The study done so far on medicinal plants and vegetables strongly supports the idea that plant constituents having antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems (Cao *et al.*, 1996). In fact, plant based drugs are an important resource of therapeutic agents with easier availability, relatively cheaper cost and non-toxic nature as compared to modern medicine. Various methods are used to investigate the antioxidant property of plant samples. In this section, merely compiled descriptions of selected Mimosoids are summarized.

Zhang *et al.* (2011) demonstrated the antioxidant activities of the methanol extracts of *M. pudica* through DPPH and FRAP assays as well as quantified the total phenolic and flavonoid contents. The results demonstrated potent antioxidant activities in the sequence of leaf> whole plant> seed> stem. Among the five flavonoids isolated, 5,7,3′,4′-tetrahydroxy-6-C-[β-D-apiose-(1→4)]-β-D-glycopyranosyl flavone revealed trolox equivalent antioxidant capacities. In a further experiment, ethanolic leaf extract of *M. pudica* exhibited moderate hydrogen peroxide and nitric oxide scavenging activity with IC_{50} value of 449.60±2.55µg/ml and 78.1±1.75µg/ml respectively (Muthukumaran *et al.*, 2011). In addition, reduced lipid peroxidation and superoxide dismutase (SOD) level was observed when leaf extract of *M. pudica* was tested in rat model (Muthukumaran *et al.*, 2010; Nazeema and Brindha, 2009). Kalaivani and Mathew (2010) studied the antioxidant and free radical scavenging activities of *A. nilotica* methanolic leaf extract, demonstrating greater potentiality of the extract to scavenge DPPH, hydroxyl radical, prevent lipid peroxidation and to possess superior reducing power. Crude 70% acetone and 50% ethanolic extracts from the leaf and bark of *Acacia nilotica* were tested for DPPH activity and found that acetone leaf and bark extract was more effective than the ethanolic extract (Gowri *et al.*, 2011). Moreover, a comparative evaluation of the antioxidant activities of 3 acacia species (*A. nilotica*, *A. seyal* and *A. laeta*) demonstrated superior bioactivities of *A. nilotica* (Abdel-Farid *et al.*, 2014). The bark extract of *Acacia catechu* exhibited significant inhibition of DPPH, H_2O_2 and reducing power activity *in-vitro*-cally followed by significant increase level of superoxide dismutase, catalase, glutathione-S-transferase and reduced glutathione at the dose of 100 and 200mg/kg bwt (Alam *et al.*, 2013). Antioxidant activity of *A. concinna* pods was determined by three different assays DPPH, ABTS-radical scavenging assay and linoleic acid peroxidation assay which reflected its

potent inhibitory activity with lower IC_{50} value (Poomanee *et al.*, 2015). Subsequently, bark extract of *Albizia lebbeck* was also proven as potent free radical scavenger (Suruse *et al.*, 2013; Vasanthi *et al.*, 2014). In addition, antioxidant activity of three enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione S transferase (GST) were assessed in diabetic rat model with decline level of damage after the administration of *A. lebbeck* (Resmi *et al.*, 2006).

2.5. Phytochemistry

Plants are the remarkable sources of chemical compounds having therapeutic effect on the body, referred to as phytochemicals. Virtually, these phytochemicals in plants perform a variety of physiological functions including defense mechanism against pathogens like bacteria, fungi as well as insects. Every plant synthesizes different types of phytochemicals which play an important role in pharmaceutical, food and chemical industries. In addition, these bioactive phytocompounds serve as templates for preparing several synthetic drugs. The phytochemicals have been divided into several groups such as, alkaloids, flavonoids, phenols, saponins, coumarins, anthocyanins, essential oils etc. A plethora of evidences revealed that these different types of phytochemicals are the main factors for several pharmacological activities. A glimpse of phytochemicals identified so far from the selected members of Mimosoideae is enlisted in this section.

M. pudica, one of the most well studied members of Mimosaceae, was accounted to have several bioactive components like flavonoids, phenols, alkaloids, terpenoids, glycosides, quinines, tannins, saponins and coumarin (Gandhiraja *et al.*, 2009). A few other phytochemicals including mimosine, tyrosine 3,4-dihydroxypiridine, mimosinamine, mimosinic acid were reported in *M. pudica* by Johnson *et al.* (2014). Further, five compounds namely 5,7,3′,4′-tetrahydroxyl-6-C- β-D-glucopyranosyl flavone, 5,7,3′,4′-tetrahydroxyl-8-C- β-D-glucopyranosyl flavone, succinic acid, β-sitosterol and stigmasterol were isolated and identified from the same plant i.e. *M. pudica* (Yuan *et al.*, 2006). The active metabolites such as gallic acid, protocatechuic acid, pyrocatechol, (+)- catechin, (-) epigallocatechin-7-gallate, (-) epigallocatechin-5,7- digallate, (-) epicatechin, (+) dicatechin, quercetin, (+) leucocyanidin gallate, sucrose and (+) catechin-5-gallate was reported to be found in bark extracts of *A. nilotica* (Anonymous, 2001; Malviya *et al.*, 2011; Singh *et al.*, 2009) followed by protocatechuic acid, ellagic acid, leucocyanidin, m-digallic dimer 3,4,5,7-tetrahydroxy flavan-3-ol, oligomer 3,4,7-

trihydroxy flavan 3,4-diol, 3,4,5,7-tetrahydroxy flavan-3-ol and (-) epicatechol from gum of *A. nilotica* (Malviya *et al*., 2011; Singh *et al*., 2009). Another important Mimosoid namely, *A. catechu* was reported to possesses epicatechin, epigallocatechin, epicatechin gallate, phloroglucin, protocatechuic acid, quercetin, poriferasterol glucosides, lupenone, procyanidin, L-arabinose, D-galactose, D-rhamnose, aldobiuronic acid, and taxifolin (Jain *et al.*, 2007; Sharma *et al.*, 1997). Further, 12 compounds namely 4-hydroxybenzoic acid, kaempferol, quercetin, 3,4',7-trihydroxyl-3', 5-dimethoxyflavone, catechin, epicatechin, afzelechin, epiafzelechin, mesquitol, ophioglonin, aromadendrin, and phenol were isolated from *A. catechu* by Li *et al.* (2010) whereas *A. concinna* was found to be own tartaric acid, oxalic acid, succinic acid, calycotomine, nicotine and rutin (Gupta and Nigam, 1970). The phytoconstituents reported in *Albizzia lebbeck* bark are melacacidin, friedelin, D-catechin, β-sitosterol, albiziahexoside, betulnic acid and echinocystic acid while the leaves contains albigenic, albigenin, kaempferol, quercetin; albizziahexoside, tannins, proteins, carbohydrates, amino acids and saponins. In addition the pods contains 3', 5 Dihydroxy 4', 7 dimethoxy flavone, and N- Benzoyl L phenyl alaninol (Rahul *et al*., 2010; Verma *et al.*, 2013). Further, eight bioactive compounds were isolated from the 95% ethanolic extract of *A. chinensis* leaves and their structures were explained as quercetin 3'-O-beta-D-glucopyranosyl-3-O-rutinoside, kaempferol 3,7-di-O-beta-D-glucopyranoside, rutin, D-pinitol, luteolin 7-O-beta-D-glucopyranoside, (+)-lyoniresinol 3alpha-O-beta-D-glucopyranoside, (-)-lyoniresinol 3-alpha-O-beta-D-glucopyranoside, syringin (Liu *et al.*, 2009). Lupeol, epilupeol, lupenone, α-spinasterol and α-spinasterone were found to be present in *Samanea saman* (Azhar *et al*., 2009; Ragasa *et al.*, 2014).

A few main active phytoconstituents of some members of Mimosaceae are summarized in Fig. 2.1.

2.6. Molecular diversity of different Mimosoids and *Rhizobium*

2.6.1. Mimosoideae

Genetic marker stands for genetic differences between individual organisms or species that are located in close proximity to genes. All genetic markers hold specific genomic locations within chromosomes known as loci. Three types of genetic markers have been discovered so far such as, morphological markers, biochemical markers and DNA markers. The morphological markers are typically characterized by phenotypic characters including flower color, seed shape, growth habits or pigmentation (Sumarani *et al.*, 2004). Biochemical markers are the differences in enzymes that are

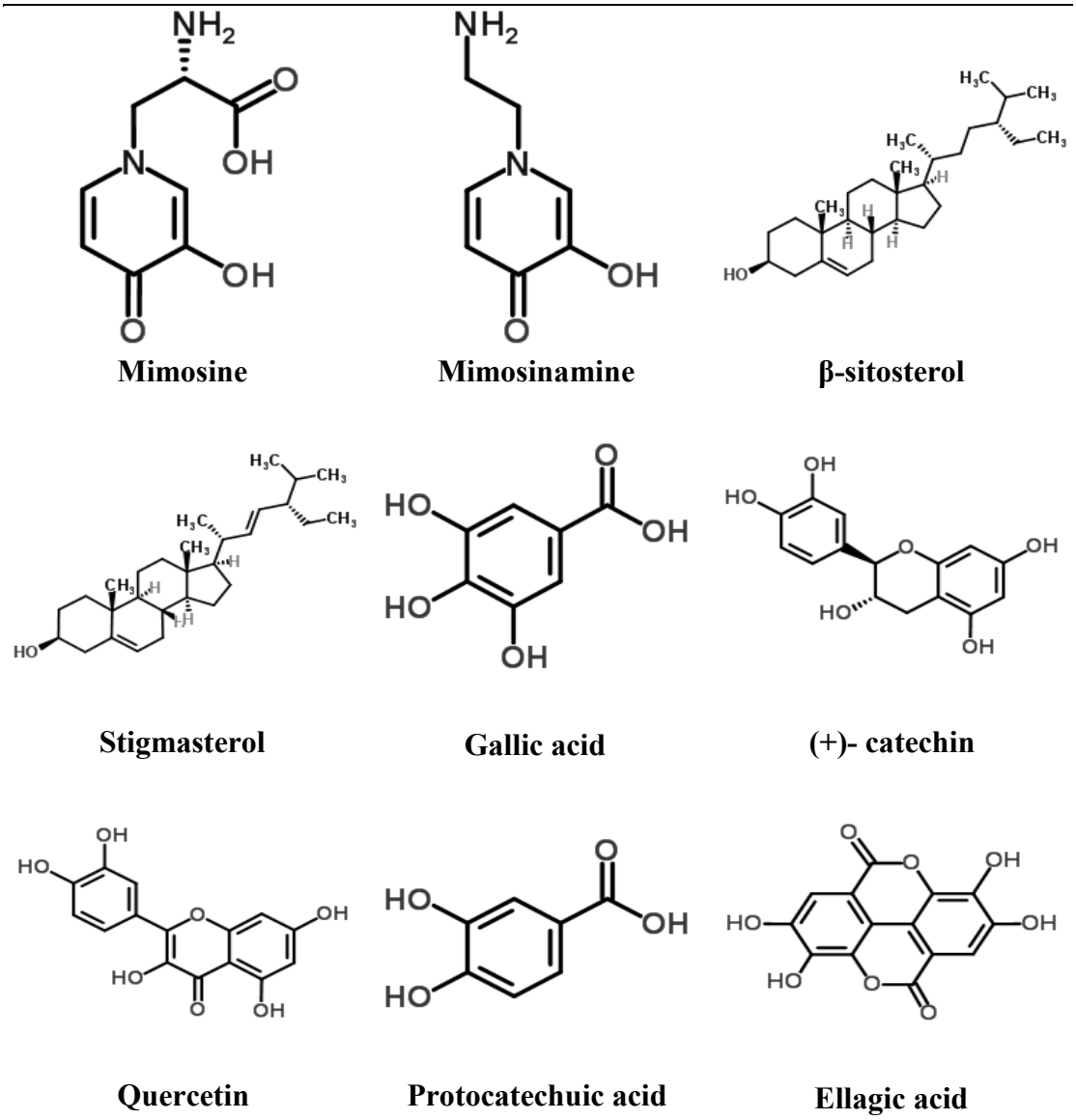


Fig. 2.1. Selected phytocompounds present in different Mimosoids.

distinguished by electrophoresis and specific staining (Pillai and Lekha, 2008). The DNA markers are the most predominant markers arise from different classes of DNA mutations such as substitutions (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA (Paterson, 1996). Besides, DNA markers are abundant in number and are not influenced by any environmental factors and developmental stages of the plant. Baring the use of DNA markers in the construction of linkage maps, they might be applied in plant breeding such as assessing the level of genetic diversity within germplasm and cultivar identity (Jahufer *et al.*, 2002; Winter and Kahl, 1995).

In essence, DNA markers might be broadly divided into three categories based on the method of detection: hybridization-based; PCR based and DNA sequence based (Winter and Kahl, 1995). In

addition, DNA markers may expose genetic differences between individuals of the same or different species which can be visualized by means of gel electrophoresis and staining with ethidium bromide or silver nitrate or detection with radioactive or colorimetric probes. A plethora of evidences suggested that DNA markers play a vital role in enhancing crop improvement and global food production by improving the efficiency of conventional plant breeding programs (Ortiz, 1998).

DNA markers or DNA fingerprinting techniques can be employed to resolve how closely related populations may be, as well as to classify the individuals providing better resolution of genetic relationships. Of the various DNA fingerprinting techniques developed for plant research, random amplified polymorphic DNA (RAPD), a PCR-based molecular technique, has become increasingly popular which are being used to evaluate the genetic relationship among species, cultivars and varieties (Williams *et al.*, 1990). A major advantage of RAPD markers is that it requires no prior sequence information and knowledge about any particular gene in a target taxon (Palumbi, 1996). Restriction fragment length polymorphism (RFLP) is another type of molecular technique in molecular biology to differentiate minor nucleotide sequence variations in homologous fragments of DNA. This technique relies on the specificity of restriction enzymes, which are extremely sequence-specific and cut the DNA only at their recognition sites (Heun *et al.*, 1991). In addition, RFLP is virtually used in the identification of genes for genetic disorders, genome mapping, determination of risk of disease, and paternity testing. RFLP markers are also frequently used to distinguish between two organisms or species. The DNA from different ecotypes, various geographical isolates, and different inbred lines of a species contain many RFLP.

However, these techniques are restricted only for genetic diversity, analysis of species, cultivars and varieties but not in plant identification. Recently, a new modified molecular technique i.e. DNA barcoding was developed offering a new dimension in the scientific community (Hebert and Gregory, 2005). In fact, DNA barcoding is a novel, modern and innovative technique which can be used to explore the evolution, identification and genetic relatedness of unknown plants and animal species by using a short stretch of DNA sequence. Recently, chloroplast and mitochondrial genes are being used to study the sequence variation at generic and species level. The chloroplast genes such as matK and TrnL-F have been utilized by various workers to study the plant evolutionary pattern as well as to solve the various anomalies in the taxonomic levels. The matK gene exhibits three times high rate of nucleotide substitution than that of

the large subunit of Rubisco (rbcL) and found to be six fold higher at the amino acid substitution rate (Johnson and Soltis, 1994; Olmstead and Palmer, 1994) elucidating advanced phylogenetic signal for resolving evolutionary relationships among the plant species at all taxonomic levels.

Therefore, despite of rapid development and widespread application of molecular techniques, very little is known regarding genetic variability within and among the taxa of Mimosoideae at the DNA level. Subsequently, there has always been a debate among the taxonomists regarding the taxonomic position of several populations within the order Fabales owing to similar types of morphological characters. Since, the morphological variation between species is difficult to distinguish; an appropriate knowledge of molecular documentation would help to understand the genetic relationship among the different genera in Mimosoideae.

The genetic relationships among the three species of *Mimosa* namely, *M. pudica*, *M. pigra* and *M. invisa* were analyzed using 30 RAPD primers. Out of which eleven primers revealed 83% of polymorphism in all three species with a total of 92 bands. The polymorphism percentage at interspecific level varied from 25% to 100% as well as *M. pudica* was found to be closely related to *M. pigra* and *M. invisa* (Sulain *et al.*, 2013). High degree of diversity (~70%) within the six tree species of *Acacia* were obtained by Nanda *et al.* (2004) employing 253 of distinct bands through RAPD. The result reflected that *A. farnesiana* and *A. catechu* were the closest member sharing about 30% of similarity whereas *A. auriculiformis* shares about 28% similarity with *A. farnesiana* and *A. catechu*. In addition, *A. mollissima* shares about 18% of similarity with *A. arabica*. Further, the genetic variability of nine *Acacia nilotica* subspecies of various origins was also analyzed using RAPD by Ndoye-Ndir *et al.* (2008) exhibiting large differences between subspecies but no correlation between geographic distances and genetic distances was established. Genetic differentiation among the six varieties of *A. caven* were examined by means of RAPD supporting similar kind of trend as found in taxonomic differentiation (Pometti *et al.*, 2010). Subsequently, several RAPD and ISSR markers were employed to study the genetic diversity in Kenyan populations of *Acacia senegal* (Josiah *et al.*, 2008). Besides, phylogenetic analysis chloroplast DNA of 22 species of *Acacia* was carried out using RFLP indicating that *A. nilotica* and *A. farnesiana* are sister species, while *A. nilotica* is Afro-Asiatic and *A. farnesiana* is America in origin (Bukhari *et al.*, 1999). A few RAPD and ISSR markers were further utilized to analyze 172 individuals representing eight populations of *Albizia lebbeck* in different geographical range and the genetic diversity was found to be

ranged from 1.23 to 1.38 while the total gene variability was 0.34 (Aparajita and Rout, 2009). During the DNA barcode analysis of *Acacia*, Robinson and Harris, (2000) documented that the tribe Acacieae and genus *Acacia* are not only monophyletic but also the subgenera *Acacia* and *Aculeiferum* are sister taxa and neither of them appeared closely related to subgenus Phyllodineae. Barcoding analysis of acacias from three different continents showed that all of three cpDNA regions (rbcL, matK and trnH-psbA) distinguished and supported the newly proposed genera of *Vachellia* from *Acacia* as well as discriminated sister species within either genera and differentiated biogeographical patterns among populations from India, Africa and Australia (Newmaster and Ragupathy, 2009). Nevill *et al.* (2013) demonstrated the novel use of DNA barcoding for seed identification and demonstrated the practical potential of DNA barcoding for the growing discipline of restoration ecology of *Acacia* in the Midwest of Western Australia.

2.6.2. Rhizobium and genetic diversity

Rhizobium is a type genus of the family Rhizobiaceae of the order Rhizobiales in the class alphaproteobacteria. The genera *Rhizobium* consists of about 44 recognized species including some latest novel species like *R. sphaerophysae*, *R. pusense*, *R. vallis* and *R. herbae* (Qin *et al.*, 2012). It can be characterized as rod shaped, heterogeneous group of gram negative, aerobic, heterotrophic, non-spore forming microbe (Hirsch *et al.*, 1993). It contains granules of poly-β-hydroxybutyrate which are refractile by phase contrast microscopy and produces an acidic reaction in mineral-salts medium containing mannitol or other carbohydrates. *Rhizobium* includes the largest number of species into the family. However, the original genus *Rhizobium* has undergone several subsequent changes in recent years giving rise to many other taxa.

In Bergey's Manual of Systematic Bacteriology, *Rhizobium* was included along with other three genera namely, *Bradyrhizobium*, *Agrobacterium*, and *Phyllobacterium* within the family Rhizobiaceae and the separation of these genera was predominantly based on the ability to stimulate the production of root or leaf nodules in host plant species (Jordan, 1984). The recent taxonomy of Rhizobiaceae, as well as of any other bacterial groups is mostly supported by the phylogenetic analyses based on 16SrDNA sequences. A revision and dismemberment of the genus *Rhizobium* and its relatives of the class alphaproteobacteria was led by their phylogenetic studies. Characterization of the *Rhizobium* genome at molecular level is the most discriminating method for assessing the variability among strains and isolates of the bacteria (Demezas *et al.*, 1991; Thies *et al.*, 2001). The workers used different

type of primers to obtain 'PCR-fingerprints' to characterize rhizobial isolates at the strain level. The primers, designed to study the genetic diversity, are RAPD, 16S ribosomal RNA genes, repetitive element sequences (REP, ERIC and BOX), 16S–23S rRNA intergenic spacer regions or genes for nitrogen fixation and nodulation (De Bruijn, 1992; Thies *et al.*, 2001).

Amongst handful of work done so far, Harrison *et al.* (1992) reported that the use of RAPD primers may produce varied amplification patterns from different *Rhizobium* isolates, especially directly from nodules providing this method as a potential one for examining genetic structures or strain differentiation as well as relationships in *Rhizobium* populations. Young and Cheng (1998) further suggested that RAPD technique is a potential tool for the construction of genetic maps and useful in identification of the genetics and systematics of different populations of rhizobia. Nine soil rhizobia were isolated from different field locations and subjected to RAPD analysis revealing RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of *Rhizobium* strains (Rajasundari *et al.*, 2009). RAPD profiling was further found to be an efficient discriminatory method when different root-nodule rhizobia were collected from chickpea and green pea roots for their genomic diversity (Qureshi *et al.*, 2014). The genetic diversity of indigenous soybean rhizobia, isolated from different soil types in eastern Croatia, was studied by using different PCR fingerprinting methods such as 16S rDNA, PCR-RFLP, rep-PCR and RAPD analysis. Highly specific and reproducible patterns were found that enabled accurate strain differentiation (Sikora and Redzepovic, 2003). Apart from these, different molecular techniques such as, rep-PCR, ERIC-PCR, BOX-PCR and (GTG)5-PCR were further utilized for genotyping of different bacterial strains of *Rhizobium* (Blazinkov *et al.*, 2007; Menna *et al.*, 2009; Versalovic *et al.*, 1991).

Chapter 3
Materials and Methods

"Experience without theory is blind, but theory without experience is mere intellectual play."

-Immanuel Kant

3.1. The study area

Various regions of northern part of the Bengal province of India, especially Malda, North Dinajpur and Darjeeling districts were covered in the present study.

Malda district comprises total geographical area of 3455.66 sq km with Latitude and Longitude of 24°40’20" N to 25°32’08" N and 88°28’10" E to 87°45’50" E respectively with an average elevation of 17 metres. The region is surrounded by Bangladesh and South Dinajpur in the east, Santal Parganas of Jharkhand state in the west, Uttar Dinajpur in the north and Murshidabad in the south (Sengupta, 1969). The district is also characterized by its diversified wetland and forest vegetation. Plenty of rain throughout the year promote the growth of natural flora. The Adh soi wetland (beel), located at Harischandrapur-II block, is one of the largest among the wetlands of the state comprising rich vegetation due to its macrophytic diversity (Chowdhury and Das, 2011). Adina and Bhalluka forests are two other important forest areas of the district.

North Dinajpur District (latitudes of 25°11' N and 26°49' N and longitudes of 87°49' E and 90°00' E) is in the gangetic belt of West Bengal, occupying an area of 3142 sq km. Bangladesh is in the East, Bihar on the West, Darjeeling and Jalpaiguri Districts are on the North and Malda District is on the South of this district. The territory is also covered by several green forests (Sengupta, 1985).

Darjeeling (Latitude 27.03° N, Longitude 88.18° E), is the most beautiful hill station of West Bengal in India with a total area of 3,149 sq. km. The district is situated at foothills of the Himalayas and famous for its spectacular tea gardens. Tea also the major cash crop and main backbone of local economy along with tourism. The district extends from the tropical tarai plains, at about 300 feet above sea level, to the Sandakphu-Phalut ridge at 12,000 feet. Darjeeling is surrounded by the borders of Sikkim to the North, Bhutan to the East and Nepal to the West. Besides, the district owns a worth resource of diversified

natural vegetation. In essence, the whole town is ablaze with a wide range of flowers such as, rhododendrons, magnolias, gladioli, tiger lilies, hydrangeas, sweet peas, corn flowers, roses and dahlias unite to make Darjeeling bloom.

3.2. Exploitation of local ethno-medicinal plants

Practice of medicinal plants is widespread among the tribal people of Malda and North Dinajpur districts. Moreover, it is deeply rooted in their socioeconomic culture. Considering immense cultural and ethnolinguistic assortment of the ethnic people of these territories, several field interviews were designed to collect the ethnic knowledge and the plant species employed in traditional remedy with special emphasis on the members of Mimosaceae. A survey was conducted during March 2012 to May 2013 in the above mentioned districts. Several interviewing procedures, including group discussion, semi-structured questionnaire, direct interview, open-ended conversations etc. were followed to obtain the information from the native traditional healers (commonly known as Kavirajs, Baidyas or Ojhas) as well as from elderly knowledgeable persons regarding the use of different medicinal Mimosoids curing several ailments. The purpose of this survey was explained to the healers in details, and prior informed consent was taken as per ethical guidelines of the International Society of Ethnobiology (Posey, 1990). Every plant was pointed out by the informants and their local names, used plant parts, formulation and dosages were also recorded for further purposes.

3.3. *In-vitro* Antioxidant profiling

3.3.1. Selection of Plant material

A total of 4 plant species under Mimosaceae were selected for the assessment of their antioxidative aptitude based on the local knowledge. The name of plant species are as follows: *Acacia catechu, A. nilotica*, *Mimosa pudica* and *M. invisa*. The species were collected from above mentioned three districts and authenticated by the Taxonomists of Department of Botany, University of North Bengal, India and voucher specimens were (Table 3.1) deposited at the herbaria of the same Department and also in the Department of Botany, Raiganj University, India. The fresh leaves of each plant were collected during the month of August-September 2013.

3.3.2. Ethical statement

3.3.2.1. Plan collection and conservation

The plant samples collected from the different parts of Malda, North Dinajpur and Darjeeling districts were not within a National Park /Reserve Forest /Govt. protected area and the study did not involve any endangered or protected species.

Table 3.1. Selected plant species selected for antioxidant activities.

Name of Plant	Abbreviation used	Voucher no.
Acacia catechu (L.f.) Willd	ACL	NBU/UD/1039
Acacia nilotica (L.) Delile	ANL	RUC/MLD/433
Mimosa pudica L.	MPD	NBU/MLD/308
Mimosa invisa Mart. ex Colla	MIN	NBU/MLD/311

3.3.2.2. Animal maintenance and approval

All the experiments using animals were reviewed and approved by the Animal Ethical Committee of the University of North Bengal (840/ac/04/CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

3.3.3. Chemicals and reagents

Chemicals and reagents in the present study for varied experiments were of analytical grade and purchased either from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), or Merck (Mumbai, India), or Sigma-Aldrich (USA), unless mentioned otherwise. Milli-Q ultrapure water (grade >1.83) was used for all the experiments.

3.3.4. Preparation of plant extracts

The disease-free leaves of each plant (15 g each) were washed twice thoroughly in double distilled water and shade-dried separately for 21-27 days and then pulverized into powder by electric grinder (Lords Hummer 1100). Extensive extraction was performed in Soxhlet apparatus for 9-11h using suitable solvent (ethanol) with dried plant material (plant:solvent, 1:10 v/v). The extracts were then concentrated under reduced vacuum pressure at 40ºC in a rotary vacuum evaporator (Buchi Rotavapor R-3, Switzerland). The concentrated extracts were further lyophilized separately using Eyela Freeze Dryer (FDU-506, USA) and weighed subsequently. The yields (13%) of lyophilized crude extracts were calculated following the formula:

$$y = \frac{w_2 - w_1}{w_0} \times 100$$

where, W_2 is the weight of lyophilized extract and container; W_1= weight of the container only; W_0= weight of the initial dried plant sample. Finally, the lyophilized extracts were stored in sterile container and placed in -20ºC until further use. The extracts were freshly dissolved in Milli-Q water prior to experiments.

3.3.5. Various *in-vitro* antioxidant assays

For each assay in the present study, comparative analyses were drawn with scavenging potentialities of the different plant extracts with that of the standards, corresponding to the assays. Besides, the concentration of each extract was selected based on the physiological standards to

reflect their dose-dependent inhibitory effects.

3.3.5.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The free radical scavenging activity through DPPH assay was performed as per Chew *et al.* (2009) with a brief modification. Different concentrations of plant extracts (0-100µg/ml) were prepared and mixed properly with freshly prepared DPPH solution (1mM; diluted in 95% methanol) and kept in dark. Optical density (OD) was measured after 30 minutes of reaction at 517nm using UV-Vis Spectrophotometer (Thermo UV1, Thermo Electron Corporation, England, UK). Ascorbic acid was taken as standard. The percent radical scavenging activity was calculated using following Equation I:

$$DPPH = \frac{A_0 - A_1}{A_0} \times 100$$

Where, DPPH= %age scavenging of DPPH; A_0= absorbance of the control and A_1= absorbance in the presence of samples and standard.

3.3.5.2. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay of each plant extract was carried out on the basis of Fenton reaction (Kunchandy and Rao, 1990) with a few changes. Briefly, reaction mixture was prepared containing 2-deoxy-2-ribose (2.8mM), monopotassium phosphate-potassium hydroxide buffer (KH_2PO_4-KOH; 20mM; pH 7.4), $FeCl_3$ (100µM), ethylenediaminetetraacetic acid (EDTA; 100µM), hydrogen peroxide (H_2O_2; 1.0mM), ascorbic acid (100µM) and various concentrations of extracts (0–200µg/ml) up to a final volume of 1ml and the reaction mixture was left for 1h incubation at 37°C. Following incubation, the incubated mixture (0.5ml) was taken into another tube and mixed with 1ml of TCA (2.8%) and 1 ml of aqueous thiobarbituric acid (TBA; 1%). The final mixture was incubated at 90°C for 15 min then cooled down to room temperature and the absorbance was measured at 532nm against a blank solution. Mannitol was used as positive control. Percentage of inhibition was evaluated following Equation- I.

3.3.5.3. Superoxide radical scavenging assay

The assay was performed by the reduction of nitro-blue tetrazolium (NBT) as described by Fontana *et al.* (2001) with brief modifications. Usually, the nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/ NADH) system generates superoxide radicals, which reduce NBT to a purple formazan. A reaction mixture (1ml) was prepared containing phosphate buffer (20mM, pH 7.4), NBT (50µM), PMS (15µM), NADH (73µM), and various concentrations (0-100µg/ml) of plant extracts and mixed properly subsequent to incubation for 5 min at ambient temperature. The absorbance at 562nm was measured against an appropriate blank

to determine the quantity of formazan generated. Quercetin was used as standard.

3.3.5.4. Singlet oxygen scavenging assay

The production of singlet oxygen ($_1O^2$) was determined by monitoring the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO) using the method of Pedraza-Chaverri *et al.* (2004) with minor modifications. The reaction mixture contained 45mM phosphate buffer (pH 7.1), 50mM NaOCl, 50mM H_2O_2, 50mM L-histidine, 10µM RNO and various concentrations (0-200µg/ml) of plant extracts to make final volume of 2ml. The mixture was then incubated for 40 min at 30°C and decrease in the absorbance of RNO was measured at 440nm. Lipoic acid was used as a reference compound. Singlet oxygen scavenging activity was calculated using the Equation I.

3.3.5.5. Nitric oxide radical scavenging assay

Nitric oxide radical quenching activity was performed following the GriessI-llosvoy reaction (Garratt, 2012) with minor modifications. In brief, phosphate buffered saline (pH 7.4), sodium nitroprusside (SNP; 10mM) and different concentrations of plant extracts (0-100µg/ml) were mixed to make final volume of 3 ml. Following incubation for 150 minutes at 25°C, 1ml of sulfanilamide (0.33%; diluted in 20% of glacial acetic acid) was added to 0.5ml of the pre-incubated reaction mixture and left for 5 min of incubation. Then, 1ml of N-(1 -Naphthyl)ethylenediamine dihydrochloride (NED; 0.1%) was added and incubated for 30min at 25°C to develop the color. The absorbance was measured spectrophotometrically at 540 nm against blank sample. Curcumin was used as standard. The percentage inhibition was calculated using Equation I.

3.3.5.6. Peroxynitrite scavenging assay

Peroxynitrite ($ONOO^-$) was prepared using the method of Beckman *et al.* (1994). An acidic solution (0.6M HCl) was prepared mixing with 5ml of H_2O_2 (0.7M) and 5ml of KNO_2 (0.6M) on an ice bath for 1 min. Subsequently, 5ml of ice-cold NaOH (1.2M) was added to the mixture. Then, excess H_2O_2 was removed by the treatment with granular MnO_2 pre-washed with NaOH (1.2M) and the reaction mixture was left overnight at -20°C. Finally, peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm (ε = 1670 M-1 cm-1).

The peroxynitrite scavenging aptitude was measured by Evans Blue bleaching assay. The assay was carried out as per the method of Bailly *et al.* (2000) with a slight modification. Briefly, a reaction mixture was prepared containing phosphate buffer (50mM; pH 7.4), DTPA (0.1mM), NaCl (90mM), KCl (5mM), 12.5µM of evans blue, various doses of plant extracts (0–200µg/ml) and 1mM of peroxynitrite in a

final volume of 1ml. After incubation at 25°C for 30 min the absorbance was measured at 611 nm. The scavenging percentage of ONOO- was calculated by comparing the results of the test and blank samples. Gallic acid was used as the reference compound.

3.3.5.7. Hypochlorous acid scavenging assay

In-vitrocally, hypochlorous acid (HOCl) was prepared freshly by mixing 10% (v/v) solution of NaOCl and 0.6M of H_2SO_4 by adjusting the pH (6.2) and the concentration was determined by measuring the absorbance at 235nm using the molar extinction coefficient of $100M^{-1}$ cm^{-1} as per Aruoma and Halliwell (1987) with few changes. In brief, a reaction mixture was prepared containing 50mM phosphate buffer (pH 6.8), catalase (7.2µM), HOCl (8.4mM) and plant extracts of different concentrations (0–100µg/ml) into a final volume of 1ml. Then, mixture was incubated for 20 min at 25°C and absorbance was measured against an appropriate blank. The quenching activity was accessed by measuring the decrease in absorbance of catalase at 404nm. Ascorbic acid was used as standard.

3.3.5.8. Iron chelation assay

Ferrous ion chelating activity was carried out as per the method of Haro-Vicente *et al.* (2006) with slight changes. Each plant extract (0–200µg/ml) was mixed properly with ferrous sulfate solutions (12.5µM) in HEPES buffer (20mM; pH 7.2) followed by the addition of ferrozine (75µM) to initiate reaction. Reaction mixture was shaken vigorously and incubated for 20 min at room temperature. Absorbance was measured at 562nm. EDTA was used as positive control.

3.3.5.9. Hydrogen peroxide scavenging assay

The assay was determined by the method of Long *et al.* (1999) with minor modifications. A mixture was prepared with H_2O_2 (50mM) and various concentrations of plant samples (0-2000µg/ml) and left for 30 min of incubation at room temperature followed by the addition of 90µl H_2O_2, 10µl of Methanol (HPLC grade) and 0.9ml of FOX reagent (prepared by mixing 9 volumes of 4.4mM BHT in HPLC grade methanol with 1 volume of 1mM xylenol orange and 2.56mM ammonium ferrous sulfate in 0.25M H_2SO_4). Entire mixture was then vortexed and left for incubation for 30 min. Absorbance was measured at 560nm. Sodiun pyruvate was used as positive control.

3.3.5.10. Lipid peroxidation inhibition assay

Lipid peroxidation assay was followed by the method of Kizil *et al.* (2008) with a few modifications. Swiss albino mice brain homogenate was prepared by centrifuging (20±2 ×g) with phosphate buffer (50mM;

pH 7.4) and potassium chloride (KCl; 120mM) at 3000 rpm for 10 min. Different concentrations of extracts (0-25µg/ml) were mixed with the homogenate (100µl) followed by addition of ferrous sulfate ($FeSO_4$; 0.1mM) and ascorbic acid (0.1mM) and incubated for 1h at 37°C. After incubation, TCA (500µl; 28%) and TBA (380µl; 2%) were added in the reaction mixture and then heated at 95°C in water bath for 30 min. After that, mixtures were cooled down to room temperature and centrifuged at 8000 rpm for 2 min. Absorbance of the supernatant was measured at 532 nm. Trolox was used as positive control.

3.3.5.11. Total antioxidant activity

Standardized modified method of Prieto *et al.* (1999) was followed to study the total antioxidant activity (TAA) of each plant extract. Various concentrations of extracts (0-150 µg/ml) were mixed with 1 mL of reaction mixture containing 0.6 M of sulfuric acid (H_2SO_4), sodium phosphate (28 mM) and ammonium molybdate (1%). Then the mixture was kept on a water bath at 95°C for 1 h and subsequent cooling to room temperature. The absorbance was read at 695 nm against a suitable blank. Ascorbic acid was used as a standard. The antioxidant activity was measured by the capacity of the yam extract to reduce molybdenum (VI) to molybdenum (V) using equation II:

$$TAA = \frac{OD_o - OD_1}{OD_0} \times 100$$

where,, TAA= % of TAA; OD_0 was the absorbance of the blank and OD_1 was the absorbance in the presence of the samples and standard (ascorbic acid).

3.3.5.12. Quantification of total phenolic content

Total phenolic content (TPC) was determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965) with slight changes. In brief, each extract (100µl) was mixed with 0.75ml of Folin-Ciocalteu reagent (previously diluted 1000-fold with distilled water) and left for 5 min at room temperature followed by the addition of sodium carbonate (Na_2CO_3; 0.06%) to the mixture. Following incubation of 90 min at room temperature, the absorbance was measured at 725nm. Phenolic content was measured against a gallic acid standard curve ($R^2 = 0.9708$).

3.3.5.13. Quantification of total flavonoid content

The total flavonoid content was measured using aluminum chloride ($AlCl_3$) method (Zhishen *et al.*, 1999) with few modifications. In brief, each extract (100µl) was added to 0.3ml of distilled water followed by addition of $NaNO_2$ (5%; 0.03ml). Following 5 min of incubation at room temperature, $AlCl_3$ (10%; 0.03ml) was added and left for 5min. Reaction mixture was then treated with 0.2ml of sodium hydroxide (NaOH; 1mM) and diluted to 1ml with water. Absorbance was

measured at 510nm. The flavonoid content was determined from a quercetin standard curve ($R^2 = 0.9891$).

3.4. PCA and HCA of different antioxidant traits

To analyze the relationship between the different antioxidant traits and the quantified phytochemicals, principal component analysis (PCA) based on the correlation matrix were drawn for selected samples. Two factors were extracted under varimax method. The data obtained from the antioxidant profiling were analyzed by multivariate statistical approach, employing hierarchical cluster analysis (HCA). The method employed was 'proximity' matrix using between group linkages. The differences between the measured variables were calculated by square Euclidean distances. Transform values of variables (average zero and S.D. 1) called Z scores was carried out as a pre-treatment of the data. Horizontal dendrogram with all clusters icicle chart was carried out to elucidate the similarity or nearness of the various measured variables.

3.5. Assessment of cytotoxicity of plant extracts

3.5.1. *In-vitro* haemolytic assay

The haemolytic consequence of each extract was evaluated according to the standardized method of Malagoli (2007) with few modifications. In brief, human blood was collected in citrated tubes and erythrocytes were then washed (150 ×g for 5 min) three times with 20mM Tris–HCl containing 144mM NaCl (pH 7.4). Then, the erythrocyte suspension (100µL) was prepared with the same solution and plated into each well of the 96-well plate. Sodium chloride solution (0.85%) containing calcium chloride (10mM) was added (100µL) to each well. First lane of the plate served as the negative control without plant extract. Various concentrations (0-200µg/ml) of extracts were added into the different wells beginning from the second lane. In another set of lanes, 100µL of Triton X-100 (0-200µg/ml) in 0.85% saline were plated and used as standard. Thereafter, the plate was incubated for 30 min at 37°C. Following incubation, the suspension was centrifuged (604 ×g for 5 min) and the supernatant was taken to a fresh 96-well plate and measured the absorbance of liberated haemoglobin at 540nm. Percentage of haemolytic activity was calculated using the following equation:

$$Haemolysis = \frac{H_0 - H_1}{H_0} \times 100$$

where, haemollysis= %age of haemolysis; H_0 was the absorbance of the blank and H_1 was the absorbance in the presence of the samples and standard (Triton X-100).

3.5.2. Erythrocyte membrane stabilizing activity (EMSA)

Standardized method of Navarro *et al.* (1993) was followed to estimate the

erythrocyte membrane stabilizing activity of juice. Briefly, a reaction mixture of 1ml was prepared containing 50mM of phosphate buffer (0.2mL; pH 7.2), 0.4ml of distilled water, 0.1ml of RBC suspension (10%; diluted in PBS), EDTA (40µl; 12mM), 60µl of nitro blue tetrazolium (NBT; 1%), 40µl of riboflavin and varying concentrations of extracts (0-200µg/ml) and mixed properly followed by bright light exposure for 30s and then incubated for 30 min at 50ºC. After incubation, the reaction mixture was centrifuged at 1000 rpm for 10 min and absorbance of the supernatant was measured at 562nm. Quercetin was taken as reference standard. The EMSA was measured using the equation:

$$protection\ (\%age) = \frac{A_s}{A_b} \times 100$$

where, A_s and A_b are the absorbance value of sample and blank, respectively.

3.5.3. MTT cell cytotoxicity assay

The assay was performed to evaluate the cytotoxic properties of each extract on murine splenocytes and macrophages. The splenocyte and macrophage cells were collected from Swiss albino mice (Dey and Chaudhuri, 2016). The mouse was sacrificed under mild ether anesthesia and the spleen was aseptically removed from the body and washed thrice (1000 rpm) with RPMI-1640 and splenocytes suspension was prepared and resuspended in 0.16M NH_4Cl (in 0.17M Tris; pH 7.2) to remove any trace of erythrocytes. The reaction was stopped after 5 min, using chilled RPMI-1640 and the cells were washed as previously mentioned. The peritoneal exudate macrophages were collected by washing the mouse peritoneal region with RPMI-1640. A cell suspension (2×10^6 cells/ml) was prepared with penicillin (50U/ml), streptomycin (50U/ml), nystatin (50U/ml) and fetal bovine serum (FBS, 10%) in RPMI-1640 medium. Hundred µl of cell suspension was added with 100µl of different concentrations (0-200µg/ml) of plant extracts (dissolved in RPMI-1640) to the wells of 96-well plate. Plates were then covered and incubated under 5% CO_2 and humidified atmosphere of 90% air at 37°C temperature for 48h. The assay was performed with EZcount™ MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions.

3.6. Neurotherapeutic effects of plant extract

3.6.1. Preparation of plant extracts and chemicals

In this study, methanol was selected as extracted solvent for *M. pudica* and *M. invisa* while *A. catechu* was extracted in ethanol and the chemicals were of analytical grade procured either from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), or Merck (Mumbai, India), or Sigma-Aldrich (USA), unless mentioned otherwise.

3.6.2. Screening of acetylcholinesterase

(AChE) inhibitory activity (*in-vitro*)

3.6.2.1. Selection of Plant material

The plant ethanolic leaf extracts of *Acacia catechu* (ACL), *Mimosa pudica* (MPD), and *M. invisa* (MIN) were chosen in this study depending on their ethnopharmacological significance.

3.6.2.2. AChE inhibitory assay

Acetylcholinesterase inhibiting activity of selected plant extracts was carried out based on Ellman *et al.* (1961) method with concise modification. Briefly, a reaction mixture was prepared containing sodium phosphate buffer (0.1mM), 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB; 0.1mM), various concentrations of plant extracts (0-200µg/ml) and acetylcholinesterase (2U/ ml) in a 96 well micro plate and incubated for 15 min at 25ºC. Following incubation, acetylthiocholin iodide (0.05mM) was added as substrate in the reaction mixture and the enzyme activity was measured immediately after 3 min in a Bio-Rad iMark™ microplate absorbance reader at 412nm. Eserine was used as positive control. Percentage of inhibition was calculated in terms of percentage by dividing the difference of sample absorbance from control with control absorbance × 100.

3.6.3. Memory-Enhancing Behavioral Study (*in-vivo*)

3.6.3.1. Selection of Plant material

Mimosa pudica (MPD) and *M. invisa* (MIN) leaf extract were chosen for performing memory impairment paradigm based on their ethnic use.

3.6.3.2. Animal maintenance

Swiss albino mice (9-10 weeks old; 30-35 gm) of both sexes were first acclimatized for a period of 7 days in the Animal House of the Department of Zoology, University of North Bengal prior to experiment. Mice were maintained at an ambient temperature of 25±2°C, humidity of 55–60%, with12h dark/light cycle and fed pellet diet and water *ad libitum*. The animal trials were evaluated and permitted by the ethical committee (840/ac/04/CPCSEA) of University of North Bengal and performed in accordance with the legislation for the protection of animals used for scientific purposes.

3.6.3.3. Acute toxicity study

Acute oral toxicity of selected plant extracts have been performed as per OECD guidelines (OECDiLibrary, 2002). No symptoms of toxicity and death were recorded at maximum dose of 1000 mg/kg body weight (BW) of respective plant extract. Therefore, it was inferred to be safe. Thus, 100 mg/kg BW was selected as the starting lower dose and a higher dose of 200 mg/kg BW were also employed for further testing.

3.6.3.4. Experimental design and drug administration

A total of seven groups of mice, six in

each group received the following treatment schedule:

Group I: normal control, received normal saline for 7 days; Group II: Scopolamine-treated mice (0.5mg/kg; dissolved in Mili-Q water), received normal saline for 7 days; Group III: Scopolamine-treated mice, received standard drug, piracetum at 200mg/kg BW per day (dissolved in Mili-Q water) for 7 days; Group IV: Scopolamine-treated mice, received low dose MPD extract (in Mili-Q water) at 100 mg/kg BW per day for 7 days; Group V: Scopolamine-treated mice, received high dose MPD extract (in Mili-Q water) at 200 mg/kg BW per day for 7 days; Group VI: Scopolamine-treated mice, received MIN dose plant extract (in Mili-Q water) at 100 mg/kg BW per day for 7 days; Group VII: Scopolamine-treated mice, received high dose MIN extract (in Mili-Q water) at 200 mg/kg BW per day for 7 days.

On 8th day, plant extracts/standard drug/ normal saline was injected after 90 min. of scopolamine treatment followed by behavioral tests performed after 45 min of injection. The retention of learned task was examined after 24h of last injection (9th day). All the trial and examining sessions were carried out at night, preferably between 19:00 and 23:00h due to restless movement of the mice during night.

3.6.3.5. Step-through Passive Avoidance Task

The task was carried out as per previously described methods of Reddy (1997), using a wooden two-compartment passive avoidance apparatus (dark and light chamber, partitioned by a wall with a openable door in the middle part), with slight alterations of the time intervals. After compliance of the trial, on day 7th, the animal groups were kept in the experimental room. One hour later, each mouse was placed in the light chamber for the attainment trial and was left to familiarize to the apparatus. After 100 s, the middle door was opened, and the mouse was allowed to enter the dark compartment. The latency time with which the animal crossed into the dark compartment was recorded. Animals that fail to enter the dark compartment within 100s were removed from the experiments. Once the mouse entered with all its four paws to the dark compartment, the middle door was closed, and the animal was transferred into its home cage. The same trial was again repeated after 30min of first test, but the door was opened after 5s. Once the animal reached to the dark compartment, the door was closed and a mild foot shock (25V, AC, 5s) was immediately delivered to the grid floor of the dark room. Afterward, the mouse was immediately transferred to its home cage. In this test, the initial latency (IL) period of entrance into the dark chamber was noted within 120s (selected as maximum time). A retention test was performed to determine short term memory of mice after

24h of last training (i.e. 8th day). Briefly, each animal was placed in the light compartment for 20s and the middle door was opened. The step through latency (STL) was measured for entering into the dark compartment and the test session ended when the animal reached the dark chamber or waited in the light compartment for 300s. During these sessions, no electric shock was applied.

3.6.3.6. Preparation of brain tissue samples

After behavioral evaluations, the animals were anesthetized and sacrificed. The brain of the sacrificed mice of each group were removed quickly from the skull and washed consciously in ice cold normal saline followed by homogenization in 1X KH_2PO_4 saline (10%, w/v, pH 8), to which BHT (0.004%, w/v) was added to prevent autoxidation of the samples. Subsequently, the homogenate was centrifuged at 10,000 ×g for 30 minutes at 4ºC and an aliquot of supernatant was separated for further biochemical studies.

3.6.3.7. Determination of AChE activity in the brain tissue

The brain AChE activity was measured as per Ellman's method (1961) using DNTB (5, 5-dithiobis (2-nitrobenzoate). The absorbance was measured at 412 nm.

3.6.3.8. Determination of SOD, CAT and GSH activity in the brain tissue

SOD activity of tissue was measured following the method of Kakkar *et al.* (1984). Briefly, a reaction mixture was prepared containing PMS (phenazine methosulphate; 200µl), sodium pyrophosphate buffer (pH 7; 2.4ml), homogenate supernatant (600µl) and NADH (400µl) by mixing properly. Glacial acetic acid (1ml) was added after 60s. of incubation to terminate the reaction. The chromogen color intensity was measured at 560nm. Results were expressed in units/mg protein. Further, CAT activity of tissue was determined as per the standardized method of Kar and Mishra (1976) and the absorbance of the reaction solution was appraised at 240nm. whereas reduced glutathione (GSH) was estimated by Ellman's method (1961) with the modified method of Jollow *et al.* (1974), using DTNB as a substrate and the yellow colour intensity was read immediately at 412nm.

3.6.3.9. Statistical analysis

For reproducibility, all data were prepared as the mean ± SD of six measurements. Statistical analysis were performed by one-way analysis of variance (ANOVA) with Dunnett's test using KyPlot version 5.0 (32 bit) for windows. $P < 0.05$ was considered as significant.

3.7. Anti-diabetic Activity of plant extract

3.7.1. Selection of Plant material

The plant, ethanolic extract of *A. nilotica* leaf (ANL) was selected in this study

based on its ethnopharmacological relevance.

3.7.2. α-amylase inhibitory activity of plant extract

The study was performed as per the standard method of Hansawasdi *et al.* (2000) with slight modifications. In brief, 1% of starch azure was suspended in Tris–HCl (0.5M; pH 6.9) containing 0.01M of $CaCl_2$ and boiled on water bath for 5min. Then the tubes were cooled down to room temperature and different concentrations (0 –200mg/ml) of plant extract and amylase enzyme (2U/ml) in Tris–HCl buffer was added to it, followed by 5 min of incubation at 37°C. Reaction was stopped by addition of 250ml of 1M HCl followed by centrifuge at 3,000 rpm for 10 min. The supernatant was collected and absorbance was read at 595nm. The percentage of inhibition was measured from the following formula:

$$PI(\alpha - amylase) = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the blank and A_1 was the absorbance in the presence of the varying concentrations of extract; PI= Percentage of inhibition.

3.7.3. Acute oral toxicity study

To investigate the acute toxicity of ANL, we followed OECD (test 423: Acute oral toxicity – Acute toxic class method; 2002) guidelines (OECDiLibrary, 2002). All the animals were kept on fasting overnight before treatment. ANL extract at the dose range of 100mg–2000mg/kg body weight (BW) were administered orally to six different groups of mice comprised of six animals in each group. The groups were kept under critical surveillance to observe about any kind of toxicological or mortality symptoms, if any, at 1, 2, 4, 8, 24, 48 and 72h of interval.

3.7.4. Induction of diabetes in experimental animals

Mice were made diabetic by a single intra-peritoneal injection (0.2ml) of freshly prepared alloxan monohydrate in saline (154mM NaCl) with a dose of 150mg/kg BW. On the 3rd day of alloxan injection, blood glucose level of each animal was recorded and the mice with blood glucose level of >200mg/dl were considered as diabetic and chosen for further study.

3.7.5. Experimental design and drug administration

A total of five groups of mice, six in each group received the following treatment schedule.

Group I: normal control (non-diabetic mice), received normal saline; Group II: type 1 diabetic control (alloxan-treated mice), received normal saline; Group III: diabetic mice (alloxan-treated mice), received standard drug, glibenclamide at 5 mg/kg BW per day; Group IV: low dose extract administrated diabetic mice, received ANL extract (in Mili-Q water) at 50mg/kg BW per day; Group V: high dose extract administrated diabetic mice,

received ANL extract (in Mili-Q water) at 200mg/kg BW per day. These treatments were continued for 20 consecutive days.

3.7.6. Blood glucose level estimation

Fasting blood glucose levels (12h after feed delivery) of mice were measured from the 1st day of extract treatment. Blood glucose estimation was done by single touch glucometer (Bayer, contour TS meter) on every 10-day interval from the tail vein.

3.7.7. Collection of blood, serum and tissue sample

On the 21st day of experiment, all the mice were sacrificed at fasting condition under mild ether anesthesia and blood was collected by puncturing the heart. Blood sample (50µl) was used to estimate glycated hemoglobin (HbA1c) level by ion -exchange HPLC using Bio-Rad D-10™ Dual HbA1c program 220-0201 following the manufactures instructions. The serum was separated by centrifugation at 1000 rpm for 10 min and kept at -20°C until further use. Liver, kidney and skeletal muscle (collected from thigh muscle) were separated from body parts followed by gentle washing in PBS (phosphate buffer saline) and stored at -20°C.

3.7.8. Study of different serum biochemical parameters

Serum insulin level was measured by the ELISA method using Accu-Bind Universal ELISA kit (Monobind Inc., USA) according to manufactures instructions. Besides, different enzymatic assays like acid phosphatase (ACP), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were carried out for all the experimental groups using commercially available kits (Crest Biosystems, Goa, India).

3.7.9. Measurement of protein and lipid metabolic disorders

3.7.12.1. Estimation of serum urea, uric acid, creatinine, cholesterol and triglyceride

Levels of serum urea, uric acid, creatinine, cholesterol and triglyceride were measured spectrophotometrically by the addition of enzymes present in the reagent kits of Crest Biosystems (Goa, India) following manufactures instruction and the values were expressed in mg/dL in all cases.

3.7.10. Estimation of glycogen level

Hepatic glycogen level was estimated according to the standard method of Carroll *et al.* (1956) with a few alterations using anthrone reagent. The intensity of the green to dark-green coloration of the reaction mixture was estimated at 630nm. Glycogen content of tissue was determined from the standard curve prepared with glucose solution.

3.7.11. Estimation of lipid peroxidation

Commercially available TBARS assay kit (Cayman Chemical, USA) was used to

measure the malondialdehyde (MDA) content in serum of lipid peroxidation in all experimental groups.

3.7.12. Determination of peroxidase (Px) and catalase (CAT) activity

The method of Sadasivam and Manickam (2008) was followed to estimate the peroxidase activity in liver, kidney and skeletal muscle tissues of experimental groups through the oxidation of guiacol and the absorbance was recorded at 436nm. The catalase activity of above said organs of each group were estimated according to a standard method of Luck (1963).

3.7.13. Statistical analysis

Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). Group-difference were measured using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Data of OGTT was analyzed using two-way ANOVA. $P<0.05$ was considered significant. All data are reported as the mean ±SD of six measurements

3.8. Chemical Characterizations of selected plant extracts

Characterizations of plant extracts provide major opportunities for new drug discovery as well as for pharmaceutical industry. In essence, natural products or phyto-chemicals are constantly being screened due to their safe and less adverse effects. Several modern techniques of isolation are being included in characterization of bioactive molecules from plant extracts. In the present study, Fourier transform infrared (FTIR) spectra, Gas Chromatography-Mass Spectroscopy (GC-MS) and Nuclear Magnetic Resonance (NMR) spectra were employed and summarized below.

3.8.1. FTIR analysis

Fourier Transform Infrared (FTIR) has been established to be a helpful tool for the characterization and detection of compounds or functional groups (i.e. chemical bonds) present in an unknown mixture of plant extracts (Eberhardt *et al.*, 2007). Briefly, previously prepared selected extract (10µg) was taken in calcium fluoride (CaF_2) vessel and placed in a sample cup of a diffuse reflectance accessory. Thereafter, IR spectrum was obtained using Shimadzu 8300 FTIR spectrophotometer at ambient temperature (25ºC). The background correction was done by taking IR spectrum of de-ionized water as the reference in identical condition. The sample was scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal to noise ratio. The functional groups were identified from the IR peaks (Silverstein *et al.*, 2006).

3.8.2. GC-MS analysis

In the present study, GC-MS analysis was done following two different procedures which are summarized below.

Procedure-I:

Sample preparation and analysis

Here, GC-MS analysis was performed following the method of Kind *et al.* (2009) after slight alteration. In brief, the leaf extract (*A. catechu*) was derivatized with 10µl of methoxyamine hydrochloride (20mg/ml in Pyridine) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS), 2µl FAME (Fatty Acid Methyl Esters) markers [a mixture of internal Retention Index (RI) markers was prepared using fatty acid methyl esters of C8, C10, C12, C14, C16, C18, C20, C22, C24 and C26 linear chain length, dissolved in chloroform (HPLC) at a concentration of 0.8mg/ml (C8-C16) and 0.4mg/ml (C18-C30)] was added.

HP-5MS capillary column (Agilent J & W; GC Columns (USA) with length of 30m plus Duraguard 10m, diameter 0.25mm narrowbore, (film 0.25µm) was used. The analysis was performed under the following oven temperature programme: Injection in sandwich mode with fast plunger speed without viscosity delay or dwell time, oven ramp 60ºC (1 minute hold) to 325ºC at 10ºC/minute, 10 minute hold before cool-down, 37.5 minute run time. The injection temperature was set at 250ºC; the MS transfer line at 290ºC and the ion source at 230ºC. Prior to analysis the method was calibrated with the FAME standards available with the Fiehn GC/MS Metabolomics library (2008) (Agilent ChemStation, Agilent Technologies Inc., Wilmington, USA). Helium was used as the carrier gas at a constant flow rate of 0.723ml/min (carrier linear velocity 31.141 cm/sec). Samples (1µl) were injected via the split mode (Split ratio 1:5) onto the GC column. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute GC-MS results and to identify chromatographic peaks. Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra, retention times and retention indices with entries of those in Agilent Fiehn Metabolomics library using Agilent retention time locking (RTL) method. Response ratio was calculated as peak area ratios of metabolite and ribitol as internal standard. Relative response ratio (RRR) is normalized response ratio per gram crude extract.

Procedure-II:

Sample preparation and Silylation derivation for GC-MS

The plant (*A. catechu*, *Mimosa pudica* and *M. invisa*) extracts (50mg) were separately mixed with 2 ml n-hexane with occasional shaking at laboratory temperature (20-22° C). After 24 h of incubation, 20µl of BSTFA and TMCS (99:1 v/v) mixture was added to the previously prepared solution and left for 60 min of incubation at 20-22° C with occasional shaking. Then the mixture was centrifuged at 15,000 rpm for 20 min at 25°C. The supernatants were

collected and passed through the anhydrous Na_2SO_4 and activated charcoal (2:1; w/w) in a 10 cm mini-column $1/10^{th}$ packed with non-absorbent cotton to remove moisture and color (Dey and Chaudhuri, 2015). The eluted liquid was re-centrifuged at 15,000 rpm for 20 min and passed through Whatman filter paper no. 1 (11µm). The resultant clear solutions were used for analysis.

Analysis

Thermo Scientific Trace 1300 gas chromatography coupled with Thermo Scientific ISQ QD single quadrupole mass spectrophotometer was used to analyze the samples. The GC was equipped with TG-5MS column (30 m × 0.25 mm × 0.25 µm). The inlet temperature was maintained at 250°C and initial temperature was set at 60°C (solvent delay 5 min) with a hold of 4 min, followed by a ramp of 5°C to 290° C with a hold of 10 min (60 min program). Each sample (1µl) was injected in a splitless mode (split flow 50ml/min) with splitless time of 0.80 min, using a Thermo Scientific AI-1310 auto-sampler. Helium (99.99%) was used as the carrier gas at a constant flow rate of 1ml/min, passed through hydrocarbon and dehydrating columns. MS transfer line temperature was set at 290°C with an Ion source temperature of 230°C (electron ionization). Individual sample was analyzed at electron energy 70 eV (vacuum pressure- 2.21e-0.5 Torr). The mass analyzer range was set to 50-650 atomic mass unit (amu). Automated mass spectral deconvolution and identification system (AMDIS; version 2.70) was used to analyze GC-MS results and to identify chromatographic peaks (Dey and Chaudhuri, 2015). The major and essential compounds were identified by mass fragmentation patterns of the reference of parent compound using MS interpreter Version 2.0 and by matching with the reference database of National Institute Standard and Technology (NIST) with a MS library version 2011.

3.8.3. NMR analysis

The ^{1}H and ^{13}C NMR-spectra of plant extracts (*Acacia catechu*, *A. nilotica*, *Mimosa pudica* and *M. invisa*) were recorded with a 300 MHz Bruker FT-NMR (Avance AV-300) spectrometer. Deuterated chloroform and dimethylsulfoxide were used as solvents. All experiments were done at room temperature (24°C). Calibrations of spectra were done on the basis of solvent residual peak and the chemical shifts were reported in δ values (Silverstein *et al*., 2006).

3.9. *In-silico* drugability prediction and Pharmacokinetic study

Following successful characterization of phytochemicals from different extracts, *in-silico* docking techniques towards the discovery of potent drug was undertaken to establish whether the phytochemicals have any role in the management of different imperative diseases including neurodegeneration and diabetes which are

explained below.

3.9.1. Drugability prediction and ROS

High resolution structures of FAS Ligand (FasL; PDB ID- 4MSV) (Liu *et al.*, 2016), NADPH oxidase (PDB ID- 1OEY) (Wilson *et al.*, 2003) and Toll like receptors (TLR; PDB ID- 5AWA) (Beesu *et al.*, 2015) of human were retrieved from RSCB Protein Data Bank (PDB). These protein structures were generated through X-ray diffraction method and all of them have resolution ranging from 2.0-2.5Å. This range of resolution is quite considerable since they are assumed of having correct folding pattern, less errors in surface loops and fewer number of sidechains in wrong rotamer (https://en.wikipedia.org/wiki/Resolution_(electron_density).

3.9.2. Drugability prediction and neurodegeneration

During this study, human dopamine receptor D3 protein (PDB ID- 3PBL) was considered (Chien *et al.*, 2010) as the target protein for performing computational docking investigations.

3.9.2.1. Pharmacokinetic study

Generally, 'Lipinski's rule of five' is considered as "rule of thumb" while predicting a chemical or compound as novel drug (http://www.rsc.org/chemistryworld/Issues/2008/June/BreakingTheRules.asp). This rule is purely based on the physiochemical properties of the ligand molecule. Therefore, I predicted physiochemical properties using number of hydrogen donor and acceptor, molecular weight as well as partition coefficient (logP or lipophicity) via ALOGPS (http://www.vcclab.org/lab/alogps/) and Chemicalize (http://www.chemicalize.org/). I further screened the target drug molecules through BBB predictor (http://www.cbligand.org/BBB/index.php) using SVM and AdaBoost algorithms with four different fingerprints, namely, MACCS, Openbabel (FP2), Molprint 2D and PubChem to verify whether the target compounds can indeed cross the blood brain barrier (BBB). Besides, another tool, BioZyne (http://pgp.biozyne.com/) was employed to study the P-glycoprotein (P-gp) substrate property of considered drug target.

3.9.3. Drugability prediction and diabetes

Nrf2 transcription factor (PDB ID: 5FNQ), responsible for defense mechanism, was employed in the present study as a target protein using the identified phytocompounds including catechol, β-sitosterol, α-tochopherol and γ- tocopherol.

3.9.4. Docking study

The PDB protein structures were prepared for docking after deletion of water atoms followed by addition of polar hydrogen atoms. A supplementary file was created with information regarding the dimensions for the purpose of docking. Gasteiger

charges were further added to both proteins and ligands on the basis of electronegativity equilibration. The torsions of all ligands (i.e. selected phytocompounds) were allowed to rotate freely to obtain better outcome. Molecular docking was performed by Autodock Vina and the docked complexes were visualized by means of PyMol protein structure visualization package through which the relative distance between the atoms of ligands and proteins were also generated [in the unit of Angstrom (Å)]. The chemical structures for the identified phytochemicals were downloaded in .sdf format from NCBI-PUBCHEM (http:// www.ncbi.nlm.nih.gothrv/pccompound) followed by converted into .pdb format through SMILES server (https:// cactus.nci.nih.gov/translate/).

3.10. Study of Molecular diversity of Mimosoids

3.10.1. Study area

Different areas of Malda, Uttar Dianpur and Darjeeling districts were chosen for germplasm collection.

3.10.2. Collection of plant material

Selected places (Chanchal, Ratua, Samsi, Raiganj, Kaliyaganj, Sibmandir and NBU campus) of above mentioned 3 districts (Table 3.2) of West Bengal in India were visited for the collection of the germplasm. A total of eleven months were taken to complete the field survey depending upon the season. Nine different plant materials (Table 3.2) under the subfamily Mimosoideae (Mimosaceae) were collected and authenticated by the plant taxonomist of Department of Botany, University of North Bengal. The voucher specimens were deposited at the Botany Department Herbarium, University of North Bengal. The habit, habitat, locality, collection time and morphology of each plant specimen were recorded in field data sheet (Fig. 3.1).

3.10.3. Molecular diversity of selected Mimosoids

Genetic diversity of 9 different Mimosoids were studied by using different types of PCR based molecular techniques namely, RAPD, PCR-RFLP and barcoding markers. Isolation of DNA is the foremost approach to carry out these molecular fingerprinting. The method developed by Doyle and Doyle (1987) was used for the isolation of genomic DNA with minor alterations as summarized below.

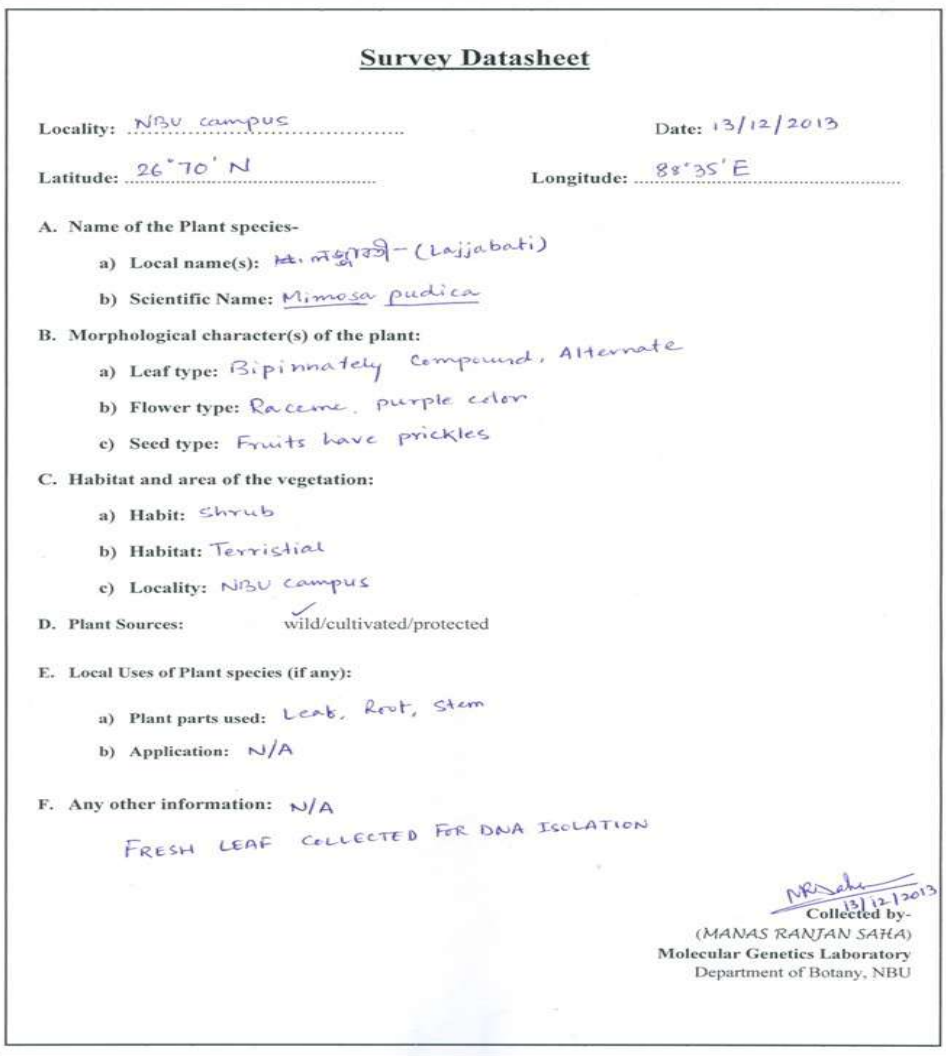
3.10.3.1. Extraction of DNA from leaf

The DNA extraction procedures from the selected plant samples are as follows:

- Fresh tender leaves (approx. 5 gm) were grounded in a mortar and pestle using liquid nitrogen followed by quick transfer of those pulverized leaf materials into Oakridge tube (Tarsons, Cat#541040; 30 ml) containing 15 ml of pre-warmed CTAB extraction buffer (Refer Appendix- B for composition) and mixed properly.

Table 3.2. Collection sites of 9 different plant samples.

Name of the plant species	Sample ID	Voucher No.	(West Bengal) District	Location	Latitude	Longitude
Mimosa pudica L.	M1	NBU/MLD/308	Malda	Chanchal	25°38′ N	88°01′ E
Mimosa invisa Mart. ex Colla	M2	NBU/MLD/311		Ratua	25°19′ N	87°92′ E
Acacia nilotica (L.) Delile	M3	RUC/MLD/433		Samsi	25°27′ N	88°00′ E
Acacia nilotica var. *indica* (Benth.) A.F.Hill.	M4	RUC/UD/454	Uttar Dinajpur	Raiganj	25°61′ N	88°12′ E
Acacia catechu (L.f.) Willd	M5	NBU/UD/1039		Kaliya-ganj	25°63′ N	88°32′ E
Acacia concinna (Willd.) DC.	M6	NBU/UD/756		Raiganj	25°61′ N	88°12′ E
Albizia lebbeck (L.) Willd.	M7	NBU/DJ/902	Darjee-ling	Siliguri	26°72′ N	88°39′ E
Albizia chinensis (Osbeck) Merr.	M8	NBU/DJ/1002		NBU campus	26°70′ N	88°35′ E
Samanea saman (Jacq.) Merr.	M9	NBU/DJ/1022		NBU campus	26°70′ N	88°35′ E



Survey Datasheet

Locality: NBU campus Date: 13/12/2013

Latitude: 26°70' N Longitude: 88°35' E

A. Name of the Plant species-

a) Local name(s): [illegible] – (Lajjabati)

b) Scientific Name: Mimosa pudica

B. Morphological character(s) of the plant:

a) Leaf type: Bipinnately Compound, Alternate

b) Flower type: Raceme, purple color

c) Seed type: Fruits have prickles

C. Habitat and area of the vegetation:

a) Habit: Shrub

b) Habitat: Terristial

c) Locality: NBU campus

D. Plant Sources: ✓ wild/cultivated/protected

E. Local Uses of Plant species (if any):

a) Plant parts used: Leaf, Root, Stem

b) Application: N/A

F. Any other information: N/A

FRESH LEAF COLLECTED FOR DNA ISOLATION

13/12/2013

Collected by-

(MANAS RANJAN SAHA)

Molecular Genetics Laboratory

Department of Botany, NBU

Fig. 3.1. Datasheet used during the collection of germplasm.

- The tube was incubated in a water bath (Rivotek, Cat#50121002) for 1h at 65ºC with occasional mixing by gentle swirling.
- Subsequently, an equal volume of chloroform (Merck India, Cat#822265): isoamyl alcohol (Merck India, Cat#8.18969.1000) (24:1) was added and gently mixed.
- The entire mixture was then centrifuged (REMI, Model No.C-24) for 20 min at about 6,500 rpm (5,000xg) at room temperature (25ºC) and the supernatant was carefully transferred to a fresh tube without disturbing the middle cell debris layer.
- Approximately 0.6 volume of ice cold isopropanol (Merck India, Cat#17813) was added to the supernatant and mixed gently.
- The mixture was then incubated for 2h at -20ºC.
- Following, the mixture was then centrifuged at about 6,500 rpm (5,000xg) for 30 minutes at 4ºC.
- Afterward, the upper aqueous layer was removed and the pellet was washed gently with chilled 70% ethyl alcohol (BDH, Cat#10107) and air dried for 1-2h.
- Then, the pellet was allowed to dissolve in 500µl of 1X TE buffer (pH 7.4) (Refer Appendix- B for composition).
- Next, the buffered DNA solution was extracted with an equal volume of equilibrated phenol (pH 8.0) (Sigma, Cat#P4557-400ML), mixed properly and centrifuged at 13,000 rpm (16,000xg) for 20 minutes.
- Following centrifugation, the upper aqueous layer was then transferred into a fresh tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 rpm (10,000xg) for 15 minutes at room temperature.
- The upper aqueous layer was again taken to a fresh tube and 0.1 volume of 3M sodium acetate (pH 5.2) (SIGMA, Cat#S-9513) was added followed by the addition of 2 volume of ice cold absolute ethyl alcohol (BDH, Cat#10107) and mixed gently for 10 min. and then centrifuged at 4ºC for 30 min at 13,000 rpm (16,000xg).
- Finally, the pellet thus obtained was washed gently with chilled 70% ethanol, air dried and dissolved in 500µl of 1X TE buffer (pH 7.4).

3.10.3.2. Purification of crude *DNA*

RNA, protein and polysaccharides are the main contaminants found in crude DNA hampering further downstream process. Therefore removal of these impurities is the main rational pathway for purification of crude DNA. Utilization of CTAB helps in elimination of polysaccharides from DNA. Subsequently, phenol: chloroform

and RNase treatment facilitate in the elimination of RNA and most of proteins to a large extent from crude DNA. The following procedure of DNA purification is as follows:

- Freshly prepared RNaseA (50µg/ml) (SIGMA, Cat#R-4875) (Refer Appendix- B for composition) was added into the genomic DNA, dissolved in 500µl of 1X TE buffer (pH 7.4) and incubated at 37ºC for 1h in a Dry water bath (GeNei™, Cat#107173).
- Then, the equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed properly and centrifuged at 10,000 rpm (10,000xg) for 15 min at room temperature.
- The upper aqueous phase was then transferred to a fresh micro centrifuge tube (Tarsons, Cat#500010) and 0.1 volume of 3M sodium acetate (pH 5.2) was then added into the tuve followed by double volume of absolute ethyl alcohol for DNA precipitation. Next, the tube was centrifuged at 13,000 rpm (16,000xg) for 30 min at 4ºC.
- Finally, the DNA pellet was collected and washed gently with chilled 70% ethyl alcohol, air dried and finally dissolved in 100µl of 1X TE buffer (pH 7.4).

3.10.3.3. Quantification of DNA

Proper DNA quantification is required in molecular biology for the amplification of target DNA by polymerase chain reaction and complete digestion of DNA by restriction enzymes. In essence, DNA quantification is carried out by spectrophotometric measurements or by agarose gel analysis. Both the methods were used in the present study.

3.10.3.3.1.Spectrophotometric measurement

- At first, spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK) was calibrated at 260nm as well as 280nm by taking 600µl 1X TE buffer in a cuvette.
- The purified DNA (6µl diluted in 594µl of 1X TE) was taken in a cuvette, mixed properly and the optical density (OD) was recorded at both 260nm and 280nm. DNA concentration was estimated by using the following formula:

$$DNA = \frac{OD_{260} \times 50 \times DF}{1000}$$

where, DNA=Amount of DNA (ng/µl), DF= Dilution factor.

- Next, the quality of DNA was considered from the OD values recorded at 260nm and 280nm. The DNA showing A_{260}/A_{280} ratio around 1.8 was chosen for further PCR amplification by using RAPD, PCR-

RFLP and barcoding techniques.

3.10.3.3.2. Gel analysis

- The agarose gel (0.8%, gelling temperature 36°C) (SIGMA, Cat#A9539) was casted in 0.5X TBE buffer [Tris-Borate-EDTA)] (Refer Appendix- B for composition) containing 0.5µg/ml Ethidium bromide (Himedia, Cat#RM813) on gel platform (100×70mm) (Tarsons, Cat#7024).
- Sample DNA (5µl) mixed with 3µl of 6X gel loading dye (Refer Appendix-B for composition) was loaded.
- The lambda DNA/*Eco*RI/*Hin*dIII double digest (1µl) (GeNei™, Cat#106000) was loaded as molecular marker to determine the molecular size of the adjacent genomic DNA.
- The gel was run at 40 Volt (V) for 1hr in a Mini Submarine Gel Electrophoresis Unit (Tarsons, Cat#7030) using Electrophoresis Power Supply Unit (Tarsons, Cat#7090).
- Afterwaerd, the run time was over the gel was visualized under UV light on a UV Transilluminator (GeNei™, Cat#SF850).
- The DNA quality was considered by the presence of a single compact band at the corresponding position to λ DNA/*Eco*RI/*Hin*dIII double digest indicating high molecular weight of the DNA.
- The quantity of the DNA was estimated by comparing the sample DNA with the control by visualizing the band intensity under UV.
- The pure DNA thus obtained was used for various fingerprinting studies.

3.10.3.4. Gel Photography

Gel photographs were taken for using gel documentation system fitted with Cannon SLR camera (EOS350D) and Marumi orange filter (58mm YA2, Marumi, Japan). Besides, the EOS utility software was used for this purpose.

3.10.4. RAPD analysis (Random Amplified Polymorphic DNA)

A total of 45 random 10 mer primers were tested for 9 different species and varieties of Mimosoids (Table 3.3).

3.10.4.1. RAPD-PCR amplification

An autoclaved sterile PCR tube (Tarsons, 0.2ml, Cat#500050) was taken containing 25 µl of PCR reaction mixture for PCR amplification. The mixture was consisted of following solutions:

- PCR master Mix (2X; Promega; Cat# M7122) - 12.5µl
- Primer - 1.25µl (0.25µM)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 9.25µl

One negative control PCR mix tube was prepared without DNA. The PCR reactions

Table 3.3. List of RAPD primers used in the Present Study.

Primer ID	Sequence (5′-3′)
OPA01	CAGGCCCTTC
OPA02	TGCCGAGCTG
OPA03	AGTCAGCCAC
OPA04	AATCGGGCTG
OPA05	ATTTTGCTTG
OPA06	GGTCCCTGAC
OPA07	GAAACGGGTG
OPA08	GTGACGTAGG
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG
OPA11	CAATCGCCGT
OPA12	TCGGCGATAG
OPA13	CAGCACCCAC
OPA16	AGCCAGCGAA
OPA17	GACCGCTTGT
OPA18	AGGTGACCGT
OPA19	CAAACGTCGG
OPA20	GTTGCGATCC
OPB01	GTTTCGCTCC
OPB02	TGATCCCTGG
OPB03	CATCCCCCTG
OPB04	GGACTGGAGT
OPB05	TGCGCCCTTC
OPB06	TGCTCTGCCC
OPB07	GGTGACGCAG
OPB08	GTCCACACGG
OPB11	GTAGACCCGT
OPB12	CCTTGACGCA
OPB13	TTCCCCCGCT
OPF09	CCAAGCTTCC
OPG19	GTCAGGGCAA
OPH04	GGAAGTCGCC
OPN04	GACCGACCCA
OPN05	ACTGAACGCC
OPN13	AGCGTCACTC
OPN19	GTCCGTACTG
MGL01	GCGGCTGGAG
MGL02	GGTGGGGACT
MGL03	GTGACGCCGC
MGL04	GGGCAATGAT
MGL05	CTCGGGTGGG
MGL06	CGTCTGCCCG
MGL07	CTGTCCCTTT
MGL08	GTATTGCCCT
MGL09	TGTACGTGAC

were performed on Applied Biosystems Thermocycler-2720 (Germany). The RAPD-PCR amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 94ºC for 4 min, Primer annealing at 37ºC for 1 min, Primer extension at 72ºC for 2 min.
- Cycle 2-44 - Denaturation at 94ºC for 1 min, Primer annealing at 37ºC for 1 min, Primer extension at 72ºC for 2 min.
- Cycle 45 - Denaturation at 94ºC for 1 min, Primer annealing at 37ºC for 1 min., Primer extension at 72ºC for 10 min.

After the compliance of total PCR cycle, the PCR products were separated on 1.8% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer.

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye (Refer Appendix- B for composition) and the samples were loaded and electrophoresis was carried out at 50V for 2.5h.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/EcoRI/HindIII double digest) (GeNeiTM, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used as a molecular size marker. All PCR reactions were run at least thrice and only reproducible and clear bands were scored and aligned by diversity data base software (NTSYSpc).

3.10.5. PCR-RFLP analysis

3.10.5.1. Primers used

Tab c-f in trnL-trnF (Taberlet *et al.*, 1991) region of the genome of selected Mimosoids was amplified. The primer sequence Tab c- 5′-CGAAATCGGTAGACGCTACG-3′ and

Tab f - 5′-ATTTGAACTGGTGACACGAG-3′

was used on the basis of the known sequence from the Taberlet region of the other plant species.

3.10.5.2. PCR amplification of trnL-trnF region

The polymerase chain reaction was carried out in a 25µl volume containing the following components:

- PCR master Mix (2X; Promega; Cat# M7122) - 12.5µl
- Primer - 1.25µl Tab c (0.25µM) and 1.25µl Tab f (0.25µM)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 8µl

One negative control PCR mix tube was prepared without DNA. The PCR reactions were performed on Applied Biosystems Thermocycler-2720 (Germany). The RFLP -PCR amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 95ºC for 5 min, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 2-34 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 35 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 1.8% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer (pH- 8.0).

Next, the PCR product (12.5µl) was mixed with 3µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50 V for 2 h 20 min.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.5.3. PCR product restriction digestion

The PCR products were subjected to restriction digestion with 8 different restriction endonucleases, namely EcoRI (Promega, Cat#R6011), HinfI (Promega, Cat# R6201), HaeIII (Promega, Cat#R6171), HpaI (Promega, Cat# R6311), MboI (Promega, Cat# R6711), AluI (Promega, Cat# R6281), MspI

Table 3.4. List of restriction endonucleases used in the present study.

Restriction Enzyme	Cutting Site	Temperature
*Alu*I	AG↓CT	37 ºC
*Hin*fI	G↓ANTC	37 ºC
*Hpa*I	GTT↓AAC	37 ºC
*Hae*III	GG↓CC	37 ºC
*Msp*I	C↓CGG	37 ºC
*Taq*I	T↓CGA	65 ºC
*Eco*RI	G↓AATTC	37 ºC
MboI	↓GATC	37 ºC

(Promega, Cat# R6401) and TaqI (Promega, Cat# R6151). All enzymes are 4 -base cutters. The list of the enzymes along with their cutting sites and other information's are described in Table 3.4.

The protocol of PCR-RFLP restriction digestion (20µl for each sample) is as follows:

- Restriction Enzyme buffer - 2µl
- Enzymes - 5units (0.5µl)
- PCR product - 5µl
- Pyrogen free water- 12.5µl

[N.B.- In case of enzyme *Taq*I, BSA (0.2µl) was also added separately in the reaction mixture.]

The reaction mixture tube was spinned for a while for proper mixing.

The tube was then incubated at specific temperature for each enzyme (as mentioned in Table 3.4) for a period of 1h in a Dry bath (GeNei^{TM}, Cat#107173).

Following 1h of incubation, the restriction digestion mix product was separated on a 2% (W/V) agarose gel and run in 0.5X TBE buffer (pH-8.0).

Next, the restriction digestion produc (20µl) was mixed with 5µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50 V for 2h.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.6. Barcoding analysis

3.10.6.1. Primers used

Two primers namely, matK and TrnL-F were used in the present study. The sequences of the primers are described in Table 3.5.

3.10.6.2. PCR amplification of matK region

The study was undertaken in a 25µl

volume containing the following components:

- PCR master Mix (2X; Promega; Cat# M7122) – 12.5µl
- Primer – 1.25µl each (forward and reverse)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 8µl

The PCR reactions were performed on Applied Biosystems Thermocycler-2720 (Germany). The amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 94ºC for 4 min, Primer annealing at 48ºC for 30 sec, Primer extension at 72ºC for 1 min.
- Cycle 2-34 - Denaturation at 94ºC for 1 min, Primer annealing at 48ºC for 30 sec, Primer extension at 72ºC for 1 min.
- Cycle 35 - Denaturation at 94ºC for 1 min, Primer annealing at 48ºC for 30 sec, Primer extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 2% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer (pH- 8.0).

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50V for 2h 20 min.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100 bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.6.3. PCR amplification of TrnL-F region

The amplification was carried out in a 25 µl volume containing the following components:

- PCR master Mix (2X; Promega; Cat# M7122) – 12.5µl
- Primer – 1.25µl each (forward and reverse)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 8µl

Table 3.5. Primers used for amplification of matK and TrnL-F gene segments.

Primer name	Binding	Primer sequence (5′– 3′)
matK F	Forward	CGATCTATTCATTCAATATTTC
matK R	Reverse	TCATGCACACGAAAGTCGAAGT
TabC	Forward	CGAAATCGGTAGACGCTACG
TabF	Reverse	ATTTGAACTGGTGACACGAG

The PCR reactions were performed on Applied Biosystems Thermocycler-2720 (Germany). The amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 95ºC for 5 min, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 2-34 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 35 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 2% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer (pH- 8.0).

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50V for 2h 20 min.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.7. DNA fingerprinting data analysis

In the present study, each polymorphic band was considered as binary character and was scored as 1 (presence) or 0 (absence) for each sample and accumulated in a data matrix. Further, a similarity matrix was calculated on the basis of band-sharing from the binary data using Dice coefficient (Nei and Li, 1979) while a dendrogram of similarities was generated using the group average agglomerative clustering tool. The analysis was done using the software package NTSYSpc (version 2.0) (Rohlf, 1998a). In addition, correspondence analysis (2D and 3D plot) of right vectors from the binary data was performed to exhibit a graphical representation among the varieties using NTSYSpc (version 2.0).

3.10.8. Sequencing of amplified products

A total of 4 species namely, *Mimosa pudica*, *M. invisa*, *Acacia concinna* and *Albizia chinensis* were subjected for DNA sequencing purposes using mat-K and TrnL-F gene. Amongst these species, *M. invisa* and *A. chinensis* were selected for the sequencing of both of the mat-K and taberlet regions of chloroplast genome of these species. In case of *M. pudica*, only taberlet region was subjected to DNA sequencing while only mat-K region of *A. concinna* was selected for sequencing purposes. The DNA sequencing was done by means of outsourcing to a private company, Chromous Biotech Pvt. Ltd. [#842, II, Floor, Shankar Bhawan, A Block, Shankar Nagar, Bangalore-5600092

(www.chromous.com)].

3.10.9. Submission of sequence

The raw sequences of all four above mentioned species of Mimosoides were rearranged with the help of Sequin Application Version 12.30 Standard Release [Nov 13, 2012] for Database submission to GenBank along with all necessary information viz., definition of the sequence (i.e. the specific region of the genome), source of the sequence (chloroplast DNA in this case; name of the plant species along with its taxonomic position, date and place of collection, tissue type etc.).

3.10.10. Sequence analysis and construction of phylogenetic tree

The six sequences of matK and TrnL-F region of selected species obtained in the present study were compared with other existed global sequences [downloaded from NCBI (http://www.ncbi.nlm.nih.gov)] in GeneBank under the subfamilies Mimosoideae, Papilionoideae and Caesalpinioideae (Table 3.6).

The DNA sequences were aligned by using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2) and T-Coffee (www.ebi.ac.uk/Tools/t-coffee) software. The phylogenetic tree was constructed by Neighbour Joining (NJ) method using Molecular Evolutionary Genetics Analysis (MEGA 4.0). The tree was analyzed by both NJ and UPGMA (Unweighed Pair Group Mean Average) methods. In addition, parsimony analysis, various clades, transition/transversion (ns/nv) ratio and variability in different regions were also determined by MEGA4.0 in accordance with the Kimura 2-Parameter (K2P) model. The tree was generated employing 16 (matK) and 15 (TrnL-F) sequences from Mimosoideae, Papilionoideae and Caesalpinioideae as well as 1 outgroup taxa from Cannabaceae (Table 3.6).

3.11. Medicinal and molecular assortment of Microsymbionts

3.11.1. Medical aspect

It was further intended to investigate the probable medicinal value of micro-symbionts in Mimosoids. Therefore, antioxidant activity between root and root nodule comprizing *Rhizobium* were subjected in this study. Three well-studied species namely, *M. pudica*, *M. invisa* and *A. nilotica* have chosen as host plant to collect root and root nodule. Initially, a few *in-vitro* antioxidant assays were performed among root and root nodule separately to evaluate their potentiality.

3.11.1.1. Preparation of extracts

The root and root nodule extract were prepared separately as mentioned in section 3.3.4.

3.11.1.2. In-vitro antioxidative tests of root and root nodule

The free radical scavenging activity of roots and root nodules through DPPH

assay was performed as per Saha *et al*. (2016). The Optical density (OD) was measured at 517 nm and the positive control was ascorbic acid. Further, hydroxyl radical scavenging assay of each extracts were carried out on the basis of Fenton reaction (Kunchandy and Rao, 1990) and mannitol was used as positive control. The nitric oxide radical quenching activity was performed following the GriessI-llosvoy reaction (Garratt, 2012) with minor modifications of Saha *et al*.

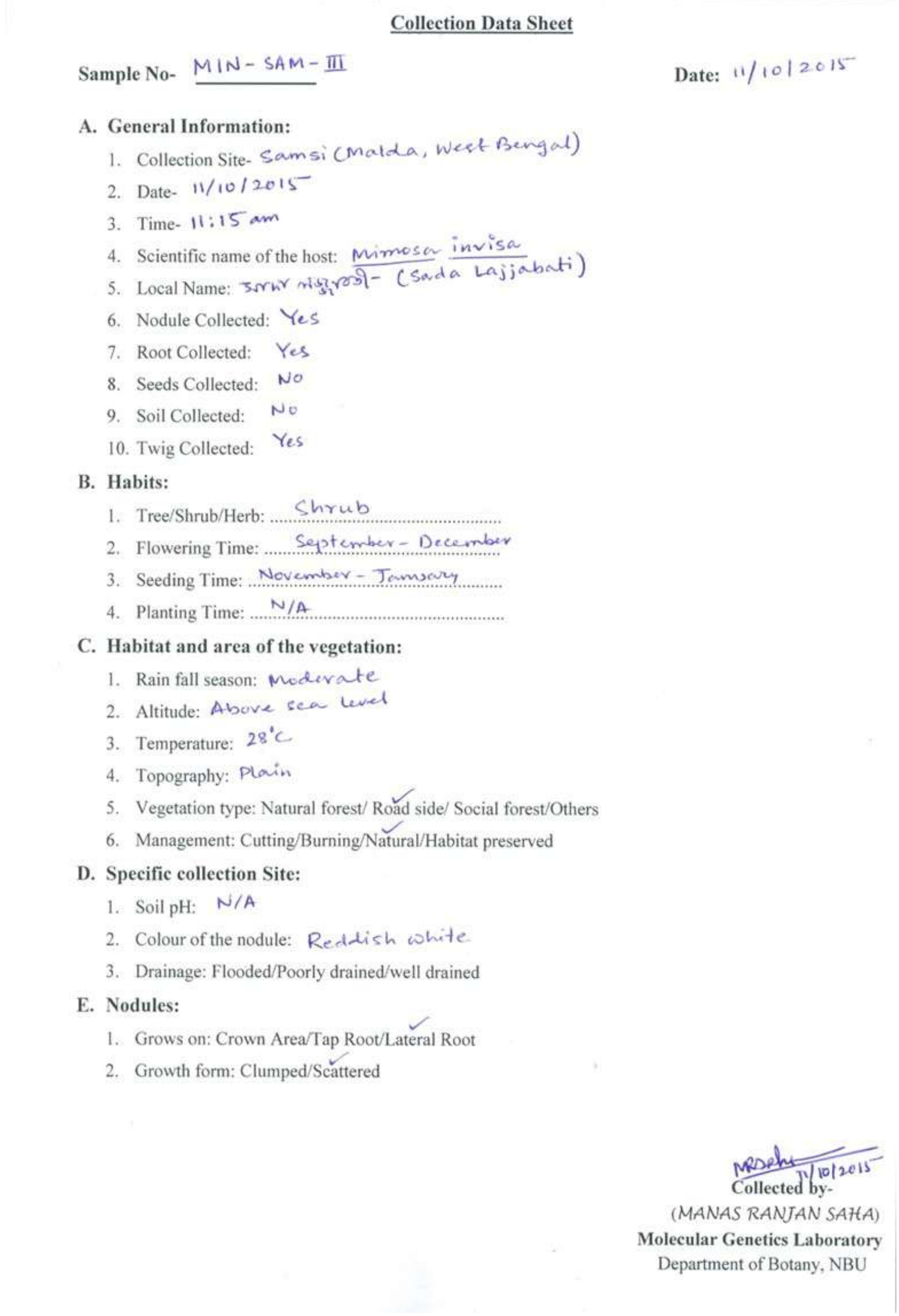
Table 3.6. Taxa, specimens and GenBank accession numbers for sequences used in the present study.

Taxa	Family	Sub-family	matK accession number	TrnL-F accession number
Mimosa invisa	Fabaceae	Mimosoideae	LM643807*	LM643811*
Mimosa pudica	Fabaceae	Mimosoideae	JQ587776	LM643810*
Acacia nilotica	Fabaceae	Mimosoideae	FJ711574	AF522973
Acacia catechu (Syn: *Senegalia catechu*)	Fabaceae	Mimosoideae	KF531964	DQ371870
Acacia concinna	Fabaceae	Mimosoideae	LM643808*	---
Samanea saman	Fabaceae	Mimosoideae	JQ587830	AF522965
Albizia lebbeck	Fabaceae	Mimosoideae	GU134994	EU440023
Albizia chinensis	Fabaceae	Mimosoideae	LM643809*	LM643812*
Abrus precatorius	Fabaceae	Papilionoideae	GU135022	EF543423
Crotalaria juncea	Fabaceae	Papilionoideae	JQ041057	---
Indigofera circinella	Fabaceae	Papilionoideae	KR735049	KR738539
Senna tora	Fabaceae	Caesalpinioideae	KJ638441	KP338336
Glycine max	Fabaceae	Papilionoideae	KF022416	EU717321
Medicago sativa	Fabaceae	Papilionoideae	HQ593363	GQ488614
Pisum sativum	Fabaceae	Papilionoideae	----	AY839473
Phaseolus vulgaris	Fabaceae	Papilionoideae	KF022429	EU717342
Senna occidentalis	Fabaceae	Caesalpinioideae	KU551092	---
Cannabis sativa	Cannabaceae		HQ619806	AY958392

*Present Study (submitted to GeneBank)

Table 3.7. Details of the collected *Rhizobium* from different location with ID.

Host plant	Collection Site	Latitude	Longitude	Sample ID
M. pudica	Samsi (Malda)	25°27′ N	88°00′ E	MPD-SAM-**I**
	Sibmandir (Darjeeling)	26°70′ N	88°35′ E	MPD-SIB-**II**
M. invisa	Samsi (Malda)	25°27′ N	88°00′ E	MIN-SAM-**III**
	Sibmandir (Darjeeling)	26°70′ N	88°35′ E	MIN-SIB-**IV**
A. nilotica	Samsi (Malda)	25°27′ N	88°00′ E	ANL-SAM-**V**
	Sibmandir (Darjeeling)	26°70′ N	88°35′ E	ANL-SIB-**VI**



Collection Data Sheet

Sample No- MIN-SAM-III **Date:** 11/10/2015

A. General Information:

1. Collection Site- Samsi (Malda, West Bengal)
2. Date- 11/10/2015
3. Time- 11:15 am
4. Scientific name of the host: Mimosa invisa
5. Local Name: সাদা লজ্জাবতী- (Sada Lajjabati)
6. Nodule Collected: Yes
7. Root Collected: Yes
8. Seeds Collected: No
9. Soil Collected: No
10. Twig Collected: Yes

B. Habits:

1. Tree/Shrub/Herb: Shrub
2. Flowering Time: September - December
3. Seeding Time: November - January
4. Planting Time: N/A

C. Habitat and area of the vegetation:

1. Rain fall season: Moderate
2. Altitude: Above sea level
3. Temperature: 28°C
4. Topography: Plain
5. Vegetation type: Natural forest/ Road side/ Social forest/Others
6. Management: Cutting/Burning/Natural/Habitat preserved

D. Specific collection Site:

1. Soil pH: N/A
2. Colour of the nodule: Reddish white
3. Drainage: Flooded/Poorly drained/well drained

E. Nodules:

1. Grows on: Crown Area/Tap Root/Lateral Root
2. Growth form: Clumped/Scattered

11/10/2015
Collected by-
(MANAS RANJAN SAHA)
Molecular Genetics Laboratory
Department of Botany, NBU

Fig. 3.2. Datasheet used during sample collection of root nodule (*Rhizobium*).

(2016). The absorbance was determined spectrophotometrically at 540 nm against blank sample. Curcumin was used as standard.

3.11.2. Molecular documentation

3.11.2.1. Collection of germplasm

The nodules of young plant species namely, *Mimosa pudica*, *M. invisa* and

Acacia nilotica (host plant) were collected from different regions (Table 3.7) in their respective growing season (August-November). Essential data were recorded during the collection of germplasm (for details see Fig. 3.2).

3.11.3. Isolation of *Rhizobium*

3.11.3.1. Surface sterilization

Prior to isolation experiment, the surface sterilization of root nodules was performed.

- Firstly, the nodulated roots were washed thoroughly with tap water to remove all the soil particles and organic materials.
- Only healthy and undamaged nodules were selected and washed with few drops of detergent (Dextran) followed by washing with distilled water.
- Then, the nodules were treated with 70% ethanol for 30 sec followed by several washes with sterile distilled water.
- The nodules were further treated with 30% sodium hypochlorite (NaOCl) for 4-6 min. Final rinsing of the nodules was done thoroughly with sterile distilled water for 4-5 times to remove the traces of NaOCl.

3.11.3.2. Media for isolation

Yeast extract mannitol (YEM) medium was used for the isolation of *Rhizobium.*

3.11.3.2.1. Isolation of Rhizobium in liquid and solid media

The following isolation procedure was carried out:

- Surface sterilized nodule lobes were crushed on a grease free sterile slide.
- Next, a loopful of the crushed nodule sap was then put into liquid YEM medium containing conical flask.
- The flask was then transferred to an orbital shaker at 200 rpm maintained at an optimum temperature of 28 ± 2ºC for 2-3 days for proper incubation. The media turned to be turbid with the growth of the bacteria.
- A loopful of culture was streaked on YEM-agar plates (1.8%).
- The YEMA plates were incubated at 28±2^{o}C for 2-3 days.
- The single pure colony obtained was then sub cultured and maintained in YEM slants for further use. The strains were sub cultured at regular interval of one month.

The pure strains were isolated from different regions (Table 3.7) were then named with their respective codes.

3.11.3.3. Morphological characterization

Fresh cultures of 24 to 48h were used for morphological characterization.

3.11.3.3.1. Colony morphology

A fresh culture from broth was streaked on the YEM-agar plates and incubated for 24-48h at 28±2ºC. The morphological

characteristics of the colonies were observed based on the diameter, shape, color, transparency, form and production of mucous as per Aneja (1996).

3.11.3.3.2. Gram staining

Gram-staining was further performed as per the standard method of Aneja (1996). The isolates were observed under light microscope to differentiate gram negative from the gram positive bacteria.

3.11.4. Genomic diversity study

The genomic DNA of *Rhizobium* strains obtained from selected plant species from different regions was isolated through following procedures:

3.11.4.1. Isolation of Genomic DNA from pure culture

- The copious amount of exopolysaccharides produced by *Rhizobium* acts as a hindrance in the isolation of genomic DNA. The CTAB/NaCl method was followed to isolate the *Rhizobium* genomic DNA using standard protocol of William and Feil (2012) with minor modifications.
- The bacterial strains were grown in YEM for 24-48h in OSI until the O.D. value reached 0.8 at 600nm.
- Thirty ml of 24-48 hr culture was put into a sterile oakridge tube and kept in the ice bucket for 1h of incubation. Then, the tubes were centrifuged (REMI, Model No.C-24) at 5000xg for 20 min at 4ºC.
- The supernatant was discarded and the pellet obtained was used for further isolation process.
- The pellet was suspended in 5.67ml 1XTE buffer (pH 8.0) (Refer Appendix- B for composition).
- Fifty five µl of lysozyme (conc. 10mg/ ml) was added to lyse the bacterial cell wall. The mixture was incubated for 5min at room temperature.
- To the mixture 300µl of 10% sodium dodecyl sulphate (SDS) was added and mixed properly.
- Afterward, 50µl of proteinase K (20mg/ml) was added and blended well with gentle swirling.
- The mixture was then incubated in water bath (Rivotek, Cat# 50121002) for 1h at 37ºC. The tubes were mixed occasionally by gentle swirling in between the incubation period.
- Following incubation, 1ml of 5M NaCl was added to the solution and mixed well. To that mixture, 0.8ml of hot (65°C) CTAB/NaCl (Refer Appendix- B for composition) was added, mixed well and incubated at 65°C for 10 min in water bath.
- The mixture was then extracted with equal volume of chloroform:isoamylalcohol (24:1) followed by gentle spinning at 5000xg for 10 min at room temperature. A white interface containing the debris

could be visible after centrifugation. The clear upper aqueous layer was transferred to a fresh oakridge tube.

- Further, equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) was added to the solutions, mixed properly and centrifuged at 5000xg for 20 min. The upper clear aqueous layer was transferred to another fresh oakridge tube.
- Approximately 0.6 vol (8ml) of chilled isopropanol stored at -20°C was added to precipitate the DNA. The tubes were shaken back and forth gently until a white cottony form of DNA was visible.
- Finally, the tubes were stored at -20°C for 2h. After incubation, the tubes were spinned at 5000xg for 30 min at 4°C. The pellet thus obtained was washed with chilled 70% ethyl alcohol, air dried and dissolved in 500µl 1X TE buffer (pH 8.0).

3.11.4.2. Purification of DNA

To eliminate the major contaminants like, RNA and protein the following procedures were followed:

3.11.4.2.1. RNase A treatment

RNase A (10mg/ml) (Refer Appendix- B for composition) was dissolved in 500µl 1X TE buffer (pH 8.0) containing the crude DNA. The mixture was incubated at 37°C for 1h in a Dry water bath (GeNei™ make, Cat#107173).

3.11.4.2.2. Protein removal

The protein impurities were removed using 500µl of phenol/chloroform/ isoamylalcohol (25:24:1) to the crude DNA and kept at 4°C. Micro centrifuge was done at 14000Xg for 2 min at room temperature. The aqueous phase was then transferred to a fresh micro centrifuge tube (Tarsons, Cat# 500010).

3.11.4.3. Ethanol precipitation of DNA

- The volume of the DNA was measured visually and 0.1 volume of 3M Sodium acetate (pH 5.2) was added and mixed well.
- It was followed by the addition of 2 volume of ice cold absolute ethyl alcohol and blended well by gentle swirling.
- The tubes were placed on ice at -20 ºC for 30 min and centrifuged at 14,000xg for 10-15 min at 4ºC.
- The DNA pellet was washed with 1 ml of chilled 70% ethanol, air dried and finally dissolved in 100µl of 1XTE buffer (pH 8.0).

3.11.4.4. Quantification of DNA

Proper DNA quantification is required in molecular biology for the amplification of target DNA by polymerase chain reaction and complete digestion of DNA by restriction enzymes. In essence, DNA quantification is carried out by spectrophotometric measurements or by agarose gel analysis. Both the methods

were used in the present study.

3.11.4.4.1. Spectrophotometric measurement

First, spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK) was calibrated at 260 nano meter (nm) as well as 280 nm by taking 600µl 1X TE buffer in a cuvette.

The purified DNA (6µl diluted in 594µl of 1XTE) was taken in a cuvette, mixed properly and the optical density (OD) was recorded at both 260nm and 280nm. DNA concentration was estimated by using the following formula:

$$DNA = \frac{OD_{260} \times 50 \times DF}{1000}$$

where, DNA=amount of DNA (ng/µl); DF=dilution factor.

Next, the quality of DNA was considered from the OD values recorded at 260nm and 280nm. The DNA showing A_{260}/A_{280} ratio around 1.8 was chosen for further PCR amplification by using RAPD techniques.

3.11.4.4.2. Gel electrophoresis

- DNase free Agarose (0.8%, gelling temperature 36°C) was used to cast the gel in 0.5X TBE (Tris-Borate-EDTA) buffer (Refer Appendix- B for composition) containing 7µl of Ethidium bromide (10mg/ml) on gel platform (100x70mm) (Tarsons, Cat # 7024).
- Five µl of DNA samples were mixed with 3µl of 6X gel loading dye (refer appendix for composition) and loaded in the wells carefully. Lambda DNA/ *Eco*RI/*Hind*III double digest (2µl) and 100 bp ladder were used as molecular markers to determine the size of genomic DNA.
- The gel was run at 50 volt (V) and 100mAmp for 1.5h in the Midi Submarine Electrophoresis Unit (Tarsons, Cat #7050) connected to the Electrophoresis Power Supply Unit (Tarsons, Cat #7090).
- Finally, the gel was viewed on a UV Transilluminator (GeNei™, Cat #SF850). The molecular sizes of the genomic DNA were detected in the form of bands. The sizes of the bands were estimated with Photo-Capt Version 12.4, (Vilber Lourmat, USA).

3.11.4.5. Gel photography

Please refer section 3.10.3.4.

3.11.5. RAPD analysis

Table 3.8. List of RAPD primers used in the Present Study.

Primer ID	Sequence (5′-3′)
OPA01	CAGGCCCTTC
OPA02	TGCCGAGCTG
OPA03	AGTCAGCCAC
OPA04	AATCGGGCTG
OPA05	ATTTTGCTTG
OPA06	GGTCCCTGAC
OPA07	GAAACGGGTG
OPA08	GTGACGTAGG
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG
OPD 03	GTCGCCGTCA
DAF 9	CCGACGCGGC
OPI 06	AAGGCGGCAG
OPY 04	AAGGCTCGAC
OPQ 01	GGGACGATGG

3.11.5.1. Primers used

A total of 15 random 10 mer primers were tested for 6 different *Rhizobium* species (Table 3.8).

3.11.5.2. RAPD-PCR amplification

An autoclaved sterile PCR tube (Tarsons, 0.2ml, Cat#500050) was taken containing 25µl of PCR reaction mixture for PCR amplification. The mixture was consisted of following solutions:

- PCR master Mix (2X; Promega; Cat# M7122) - 12.5µl
- Primer - 1.25µl (0.25µM)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 9.25µl

The ingredients were mixed evenly in a SpinWin PCR micro centrifuge (Tarsons, Cat# 1000).

The PCR reactions were performed in Applied Biosystems, Thermal Cycler, 2720 PCR machine.

The amplification cycle (modified protocol of Elboutahiri *et al.,* 2009) set at 35 cycles were as follows:

- Cycle 1: Denaturation at 94ºC for 4 min, primer annealing at 36ºC for 1 min, primer extension at 72ºC for 2 min.
- Cycle 2-34: Denaturatin at 94ºC for 1 min, primer annealing at 36ºC for 1 min, primer extension at 72ºC for 2 mins.
- Cycle 35: Denaturatin at 94ºC for 1 min, primer annealing at 36ºC for 1 min, final extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 1.8% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer.

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye (Refer Appendix- B for composition) and the samples were loaded and electrophoresis was carried out at 50V for 2.5h.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used as a molecular size marker. All PCR reactions were run at least thrice and only reproducible and clear bands were scored and aligned by diversity data base software (NTSYSpc).

3.11.5.2. DNA fingerprinting data analysis

Please refer section 3.10.7 for more details.

Chapter 4
Results and Discussion

"A positive attitude causes a chain reaction of positive thoughts, events and outcomes. It is a catalyst and it sparks extraordinary results".
-Wade Boggs

4.1. Utilization of local plants of selected Mimosoids

The knowledge of medicinal plants in India has been accumulated in course of many centuries based on several ancient medicinal systems, including ayurveda, unani and siddha (Lone and Bhardwaj, 2013). According to the survey report of World Health Organization (WHO, 2002), 80% people of the developing world use plant remedies for several therapeutic purposes. India, one of the richest floristic regions of the world has diverse socioeconomic, ethnic, linguistic and cultural areas. Therefore, the indigenous knowledge of medicinal plants and their use in treating several ailments might reasonably be expected in this country. Chandel *et al*. (1996) have reported that nearly about 70% of tribal and rural inhabitants of India are to a large extent depended on medicinal plants for their primary healthcare management due to either insufficient or inaccessible or less availability of modern healthcare system.

Despite of having different indigenous races and their own inherited knowledge of practices, not much attention was paid to explore the ethno-botanical information lies in Malda and Uttar Dinajpur districts. Hence, an initiative was undertaken to obtain those unrevealed information of medicinal practices by means of semi-questionnaire process, direct and group discussion with local people, practitioners and traditional healers. It was observed that the local people use those polyherbal formulations pleasantly rather than taking standardized drug. A total of 337 medicinal plants were identified (Saha *et al*., 2014a, 2014b, 2014c; De Sarker *et al*., 2015) to be used to treat different ailments in those two districts during survey (data is not presented here). Since the focus of the present endeavor was on the utilization of Mimosaceae, only local ethno-botanical knowledge of Mimosoids was represented here. A total of six species of Mimosaceae (Mimosoideae) were found to have different ethnopharmacological activities combating various types of ailments in the present study (Fig. 4.1). Of these, the root

decoction of *Mimosa pudica* was reported to heal leucorrhoea while leaf decoction is effectively used in the management of breast cancer or tumor (Saha *et al*., 2014c). *M. invisa* root juice was also administrated to treat leucorrhoea by the aboriginal tribe of Malda districts. Further, tender leaves of *Acacia nilotica* were found to be used to treat diabetes (Saha *et al*., 2014c). The bark of *A. catechu* along with other ingredients was reported to be effective against bone crack, ankle sprain and leucorrhea (Saha *et al*., 2014a). Consequently, the leaf and fruits infusion of *Acacia concinna* are mixed together and applied externally on head to promote hair growth (De Sarker *et al.*, 2015) while root is used as anti-dysenteric. Furthermore, the bark of *Albizia lebbeck* is used as anthelmintic and in case of eczema, leucoderma and other skin disorders. Apart from these, one taxa from Mimosaceae i.e. *A. catechu* was identified to be used as ethno-veterinarian purposes (Saha *et al*, 2014b).

4.2. *In-vitro* antioxidant activities

The endogenous free radical forming pathway demonstrates a cascade of diverse free radicals originating from molecular oxygen. Therapeutic benefits of medicinal plants are usually attributed to their antioxidant properties preventing the deleterious effects of oxidative stress by scavenging free radicals (Gandhi and Abramov, 2012). Hence, the present study was intended to investigate the free radical scavenging and reducing capabilities of selected plant species. The plants were chosen on the basis of preliminary screening of DPPH scavenging activity of all the extracts (Table 4.1). Results showing more than 80% of free radical inhibitory activity at 100 µg/ml concentrations through DPPH were only selected for further comprehensive analysis. Herein, four plant species, namely *Acacia catechu* (ACL), *A. nilotica* (ANL), *Mimosa pudica* (MPD) and *M. invisa* (MIN) were responded to this test, thereby rest were discarded.

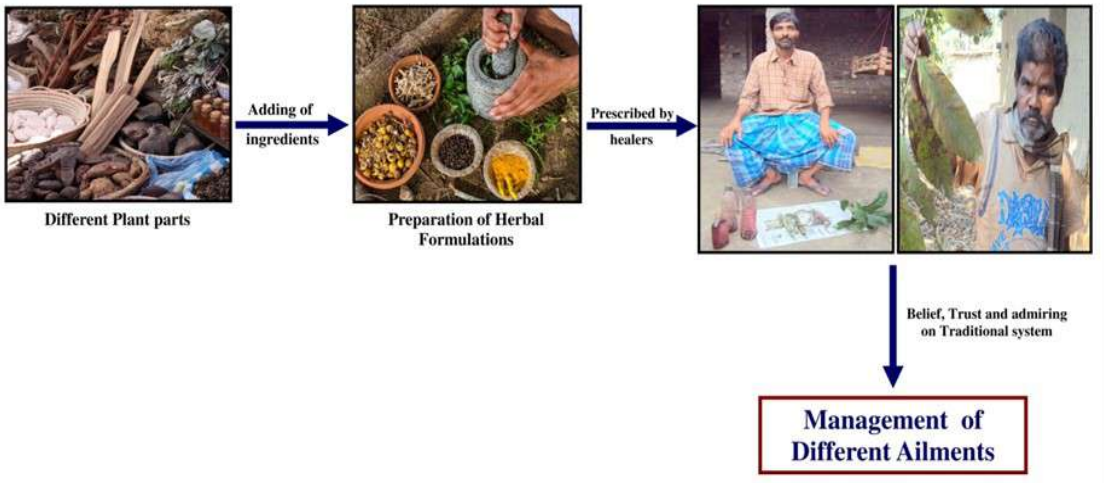


Fig. 4.1. Preparation of herbal-formulations by traditional healers.

The *in-vitro* DPPH free radical scavenging analysis of all four extracts revealed significant antioxidant potential compared to standard ascorbic acid. ACL extract exhibited higher quenching activity of than the ANL, MPD, MIN as well as standard at a dose of 100µg/ml (Fig. 4.2.A; Table 4.2).

ACL revealed 86.30±0.18% of inhibition while ANL, MPD and MIN showed 83.79±0.92%, 82.29±0.85% and 80.63±1.25% of inhibition at 100µg/ml respectively with greater inhibitory effect than the standard (28.32±1.39% at 100µg/ml). Actually, a decrease in the absorbance of the reaction mixture signifies decent free radical scavenging activity of the compound (Krishnaiah *et al.*, 2011). In fact, all the extracts exhibited higher DPPH scavenging activity compared to many other studied medicinal and aromatic plants, reported so far (Miliauskas *et al.*, 2004). This may be due to elevated levels of the active phytocompounds together with the polyphenolics in the extracts which scavenged DPPH radicals and thus attributed to their higher antioxidant activity (Cheung *et al.*, 2003). In hydroxyl radical ($OH^{\bullet}$) quenching activity, ANL extract was found to be much higher inhibitory activity in all studied doses (94.98±0.39% at 200µg/ml) than the others.

Though, at 200µg/ml, ACL, MPD and MINV were found to display lower hydroxyl radical quenching activity of 54.90±1.75%, 54.04±1.09% and 52.25±0.95% respectively (Fig. 4.2.B), they are strong enough to establish their prominent role as a potent source of antioxidants as evident from other studies (Miliauskas *et al*., 2004). Superoxide anion ($O_2^{\bullet-}$) is a weak oxidant but it gives rise to highly reactive cell-damaging free radicals and oxidizing agents namely, $OH^{\bullet}$ and singlet oxygen ($^1O_2$), both of which in turn

Table 4.1. DPPH scavenging activity of all 9 Mimosoids

Name of the plant	Percentage of inhibition			
	10 µg/ml	**20 µg/ml**	**80 µg/ml**	**100 µg/ml**
Mimosa pudica	22.56±1.93	42.36±1.93	67.88±0.98	**82.29±0.85**
Mimosa invisa	19.68±3.34	34.18±0.63	62.15±0.47	**80.63±1.25**
Acacia nilotica	33.72±2.12	43.13±1.38	78.91±0.63	**83.79±0.92**
Acacia nilotica var. indica	21.02±0.62	30.95±2.73	69.40±0.84	74.85±0.61
Acacia catechu	38.28±2.22	48.54±3.63	81.68±0.66	**86.30±0.18**
Acacia concinna	23.10±0.82	33.00±1.48	72.93±0.53	76.07±0.66
Albizia lebbeck	20.19±1.23	33.63±1.74	66.96±0.88	72.27±0.61
Albizia chinensis	18.38±0.51	31.74±0.72	64.29±0.88	70.23±1.16
Samanea saman	20.99±2.71	30.09±1.65	62.57±0.30	68.77±1.07

cause oxidative stress in biological systems. In fact, $^1O_2$ induces hyper-oxidation and oxygen cytotxicity and decreases antioxidative activity in cells (Halliwell and Gutteridge, 2015). ANL extract was found to possess better superoxide anion scavenging activity (40.97±0.34% at 100µg/ml) in dose dependent manner followed by ACL, MPD and MIN with lower IC_{50} value (121.07±2.24µg/ml) compared to others (Fig. 4.2.C; Table 4.2).

Decent amount of singlet oxygen ($^1O_2$) scavenging activity was observed in the following order MIN> ACL> MPD > ANL (Table 4.2) as given in Figure 4.2.D. The results were much lower than the inhibitory activity of standard lipoic acid, nevertheless, they have still great importance as therapeutic agents in preventing or lowering the progress of free radical like, $^1O_2$. Hence, the result indicated presence of certain active metabolites which reacted with singlet oxygen revealing potent scavenging activity. On the other hand, H_2O_2 has the capacity to inactivate several enzymes by means of oxidation of essential thiol groups. In addition, $O_2^{\bullet-}$ converts into H_2O_2 by the action of superoxide dismutase (SOD) as well as reacts with Fe^{2+} or Cu^{2+} ions inside the cells which leads to the generation of $OH^{\bullet}$ causing DNA damage and lipid peroxidation (Halliwell and Gutteridge, 1999).

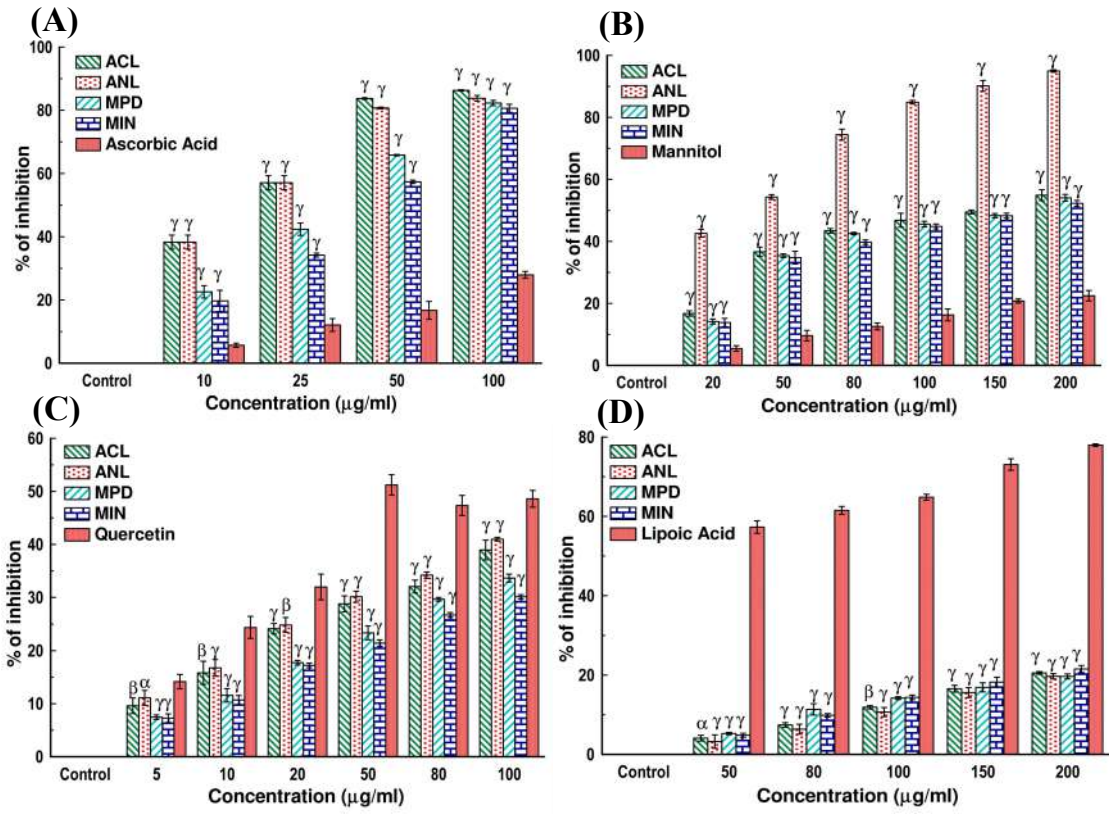


Fig. 4.2. Free radical scavenging activity through **(A)** DPPH, **(B)** Hydroxyl radical, **(C)** Superoxide anion and **(D)** Singlet oxygen scavenging activity of 4 different Mimosoids and their respective standards. [Each value represents mean ±SD (n=6); Where, γ= *P*<0.001, β= *P*<0.01 and α= *P*<0.05 Vs standard (µg/ml)].

Table 4.2. Percentage of inhibition of all four plant species.

Parameters	ACL (%)	ANL (%)	MPD (%)	MIN (%)	Standard (%)
DPPH (at 100 µg/ml)	86.30±0.18	83.79±0.92	82.29±0.85	80.63±1.25	27.93±1.10 (Ascorbic Acid)
Hydroxyl Radical (at 200 µg/ml)	54.90±1.75	94.98±0.39	54.04±1.09	52.25±0.95	22.48±1.68 (Mannitol)
Hydrogen Peroxide (at 2 mg/ml)	11.57±1.49	21.99±0.99	15.12±0.63	11.88±1.45	54.43±4.72 (Sodium Pyruvate)
Nitric Oxide (at 100 µg/ml)	63.04±0.36	60.55±0.84	57.91±0.30	60.72±0.44	55.08±1.93 (Curcumin)
Superoxide Anion (at 100 µg/ml)	38.94±1.86	40.97±0.34	33.64±0.73	30.13±0.38	48.59±1.58 (Quercetin)
Hypochlorous Acid (at 100 µg/ml)	41.37±2.56	44.17±1.06	39.27±1.11	37.91±1.04	36.10±2.80 (Ascorbic Acid)
Total Antioxidant Activity (at 200 µg/ml)	92.66±0.05	94.82±0.07	92.58±0.03	91.12±0.12	68.95±0.50 (Ascorbic Acid)
Peroxynitrite (at 200 µg/ml)	17.52±0.56	20.12±1.04	19.05±0.42	16.80±0.35	16.72±0.91 (Gallic Acid)
Singlet Oxygen (at 200 µg/ml)	20.56±0.35	16.69±0.68	19.67±0.68	21.53±0.88	77.97±0.30 (Lipoic Acid)
Lipid Peroxidation (at 25 µg/ml)	50.28±0.51	45.15±2.30	37.09±0.51	33.46±0.34	77.58±1.03 (Trolox)
Iron chelation (at 200 µg/ml)	35.14±0.55	33.67±1.54	33.25±1.07	32.52±0.79	99.57±0.15 (6 µg/ml) (EDTA)

Total Phenol and Flavonoid Content :

ACL	Total phenol content was recorded as 89.59 ± 2.71 mg gallic acid equivalent per 100 mg of plant extract while total flavonoid content was observed as 13.92 ± 1.60 mg quercetin equivalent per 100 mg of plant extract.
ANL	Total phenol content was 83.21 ± 1.32 mg gallic acid equivalent per 100 mg of plant extract and total flavonoid content was noted as 16.56 ± 2.36 mg quercetin equivalent per 100 mg of plant extract.
MPD	Total phenol content was 76.6±0.02 mg gallic acid equivalent per 100 mg of plant extract and total flavonoid content was found as 35.2±0.5 mg quercetin equivalent per 100 mg of plant extract.
MIN	Total phenol content was 61.9±0.5 mg gallic acid equivalent per 100 mg of plant extract and total flavonoid content was recorded as 30.4±0.2 mg quercetin equivalent per 100 mg of plant extract.

It was observed that all the extracts significantly scavenged H_2O_2 (Fig. 4.3.A), which might be attributed to the presence of phenolic groups that could donate electrons to H_2O_2 thereby, neutralizing it into water. Amongst four extracts, ANL showed better H_2O_2 inhibitory potentiality (P <0.001) than the others as evident from Table 4.2. Further, iron chelating capacity assay was employed to evaluate the ability of extracts to disrupt the formation of the ferrozine-complexes (reaction between ferrous ion and ferrozine). The iron chelating activity of ACL was observed to be of 35.14±0.55% at 200µg/ml followed by ANL> MPD> MIN (Fig. 4.3.C) showing the inhibition percentage of 33.67±1.54%, 33.24±1.07% and 32.51±0.79% respectively at 200µg/ml (Table 4.2) which reflected their decent chelating activity.

Accordingly, nitric oxide (NO) is a potent mediator of pro-inflammatory cellular activation causing subsequent inflammatory cellular injury. Moreover, spontaneous coupling of NO with superoxide radicals give rise to highly reactive peroxynitrite ($ONOO^-$), which is responsible for causing inflammation in cognitive disorders (Gimenez-Garzo *et al.*, 2015). Besides, hypochlorous acid (HOCl) is produced at the sites of inflammation due to oxidation of Cl^- ions by the neutrophil enzyme, myeloperoxidase and induces target cell lysis (Aruoma *et al.*, 1989). Result exhibited that ACL, ANL, MPD and MIN extracts not only possess higher inhibitory capacities with lower IC_{50} value (Table 4.3) to scavenge NO, $ONOO^-$ and HOCl, the quenching activities (Table 4.2) are also better (Fig. 4.3.B; 4.4.A and 4.4.B) than the respective standards such as curcumin, gallic acid and ascorbic acid respectively indicating potent antioxidant agents.

Therefore, it may be inferred that the extracts might be regarded as a potent antioxidative agents that probably reacted with reactive nitrogen species (RNS) preventing NO-mediated tissue damage or inflammation. Lipid peroxidation is another complex chain process that involves a variety of free radicals especially $^1O_2$, H_2O_2 and $OH^\bullet$, where in

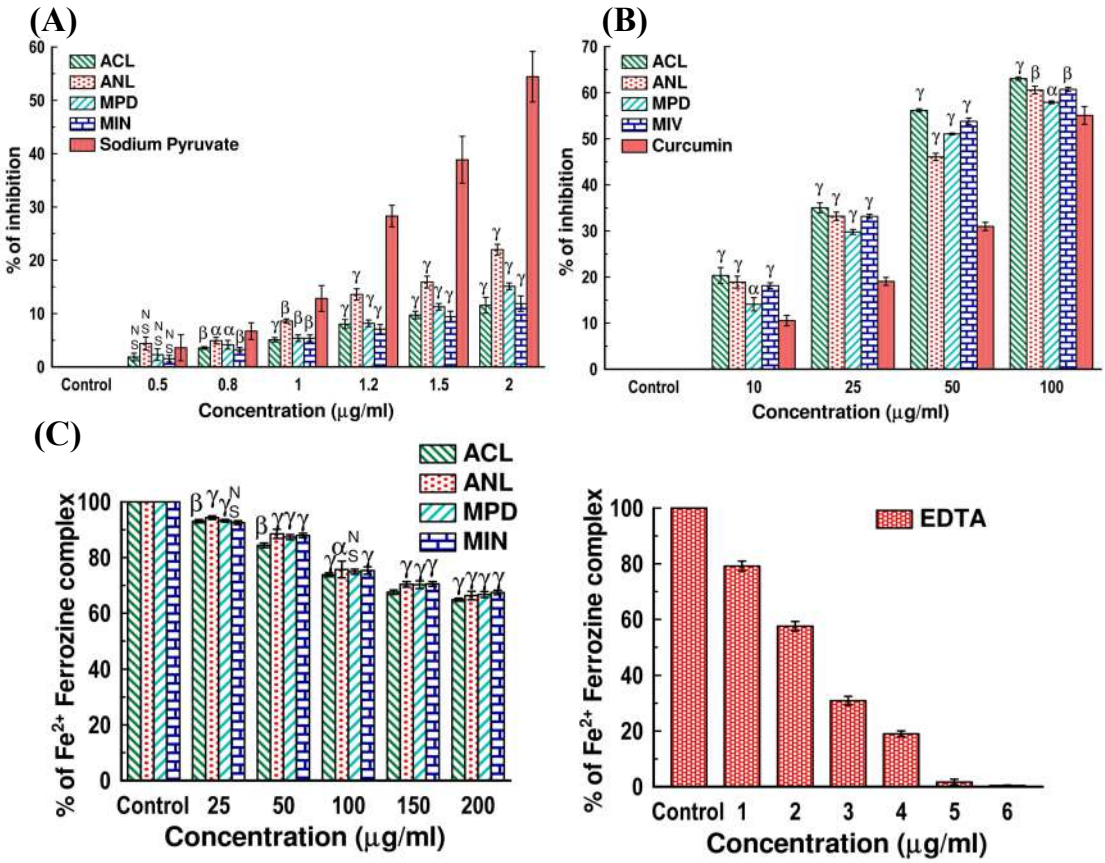


Fig. 4.3. (A) H_2O_2, **(B)** NO quenching activity and **(C)** Iron chelating properties of different Mimosoids and positive control. [Each value represents mean ±SD (n=6); Where, γ= *P*<0.001, β= *P*<0.01 and α= *P*<0.05 Vs standard (µg/ml)].

presence of antioxidants, lipid peroxidation becomes minimal. Figure 4.4.C exhibited significant lipid peroxidation inhibitory activity of each extracts with lower IC_{50} value (Table 4.2 and Table 4.3) revealing their adequate potency against alleged free radicals.

Such observation seems well justified by the possibility that the extracts may possess some bioactive compounds or anti-oxidative agents which react with the free radicals reducing lipid oxidation.

Results (Fig. 4.4.D) obtained from total antioxidant activity, clearly specified that the leaf extracts of *Acacia nilotica*, displayed a higher reduction potential (reduction of Mo(VI) to Mo(V) by electron donation) than ACL> MPD> MIN extracts and standard ascorbic acid thus, emphasizing its strong antioxidant nature. Eventually, the total phenolic content of ACL extract was found to be highest (89.59 ± 2.71 mg gallic acid equivalent per 100 mg of plant extract) than the other extracts while MPD extracts exhibited highest (Table 4.2) flavonoid content (35.2±0.5 mg quercetin equivalent per 100 mg of plant extract) suggesting their probable role as a chief contributors for such antioxidant activities.

4.3. Principal component analysis (PCA) and hierarchical cluster analysis (HCA)

4.3.1. PCA and HCA analysis of *A. catechu* (ACL) extract among the

Table 4.3. IC_{50} values of each extract with their respective standard used in the present study.

Name of the assays	IC_{50} values[$] (µg/ml)				
	ACL	ANL	MPD	MIN	Standard
DPPH	15.5 ±0.4$^{\beta}$	15.5±0.5$^{\beta}$	29.6±0.7$^{\beta}$	38.5±1.0$^{\beta}$	Ascorbic acid (240.1±28.3)
Hydroxyl Radical	121.2 ±1.2$^{\beta}$	6.9±0.2$^{\beta}$	128.4±1.6$^{\beta}$	135.4±5.2$^{\beta}$	Mannitol (589.0±46.5)
Hydrogen Peroxide	15604.9±613.8$^{\alpha}$	8.3±0.2$^{\gamma}$	12984.6±340.6$^{\gamma}$	16027.2± 1013.2$^{\beta}$	Sodium Pyruvate (3176.4±140.2)
Hypochlorous	130.6±4.7$^{\alpha}$	117.5±3.8$^{\alpha}$	140.2±2.7$^{\delta}$	156.9±2.1$^{\delta}$	Ascorbic acid (165.9±16.3)
Iron Chelation	320.6 ± 10.8$^{\gamma}$	363.9±30.4$^{\beta}$	356.7±3.5$^{\beta}$	364.8±3.1$^{\gamma}$	EDTA (1.4±0.01)
Lipid Peroxidation	32.1 ± 0.9$^{\gamma}$	210.6±37.3$^{\alpha}$	60.4±0.4$^{\gamma}$	57.5±2.0$^{\gamma}$	Trolox (11.1±0.2)
Nitric Oxide	45.5±1.3$^{\beta}$	56.4±2.1$^{\beta}$	58.7±0.6$^{\beta}$	50.5±0.2$^{\beta}$	Curcumin (96.8±5.0)
Peroxynitrite	854.0±59.9$^{\alpha}$	747.5±53.0$^{\delta}$	772.8±23.0$^{\beta}$	918.8±16.3$^{\beta}$	Gallic acid (734.8±28.3)
Singlet Oxygen	1103.7±24.6$^{\gamma}$	866.0±8.7$^{\gamma}$	735.0±41.3$^{\beta}$	698.4±23.1$^{\gamma}$	Lipoic acid (48.4±2.0)
Superoxide Radical	131.9±4.4$^{\gamma}$	121.0±2.2$^{\gamma}$	170.8±4.2$^{\gamma}$	197.2±0.7$^{\gamma}$	Quercetin (63.9±4.1)
Total Antioxidant	9.3±0.0$^{\beta}$	5.6±0.10$^{\beta}$	10.1±0.4$^{\gamma}$	9.9±0.1$^{\beta}$	Ascorbic acid (93.1±4.9)

[$] Data are expressed as mean ± S.D; $^{\alpha}p < 0.05$, $^{\beta}p < 0.01$, $^{\gamma}p < 0.001$ and $^{\delta}p>0.05$ vs Standard.

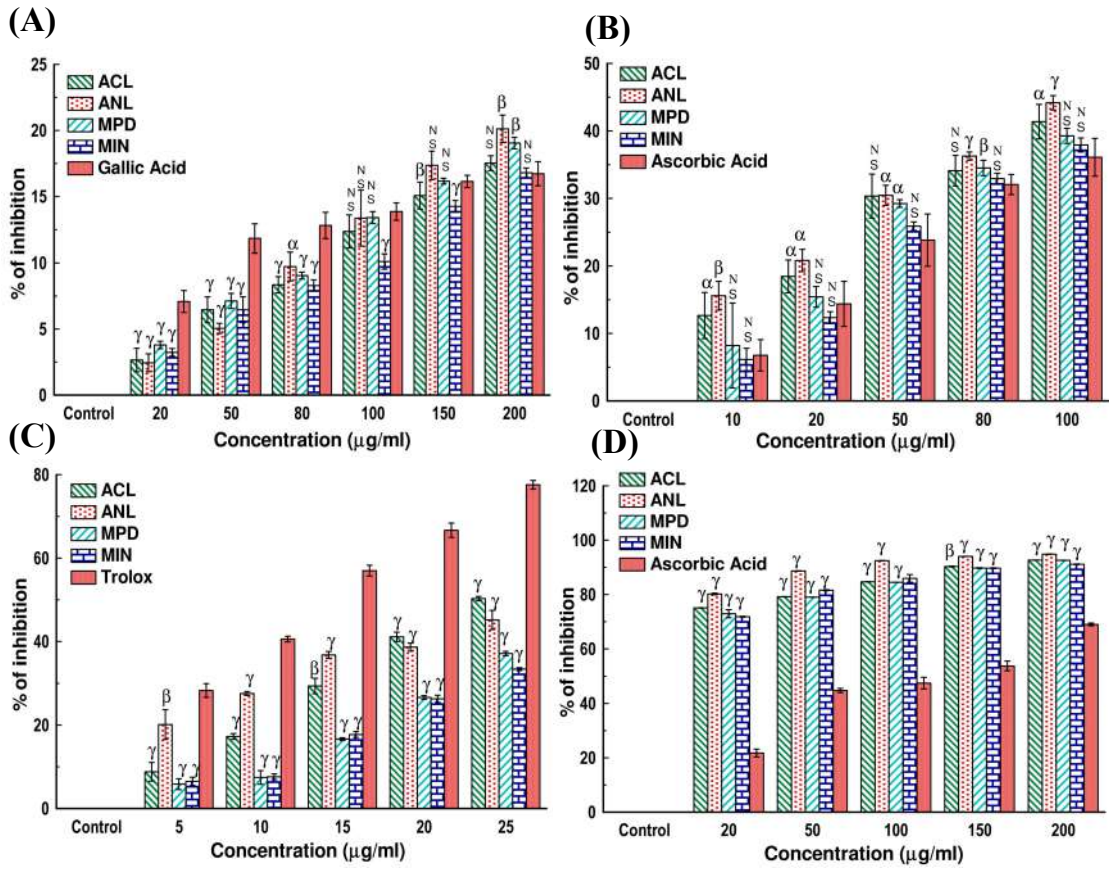


Fig. 4.4. Antioxidant properties of 4 Mimosoids by means of **(A)** Peroxynitrite, **(B)** HOCl, **(C)** Lipid peroxidation and **(D)** Total antioxidant activity assay. [Each value represents mean ±SD (n=6); Where, γ= *P*<0.001, β= *P*<0.01 and α= *P*<0.05 Vs standard (µg/ml)].

selected antioxidant traits

In the present study, principal component analysis (PCA) of *A. catechu* was performed to determine how the seven parameters namely DPPH, Hydroxyl radical, NO, iron chelation, lipid peroxidation, phenol and flavonoid content correlate with each other under univariate analysis. The first and second principal component (PC1 and PC2) accounted for 57.38% and 34.42% of the variance, respectively. The loading plot revealed that hydroxyl radical and DPPH were firmly located positively on the PC1 with cosine value of 0.945 and 0.962 respectively while lipid peroxidation was firmly located positively on the PC2 with cosine value of 0.967. The correlation matrix (Table 4.4) generated from PCA loading plot revealed highest positive correlation between hydroxyl radical and DPPH activity (0.843) while highest negative correlation had been observed between flavonoid with and hydroxyl radical (-0.990) followed by iron chelation and hydroxyl radical scavenging activity (-0.934).

A dendrogram was prepared through hierarchical cluster analysis (HCA) grouping the antioxidant capacities of ACL into statistically significant clusters (Fig. 4.5). The data of HCA virtually corroborated with the results of PCA. The

present dendrogram revealed a broad cluster between lipid peroxidation, NO, hydroxyl radical, DPPH and phenolic content which were located positively on PC1. The iron chelation was found to be closely associated with flavonoid content whereas the hydroxyl radical and flavonoid content were merged at highest distance (proximity matrix value of 19.90) among all the variables. Interestingly, there was no cluster formation between flavonoid and phenolic content which are said to be chiefly responsible for antioxidant activities. Therefore, the bioactivity of EKH may be due to the presence of other class of phytochemicals apart from phenolics and flavonoids.

4.3.2. PCA and HCA analysis of *M. pudica* and *M. invisa* extract among the selected antioxidant traits

The results obtained from antioxidant and free-radical scavenging assays were further analyzed based on their dose-dependent activities, to interpret the underlying inter-correlation patterns in MPD and MIN. The results revealed that the overall bioactivities of both extracts are highly comparable, demonstrating similarly in both the distribution of variables in the component plot (Fig. 4.6) as well as dose-dependent percentage of inhibition in all the assay. The extent of similarity was reflected by the closeness of traits in the component plot. For instance, optimum correlation was found between DPPH with both inhibition of lipid peroxidation and NO (0.998) whereas, lowest correlation resided between H_2O_2 and TAA (-0.623) in *M. pudica* (Fig. 4.6.B). Concurrently, in case of *M. invisa*, maximum correlation was observed between DPPH and lipid peroxidation (0.999) whereas, lowest correlation was found between superoxide and TAA (-0.642). This finding was further supported by the data obtained from hierarchical clustering (Fig. 4.6.E). Interestingly, the most prominent difference between MPD and MINV was the divergence of superoxide radical scavenging activity, representing its independent allocation in the component plot (Fig. 4.6.A and 4.6.D). The

Table 4.4. Correlation Matrix of different antioxidant assays in ACL extract.

	DPPH	Hydroxyl	NO	Iron	Lipid	Phenol	Flavonoid
DPPH	1.000						
Hydroxyl	0.843	1.000					
NO	0.540	0.339	1.000				
Iron	-0.595	-0.934	-0.147	1.000			
Lipid	0.765	0.300	0.553	0.062	1.000		
Phenol	-0.294	0.266	-0.366	-0.593	-0.840	1.000	
Flavonoid	-0.910	-0.990	-0.402	0.875	-0.430	-0.129	1.000

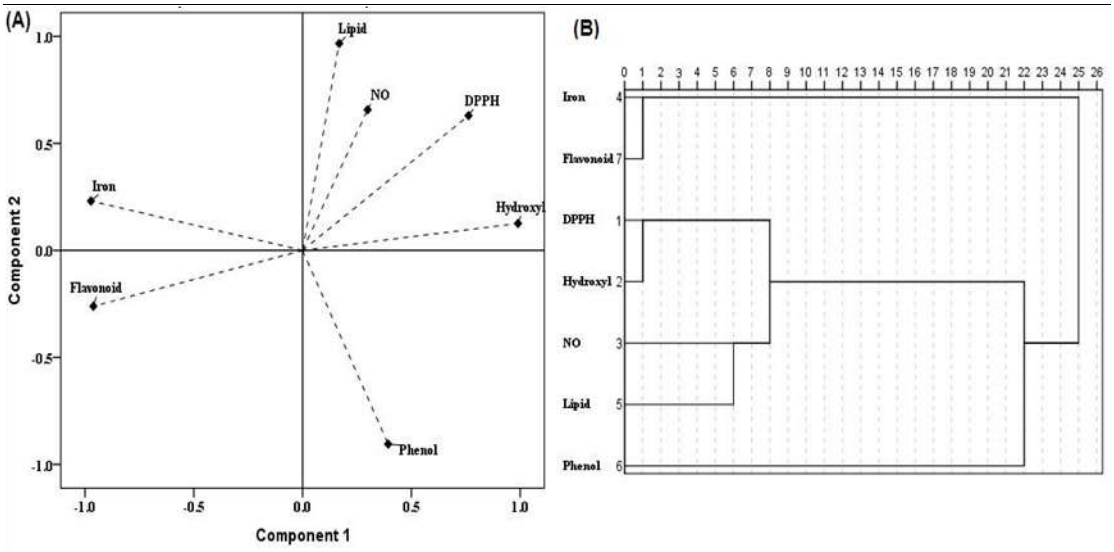


Fig. 4.5. PCA **(A)** and HCA **(B)** analysis of ACL extract among selected antioxidant traits.

component plot reveals that the highly reactive hydroxyl radical is probably generated from H_2O_2 under the presence of transition metal such as iron. Clustering pattern in HCA reveals that there is no intervening effect of iron chelation on the hydroxyl radical scavenging activities and the activity is marginally influenced by inhibition of H_2O_2. The individual proximity scores has been depicted through heat map, where red to green represents high to low proximity scores (Fig. 4.6.C and 4.6.F). The details of correlation matrix, proximity scores and screet plots of MPD and MINV are provided in Table 4.5 and 4.6 respectively.

4.4. Evaluation of cytotoxicity

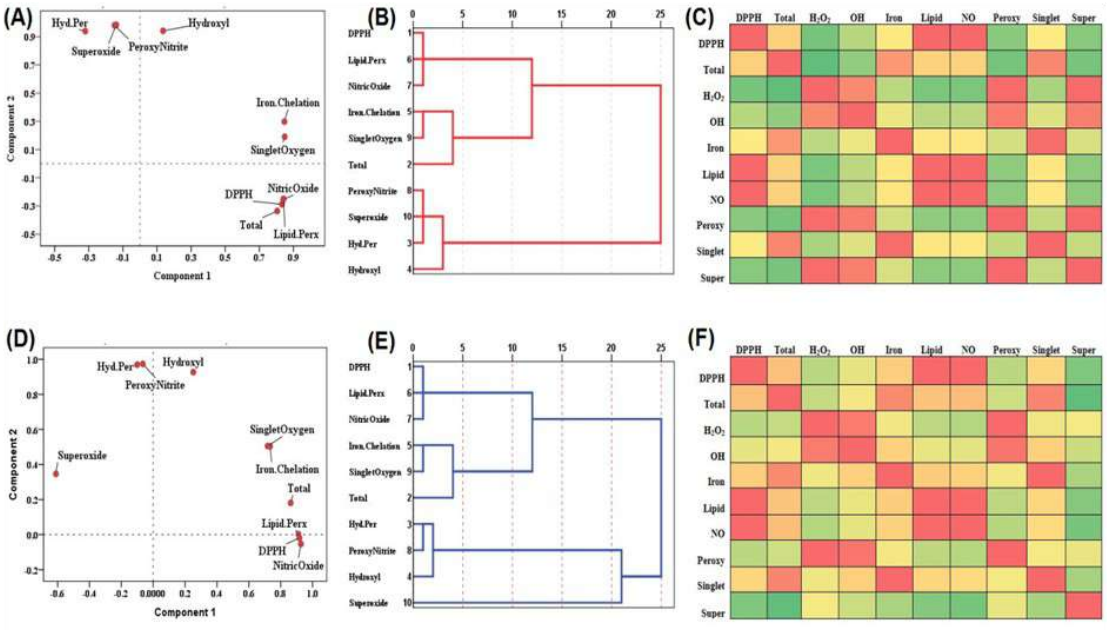


Fig. 4.6. PCA, HCA and heat map analysis different antioxidant traits used in MPD and MIN extracts. PCA **(A)**, HCA **(B)** and Heat Map **(C)** of MPD extract; PCA **(D)**, HCA **(E)** and Heat Map **(F)** of MIN extract.

In order to investigate probable cytotoxic and detrimental effects of ACL, ANL, MPD and MINV extracts on normal body cells upon consumption, haemolytic activity, erythrocyte membrane stabilizing activity and MTT assay on splenocyte and macrophage cells were further considered.

4.4.1. Assessment of haemolytic activity

The haemolytic activity of plant extracts or any compounds is an indication towards the cytotoxicity of normal healthy cells (Da Silva *et al.*, 2004). The hemolysis process is actually related to the concentration and potency of extract. Plant derived metabolites showed haemolytic activity by altering changes in the erythrocyte membrane by means of destruction of red blood cells. Hence, *in-vitro* haemolytic assay by spectroscopic method represents a simple and effectual method for the quantitative measurement of hemolysis. Figure 4.7.A exhibited that all of four samples possess very low haemolytic effect towards human erythrocytes. A dose dependant increase of inhibition was observed; for instance, MIN was found to be showed 8.79±0.29%, MPD revealed 8.20±0.84% while ACL and ANL displayed 7.79±0.55% and 6.74±0.43% of inhibition respectively at 100µg/ml of each concentration which is virtually a negligible one. Therefore, these data suggested the non-toxic effect of the extracts making it suitable for the preparation of drugs involved in the treatment of various diseases.

4.4.2. Erythrocyte membrane stabilizing activity (EMSA)

EMSA indirectly evaluates the antioxidant capacity of test sample against the superoxide radical mediated destruction of the erythrocyte membrane. Actually, erythrocytes are filled with haemoglobin and their membranes are composed of highly unsaturated fatty acids. The auto-oxidation of riboflavin takes place in

Table 4.5. Correlation matrix of different antioxidant assays in MPD extract.

	DPPH	Total	Hydro Perx.	Hydroxyl	Iron Chelation	Lipid Perx.	Nitric Oxide	Peroxy Nitrite	Singlet Oxy	Super-oxide
DPPH	1.00									
Total	0.53^{NS}	1.00								
Hydro Perx.	-0.48^{NS}	-0.62^{NS}	1.00							
Hydroxyl	-0.06^{NS}	-0.30^{NS}	0.84^{**}	1.00						
Iron Chelation	0.41^{NS}	0.78^{**}	-0.03^{NS}	0.28^{NS}	1.00					
Lipid Perx.	0.99^{***}	0.52^{NS}	-0.45^{NS}	-0.02^{NS}	0.43^{NS}	1.00				
Nitric Oxide	0.99^{***}	0.52^{NS}	-0.46^{NS}	-0.02^{NS}	0.43^{NS}	0.99^{***}	1.00			
Peroxy Nitrite	-0.36^{NS}	-0.49^{NS}	0.98^{***}	0.90^{**}	0.13^{NS}	-0.32^{NS}	-0.33^{NS}	1.00		
Singlet Oxy	0.41^{NS}	0.86^{**}	-0.14^{NS}	0.20^{NS}	0.97^{***}	0.43^{NS}	0.43^{NS}	0.01^{NS}	1.00	
Superoxide	-0.37^{NS}	-0.47^{NS}	0.97^{***}	0.90^{**}	0.16^{NS}	-0.33^{NS}	-0.33^{NS}	0.99^{***}	0.03^{***}	1.00

The matrix is based on the PCA performed by SPSS statistics. NS= Non-significant (1-tailed), ***p<0.001; **p<0.01 (1-tailed).

Table 4.6. Correlation matrix of different antioxidant assays in MIN extract.

	DPPH	Total	Hydro Perx.	Hy-droxyl	Iron Chelation	Lipid Perx.	Nitric Oxide	Peroxy Nitrite	Singlet Oxy	Superox-ide
DPPH	1.00									
Total	0.60^{NS}	1.00								
Hydro Perx.	-0.04^{NS}	0.02^{NS}	1.00							
Hydroxyl	0.25^{NS}	0.33^{NS}	0.91^{**}	1.00						
Iron Chelation	0.54^{NS}	0.84^{**}	0.32^{NS}	0.53^{NS}	1.00					
Lipid Perx.	0.99^{***}	0.59^{NS}	-0.03^{NS}	0.27^{NS}	0.55^{NS}	1.00				
Nitric Oxide	0.99^{***}	0.62^{NS}	-0.08^{NS}	0.24^{NS}	0.53^{NS}	0.98^{***}	1.00			
Peroxy Nitrite	-0.04^{NS}	0.09^{NS}	0.99^{***}	0.93^{***}	0.35^{NS}	-0.02^{NS}	-0.07^{NS}	1.00		
Singlet Oxy	0.50^{NS}	0.86^{**}	0.31^{NS}	0.53^{NS}	0.99^{***}	0.51^{NS}	0.49^{NS}	0.35^{NS}	1.00	
Superoxide	-0.43^{NS}	-0.64^{NS}	0.35^{NS}	0.06^{NS}	-0.15^{NS}	-0.41^{NS}	-0.49^{NS}	0.27^{NS}	-0.18^{NS}	1.00

The matrix is based on the PCA performed by SPSS statistics. NS= Non-significant (1-tailed), ***p<0.001; **p<0.01 (1-tailed).

presence of light, generating superoxide radicals which induce the haemolysis of RBC (Dey *et al.*, 2013). In the present study, Fig. 4.7.B revealed significant (P <0.001) percentage of erythrocyte membrane protection in all the doses indicating ANL (66.21 ± 1.34% at 200µg/ml) as a potent natural dietary supplement than the others whereas MIN extract was also found to be exhibited 63.51±2.69% of inhibition at the same concentration (200µg/ml) followed by ACL > MPD which suggest all the extracts might be treated as effective antioxidants to stabilize the erythrocyte membrane.

4.4.3. MTT cell viability assay

To investigate further probable cytotoxic and detrimental effects of ACL, ANL, MPD and MINV on normal body cells upon consumption, we further considered routine MTT assay on murine splenocyte and peritoneal macrophage. Result (Fig. 4.7.C and 4.7.D) demonstrated no cytotoxic effect on either of splenocyte or macrophage cells as no drastic change in metabolic activity of cells was observed up to the highest dose. The effect of extracts on the cell viability was found to be non-significant ($P > 0.05$) compared to the control. Hence, both extracts could well be treated as consumable bio-safety stuff.

Hence, from the above experiments it could be inferred that all the extracts revealed negligible cytotoxic activity up to certain consumable doses, and therefore, can be safely used as bio-safety nutrient supplement for future purposes.

4.5. Neurotherapeutic effects of plant extract

4.5.1. AChE inhibitory activity of ACL, ANL, MPD and MIN extracts (*in-vitro*)

Despite of having potent antioxidative function of extracts claiming their indirect

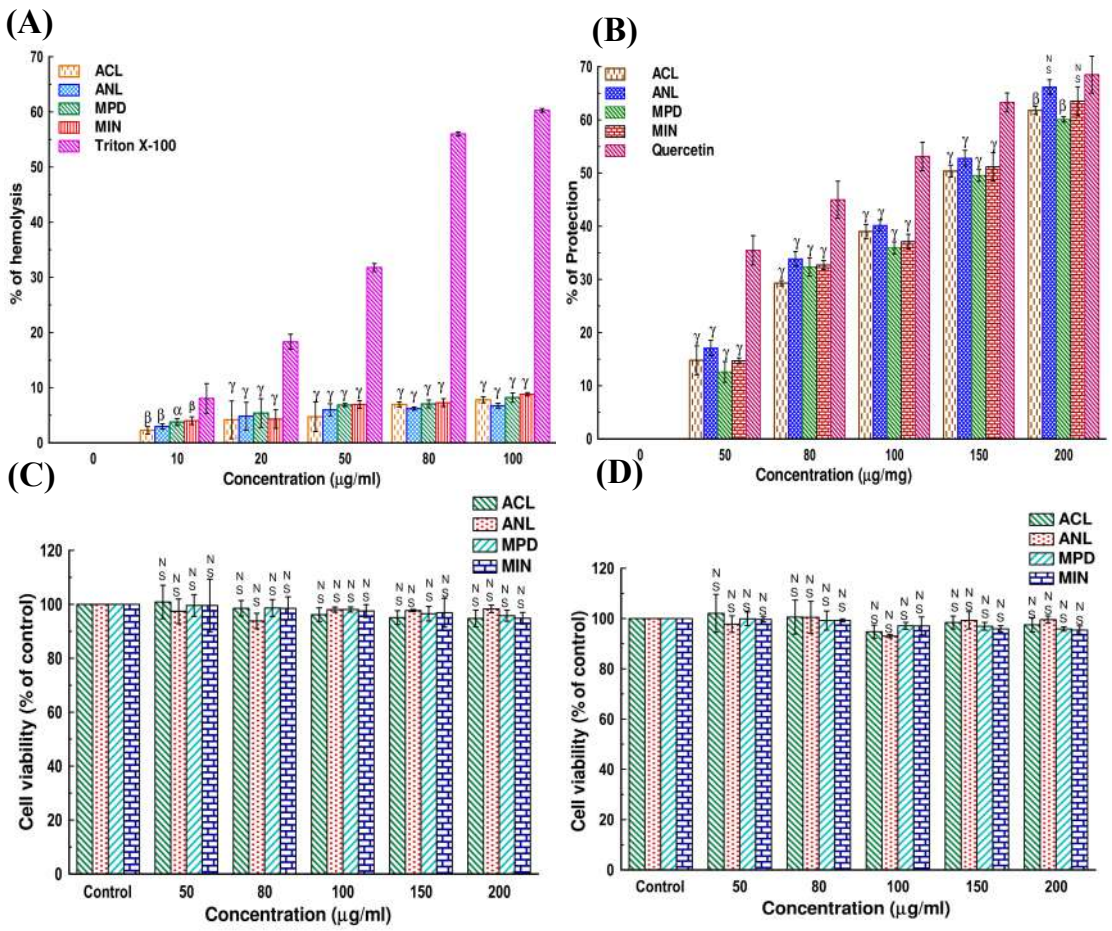


Fig. 4.7. Haemolytic activity of selected Mimosoids and standard Triton X-100 **(A);** Erythrocyte membrane stabilizing activity **(B);** MTT cell viability activity of splenocyte **(C)** and macrophage **(D)** [Each value represents mean ±SD (n=6); Where, γ= *P*<0.001, β= *P*<0.01 and α= *P*<0.05 Vs standard (µg/ml)]

role against neurodegenerative disorders or NDs (Barnham *et al.*, 2004), it was further intended to assess the AChE inhibitory activity of ACL, MPD and MIN over NDs for the first time. The experiment was undertaken considering the complexities of several age-related disorders (e.g., dementia, AD and PD) occurring due to oxidative stress which lowers the function of acetylcholine (ACh) and dopamine in brain (Chattipakorn *et al.*, 2007). Synthesis of ACh by AChE is the most crucial pathway for the pathophysiology of AD. Therefore, use of acetylcholinesterase inhibitor (AChEI) to suppress the degradation of ACh seems to be a rational approach which would maintain the balance of ACh in synaptic cleft (Birks, 2006). In essence, acetylcholinesterase enzyme hydrolyses the substrate (acetylthiocholine iodide) and produces thiocholine which in turn reacts with Ellamn's reagent (5, 5'-dithiobis-2-nitrobenzoic acid) and a yellow color compound, 5-thio-2-nitrobenzoic acid is thus produced (Chattipakorn *et al.*, 2007). The inhibition of cholinesterase enzyme activity is evident by fading of yellow color of the compound. In the present study, the AChE inhibitory activity of

ACL was found to be 73.47±0.303% at 200µg/ml with lower IC_{50} value of 75.91 ±2.28µg/ml. ANL yields 68.21±0.47% of inhibitory activity at 200µg/ml and the IC_{50} value was found to be 86.27±1.32 µg/ml whereas MPD revealed 74.40±0.52% of inhibition at 200µg/ml with IC_{50} value of 109.41±2.46µg/ml and MIN showed quite similar inhibitory activity of 75.24±0.85% at 200µg/ml with the IC_{50} value of 107.25±1.46µg/ml. However, the IC_{50} values of ACL, MPD and MIN were found to be relatively higher than standard eserine (0.023±0.0005µg/ml). Even although, the results reflected better AChE inhibitory activities than other reported medicinal plants (Mathew and Subramanian, 2015; Mukherjee *et al.*, 2007), thereby suggesting the potent role of ACL, MPD and MIN as cholinesterase inhibitors (AChEI) and might be useful as anti-cholinesterase drug against AD and PD.

4.5.2. Effects of MPD and MIN extracts on scopolamine-induced memory impairment in mice

Since enhanced result was observed from *in-vitro* AChE inhibitory assay, it was intended to perceive whether MPD and MIN extracts have any memory improvement or retention activity on scopolamine-induced rodent model. In essence, scopolamine causes oxidative stress by means of interference with acetylcholine in brain leading to cognitive impairment as well as increases the levels of AChE (Rahnama *et al.*, 2015). The oxidative stress contributes to pathogenesis and histological changes in patients with NDs (Gilgun-Sherki *et al.*, 2001). In this context, MPD and MIN had already been exposed to be potent anti-oxidative agents in the present study. Therefore, the neurotherapeutic effect of MPD and MIN on memory deficits in a mouse model of amnesia (passive avoidance test) induced by scopolamine was evaluated. As a result, passive avoidance test, a fear-motivated avoidance test, was employed to describe the way in which the animal learns to avoid an aversive stimulus (electric foot-shock) as a part of long-term memory. Table 4.7 revealed that the initial latency time to enter the dark chamber was significantly longer in the mice given only scopolamine as compared to the control group suggesting amnesic effect of mice. The treatment with MPD and MIN extracts significantly (P<0.001) attenuated the scopolamine-induced memory deficit in mice to a great extent and also associated with the short-term memory (STL) improvement (Table 4.7) suggesting anti-amnesic effect of extracts in the scopolamine-induced rodent model. While considering brain AChE-inhibitory activity, MPD and MIN were also recorded to be reversed the scopolamine-induced memory impairment in mice by increasing cholinergic activity through the inhibition of AChE (Fig. 4.8.A). Hence, it can be inferred that MPD and MIN could

Table 4.7. Effect of MPD and MIN extracts on scopolamine-induced memory impairment in the passive avoidance test.

Groups	IL (Sec.)	STL (Sec.)
Group I (Control)	20.33±2.25	141.33±8.16
Group II (SCP)	103.16 ± 5.03^{a}	83.16 ± 9.45^{a}
Group III (SCP + Donepezil)	$28.33\pm2.33^{a\,\alpha}$	$178.16\pm9.86^{a\,\alpha}$
Group IV (SCP + MPD-low)	$67.83\pm4.62^{a\,\alpha\,A}$	$136.33\pm10.48^{d\,\alpha\,A}$
Group V (SCP + MPD-high)	$34.33\pm4.17^{a\,\alpha\,C}$	$167.83\pm9.62^{a\,\alpha\,D}$
Group VI (SCP + MIN-low)	$73.16\pm4.11^{a\,\alpha\,A}$	$131.50\pm11.97^{d\,\alpha\,A}$
Group VII (SCP + MIN-high)	$39.66\pm3.20^{a\,\alpha\,A}$	$160.83\pm4.95^{a\,\alpha\,B}$

IL- Initial latency; STL- Step Through Latency; SCP- Scopolamine; $^{a}p<0.001$; $^{b}p<0.01$; $^{c}p< 0.05$; ^{d}p=non-significant (p>0.05) vs control group; $^{\alpha}p<0.001$; $^{\beta}p<0.01$; $^{\gamma}p< 0.0$; $^{\Psi}p$=non-significant (p>0.05) vs Scopolamine group; $^{A}p<0.001$; $^{B}p<0.01$; $^{C}p< 0.05$; ^{D}p=non-significant (p>0.05) vs SCP+Donepezil group [Data represented as mean ± SD]

be a potent AChE-inhibitors by hindering the destruction of ACh (McGleenon *et al.*, 1999), thereby confirm the results as found in *in-vitro* tests. This result also supports the ideas which might be due to a decrease in gene transcription, translation and enhance cholinergic activity thereby improving cognitive function (Shahidi *et al.*, 2008).

It has been well-speculated that every cellular organism sustains its own antioxidant stability to protect tissues from oxidative damage at a certain stage. SOD, catalase, GSH etc. are the fundamental antioxidant enzymes that protect tissues from highly reactive hydroxyl radicals and superoxide anions, linked with NDs (Gilgun-Sherki *et al*., 2001). In the present study, scopolamine treatment significantly (P<0.001) depleted antioxidant capacity of SOD, catalase and GSH system in brain tissues (Fig. 4.8.B-D). At the same time, these alterations were further ameliorated significantly by the administration of MPD and MIN extracts. High dose of MPD was found to be more protective one in all cases than others; for instance, 85.48±5.35% of control was observed in catalase, 81.80±3.77% in SOD and 75.11±4.39% in GSH activity. However, MIN showed lower percentage of control in every case than MPD, it is defensive enough combating oxidative stress prompted by scopolamine. Hence, the results suggest antioxidative prospective of MPD and MIN that contributed to effective neuronal plasticity and memory function. Thus, most importantly, we provide first evidence for a potent neurotherapeutic role of MPD and MIN in the protection from ROS-mediated neuronal damage as well as we identified some of the responsible target phytocompounds that could be treated as future CNS drug.

4.6. Anti-diabetic activity of *Acacia nilotica* (ANL) extract

Diabetes is progressively affecting a large number of populations and fatally reducing their quality of life. The use of conventional medicines in diabetes

management is quite expensive, thus, unreasonable to most of patients. Furthermore, most of these conventional drugs exert adverse side-effects. Inclusion of herbal remedy into conventional healthcare system may considerably improve the overall healthcare system. The advantage of herbal remedy over modern medicines is that most of the herbal medicines are plant-based and comparatively cheaper, possess fewer side-effects owing easy acceptability.

In the present study, anti-diabetic appraisal of ANL extract was investigated. Initially, no mortality has been observed upon oral administration of 2000 mg/kg BW ANL extract. Hence, ANL extract may be safe for consumption.

Alloxan, a β-cytotoxin, induces diabetes in a wide variety of animal species by

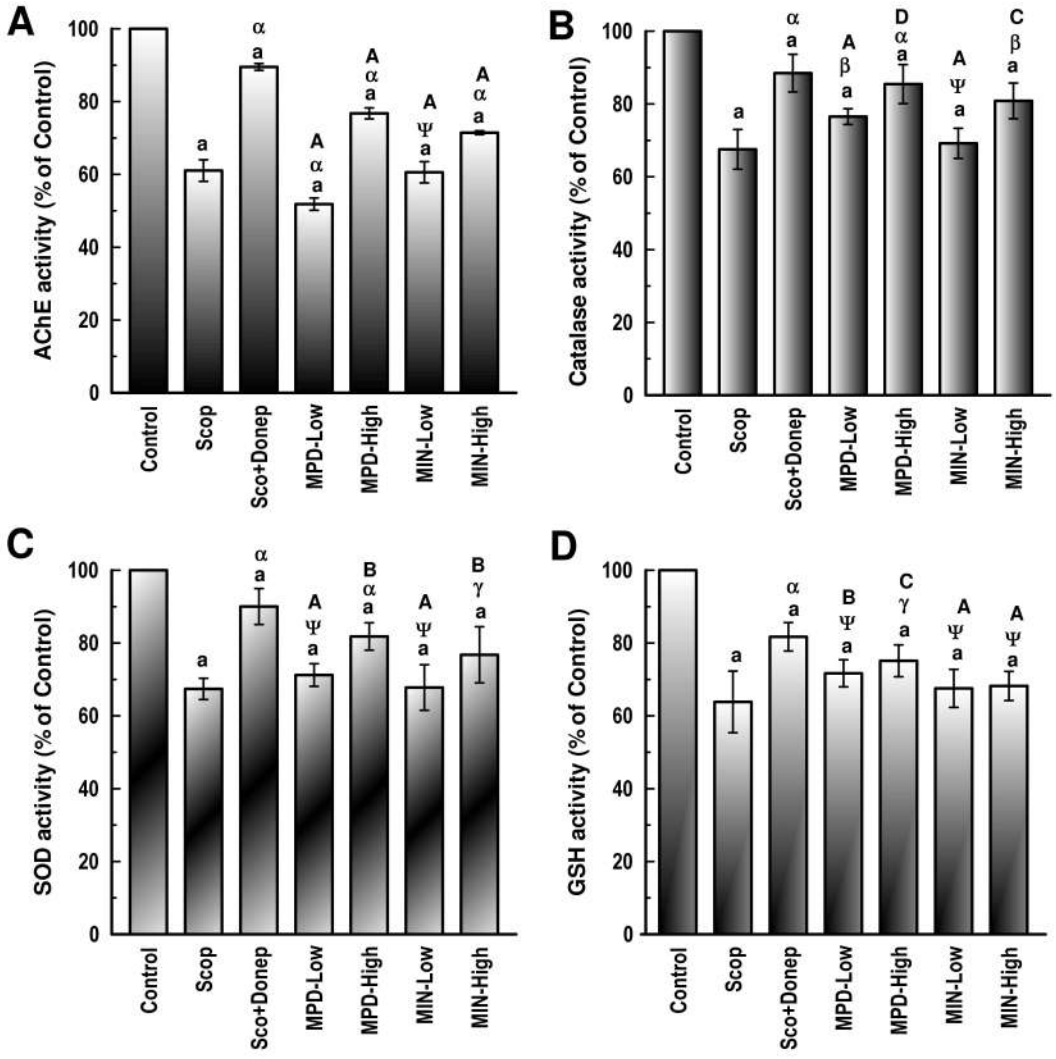


Fig. 4.8. Effect of MPD and MIN extracts on scopolamine-induced memory impairment in the passive avoidance test. **(A)** AChE, **(B)** Catalase, **(C)** SOD and **(D)** GSH activity of MPD and MIN extracts. Scop= Scopolamine, Donep= Donepezil. $^{a}p<0.001$; $^{b}p<0.01$; $^{c}p< 0.05$; ^{d}p=non-significant ($p>0.05$) vs control group; $^{\alpha}p<0.001$; $^{\beta}p<0.01$; $^{\gamma}p< 0.0$; $^{\Psi}p$=non-significant ($p>0.05$) vs Scopolamine group; $^{A}p<0.001$; $^{B}p<0.01$; $^{C}p< 0.05$; ^{D}p=non-significant ($p>0.05$) vs Sco+Donep group [Data represented as mean ± SD]

damaging the insulin secreting pancreatic β-cells via formation of reactive oxygen species like nitric oxide leading to a reduction of endogenous insulin release and increase blood glucose level in tissues (Szkudelski, 2001). This hyperglycemic condition was found to be lowered significantly (P <0.001) after 20 consecutive days of high-dose (200mg/kg BW) ANL treatment (Fig. 4.9.A). It was observed that the glucose level was lowered from 237.2±9.23mg/dl on day 1 to 74±14.37mg/dl on day 20 in HD-ANL group with a decrease of 68.80%. In fact, this anti-hyperglycaemic activity of HD-ANL was quite similar to standard drug, glibenclamide (78.8±12.51mg/dl) after 20 days of administration indicating a significant anti-diabetic activity of ANL in mice. It might be due to increasing glycogenesis, inhibiting gluconeogenesis in the liver or inhibiting the absorption of glucose from the intestine. In another study, reduced blood glucose level was also observed in alloxan-treated rabbits after administration of *A. nilotica* pod extracts at 400mg/kg BW dose (Ahmad *et al.*, 2008). Vats *et al.* (2002) also showed a significant reduction in the levels of blood glucose after the treatment of *Trigonella foenum-graecum*, *Ocimum sanctum* and *Pterocarpus marsupium* extract in normal and alloxanized diabetic rats. Subsequently, decline in body weight is often found to be coupled with diabetic conditions. Therefore, body weight of each group was also measured during dose administration to find out whether any alterations occurred or not. Result (Table 4.8) exhibited that the body weight of diabetic group was decreased after 20 days of alloxan administration while LD-ANL and HD-ANL group exhibited almost at per body weight with standard glibenclamide group. Hence, it may be inferred that ANL extract is help in maintaining the body weight after affected by diabetes.

Besides, insulin plays a major role in glucose homeostasis along the side of a counter regulatory hormone, glucagon, which elevates serum glucose. The reduced level of insulin (64%) in the diabetic group leads to the pathogenesis of diabetes and its several complications thereby enhancing glucose level in blood. Even though the insulin level was not restored upto normal level, it was significantly (P<0.01) improved 85% by high ANL treatment. Thus, present study revealed a distinct hypoglycaemic action through the stimulation of surviving β-cells of islets of langerhans to release more insulin. Treatment with ANL extract and glibenclamide probably increased the insulin secretion (Fig. 4.9.B), which in turn, activated the glucokinase, thereby increasing utilization of glucose and thus, the increased utilization leads to decreased blood sugar level.

On the other hand, it is well-known that glycogen is the major intracellular storable

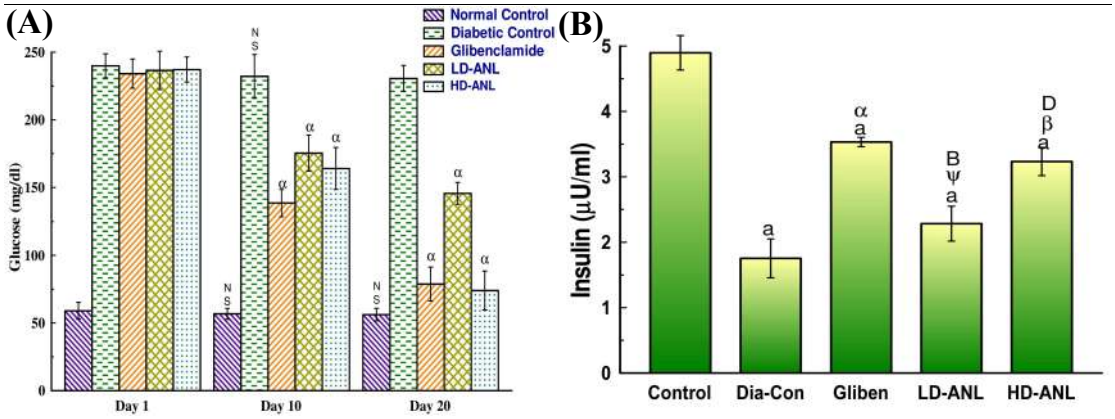


Fig. 4.9. (A) Effect of different groups on blood glucose (mg/dl) level in alloxan-induced diabetic mice on 20 days of treatment [$^{NS}P > 0.05$, $^{\alpha}P < 0.001$ vs day 1 of corresponding group]. **(B)** Insulin level of different groups after 20 days of ANL treatment. Dia-Con= Diabetic control, Gliben= Glibenclamide group. Here, $^{a}p<0.001$; $^{b}p<0.01$; $^{c}p< 0.05$; ^{d}p=non-significant (p>0.05) vs control group; $^{\alpha}p<0.001$; $^{\beta}p<0.01$; $^{\gamma}p< 0.0$; $^{\psi}p$=non-significant (p>0.05) vs diabetic control group; $^{A}p<0.001$; $^{B}p<0.01$; $^{C}p< 0.05$; ^{D}p=non-significant (p>0.05) vs Glibenclamide group [Data represented as mean ± SD]

Table 4.8. Role of ANL extract on body weight (gm) of diabetic mice.

	Control gr.	Diabetic gr.	Glibenclamide gr.	LD-ANL gr.	HD-ANL gr.
1st Day	27.16±0.53	30.35±0.75	26.18±0.85	25.89±1.28	26.25±1.61
10th Day	27.24±1.13$^{N.S.}$	25.57±0.63$^{N.S.}$	26.58±1.07$^{N.S.}$	25.21±1.06$^{N.S.}$	26.09±1.29$^{N.S.}$
20th Day	29.60±1.12$^{N.S.}$	22.34±1.31$^{N.S.}$	27.27±0.83$^{N.S.}$	25.68±1.24$^{N.S.}$	27.56±0.59$^{N.S.}$

Here, $^{N.S.}P > 0.05$ vs day 1 of corresponding group

form of glucose in various body tissues and its level in such tissues, especially liver is a direct reflection of insulin activity (Sharma *et al.*, 1993). Actually, insulin enhances glycogen deposition by means of regulating glycogen synthase and by inhibiting glycogen phosphorylase. It was observed that the hepatic glycogen level in diabetic group was dramatically deceased by 56% comparison to control group which was significantly ($P < 0.001$) improved by 45%, 15% and 30% in glibenclamide, low and high ANL group respectively (Fig. 4.10.A). The possible mechanism underlying restored glycogen content involves the activation of glycogen synthase system and inhibition of glycogen phosphorylase by the extract (Bansal *et al.*, 1981).

During diabetes, the excess amount of glucose present in the blood reacts with haemoglobin reducing its amount to form glycosylated haemoglobin or HbA1c (Koenig *et al.*, 1976) which is monitored as a reliable index of glycemic control in diabetes. In fact, HbA1c was found to increase in patients and/or animals with the onset of diabetes and this increasing ratio was directly proportional to the fasting blood glucose levels (Sheela and Augusti,

1992). Administration of ANL extract for 20 consecutive days to diabetic mice reduced the glycosylation of haemoglobin and thus decreased the levels of HbA1c was observed in diabetic animals (Fig. 4.10.B). In the present study, the diabetic control group revealed higher levels of glycosylated haemoglobin compared to control mice which was further decreased in LD-ANL and HD-ANL group indicating their potent anti-hyperglycaemic effect.

An imbalance between oxidants and antioxidants arise during diabetes mellitus initiating several diabetes-induced complications (Smirnoff, 2001). Higher levels of reactive oxygen species (ROS) or free radicals in diabetic subject are capable of chemically altering all major types of biomolecules including lipids, proteins and nucleic acids, by changing their structure and functions, thus leading to cell damage (Sinclair, 1993). Malondialdehyde (MDA), a marker of lipid peroxidation, results due to the peroxidation of polyunsaturated fatty acids (PUPA) in the cell membrane. Presence of elevated MDA in the serum signifies the onset of diabetes mellitus caused by oxidative stress (Ceriello, 2000). Fig. 4.11.A revealed increased level of MDA in diabetic control group while it was normalized upon administration of ANL extract. It may be inferred that certain phytochemicals present in the extract which reacted with free radicals, thus avoided lipid peroxidation as well as the risk of cordiovascular diseases (Pari and Umamaheswari, 2000).

In addition, catalase (CAT) and peroxidase (PX) are two significant indices of oxidative stress. Autoxidation of alloxan-induced diabetes generates more ROS like superoxide radical ($O_2^{\bullet-}$), H_2O_2 and hydroxyl radical ($OH^{\bullet-}$) that accumulate

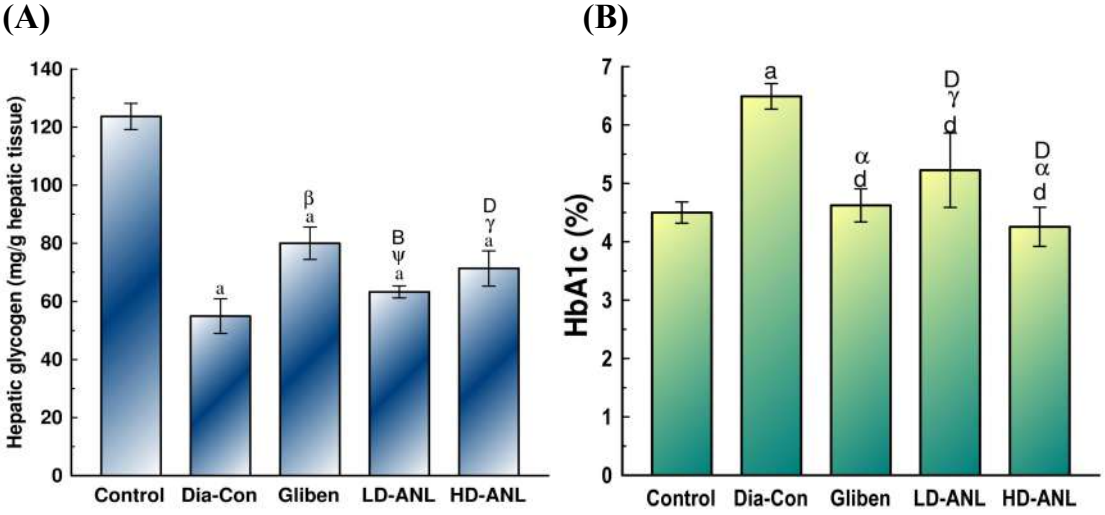


Fig. 4.10. (A) Hepatic glycogen level and **(B)** HbA1c level of different groups after 20 days of ANL treatment. [Dia-Con= Diabetic control, Gliben= Glibenclamide group. Here, ^{a}p<0.001; ^{b}p<0.01; ^{c}p< 0.05; ^{d}p=non-significant (p>0.05) vs control group; $^{\alpha}p$<0.001; $^{\beta}p$<0.01; $^{\gamma}p$< 0.0; $^{\Psi}p$=non-significant (p>0.05) vs diabetic control group; ^{A}p<0.001; ^{B}p<0.01; ^{C}p< 0.05; ^{D}p=non-significant (p>0.05) vs Glibenclamide group; Data represented as mean ± SD]

over time in liver, kidney and skeletal muscle, thereby contribute cell injury and development of human diseases (Sinclair, 1993). Present findings exhibited that treatment with ANL extract upregulated the activity of CAT (Fig. 4.11.B) and PX (Fig. 4.11.C) enzymes compared to diabetic mice and thus may help to counteract the damage by the ROS generated during diabetes. In fact, a significant decrease (P <0.01-0.001) in CAT and PX activities were seen in liver, kidney and skeletal muscle, which were subsequently normalized due to glibenclamide and ANL treatment. A 34%, 30% and 38% lowered PX activity and 48%, 44% and 57% lowered CAT activity was measured in liver, kidney and skeletal muscle. When treated with high ANL, highest increase (51%, P <0.001) of peroxidase and catalase activities were (123%, P <0.001) was seen in case of skeletal muscle. Similar type of activity of these enzymes in diabetic group was also documented by several investigators (Montefusco-Pereira *et al.*, 2013; Sekeroglu *et al.*, 2000) suggesting ANL could be beneficial for correcting the

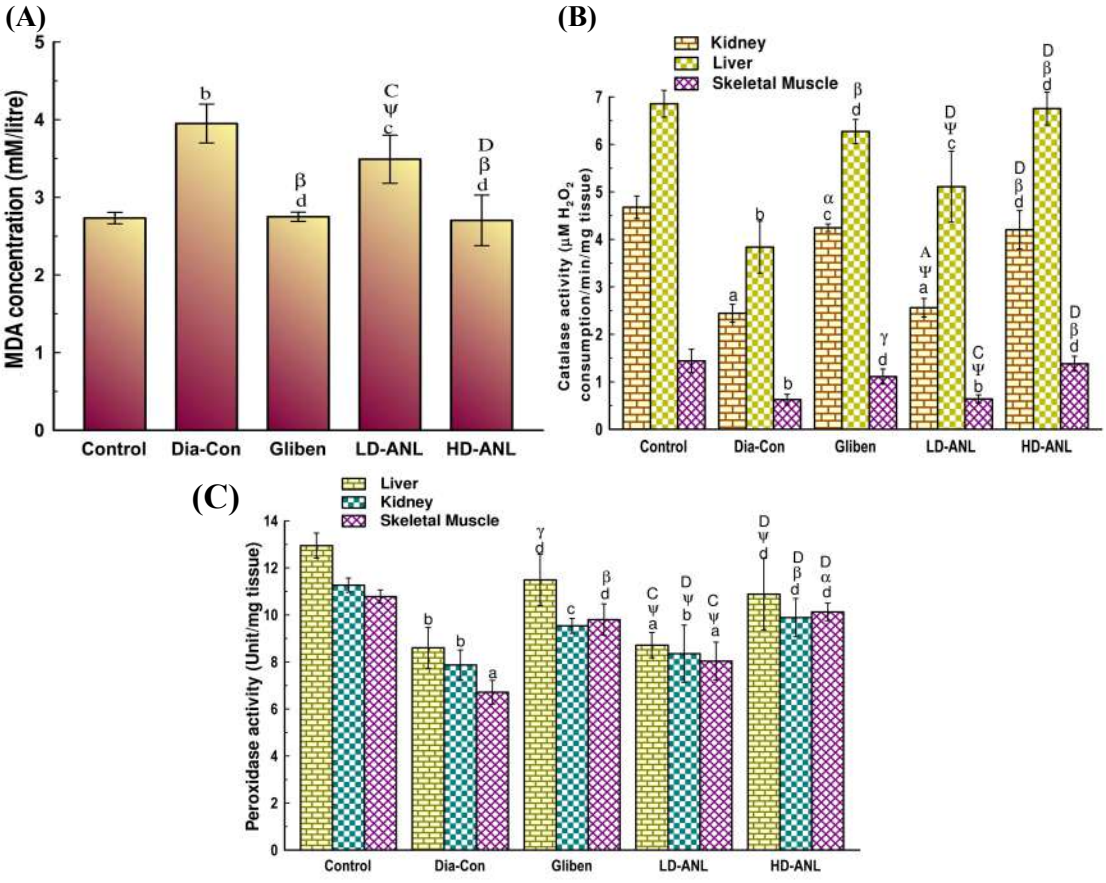


Fig. 4.11. (A) MDA concentration level, **(B)** Catalase activity and **(C)** Peroxidase activity in liver, kidney and skeletal muscle of different groups after 20 days of ANL administration. Dia-Con= Diabetic control, Gliben= Glibenclamide group. Here, ^{a}p<0.001; ^{b}p<0.01; ^{c}p< 0.05; ^{d}p=non-significant (p>0.05) vs control group; $^{\alpha}p$<0.001; $^{\beta}p$<0.01; $^{\gamma}p$< 0.0; $^{\Psi}p$=non-significant (p>0.05) vs diabetic control group; ^{A}p<0.001; ^{B}p<0.01; ^{C}p< 0.05; ^{D}p=non-significant (p>0.05) vs Glibenclamide group [Data represented as mean ± SD]

hyperglycemia and it may prevent diabetic complications due to lipid peroxidation and free radical oxidation.

In a further attempt to gain an insight into the underlying biochemical mechanisms involved in anti-diabetogenic activities of plant extract, several renal parameters were investigated associated with diabetes. Kidneys are such major organs of the body that remove metabolic wastes such as urea, uric acid, creatinine and thereby optimum chemical composition of body fluids is maintained. The elevation in the levels of blood urea, uric acid and creatinine is considered as significant markers of renal or kidney dysfunction associated with diabetes. During diabetic condition, continuous catabolism of amino acid results high quantity of urea from urea cycle and eventually produces uric acid in the blood (Landin *et al.*, 1991). Creatinine, the metabolite of muscle creatine, is easily excreted by the kidneys and its elevated level in the serum signifies kidney impairment (Landin *et al*, 1991). Administration of ANL extract, however, produced a significant reduction in the levels of these three metabolites (Table 4.9), thereby indicating protection against impairment of kidneys due to diabetes. Simultaneously, high concentration of cholesterol and triglycerides in the blood becomes a risk factor for coronary heart disease occurring due to the increase in the mobilization of free fatty acids from the peripheral depots, since insulin inhibits the hormone-sensitive lipase (Al-Shamaony *et al.*, 1994). Table 4.9 clearly reflected that the treatment with ANL to diabetic animals significantly reduced the plasma cholesterol level to near normalcy and thus minimized the risk of cardiovascular disease (Rhoads *et al.*, 1976). Correspondingly, ANL was also able to bring down the levels of triglycerides in diabetic animals to an appreciable extent (Table 4.9) when compared to the untreated diabetic group.

Furthermore, acid phosphatase (ACP) and alkaline phosphatase (ALP) are two liver marker enzymes often employed to evaluate the integrity of plasma membrane and endoplasmic reticulum (Akanji *et al.*, 1993). In essence, elevation level of these two enzymes in the serum reflects obliteration of structural integrity of the liver during diabetes. In addition, transaminases i.e. aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are two recognized enzymes used as biomarkers to assess possible toxicity to the liver (Rahman *et al.*, 2001). An increase level of AST and ALT in the serum may chiefly be due to the leakage of these enzymes from the liver cytosol into the blood stream providing an indication of liver damage as well. Present study has documented that ACP, ALP, AST and ALT levels were significantly elevated in the diabetic control animals and subsequently reduced (Table 4.9) in diabetic groups after the

Table 4.9. Different enzymatic and biochemical parameters of the serum of different five experimental groups.

Parameters (units)	Control	Diabetic control	Glibenclamide	LD-ANL	HD-ANL
ACP (K.A.)	3.19±0.11	7.19±0.14^{b}	5.7±0.57$^{c\ \psi}$	6.95±0.10$^{a\ \psi\ C}$	5.93±0.27$^{b\ \gamma\ D}$
ALP (K.A.)	11.0±1.01	21.23±1.42^{a}	12.34±0.42 $^{d\ \alpha}$	16.02±1.51$^{b\ \gamma\ C}$	13.53±1.74$^{d\ \beta\ D}$
AST (U/ml)	66.58±0.86	99.64±3.78^{a}	75.62±1.01$^{a\ \alpha}$	86.61±1.19$^{a\ \beta\ A}$	76.97±3.89$^{c\ \beta\ D}$
ALT (U/ml)	42.19±1.48	87.46±2.45^{a}	53.82±2.66$^{b\ \alpha}$	74.87±2.02$^{a\ \beta\ A}$	72.19±13.95$^{c\ \psi\ D}$
Creatinine (mg/dl)	0.19±0.005	0.35±0.01^{a}	0.19±0.004$^{d\ \alpha}$	0.25±0.03$^{c\ \gamma\ C}$	0.17±0.01$^{d\ \alpha\ D}$
Triglyceride (mg/dl)	90.36±6.45	137.83±6.93^{a}	89.07±7.73$^{d\ \beta}$	117.45±17.79$^{d\ \psi\ D}$	112.89±25.74$^{d\ \psi\ D}$
HDL Cholesterol (mg/dl)	82.17±8.02	129.87±3.50^{a}	83.30±15.80$^{d\ \beta}$	133.14±44.61$^{d\ \psi\ D}$	93.29±10.91$^{d\ \beta\ D}$
Urea (mg/dl)	21.24±0.05	26.10±1.2^{b}	22.72±2.52$^{d\ \psi}$	24.32±1.00$^{d\ \gamma\ D}$	22.52±1.35$^{b\ \psi\ D}$
Urea N_2 (mg/dl)	9.92±0.23	22.37±1.85^{a}	10.61±1.17$^{d\ \alpha}$	10.52±0.63$^{d\ \alpha\ D}$	11.36±0.47$^{b\ \alpha\ D}$
Uric acid (mg/dl)	1.57±0.14	2.26±0.12^{b}	1.90±0.14$^{d\ \gamma}$	2.70±0.10$^{a\ \beta\ B}$	2.03±0.11$^{c\ \psi\ D}$

^{a}p<0.001; ^{b}p<0.01; ^{c}p< 0.05; ^{d}p=non-significant (p>0.05) vs control group; $^{\alpha}p$<0.001; $^{\beta}p$<0.01; $^{\gamma}p$< 0.0; $^{\psi}p$=non-significant (p>0.05) vs diabetic control; ^{A}p<0.001; ^{B}p<0.01; ^{C}p< 0.05; ^{D}p=non-significant (p>0.05) vs Glibenclamide group [Data represented as mean ± SD]

administration of ANL leading towards the alleviation of liver injury (El-Demerdash *et al.*, 2005).

In conclusion, present study is significant as it covers different biochemical and metabolic aspects responsible for the progression of diabetes. What's more, ANL extract exhibited significant increase in ameliorating varied diabetic complications and blood glucose control compared to glibenclamide. However, at this stage it is difficult to predict whether any phytochemical compound acted independently or in synergetic manner because active principles or biomolecules are always responsible for their antidiabetogenic effect (Bhat *et al.*, 2011; Coman *et al.*, 2012). Hence, it may be inferred that ANL extract possibly helped in islets regeneration/protection and insulin production (Kanter *et al.*, 2003) which indirectly modulated all other biochemical parameters, thus ANL holds a hope towards the discovery of new anti-diabetic drug.

4.7. Chemical Characterizations of selected plant extracts

Since the extracts exhibited potent antioxidant, anti-neurodegenerative and anti-diabetic activity, it would be an amicable one to identify the active phytocompounds responsible for those activities present in the extracts. In this regard, FTIR, GC-MS and NMR analysis have been considered.

4.7.1. FT-IR analysis

Fourier transform infrared spectroscopy (FT-IR) analysis, one of the most widely

accepted methods, is utilized preliminary to characterize the functional groups based on the peak value in the region of infrared radiation. Besides, it is a required process to identify medicines in Pharmacopoeia (Liu *et al.*, 2006). In the present study, FTIR analysis of ACL and ANL extracts were employed. Figure 4.12 and 4.13 revealed that the major peaks indicated the presence of phenols, alkanes, aldehydes, aromatics, amides, alkenyls etc (Table 4.10). Hence, it may be inferred that *A. catechu* (ACL) and *A. nilotica* (ANL) extracts may be the potent source of many natural flavonoids and sterols including quercetin, iso-quercetrin, kaempferol, myricetin, isorhamnetin, stigmasterol, campesterol etc. inhibiting or suppressing the formation of free radicals by binding to the metal ions or quenching singlet oxygen.

4.7.2. GC-MS analysis

4.7.2.1. GC-MS analysis of A. catechu (ACL) extract

GC-MS analysis (Fig. 4.14) of ACL extract was performed to identify the presence of various bioactive metabolites and neurotransmitters, if any. A total of 41 different bioactive compounds (Table 4.11) have been identified by GC-MS analysis. Of the 41 compounds, 5 compounds have already been proven as potent antioxidants including gallic acid, D -mannitol, catechin, epicatechin and isoquercitrin (Fig. 4.15).

Amongst these phytometabolites, catechin, D-mannitol, epicatechin and isoquercitrin

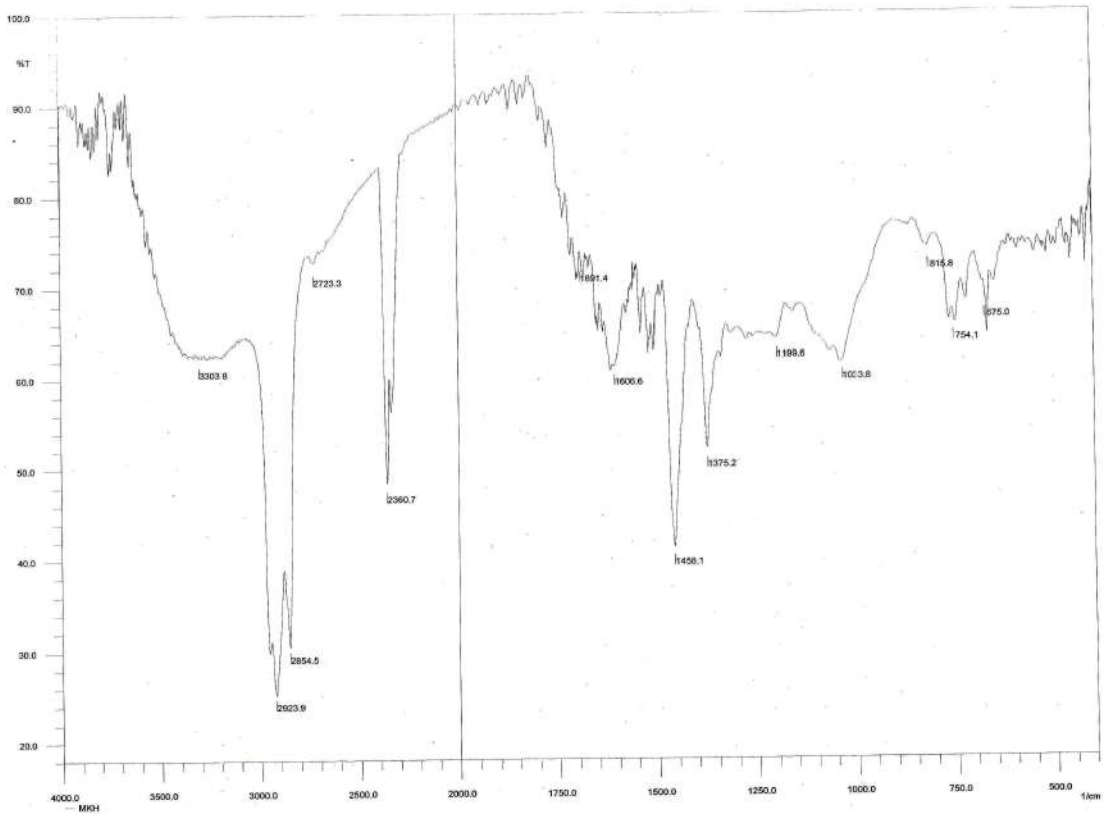


Fig. 4.12. FT-IR spectroscopic analysis of ACL.

Table 4.10. FTIR absorption values and functional groups of ACL and ANL extracts.

Plant Sample	Wave number (cm^{-1})	Types of Bond	Functional groups	Absorption intensity
ACL	3303.8	C≡C-H stretch	Alkynes	Strong
		O-H stretch, H-bonded	Alcohols	Strong
	2923.9	C-H stretch	Alkanes	Strong
	2854.5	C-H stretch	Alkanes	Strong
	2723.3	O=C-H stretch	Aldehydes	Weak
	2360.7	Unknown	Unknown	Unknown
	1691.4	C=O Stretch	Aldehydes	Strong
	1606.6	C=O Stretch	Amides	Weak
	1458.1	C-H Stretch	Alkanes	Strong
	1375.2	C-H Stretch	Alkanes	Strong
	1199.6	C=C Stretch	Alkenyl	Medium
	1033.8	C-O Stretch	Primary alcohol	Medium
	815.8	C-H Stretch	Alkanes	Strong
	754.1	Unknown	Unknown	Unknown
	675.0	C–H "oop"	Aromatics	Medium
ANL	3296.1	O-H stretch	Alcohols	Strong
	2922.0	C-H stretch	Alkanes	Strong
	2852.5	C-H stretch	Alkanes	Strong
	2723.3	O=C-H stretch	Aldehydes	Weak
	2358.8	Unknown	Unknown	Unknown
	1687.6	C=O Stretch	Aldehydes	Strong
	1614.3	C=C Stretch	Alkenes	Medium
	1454.2	C-H Stretch	Alkanes	Strong
	1317.3	C=C Stretch	Alkenyls	Medium
	1203.5	C=C Stretch	Alkenyls	Medium
	1134.1	C=O Stretch	Ketones	Strong
	1033.8	C-O Stretch	Primary alcohol	Medium
	864.0	Unknown	Unknown	Unknown
	815.8	C-H Stretch	Alkanes	Strong
	721.3	Unknown	Unknown	Unknown
	669.3	C–H "oop"	Aromatics	Medium

are known to be effective scavengers of hydroxyl, peroxyl, superoxide and DPPH radicals with significant anti-cancerous activity (Desesso *et al.*, 1994; Valcic *et al.*, 2000; Vellosa *et al.*, 2011) while gallic acid is known as strong antioxidative agent (Yen *et al.*, 2002). In addition, catechin and epicatechin reveal monosamine

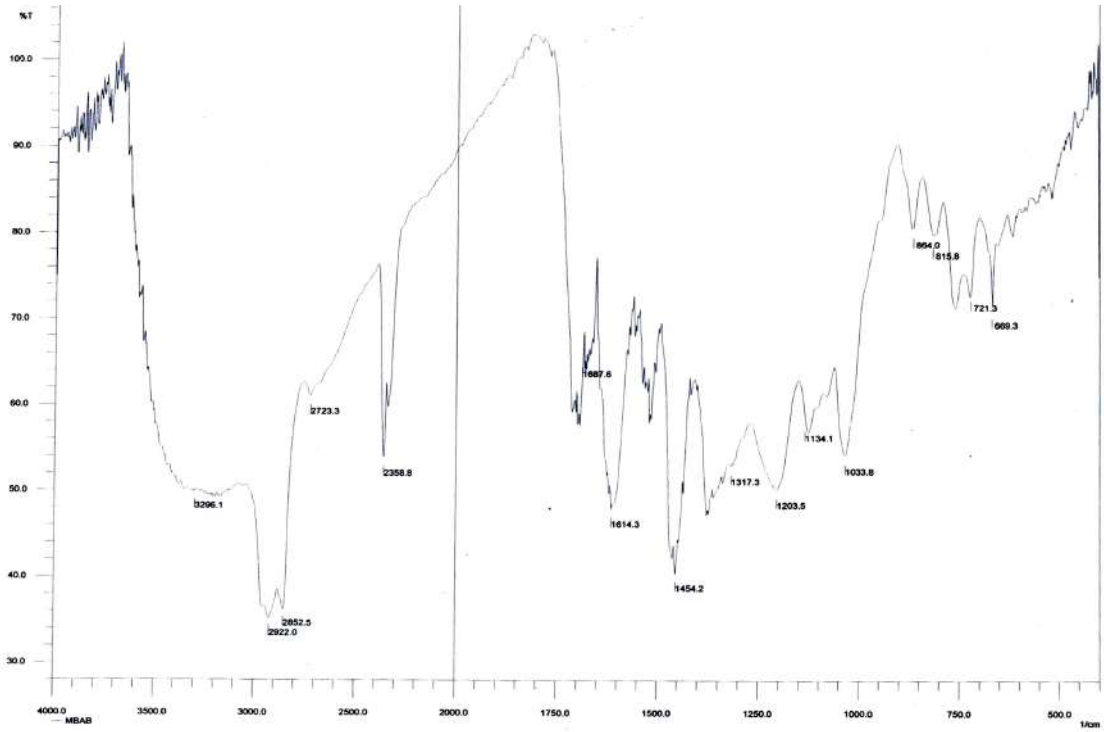


Fig. 4.13. FT-IR spectroscopic analysis of ANL.

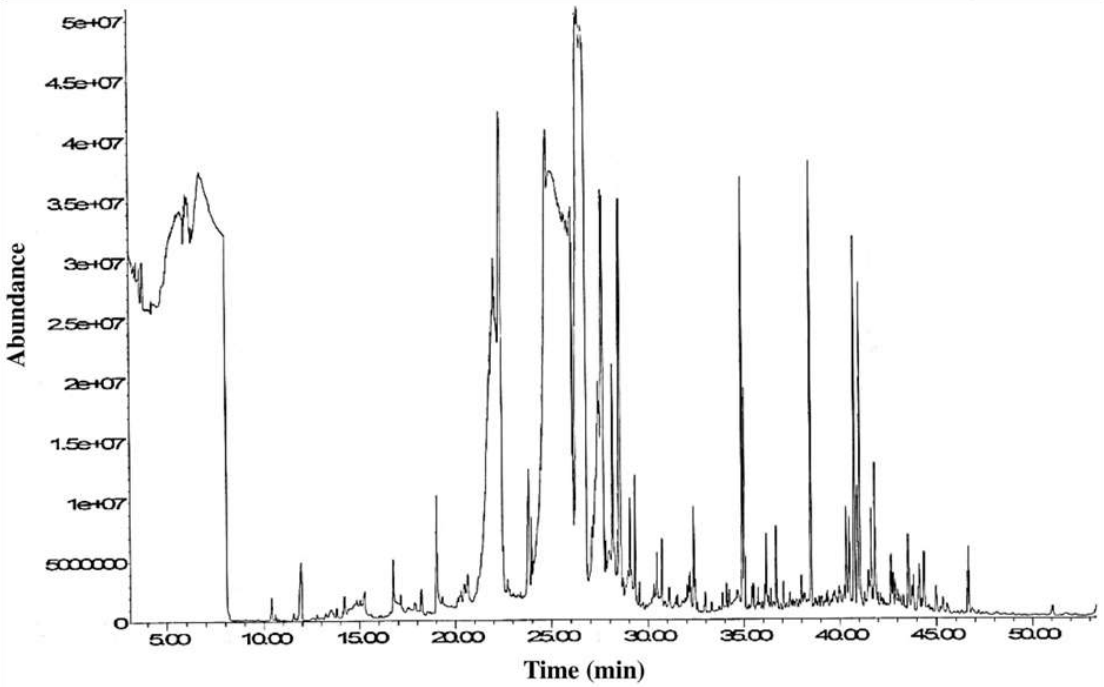


Fig . 4.14. Gas chromatogram-Mass spectroscopy of *A. catechu* leaf extract.

oxidase inhibitory activity responsible partly for Parkinson's disease (PD), Alzheimer's disease (AD) and others cognitive disorders (CDs).

Concurrently, a few other bioactive compounds namely, phenylalanine, tyramine, dopamine, and serotonin have been identified which have either anti-neurodegenerative properties or are known as the precursor of compounds which are

Table 4.11. List of metabolites detected in *A. catechu* leaf extract by GC-MS analysis.

Name of the Metabolites	Log of RRR*	
	Average	SD
L-(+) lactic acid	1.07	0.32
L- alanine	0.55	0.19
L- valine	0.94	0.12
Urea	0.23	0.33
Pipecolic acid	1.28	0.42
Glycerol	2.32	0.02
Phosphoric acid	1.30	0.02
L-threonine	1.10	0.17
Glycine	0.56	0.09
Succinic acid	0.77	0.19
Glyceric acid	1.22	0.09
Beta-alanine	0.90	0.10
D- malic acid	1.53	0.03
O-acetylsalisylic acid	0.40	0.21
L- glutamic acid 3 (dehydrated)	1.64	0.13
4-guanidinobutyric acid	1.57	0.25
Phenylalanine	0.59	0.17
Phenylethylamine	1.09	0.08
Meleamic acid	0.48	0.17
L-glutamic acid	0.16	0.05
Lauric acid	1.33	0.50
L- asparagine	1.77	0.05
Xylitol	2.14	0.25
Arabitol	0.79	0.05
Putrescine	1.04	0.09
Methyl-beta-D-galactopyranoside	1.25	0.05
Quinic acid	1.41	0.48
Allantoin	1.69	0.06
Tyramine	3.22	0.11
D-sorbitol	1.19	0.07
D-mannitol	1.13	0.09
Gallic acid	3.19	0.02
Palmitic acid	1.80	0.08
Dopamine (hydroxytyramine)	1.02	0.07
L-tryptophan	1.52	0.02
Stearic acid	1.10	0.22
Serotonin	1.01	0.04
Sucrose	2.28	0.00
(-)- epicatechin	2.14	0.02
Catechin	2.05	0.07
Isoquercitrin	1.69	0.03

*RRR: Relative Response Ratio; SD: Standard Deviation.

anti-CDs. Out of these bioactive compounds, dopamine along with nor-epinephrine (noradrenaline) are collectively called catecholamines which are synthesized following the two equally active routes (Kulma and Szopa, 2007). In one of the routes, phenylalanine transfers into tyrosine by means of amino acid hydroxylase. Afterward, tyrosine decarboxylated to tyramine through the action of tyrosine decarboxylase and subsequently generates dopamine and norepinephrine (noradrenaline) catalysed by enzyme monophenol hydroxylase and dopamine beta- hydroxylase respectively. Therefore, it might be suggested that the synthesis of catecholamines in ACL probably follows the described route (Fig. 4.16). Previous studies (Basu and Dasgupta, 2000; Chinta and Andersen, 2005; Kulma and Szopa, 2007) exhibited that catecholamines and other neurotransmitters have distinct function to combat against neurodegeneration or cognitive disorders like AD, PD and dementia.

Hence from the above illustration, it might be inferred that *A. catechu* or ACL extract could be regarded as a potent future antioxidative stuff as well anti-psychiatric drug especially for Alzheimer's and Parkinson's disease.

4.7.2.2. GC-MS analysis of A. nilotica (ANL) extract

GC-MS data of ANL (Fig. 4.17) revealed

the exploration of a total of 29 phyocompounds (Table 4.12). Amongest this catechol, pyrogallol, γ-tocopherol, α-tocopherol, stigmasterol, β-sitosterol were reported to be as potent antioxidant (Avase *et al.*, 2015; Dillard *et al.*, 1983; Gupta *et al.*, 2011; Huang *et al.*, 1994; Justino *et al.*, 2006). Apart from it, result also yieleds that γ-tocopherol, α-tocopherol, β-sitosterol, stimasterol hold effectual anti-diabetic activity (Jamaluddin *et al.*, 1994). As evidenced from anti-diabetic appraisal

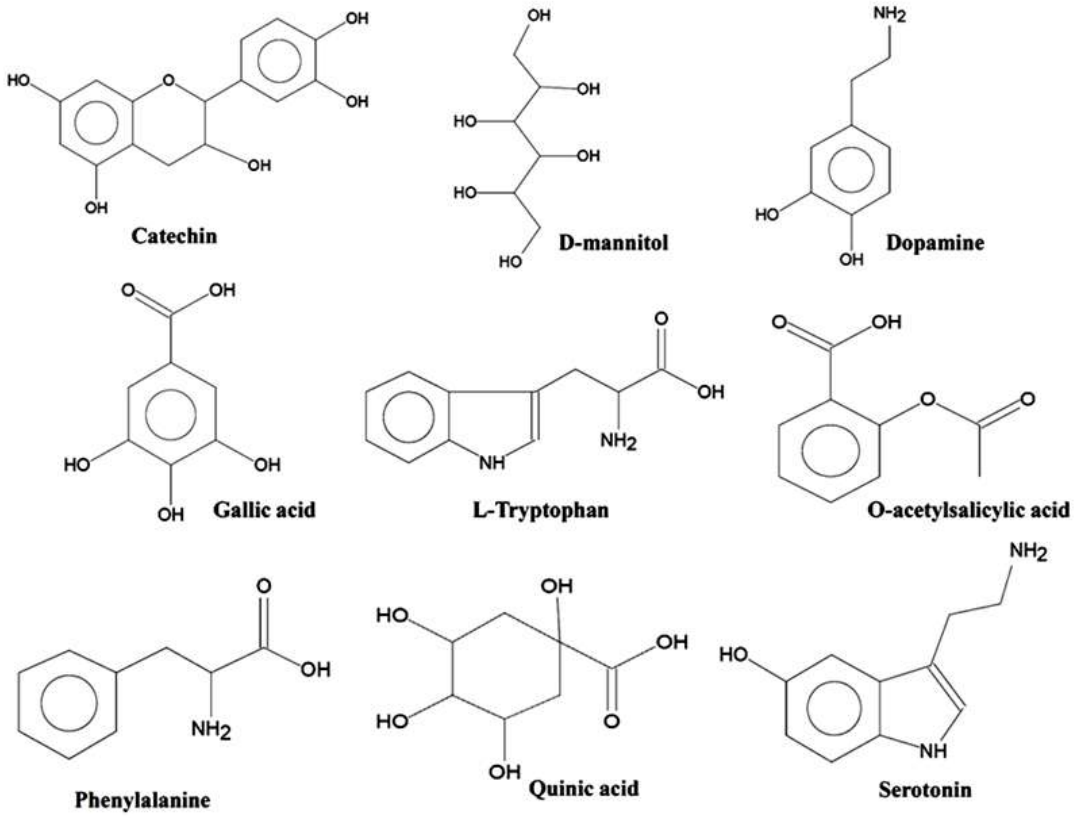


Fig. 4.15. Chemical structures of some essential bioactive metabolites identified in ACL extract by GC-MS.

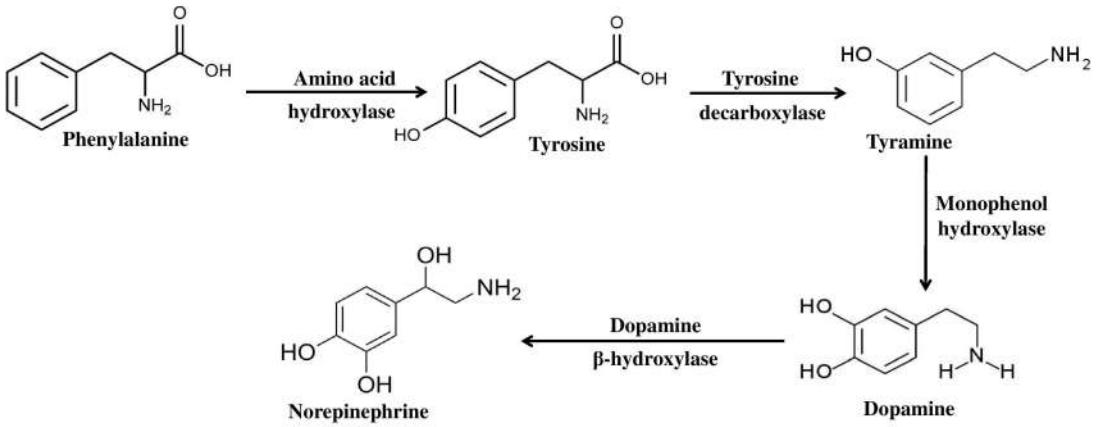


Fig. 4.16. Schematic representation of possible biosynthetic pathway of catecholamines identified in ACL extract.

of ANL, it may be presumed that these compounds might play significant role in ameliorating diabetes and its complications in mice model.

4.7.2.3. *GC-MS analysis of M. pudica (MPD) and M. invisa (MIN) extracts*

During GC-MS analysis, MPD and MIN extracts were first derived through Silylation method, which revealed the presence of several bioactive phytochemicals with known physiological implications in diverse pathological conditions. Silylation primarily substitutes the active hydrogen present in different functional groups (-OH, -COOH, -NH, -NH2, and -SH) in a molecule and introduces a silyl group in the form of dimethylsilyl [$SiH(CH_3)_2$], t-butyldimethylsilyl [$Si(CH_3)_2C(CH_3)_3$] and chloromethyldimethylsilyl [$SiCH_2Cl(CH_3)_2$] (Orata, 2012). This substitution of terminal hydrogen by silyl group in the sample lowers the hydrogen bonding and thus, reduces polarity of the sample, resulting in a comparatively more volatile sample, easy to detect in GC. The bioactive phytocompounds identified in MPD and MIN (Fig. 4.18 and Fig. 4.19) corresponding to GC chromatogram have been enlisted in Table 4.13 and Table 4.14 respectively.

A total of 34 bioactive metabolites were identified in MPD extract by GC-MS whereas MIN extract exhibited a total of 40 bioactive compounds (Fig. 4.20). Most of the compounds were identified in their TMS (C_3H_9Si) derivated form, resulting in

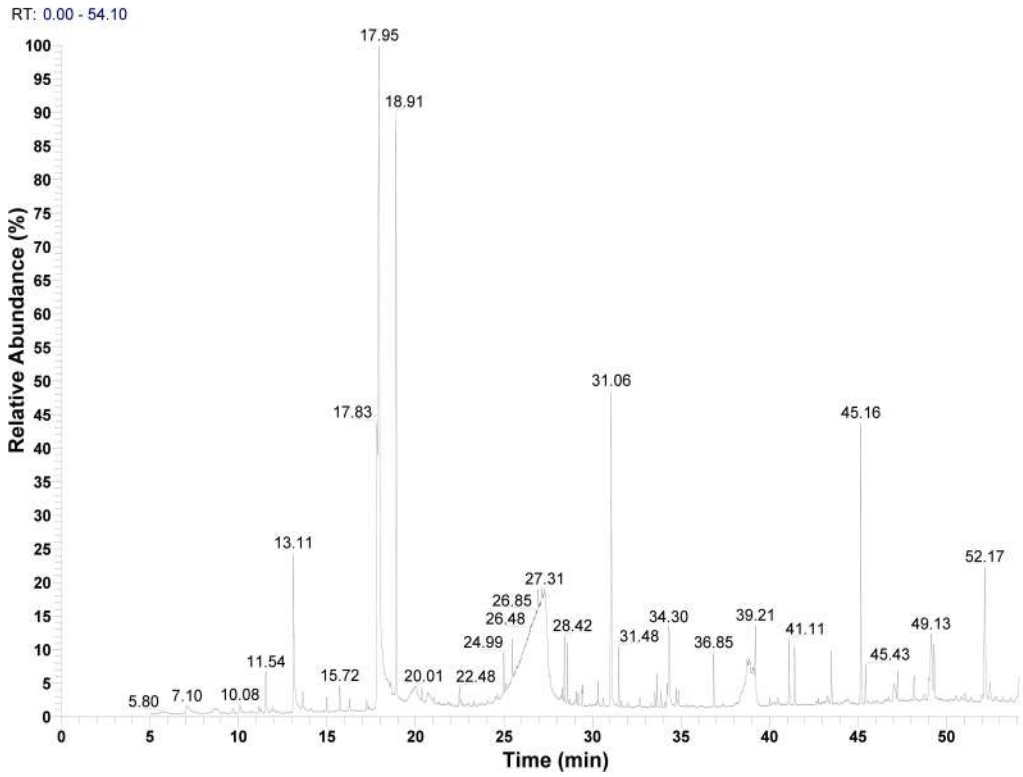


Fig. 4.17. GC-MS chromatogram of ANL extract.

Table 4.12. List of metabolites detected in *A. nilotica* leaf extract by GC-MS analysis.

Identified Compounds	MW	RT (min)	Formula
2-Propanone, 1,1-diethoxy-	146	7.10	$C_7H_{14}O_3$
Epomediol	186	10.08	$C_{10}H_{18}O_3$
Pyranone	144	11.54	$C_6H_8O_4$
Catechol	110	13.11	$C_6H_6O_2$
Phenol, 4-ethenyl-, acetate	162	13.63	$C_{10}H_{10}O_2$
5H-1-Pyrindine	117	15.72	C_8H_7N
p-Vinylguaiacol	150	16.27	$C_9H_{10}O_2$
Pyrogallol 1,3-dimethyl ether	154	17.25	$C_8H_{10}O_3$
Resorcinol	110	17.83	$C_6H_6O_2$
Pyrogallol	126	17.95	$C_6H_6O_3$
Bisphenol C	256	18.91	$C_{17}H_{20}O_2$
Dodecanoic acid	200	22.48	$C_{12}H_{24}O_2$
Hexadecanoic acid, methyl ester	270	30.32	$C_{17}H_{34}O_2$
9-Hexadecenoic acid	254	30.59	$C_{16}H_{30}O_2$
Estradiol, 3-deoxy-	256	32.01	$C_{18}H_{24}O$
Hexadecanoic acid	256	32.68	$C_{16}H_{32}O_2$
2-Hexadecanol	242	33.32	$C_{16}H_{34}O$
7,10-Octadecadienoic acid, methyl ester	294	33.53	$C_{19}H_{34}O_2$
Linolenic acid, methyl ester	292	33.65	$C_{19}H_{32}O_2$
Phytol	296	33.88	$C_{20}H_{40}O$
9,12-Octadecadienoic acid (Z,Z)-	280	34.22	$C_{18}H_{32}O_2$
Oleic Acid	282	34.73	$C_{18}H_{34}O_2$
8,11,14-Eicosatrienoic acid, (Z,Z,Z)-	306	34.86	$C_{20}H_{34}O_2$
Squalene	410	45.16	$C_{30}H_{50}$
γ-Tocopherol	416	48.16	$C_{28}H_{48}O_2$
Pregnan-18-oic acid, 20-hydroxy-, γ-lactone, (5α)-	316	49.13	$C_{21}H_{32}O_2$
dl-α-Tocopherol	430	49.30	$C_{29}H_{50}O_2$
Stigmasterol	412	52.17	$C_{29}H_{48}O$
β-Sitosterol	414	52.46	$C_{29}H_{50}O$

MW= Molecular weight; RT=Retention time.

an increase of m/z 73. Almost similar kinds of compounds were identified in both of cases belonging to the same genus of *Mimosa*.

Wide arrays of organic acids were identified in both MPD and MIN extracts namely, lactic acid, succinic acid, glutaric acid, malic acid, lauric acid, etc. Out of other bioactive metabolites characterized, azelaic acid have already been reported as an approved therapeutic agent for the skin condition rosacea (Jones, 2009) while salicylic acid, another identified metabolite, plays an important role in cytoprotection (De La Cruz *et al.*, 2004). Among the other indentified compounds gallic acid, squalene, α-tocopherol, campesterol, β-sitosterol and stigmasterol have also been established to display strong antioxidative features and protective properties against several disorders like cardiovascular disorders, cancer and NDs (Badhani *et al.*, 2015; Yoshida and Niki, 2003). Amongst these, gallic acid, a well-known polyphenolic constituent, was reported to scavenge ROS and RNS remarkably and inhibit lipid peroxidation and metal chelation (Badhani *et al*., 2015). Squalene and α-tocopherol are also excellent natural scavengers of free radicals found in vegetative oils. On the other hand campesterol, β-sitosterol and stigmasterol, collectively known as phytosterols (PS), have immense antioxidant activities (Li *et al.*, 2007; Yoshida and Niki, 2003). Moreover, they

Table 4.13. List of bioactive metabolites detected in MPD leaf extract by GC-MS analysis.

Compound Name	Formula	MW	Mass fragments (m/z)	RT(min)	RA(%)
Lactic acid, (2TMS)	$C_9H_{22}O_3Si_2$	234	147 ($C_5H_{11}O_3Si$), 117 ($C_5H_{13}OSi$), 73* (C_3H_9Si)	9.41	3.64 ± 0.86
2-acetoxyacetate (TMS)	$C_7H_{14}O_4Si$	190	133 (C_4H_9O3Si), 117 ($C_4H_5O_4$), 73* (C_3H_9Si)	12.74	5.71 ± 0.57
Propylene glycol, di-TMS	$C_9H_{24}O_2Si_2$	220	147 ($C_6H_{15}O_2Si$), 117* ($C_5H_{13}OSi$), 73 (C_3H_9Si)	13.19	0.48 ± 0.04
Glycerol, tris-TMS	$C_{12}H_{32}O_3Si_3$	308	205 ($C_8H_{21}O_2Si_2$), 147 ($C_6H_{15}O_2Si$), 73* (C_3H_9Si)	15.58	7.85 ± 0.53
3-Butenoic acid, 3-(trimethylsiloxy)-, TMS ester	$C_{10}H_{22}O_3Si_2$	246	231 ($C_9H_{19}O_3Si_2$), 147 ($C_7H_{15}O_3$), 73* (C_3H_9Si)	15.83	0.43 ± 0.10
Succinic acid (2TMS)	$C_{10}H_{22}O_4Si_2$	262	147 ($C_7H_{19}OSi$), 129 ($C_5H_9O_2Si$), 73* (C_3H_9Si)	16.43	3.25 ± 0.48
Glyceric acid, (3TMS)	$C_{12}H_{30}O_4Si_3$	322	189 ($C_7H_{17}O_2Si_2$), 147 ($C_5H_{10}O_3Si$), 73* (C_3H_9Si)	17.10	0.76 ± 0.22
Glutaric acid, bis-TMS	$C_{11}H_{24}O_4Si_2$	276	158 ($C_7H_{14}O_2Si$), 147* ($C_8H_{19}O_2$)	18.80	0.49 ± 0.07
Malic acid (3TMS)	$C_{13}H_{30}O_5Si_3$	350	233 ($C_9H_{31}O_3Si2$), 147 ($C_7H_{15}O_3$), 73 (C_3H_9Si)	21.15	3.56 ± 0.18
Adipic acid (TMS)	$C_{12}H_{26}O_4Si_2$	290	147 (C_7H_9OSi), 111 ($C_6H_{19}O_2Si$), 73* (C_3H_9Si)	21.34	0.83 ± 0.24
Salicylic acid, bis-TMS	$C_{13}H_{22}O_3Si_2$	282	267 ($C_{12}H_{19}O_3Si_2$), 149 (C_8H_9OSi), 73* (C_3H_9Si)	21.47	0.86 ± 0.18
Erythronic acid, (4TMS) deriv.	$C_{16}H_{40}O_5Si_4$	424	292 ($C_{11}H_{28}O_3Si_3$), 205 ($C_7H_{17}O_3Si_2$), 73* (C_3H_9Si)	23.09	0.34 ± 0.04
Pimelic acid (2TMS)	$C_{13}H_{28}O_4Si_2$	304	155 ($C_8H_{15}OSi$), 125 ($C_7H_9O_2$),73* (C_3H_9Si)	23.64	0.51 ± 0.11
Lauric acid (TMS)	$C_{15}H_{32}O_2Si$	272	257 ($C_{14}H_{29}O_2Si$), 117*	24.63	0.75 ± 0.32
Suberic acid (2TMS)	$C_{14}H_{30}O_4Si_2$	318	169 ($C_9H_{17}OSi$), 73* (C_3H_9Si)	25.77	0.93 ± 0.06
Nonadecane	$C_{19}H_40$	268	85 (C_6H_3), 71 (C_5H_{11}), 57 (C_4H_9)	27.75	0.26 ± 0.04
Azelaic acid, bis-TMS	$C_{15}H_{32}O_4Si_2$	332	201 ($C_{10}H_{21}O_2Si$), 73* (C_3H_9Si)	27.87	2.09 ± 0.25
Myristic acid, TMS ester	$C_{17}H_{36}O_2Si$	300	117 ($C_4H_9O_2Si$), 73* (C_3H_9Si), 55 (C_4H_7)	28.83	1.14 ± 0.14
Tridecanoic acid, 4,8,12-trimethyl-, methyl ester	$C_{17}H_{34}O_2$	270	197 ($C_{14}H_{29}$), 87* ($C_4H_7O_2$)	30.31	1.52 ± 0.06
n-Pentanoic acid, TMS ester	$C_{18}H_{38}O_2Si$	314	299 ($C_{17}H_{35}O_2Si$), 117* ($C_{18}H_9O_2Si$)	30.80	0.94 ± 0.33
Gallic acid, tetraTMS	$C_{19}H_{38}O_5Si_4$	458	280 ($C_{13}H_{21}O_3Si_2$), 73* (C_3H_9Si)	31.39	0.47 ± 0.52
Palmitic acid, TMS	$C_{19}H_{40}O_2Si$	328	313 ($C_{18}H_{37}O_2Si$), 117* ($C_{18}H_9O_2Si$)	32.69	61.26 ± 1.08
Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292	236 ($C_{15}H_{24}O_2$), 79 (C_6H_7)	33.64	1.48 ± 0.04
Margaric acid, TMS ester	$C_{20}H_{42}O_2Si$	342	327 ($C_{19}H_{39}O_2Si$), 117 ($C_4H_9O_2Si$), 73* (C_3H_9Si)	34.47	0.82 ± 0.26
1-Trimethylsiloxy-3,7,11,15-tetramethyl-2-hexadecene	$C_{23}H_{48}OSi$	368	143* ($C_7H_{15}OSi$), 123 (C_9H_{15}),	35.08	11.25 ± 0.78
Linoleic acid, TMS	$C_{21}H_{40}O_2Si$	352	337 ($C_{20}H_{37}O_2Si$), 73* (C_3H_9Si)	35.66	28.74 ±2.06
α-Linolenic acid, TMS	$C_{21}H_{38}O_2Si$	350	335 ($C_{20}H_{35}O_2Si$), 129 ($C_5H_9O_2Si$), 73 (C_3H_9Si)	35.80	99.08 ± 1.34
Octadecanoic acid, TMS	$C_{21}H_{44}O_2Si$	356	341 ($C_{20}H_{41}O_2Si$), 117 ($C_4H_9O_2Si$)	36.21	24.35 ± 2.58
Behenic acid, TMS ester	$C_{25}H_{52}O_2Si$	412	145 ($C_6H_{13}O_2Si$), 117* ($C_4H_9O_2Si$), 55 (C_4H_7)	42.47	0.53 ± 0.21
Squalene	$C_{30}H_{50}$	410	95 (C_7H_{11}), 81 (C_6H_9), 69* (C_5H_9)	45.15	9.79 ± 0.68
(+)-α-Tocopherol, O-TMS	$C_{32}H_{58}O_2Si$	502	277 ($C_{16}H_{25}O_2Si$), 237 ($C_{17}H_{33}$)	49.52	5.38 ± 0.74
Campesterol, TMS ether	$C_{31}H_{56}OSi$	472	382 ($C_{26}H_{42}Si$), 343 ($C_{25}H_{43}$), 129* ($C_6H_{13}OSi$)	51.43	2.46 ± 0.46
Stigmasterol, TMS	$C_{32}H_{56}OSi$	484	255 ($C_{19}H_{27}$), 129 ($C_6H_{13}OSi$), 83* (C_6H_{11})	51.99	5.26 ± 0.51
β-Sitosterol TMS	$C_{32}H_{58}OSi$	486	396* ($C_{29}H_{48}$), 357 ($C_{26}H_{45}$), 129* ($C_6H_{13}OSi$)	53.11	5.58 ± 0.88

MW= Mol. Wt.; RT= Retention Time; RA= Relative Abundance

accumulate in the brain by crossing the blood-brain barrier (BBB) which is one of the crucial factors for becoming a novel drug and thus, they might facilitate with cell signaling, membrane protein trafficking and neurotransmission (Burg *et*

Table 4.14. List of bioactive metabolites detected in MIN leaf extract by GC-MS analysis.

Compound Name	Formula	MW	Identifying MS (m/z)	RT	RA (%)
Lactic acid, (2TMS)	$C_9H_{22}O_3Si_2$	234	147 ($C_5H_{11}O_3Si$), 117 ($C_5H_{13}OSi$), 73* (C_3H_9Si)	9.41	5.22 ± 0.41
L-Valine, TMS ester	$C_8H_{19}NO_2Si$	189	146 ($C_5H_{12}NO_2Si$), 72* (C4H10N)	10.01	2.07 ± 0.26
l-Proline, TMS ester	$C_8H_{17}NO_2Si$	187	103 ($C_3H_7O_2Si$), 70* (C4H8N)	12.47	2.14 ± 0.51
2-Hydroxyisocaproic acid, TMS ester	$C_9H_{20}O_3Si$	204	117 ($C_5H_9O_3$), 73* (C_3H_9Si)	13.81	1.16 ± 0.25
2-Propanone, bis(trimethylsilyloxy)-	$C_9H_{22}O_3Si_2$	234	147 ($C_5H_{11}O_3Si$), 103 ($C_4H_{11}OSi$), 73* (C_3H_9Si)	13.95	1.56 ± 0.08
L-Serine, O-(TMS)-, TMS este	$C_9H_{23}NO_3Si_2$	249	147 ($C_5H_{13}NO_2Si$), 116 ($C_4H_{10}NOSi$), 73 (C_3H_9Si)	14.93	0.97± 0.12
Glycerol, tris-TMS	$C_{12}H_{32}O_3Si_3$	308	205 ($C_8H_{21}O_2Si_2$), 147 ($C_6H_{15}O_2Si$), 73* (C_3H_9Si)	15.58	28.21 ± 0.98
L-Threonine, O-(TMS)-, TMS ester	$C_{10}H_{25}NO_3Si_2$	263	147 ($C_5H_{13}NO_2Si$), 130 ($C_5H_{12}NOSi$), 73* (C_3H_9Si)	15.97	0.79 ± 0.04
Succinic acid, bis-TMS	$C_{10}H_{22}O_4Si_2$	262	147 ($C_7H_{19}OSi$), 129 (C5H9O2Si),	16.43	2.38 ± 0.63
Glyceric acid, (3TMS)	$C_{12}H_{30}O_4Si_3$	322	189 ($C_7H_{17}O_2Si_2$), 147 ($C_5H_{10}O_3Si$), 73* (C_3H_9Si)	17.10	0.58 ± 0.05
Glutaric acid, bis-TMS	$C_{11}H_{24}O_4Si_2$	276	158 ($C_7H_{14}O_2Si$), 147*	18.80	0.72 ± 0.12
Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$	212	85 (C_6H_{13}), 71 (C_5H_{11}), 57*	20.91	4.05 ± 0.86
Malic acid (3TMS)	$C_{13}H_{30}O_5Si_3$	350	233 ($C_9H_{31}O_3Si_2$), 147	21.15	2.62 ± 0.45
Adipic acid, (2TMS)	$C_{12}H_{26}O_4Si_2$	290	147 (C_7H_9OSi), 111	21.33	2.18 ± 0.38
Pyroglutamic acid, di(TMS)-	$C_{11}H_{23}NO_3Si_2$	273	156 ($C_7H_{14}NOSi$), 147 ($C_7H_{17}NO_2$), 73* (C_3H_9Si)	21.77	4.42 ± 0.72
DL-Phenylalanine, TMS ester	$C_{12}H_{19}NO_2Si$	237	146 (C_9H_8NO), 120 (C8H10N)	22.20	1.41 ± 0.06
Hexadecane	$C_{16}H_{34}$	226	71 (C_5H_{11}), 57 (C_4H_9)	23.36	2.19 ± 0.31
Glycerol, tris-TMS ether	$C_{12}H_{32}O_3Si_3$	308	205 ($C_8H_{21}O_2Si_2$), 147 (C6H15O2Si), 73* (C3H9Si)	23.68	12.13 ± 0.68
Lauric acid (TMS)	$C_{15}H_{32}O_2Si$	272	257 ($C_{14}H_{29}O_2Si$), 117*	24.64	1.04 ± 0.24
Suberic acid (2TMS)	$C_{14}H_{30}O_4Si_2$	318	169 (C9H17OSi), 73* (C3H9Si)	25.77	3.25 ± 0.68
Azelaic acid, bis-TMS	$C_{15}H_{32}O_4Si_2$	332	201 ($C_{10}H_{21}O_2Si$), 73* (C3H9Si)	27.88	5.96 ± 0.46
Tetradecanoic acid, TMS	$C_{17}H_{36}O_2Si$	300	285* ($C_{16}H_{33}O_2Si$), 129	28.84	4.08 ± 0.69
2,5-Dihydroxyacetophenone, bis(TMS) ether	$C_{14}H_{24}O_3Si_2$	296	281* ($C_{13}H_{21}O_3Si_2$), 73 (C_3H_9Si)	30.04	32.55 ± 1.31
Gallic acid, tetraTMS	$C_{19}H_{38}O_5Si_4$	458	280 ($C_{13}H_{21}O_3Si_2$), 73* (C3H9Si)	31.40	11.13 ± 0.58
Hexadecanoic acid, TMS	$C_{19}H_{40}O_2Si$	328	132 ($C_5H_{12}O_2Si$), 117	32.72	75.64 ± 2.06
Linoleic acid, TMS	$C_{21}H_{40}O_2Si$	352	337 ($C_{20}H_{37}O_2Si$), 73* (C3H9Si)	35.68	23.81 ± 1.64
α-Linolenic acid, TMS	$C_{21}H_{38}O_2Si$	350	335 ($C_{20}H_{35}O_2Si$), 129 ($C_5H_9O_2Si$), 73 (C_3H_9Si)	35.84	98.52 ± 1.32
Stearic acid, TMS ester	$C_{21}H_{44}O_2Si$	356	341 ($C_{20}H_{41}O_2Si$), 117 ($C_4H_9O_2Si$), 73* (C_3H_9Si)	36.23	43.52 ± 0.83
Arachidic acid, TMS ester	$C_{23}H_{48}O_2Si$	384	132 ($C_5H_{12}O_2Si$), 117 ($C_4H_9O_2Si$), 73 (C_3H_9Si), 55 (C4H7)	39.46	2.31 ± 0.41
1-Monopalmitin TMS ether	$C_{25}H_{54}O_4Si_2$	474	371 ($C_{21}H_{43}O_3Si$), 147 ($C_6H_{15}O_2Si$), 73* (C_3H_9Si)	41.92	3.85 ± 0.22
Behenic acid, TMS ester	$C_{25}H_{52}O_2Si$	412	145 ($C_6H_{13}O_2Si$), 117*	42.48	1.68 ± 0.45
9-Octadecenoic acid, 1,3-bis-(OTMS)-2-propyl ester	$C_{27}H_{56}O_4Si_2$	500	203 ($C_8H_{19}O_2Si_2$), 129 ($C_6H_{13}OSi$), 73* (C_3H_9Si), 67	44.45	12.59 ± 0.98
Squalene	$C_{30}H_{50}$	410	95 (C_7H_{11}), 81 (C_6H_9), 69* (C5H9)	45.17	18.37 ± 1.05
tert-Hexadecanethiol	$C_{16}H_{34}S$	258	187 ($C_{11}H_{23}S$), 57* (C_4H_9)	46.71	0.94 ± 0.35
Heptacosane	$C_{27}H_{56}$	380	85 (C_6H_{13}), 71 (C_5H_{11}), 57 (C4H9)	48.69	0.78 ± 0.12
(+)-α-Tocopherol, O-TMS-	$C_{32}H_{58}O_2Si$	502	277 ($C_{16}H_{25}O_2Si$), 237 (C17H33)	49.54	14.52 ± 0.59
Campesterol, TMS ether	$C_{31}H_{56}OSi$	472	382 ($C_{26}H_{42}Si$), 343 ($C_{25}H_{43}$), 129* ($C_6H_{13}OSi$)	51.55	2.17 ± 0.46
Stigmasterol, TMS	$C_{32}H_{56}OSi$	484	255 ($C_{19}H_{27}$), 129 (C6H13OSi),	52.01	8.22 ± 0.63
Triacontyl TMS ether	$C_{33}H_{70}OSi$	510	495* ($C_{32}H_{67}OSi$)	52.77	34.62± 0.80
β-Sitosterol TMS	$C_{32}H_{58}OSi$	486	396* ($C_{29}H_{48}$), 357 ($C_{26}H_{45}$), 129*	53.16	15.45 ± 0.62

MW= Mol. Wt.; MS= Mass fragment; RT= Retention Time (Min.); RA= Relative Abundance

al., 2013).

Therefore, it may be conclude that the present study has made a significant contribution in making MPD and MIN as powerful candidates against neuro-degenerative activities.

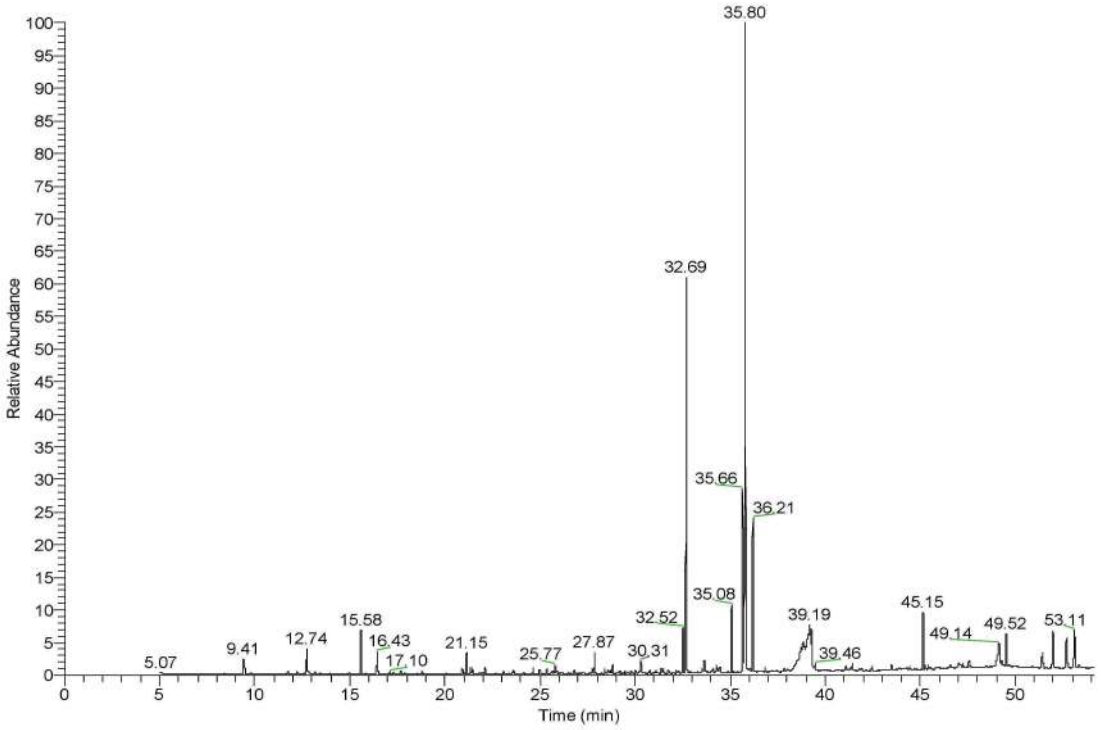


Fig. 4.18. Gas chromatogram-Mass spectroscopy of *M. pudica* leaf extract.

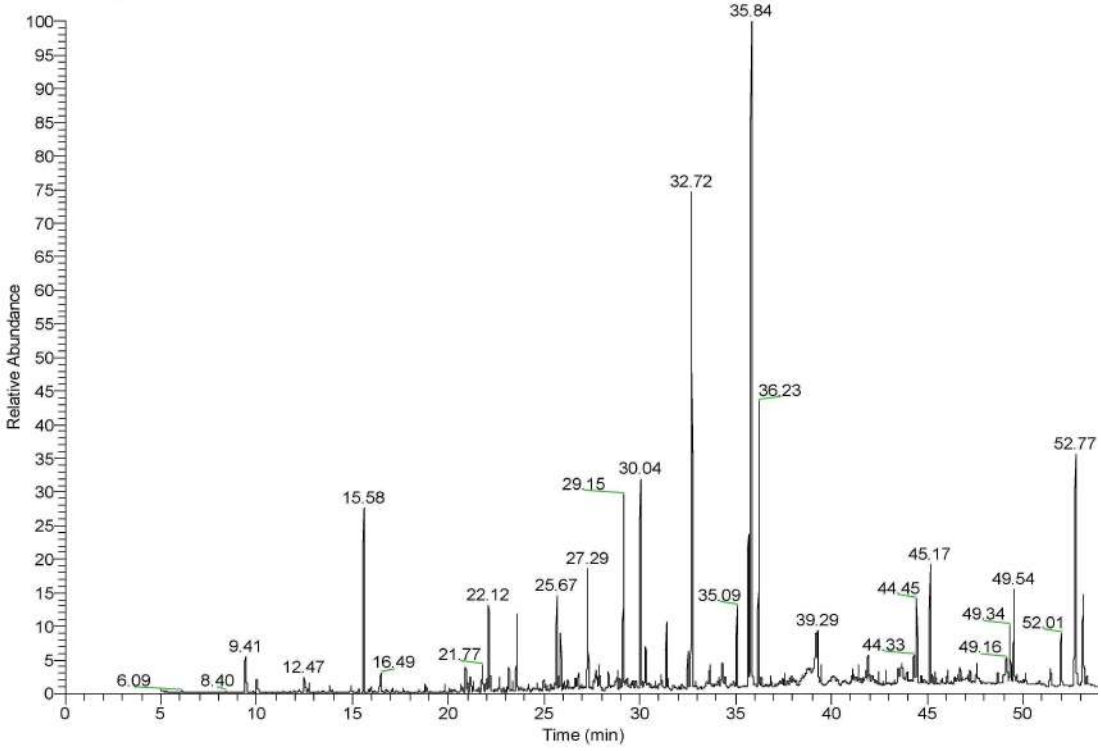


Fig. 4.19. Gas chromatogram-Mass spectroscopy of *M. invisa* leaf extract.

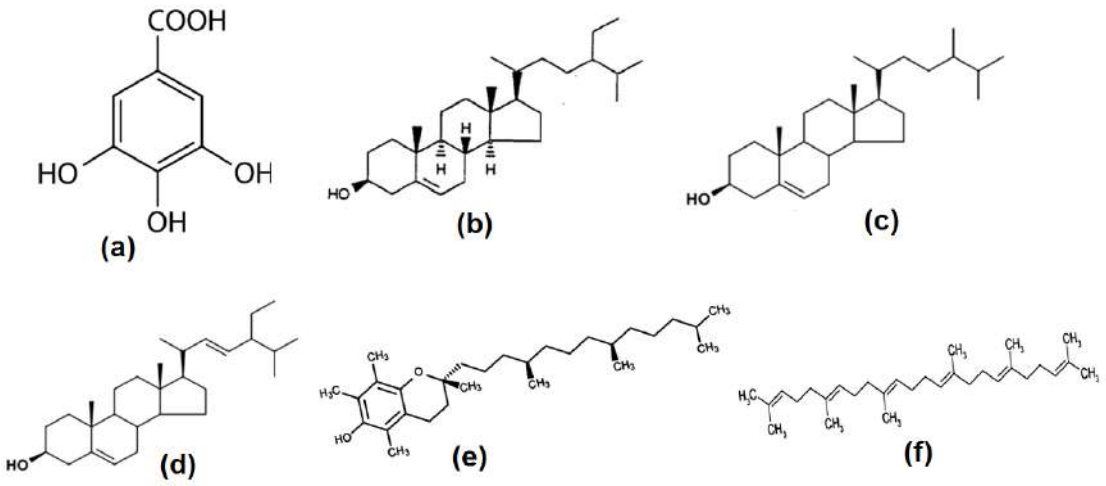


Fig. 4.20. Chemical structures of few active metabolites identified in *M. pudica* and *M. invisa* extracts by GC-MS: gallic acid (a); β-sitosterol (b); campesterol (c); sigmasterol (d); α-tocopherol (e); squalene (f).

4.7.3. NMR analysis

4.7.3.1. NMR analysis of ACL extract

^{13}C NMR spectra of ACL extract (Fig. 4.21.A) revealed several peaks in the aliphatic (δ=19.0–84.2) and aromatic region (δ=115.8–156.6) confirming the presence of both aliphatic and aromatic carbons (Silverstein *et al*., 2006). Likewise, ^{1}H NMR spectra (Fig. 4.21.B) exhibited peaks related to aliphatic, aromatic and olefinic protons. Besides, a hump near δ=8.75 indicates the presence of amine functional group supporting the result found in GC-MS analysis of ACL extract which might be responsible for the bioactive properties of the extract.

4.7.3.2. NMR analysis of MPD and *MIN extracts*

NMR spectra of MPD extract (Fig. 4.22) clearly indicated the peaks of squalene, a natural antioxidative agent, while MIN extract exhibited the structure (Fig. 4.23) of gallic acid, another strong antioxidant having potent lipid peroxidation property. The NMR spectral analysis of squalene is as follows: ^{1}H NMR (300 MHz, $CDCl_3$) δ5.14 (m, internal vinylic protons), δ2.0 (m, methylene protons), δ1.68 (s, methyl protons), δ1.60 (s, methyl protons); ^{13}C NMR (75 MHz, $CDCl_3$) δ16.0–25.6 (eight methyl carbon), δ26.7–39.7 (ten methylene carbon), δ124.3–135.2 (twelve double bonded carbon). In case of gallic acid, the NMR spectral analysis is as follows: ^{1}H NMR (300 MHz, DMSO-d_6) δ6.94 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ173.0 (C-7), δ146.2 (C-3 and C-5), δ139.2 (C-4), δ128.4 (C-1), δ116.5 (C-2 and C-6).

4.8. *In-silico* drug targeting and Pharmacokinetic study

Since the phytochemicals, identified in the present study, were found to be effective as potent free radical scavengers or antioxidants, anti-neurodegenerative stuffs, and anti-diabetic agents, it further prompted towards the discovery of new drugs through *in-silico* computational

approach opening a new door for pharmaceutical industry.

4.8.1. In-silico drugability prediction and ROS (Reactive Oxygen Species)

Interestingly, it has been observed that FAS Ligand protein (FasL; ID- 4MSV), Toll like receptors (TLR; ID- 5AWA) and NADPH oxidase (NOX; ID- 1OEY) have a crucial role on ROS generation causing most of ROS-induced diseases and/or disorders in human body. Therefore, suppressing or avoiding of these proteins could be a rational pathway to combat against ROS. Phytochemicals like stigmasterol, β-sitosterol, and campesetrol

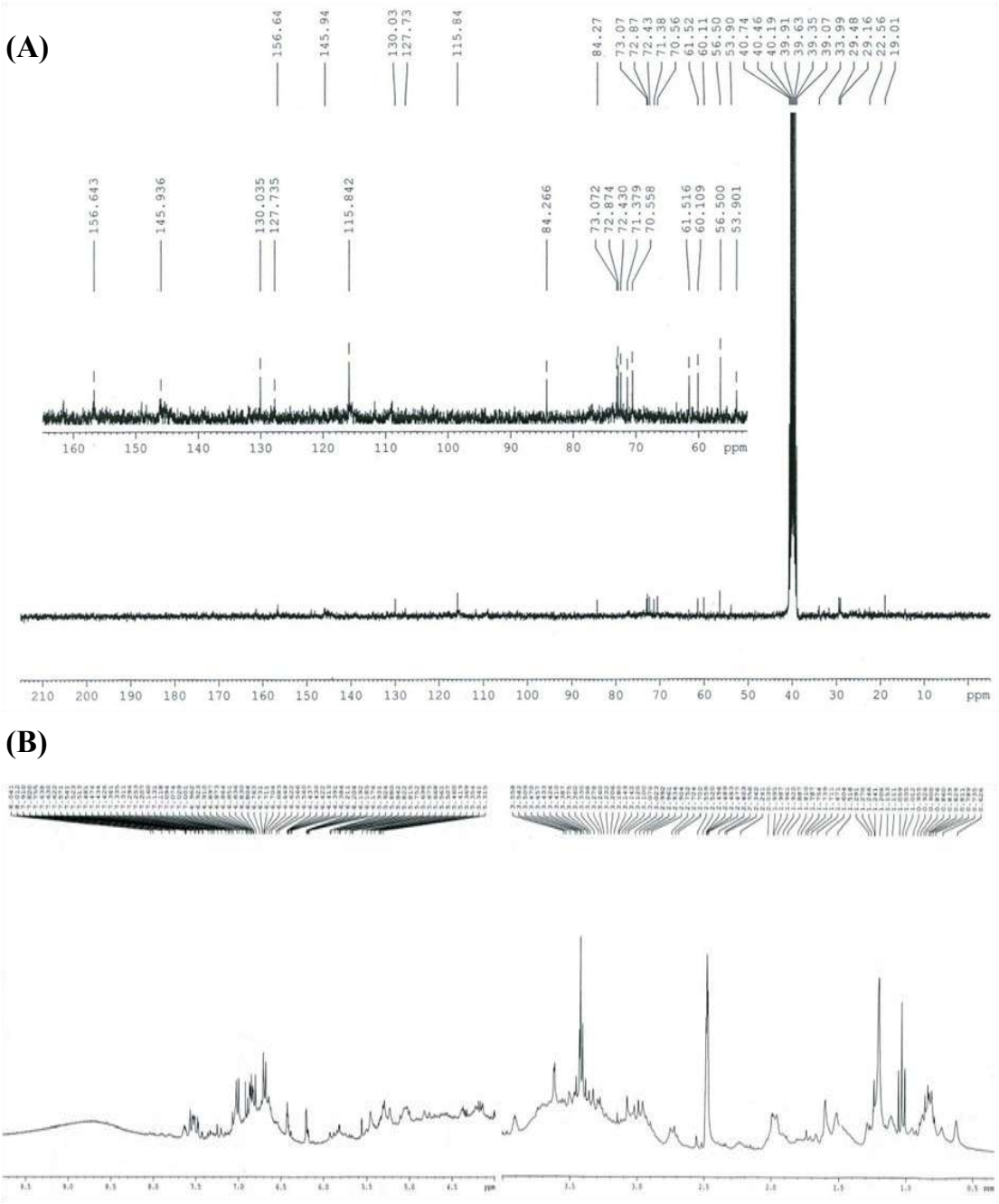


Fig. 4.21. ^{13}C NMR spectra **(A)** and ^{1}H NMR spectra **(B)** of ACL extract.

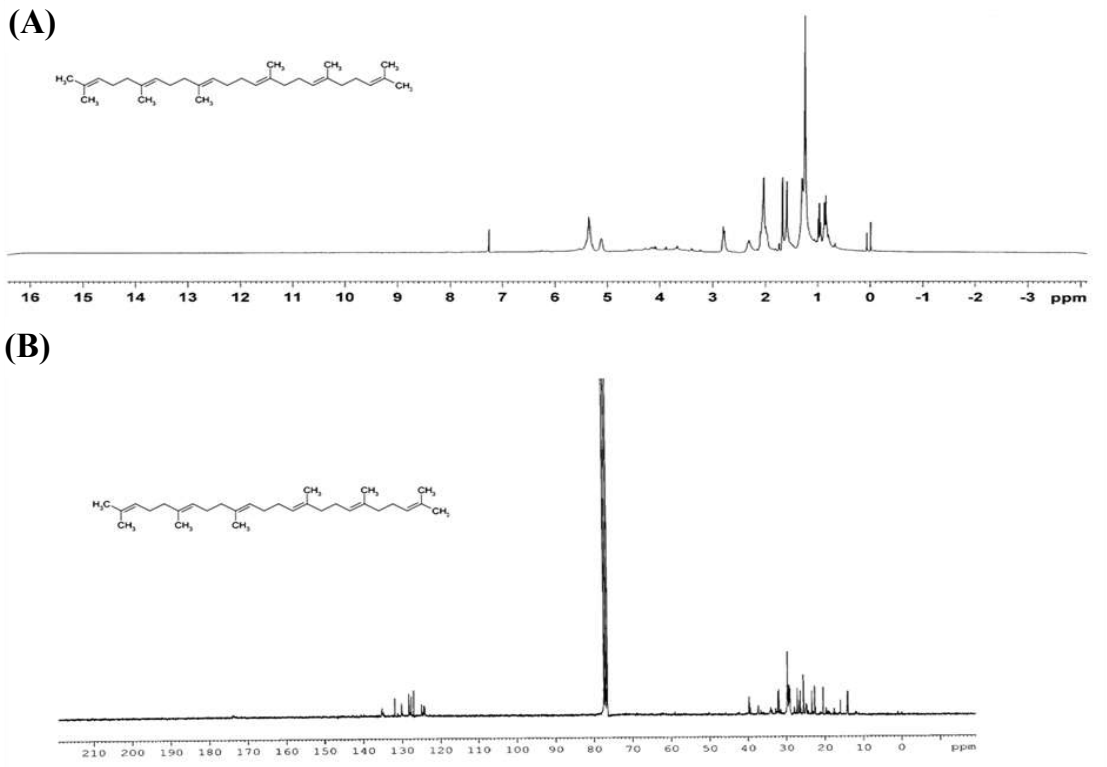


Fig. 4.22. ^{1}H NMR spectra **(A)** and ^{13}C NMR spectra **(B)** of MPD extract representing the structure of squalene.

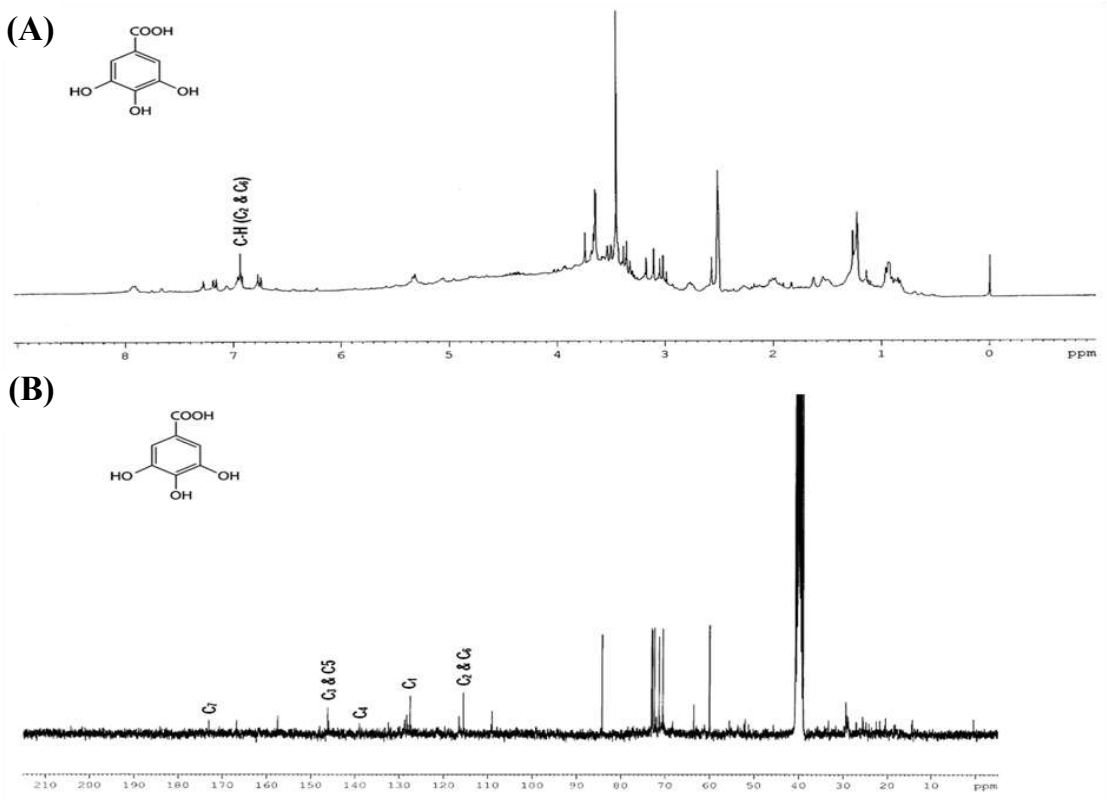


Fig. 4.23. ^{1}H NMR spectra **(A)** and ^{13}C NMR spectra **(B)** of MIN extract representing the structure of gallic acid.

have already been identified in the MPD and MIN extracts (please refer section 4.7.) exerting antioxidative properties. In fact, these metabolites are the best known antioxidants against different free radicals including hydroxyl radical ($OH^{\bullet-}$), singlet oxygen ($^1O_2^{\bullet-}$), superoxide radical ($O_2^{\bullet-}$), H_2O_2, nitric, oxide (NO), peroxynitrite ion ($ONOO^-$) etc., as well as they may be act as suppressors of free radical generating proteins. Hence, a first initiative step was undertaken to gain an insight into the underlying mechanisms between the above mentioned proteins and the active metabolites through *in-silico* computational approach.

In essence, FasL, a type II membrane protein belonging to the tumor necrosis factor (TNF) family, facilitates signaling of cell apoptosis (Lee *et al.*, 1999) which involves caspase 8 activation and a subsequent cascade of events via Fas-associated death domain (FADD/Mort1) adaptor protein leading to DNA fragmentation and cell death (Fuchs *et al.*, 1997). Besides, FasL activation has also been reported to be associated with ROS generation causing necrotic cell death (Medan *et al.*, 2005; Vercammen *et al.*, 1998). The NADPH oxidase, a membrane bound enzyme complex, transfers an electron of the complex to the oxygen molecule in the phagosome or in the cytosol, giving rise to different ROS including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide, hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) etc (Han *et al.*, 1998; Pham-Huy *et al.*, 2008). What's more, toll like Receptors (TLR), the significant intra- and extra- cellular ligand-recognition receptors, can also induce the ROS production (Marcato *et al.*, 2008). Therefore, suppressing the activity of these proteins via ligands could stop the generation of ROS damaging the cells. In this context, probable function of some identified antioxidative metabolites (e.g. stigmasterol, β-sitosterol and campesterol; found in MPD and MIN extracts) was investigated in suppressing the ROS generation by means of *in-silico* approach. Result revealed that stigmasterol effectively binds (Fig. 4.24.A-C) with FasL protein, NADPH Oxidase and TLR protein with an affinity of -5.2 kcal/mol, -5.8 kcal/mol and -6.8 kcal/mol respectively (Table 4.15). Interestingly, in the plant extracts like MPD and MIN with its bioactive compounds had already been found to be efficient free radical scavenger in different *in-vitro* antioxidant analysis (please refer 4.2 section). Hence, it could be presumed that stigmasterol probably made the protein inactive, thereby blocked the generation of ROS. Subsequently, β-sitosterol (Fig. 4.25.A-C) and campesterol (4.25.D-F) also exhibited noteworthy binding affinities (Table 4.15) indicating their potent role to suppress the production of ROS. Therefore, MPD and MIN extracts might be treated as ROS-

Table 4.15. Binding affinities between the ligands and proteins.

Ligands	Binding Affinities (kcal/mol)		
	FasL (4MSV)	NADPH (1OEY)	TLR (5AWA)
Stigmasterol	-5.2	-5.8	-6.8
β-Sitosterol	-5.0	-5.8	-6.1
Campesterol	-5.6	-6.6	-5.9

suppressor materials enlightening an innovative idea towards new drug discovery against free radicals.

4.8.2. In-silico drugability prediction and neurodegeneration

Despite of having immense antioxidant and anti-neurodegenerative properties found in selected plant extracts (MPD and MIN) due to presence of several active metabolites including gallic acid, β-sitosterol, stigmasterol and campesterol etc., these were considered as the potential drug target ligands against neurodegenerative disorders (NDs). As a part of drug target establishment, molecular docking was first performed to explore the binding pattern of the above mentioned target compounds with human brain membrane protein (dopamine receptor D3 protein; ID- 3PBL). The D3 protein, localized to the limbic areas of the brain, was selected due to its prominent role in cognition, emotional and endocrine functions and hence, it might be the chief target of antipsychotic therapy involving dopamine antagonists (Nakajima *et al.*, 2013). Results obtained from docking

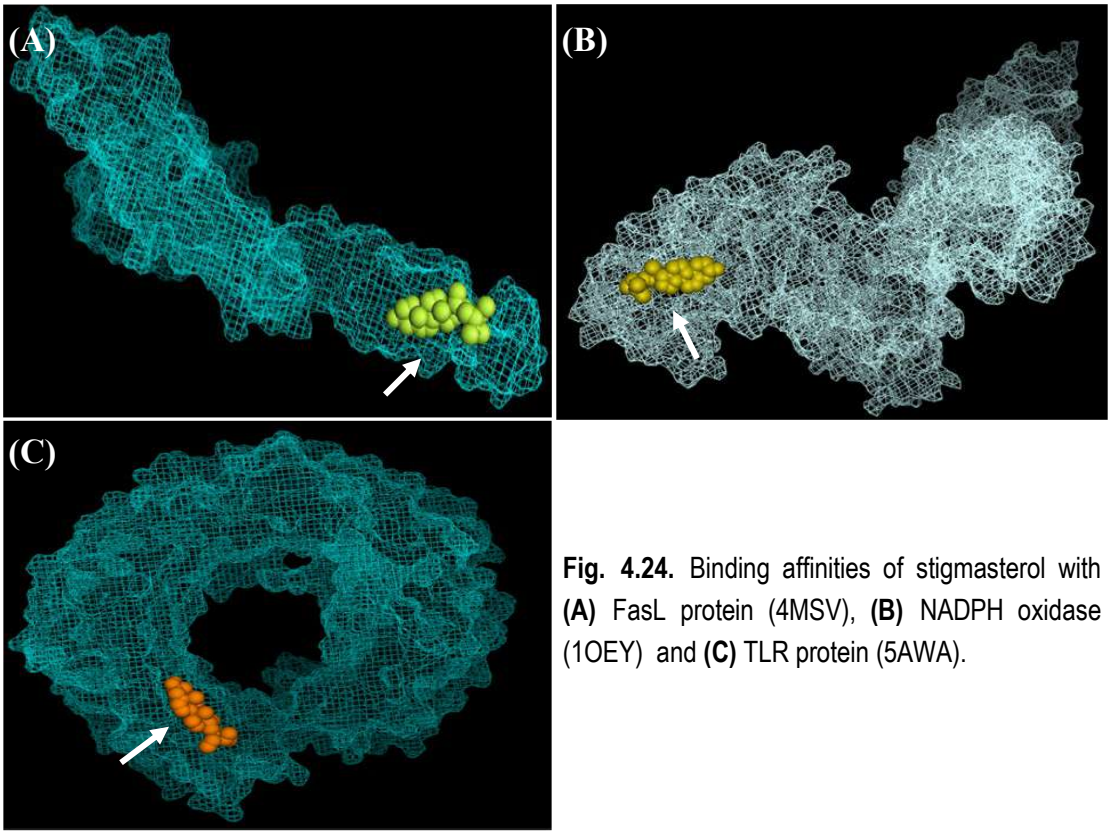


Fig. 4.24. Binding affinities of stigmasterol with **(A)** FasL protein (4MSV), **(B)** NADPH oxidase (1OEY) and **(C)** TLR protein (5AWA).

revealed different binding sites with different affinities. Only the most suitable binding sites with highest affinities were considered. Out of 5 selected ligands, gallic acid (-5.5 kcal/mol) and squalene (-5.3 kcal/mol) displayed comparatively poor binding pattern and affinity towards dopamine D3 protein than the rest of ligands, thereby discarded them. However, other selected ligand compounds i.e., β-sitosterol, stigmasterol and campesterol exhibited higher binding affinities; for instance, campesterol was found to display an affinity of -7.4 kcal/mol of affinity, β-sitosterol revealed -7.7 kcal/mol and stigmasterol displayed -8.4 kcal/mol of

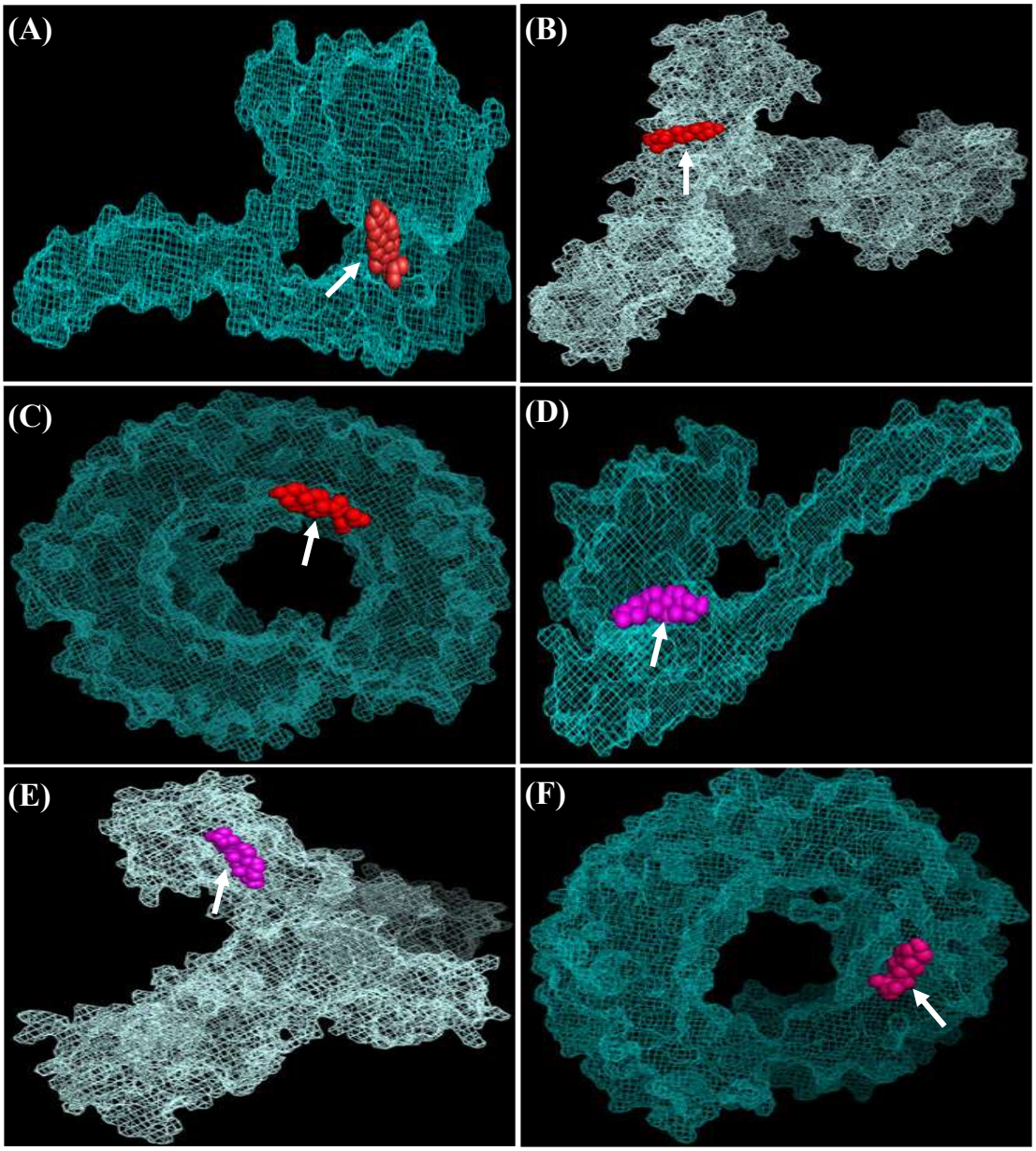


Fig. 4.25. Binding affinities of FasL protein (4MSV), NADPH oxidase (1OEY) and TLR protein (5AWA) with β-sitosterol **(A-C)** and affinities of campesterol with same proteins **(D-F)** respectively.

affinity (Fig. 4.26). Strong binding pattern of these ligand compounds with dopamine receptor D3 protein signified their possible role in the treatment of neurodegeneration.

Since remarkable binding pattern of target compounds was obtained from molecular docking, it was intended to perceive whether those compounds assure to be novel drugs in treating NDs. Screenings of 'Lipinski's rule of five' as well as 'ADMET' (Absorption, Distribution, Metabolism, Excretion and Toxicity) property of a compound are the foremost approaches to establish a compound as an effective drug. In fact, the probability of success of a compound in becoming a realistic CNS (central nervous system) drug depends on some factors that include penetration ability of molecule to cross blood brain barrier (BBB), optimum pharmacokinetic profiling and the effect of the components on brain. Hence, we followed 'Lipinski's Rule of Five' to screen the physiochemical properties of the selected ligand compounds using mechanistic *in-silico* tools, such as, ALOGPS and Chemicalize. The properties were: (1) molecular weight (≤ 500 Da), (2) lipophilicity or logP value (≤ 5), (3) H-bond donor (≤5), and (4) H-bond acceptor (≤ 10) (Table 4.16). Pharmacokinetic (PK) properties of target drugs were further assessed by means of BBB predictor analysis (using fingerprints of MACCS, Openbabel (FP2), Molprint 2D and PubChem) and BioZyne.

It was revealed from Table 4.16 that three out of four rules of Lipinski were

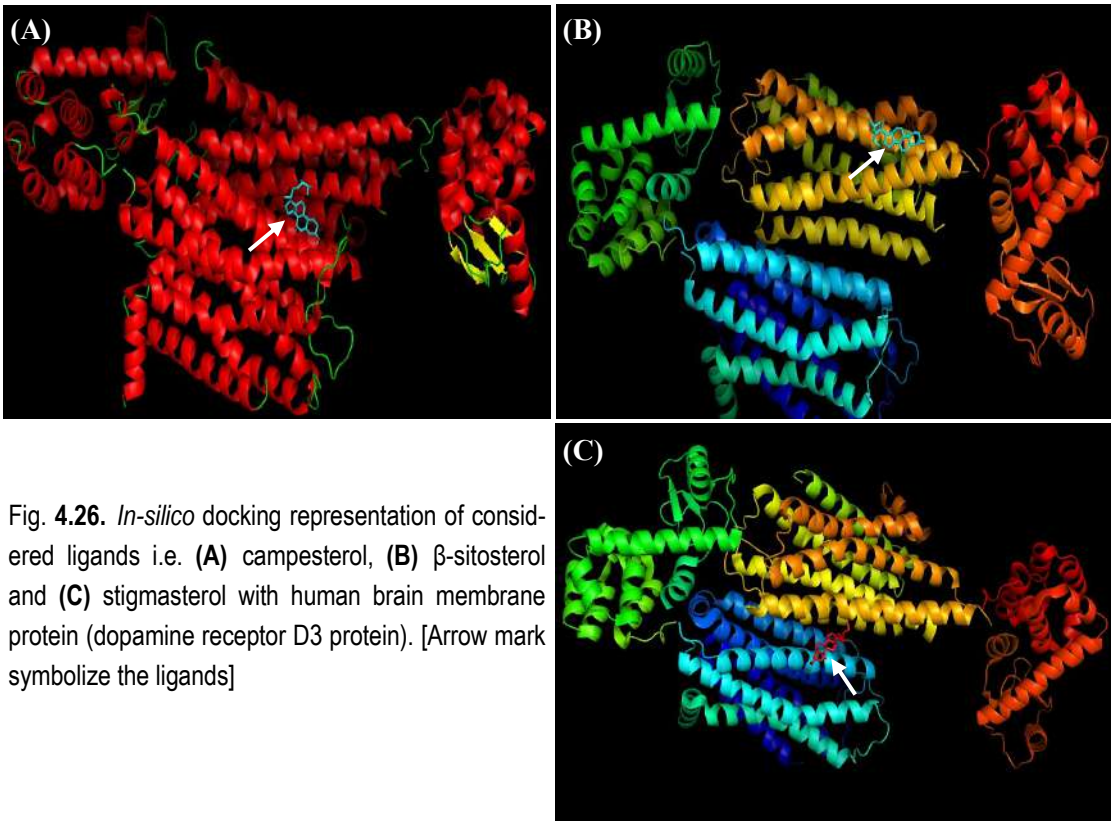


Fig. **4.26.** *In-silico* docking representation of considered ligands i.e. **(A)** campesterol, **(B)** β-sitosterol and **(C)** stigmasterol with human brain membrane protein (dopamine receptor D3 protein). [Arrow mark symbolize the ligands]

Table 4.16. Physiochemical properties of selected ligands as per Lipinski's rule.

Properties	Stigmasterol	β-sitosterol	Campesterol	Lipinski's rule (Present study response)
H-donor	1	1	1	P
H-acceptor	1	1	1	P
Molecular weight (Da)	412.69	414.70	400.68	P
logP	9.43	7.84	7.40	×

religiously obeyed by the selected ligand compounds. However, the logP values in all the cases were found to be way above the prescribe value of Lipinski's rule of Five. Hence, pharmacokinetic properties (i.e., ADMET) of the target ligands were investigated. The analysis involving 'BBB permeability', which is one of the mandatory factors for effectiveness of CNS drug, confirmed that these compounds were BBB+ i.e., they can cross the BBB effectively (Fig. 4.27).

Concurrently, several junctional-proteins like, P-glycoprotein (P-gp) that lies in BBB, block the diffusion of polar solutes from blood to CNS (Alavijeh *et al.*, 2005). In fact, P-gp restricts the entry of most of the free molecules from blood to brain except, some selected small and lipophilic compounds (Miller *et al.*, 2008).

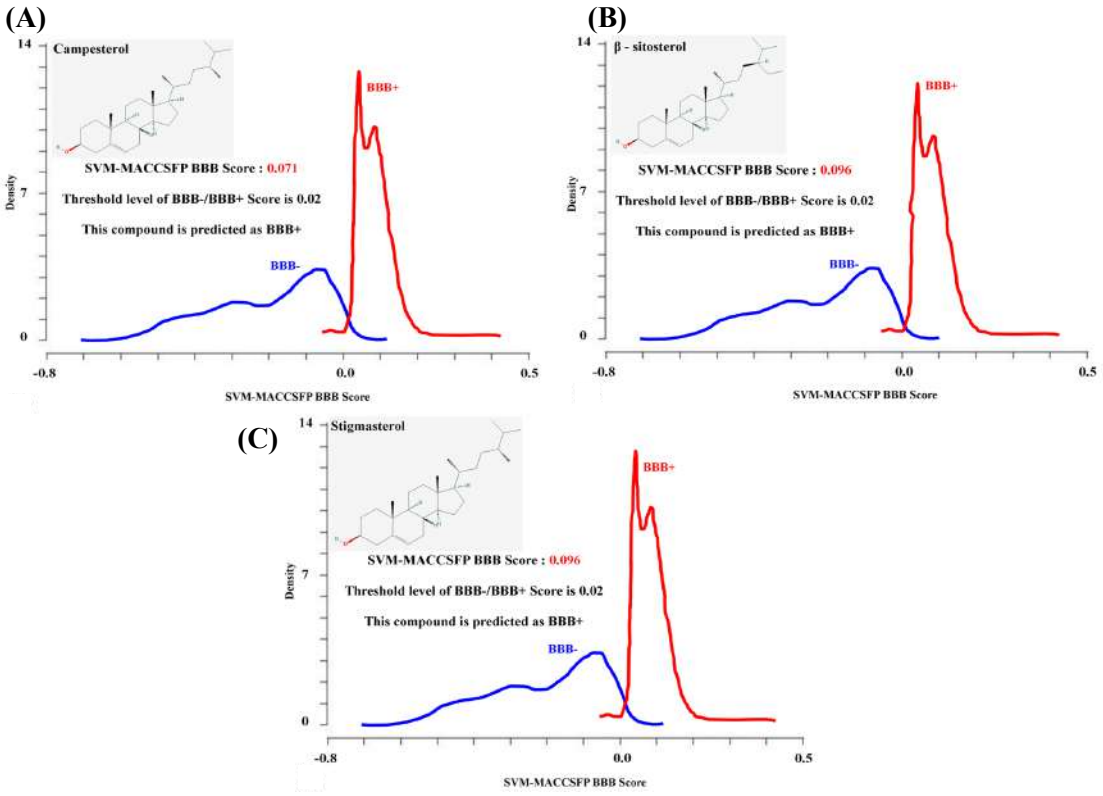


Fig. 4.27. Blood brain barrier (BBB) permeability of ligands i.e. **(A)** campesterol, **(B)** β-sitosterol and **(C)** stigmasterol; where, BBB score of each compound is more than threshold level (0.02) indicating the BBB permeability of selected ligands.

Regarding central nervous system (CNS) treatment, the penetration of drug through the lipid bilayer of BBB and BSB (blood spinal-cord barrier) plays a key role and it is directly dependent on the logP value (lipophilic character) of that molecule (Alavijeh *et al*., 2005). It has been suggested that higher lipophilicity of a drug enhances its permeability through lipid membrane and also paves way for a better distribution and improved metabolic clearance (Riley *et al.*, 2001). Therefore, higher logP value may actually be a blessing in disguise in this case.

The efficacy of an anti-ND agent not only depends on its permeability through BBB but also relies largely on its capability to remain in the brain until its desired action is aptly achieved. In this regard, the intention was to find out whether the target ligands act as P-glycoprotein (P-gp) substrates (Alavijeh *et al*., 2005). The analysis with BioZyne tool proved that the selected ligands were indeed P-gp substrates (Fig. 4.28) that allow successful transport through BBB, thus establishing PS as effective drugs against CNS disorders or NDs. Thus, high logP values and P-gp substrate nature of selected ligands (i.e., PS) indicated a better absorption through CNS (Wils *et al.*, 1994).

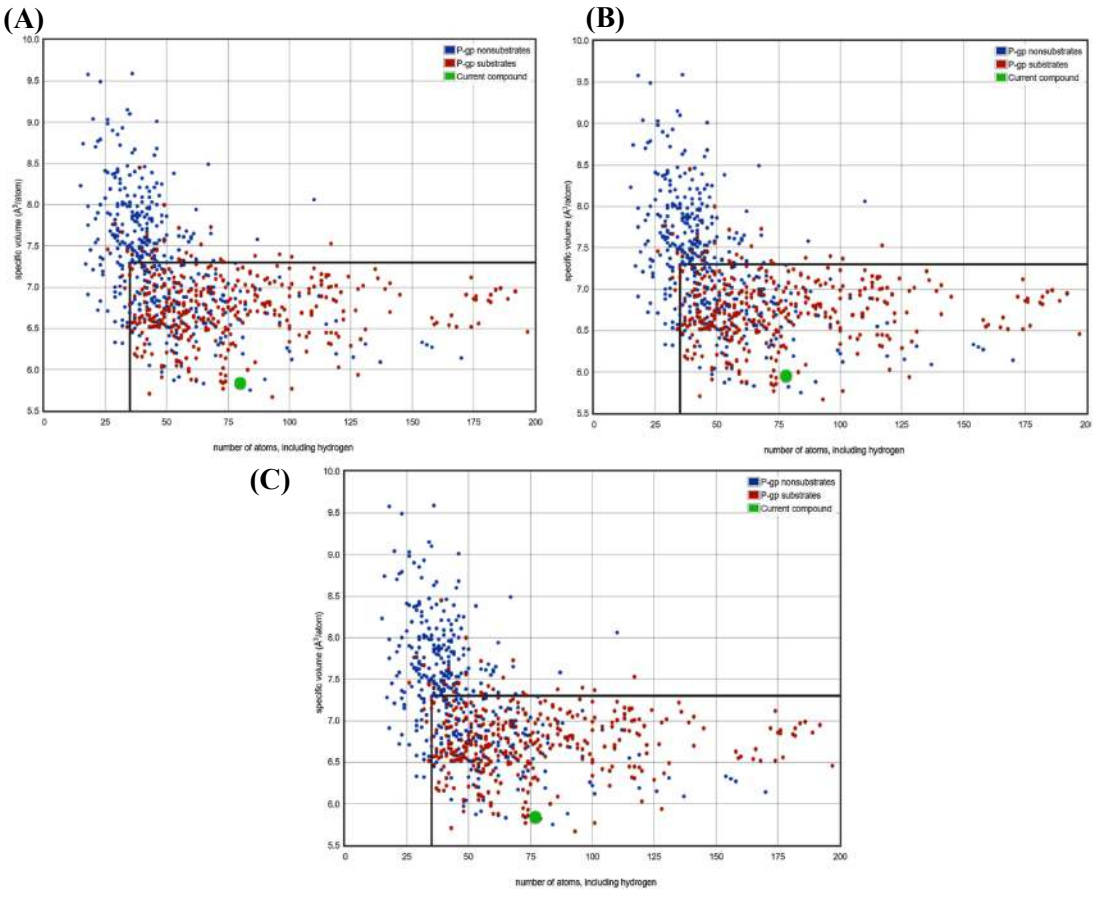


Fig. 4.28. Phospho-glycoprotein (P-gp) substrate property of selected ligands; where, considered ligands (green colored) β-sitosterol **(A)**, stigmasterol **(B)** and campesterol **(C)** clustered with P-gp substrates (red colored).

While considering a phytochemical as a novel CNS drug, lipophilic property of that bioactive compound plays a pivotal role having direct effect on the distribution and metabolism prototype of that compound (Alavijeh *et al*., 2005). It has been observed that the molecules with higher lipophilic nature tend to bind with target proteins more strongly enhancing their "distribution rate" as well as "affinity" towards metabolic enzymes (Alavijeh *et al*., 2005). Thus, higher lipophicity of the target phytocompounds might suggest smooth absorption, effortless distribution and appropriate metabolism in CNS. Besides, smooth transportation (in both directions) of these lipophilic ligands through BBB and BSB ensure their satisfied excretion from CNS making the treatment therapy against NDs more promising. Thus, it might be well justified that the target ligands i.e., phytocompounds namely, stigmasterol, β-sitosterol and campesterol being highly lipophilic, displaying decent P-gp substrate nature, may be rightfully considered as CNS drugs for treating NDs like AD and PD.

4.8.3. In-silico drugability prediction and diabetes

Amongst several phytochemicals identified in *A. nilotica* (ANL), γ-tocopherol, α-tocopherol, β-sitosterol and stigmasterol were found to have effectual anti-diabetic chattels (Jamaluddin *et al*., 1994; Gupta *et al*., 2011). In essence, Majority of the antioxidant enzymes remains under the transcription control of Nrf2 (Ma, 2013) and known to protect against the onset of diabetes (Uruno *et al.*, 2013) and diabetes-related progressive fatty liver diseases (Bataille and Manautou, 2012). Therefore, binding patterns of selected phytochemicals with Nrf2 protein (5FNQ) could be a rational way to investigate anti-diabetic activities of the extracts using molecular docking studies. It has been well-exposed that being inactivated Nrf2 remain seized in cytosol binding with kelch-like ECH-associated protein-1 (Keap1) causing ubiquitination and proteasomal degradation of Nrf2 under physiological condition. During oxidative stress, Nrf2 dissociates form Keap1 by means of Nrf2 phosphorylation or Keap1 modification, thereby activation of Nrf2 take place (Bryan *et al.*, 2013; Klaassen and Reisman, 2010). Once Nrf2 activates, it cannot be ubiquitinilated and translocates into the nucleus interacting with antioxidant response element (ARE) forms a heterodimer with Maf protein which promote the expression of cytoprotective target genes including antioxidant enzymes such as, catalase, SOD and phase II detoxifying enzymes (Zhang *et al.*, 2013). Enhanced activation of Nrf2 by pharmacologic molecules such as BHA, oleanolic acid, ursolic acid and CDDO-Im has been reported to show hepatoprotection against oxidative stress-

Table 4.17. Configurations employed in docking technique for anti-diabetic drugability prediction.

Ligand(s)	X-dimension	Y-dimension	Z-dimension	X-center	Y-center	Z-center
Alpha-tocopherol	120	120	120	15.833	25.778	2.496
Gamma-Tocopherol	120	120	120	12.522	24.23	2.69
β-sitosterol	120	120	120	13.325	25.326	2.36
Catechol	120	120	120	15.268	26.358	5.369

mediated liver damage (Klaassen and Reisman, 2010). Based on this view, a selective identified phytocompounds (catechol, β-sitosterol, α-tocopherol and γ-tocopherol) were docked with 5FNQ protein (Table 4.17) to obtain a clear picture of their underlying mechanism.

Result revealed found α-tocopherol (-7.2 kj/mol) and γ- tocopherol (-7.1 kj/mol) to be the most favored lead molecule (Table 4.18) to bind with 5FNQ at its active site determined by active site prediction server (http://www.scfbio-iitd.res.in/dock/ActiveSite.jsp). In addition to tocopherols, β-sitosterol and catechol were also accounted to be the potent candidates against diabetes (Fig. 4.29) and ROS damage (Gupta *et al*., 2011).

The docking showed that both α- and γ-tocopherols bind at "cavity 1" with amino acid sequence "RHFPTQGSYDLNMAVCI", whereas beta-sitosterol bind (-6.3 kj/mol) with "cavity 3" and cathechol bind (-5.2 kj/mol) with "cavity 6". Henceforth, it may postulate that stiff binding of these selected lead molecules with their active sites or cavities stimulates Nrf2 claiming an effectual protein-ligand interaction (Niture *et al.*, 2014). This result was well-justified by the findings of Niture *et al.* (2014) and Huang *et al.* (2012) who showed that α-tocopherol and γ-tocopherol may act as antagonist to Keap1-Nrf2 interaction resulting dissociation of Nrf2, thereby activate Nrf2 and neutralize the oxidative stress. Therefore, it could be assumed that due to presence of these phytochemicals, ANL could activate Nrf2 facilitating anti-oxidative functions (Fig. 4.30) which ultimately counteract liver injury or diabetic complications and eventually, *A. nilotica* could be treated as future anti-diabetic candidate.

Table 4.18. Chemical properties of selected ligands chosen to be docked with 5FNQ protein.

Docked Protein	Ligand(s)	Molecular weight	Hydrogen bond donor	Hydrogen bond acceptor count	XLogP3-AA
5FNQ	α-tocopherol	430.717 g/mol	1	2	10.7
	γ-Tocopherol	416.69 g/mol	1	2	10.3
	β-sitosterol	414.718 g/mol	1	1	9.3
	Catechol	110.112 g/mol	2	2	0.9

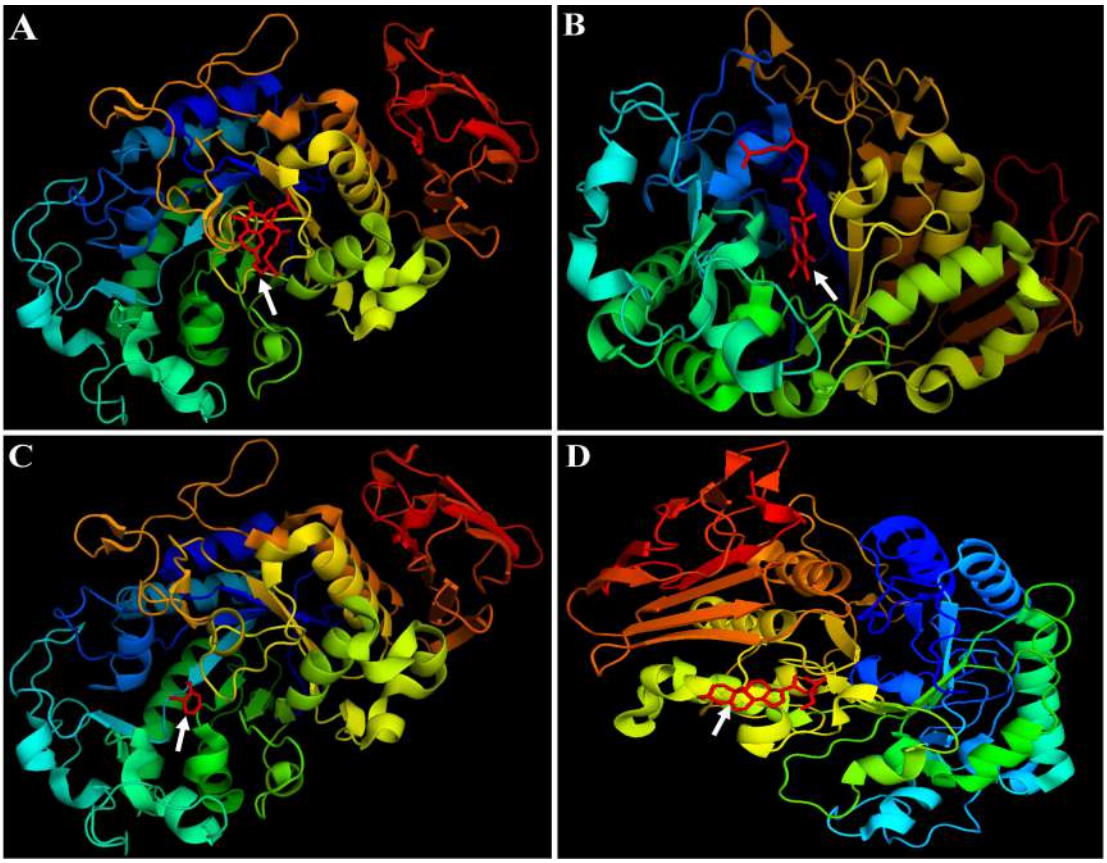


Fig. 4.29. *In-silico* docking representation of ligands such as α-tocopherol **(A)**, γ- tocopherol **(B)**, β-sitosterol **(C)** and catechol **(D)** with Nrf2 transcriptor protein (5FNQ).

4.9. Molecular diversity of different Mimosoids

4.9.1 DNA extraction, purification and quantification

The DNA from 9 Mimosoids was isolated using the standard protocol of Doyle and Doyle (1987) with minor modifications. The DNA-CTAB complex provided a network of whitish precipitate of nucleic acids after proper removal of impurities and further used for downstream processing. Agarose gel analysis of those DNA thus obtained exhibited distinct and

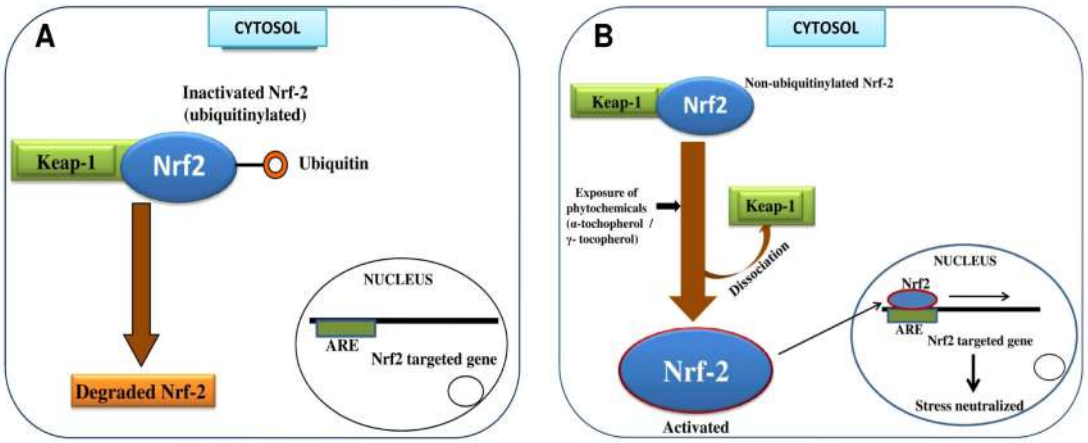


Fig. 4.30. Probable diagrammatic representation of Nrf2 action in the management of diabetes. **(A)** Inactivated Nrf2 and **(B)** Activated Nrf2.

clear bands.

Crude DNA is basically mixed with many contaminants including RNA, protein, polysaccharides etc. which lead to enzymatic reaction with DNA. Therefore, DNA purification is prerequisite step before performing downstream analysis like PCR amplification, DNA restriction and gene cloning. Inclusion of CTAB method in DNA extraction process helps to eliminate polysaccharides from DNA precipitations to a large extent. Subsequently, extraction with phenol:chloroform:isoamyl alcohol indicates the removal of protein impurities from the DNA samples. Further, RNAase enzyme is used to remove RNAs from samples.

In the present study, 2 different types of quantification methods were followed to analyze the quality of DNA. First one is spectrophotometric method and the other one is agarose gel electrophoresis. In spectrophotometric method, the DNAs were quantified in a UV spectrophotometer with 260 nm and 280 nm filters. The results were calculated as the ratio of A_{260}/A_{280} after performing of six replicates and the samples considered only showing a ratio of around 1.8 (Table 4.19).

In gel electrophoresis, the intactness of the DNA was determined with the help of 0.8% Agarose gel electrophoresis using λ DNA/*Eco*RI/*Hind*III double digest indicating molecular weight of the sample DNA (Fig. 4.31). The samples with relatively larger bands were chosen for further downstream process.

Hence, the combination of the above mentioned three steps i.e. extraction, purification and quantification allowed sufficient amount of pure DNA from the leaves of different Mimosoids for PCR amplification.

Table 4.19. List of samples showing their purity

Sample ID	A_{260}/A_{280} ratio for purity
M1	1.75
M2	1.77
M3	1.84
M4	1.78
M5	1.81
M6	1.85
M7	1.72
M8	1.81
M9	1.72

4.9.2 Random Amplified Polymorphic DNA (RAPD) analysis

RAPD is routinely used technique to evaluate the genetic relationship among species, varieties and cultivars. Initially, 45 different decamer primers have been used to study the genetic diversity (RAPD analysis) of 9 species under Mimosoideae (Raj *et al.*, 2011). Out of the 45 primers screened, 23 resulted distinct and scorable bands ranging from 190 bp to 1763 bp (Table 4.20).

A total of 330 bands were generated of which all are polymorphic bands. Interestingly, the percentage of polymorphism was found to be 100% and the number of polymorphic bands (Table 4.20) generated by each decamer primers ranged in between 2 (OPB13) and 20 (OPA01). The RAPD profile of the 9 accessions of Mimosoideae generated using primers OPA 01, OPA 16, OPB 13 and OPN 05 are represented in Fig. 4.32.A -D. A similarity matrix was further drawn using Dice coefficient of similarity (Nei and Li, 1979) ranging from 0.528 to 0.867 (Table 4.21).

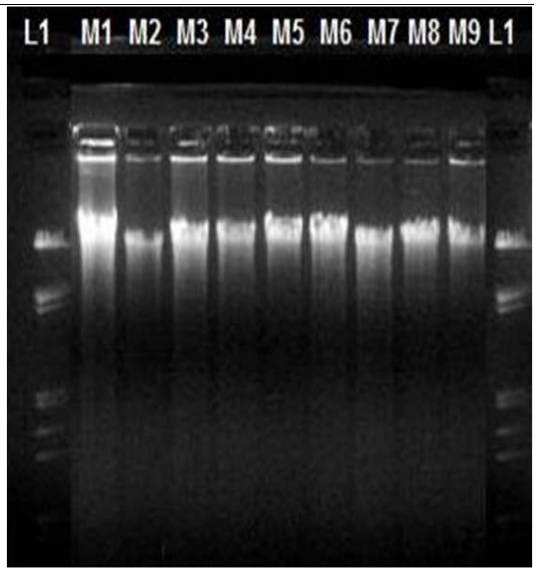


Fig. 4.31. Crude DNA of different Mimosoid samples (Lane M1-M9: different samples of Mimosoids under study (Refer **Table 3.2** for the name of species); L1: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder.

Table 4.20. Total number and size of amplified bands, number of monomorphic and polymorphic bands generated and percentage of polymorphism generated by the RAPD primers.

Primer ID	Sequence (5'-3')	Total bands amplified	Monomorphic bands	Polymorphic bands	Percentage of polymorphism	Band size (bp)
OPA01	CAGGCCCTTC	**20**	0	20	100%	1327-269
OPA02	TGCCGAGCTG	18	0	18	100%	1445-247
OPA03	AGTCAGCCAC	13	0	13	100%	1486-336
OPA04	AATCGGGCTG	12	0	12	100%	1429-455
OPA07	GAAACGGGTG	18	0	18	100%	1600-279
OPA10	GTGATCGCAG	14	0	14	100%	1471-274
OPA11	CAATCGCCGT	11	0	11	100%	1314-214
OPA12	TCGGCGATAG	10	0	10	100%	1422-421
OPA13	CAGCACCCAC	15	0	15	100%	1244-205
OPA16	AGCCAGCGAA	11	0	11	100%	1500-315
OPA17	GACCGCTTGT	16	0	16	100%	1613-232
OPA18	AGGTGACCGT	16	0	16	100%	1450-285
OPA19	CAAACGTCGG	8	0	8	100%	1500-225
OPA20	GTTGCGATCC	19	0	19	100%	1638-196
OPB01	GTTTCGCTCC	14	0	14	100%	1422-413
OPB11	GTAGACCCGT	10	0	10	100%	1485-320
OPB12	CCTTGACGCA	5	0	5	100%	1125-**190**
OPB13	TTCCCCCGCT	**2**	0	2	100%	1085-250
OPF09	CCAAGCTTCC	15	0	15	100%	1500-248
OPG19	GTCAGGGCAA	15	0	15	100%	1525-210
OPN05	ACTGAACGCC	12	0	12	100%	1571-266
OPN13	AGCGTCACTC	18	0	18	100%	1725-317
OPN19	GTCCGTACTG	8	0	8	100%	**1763**-524
	Total	**330**	**0**	**330**	**100%**	

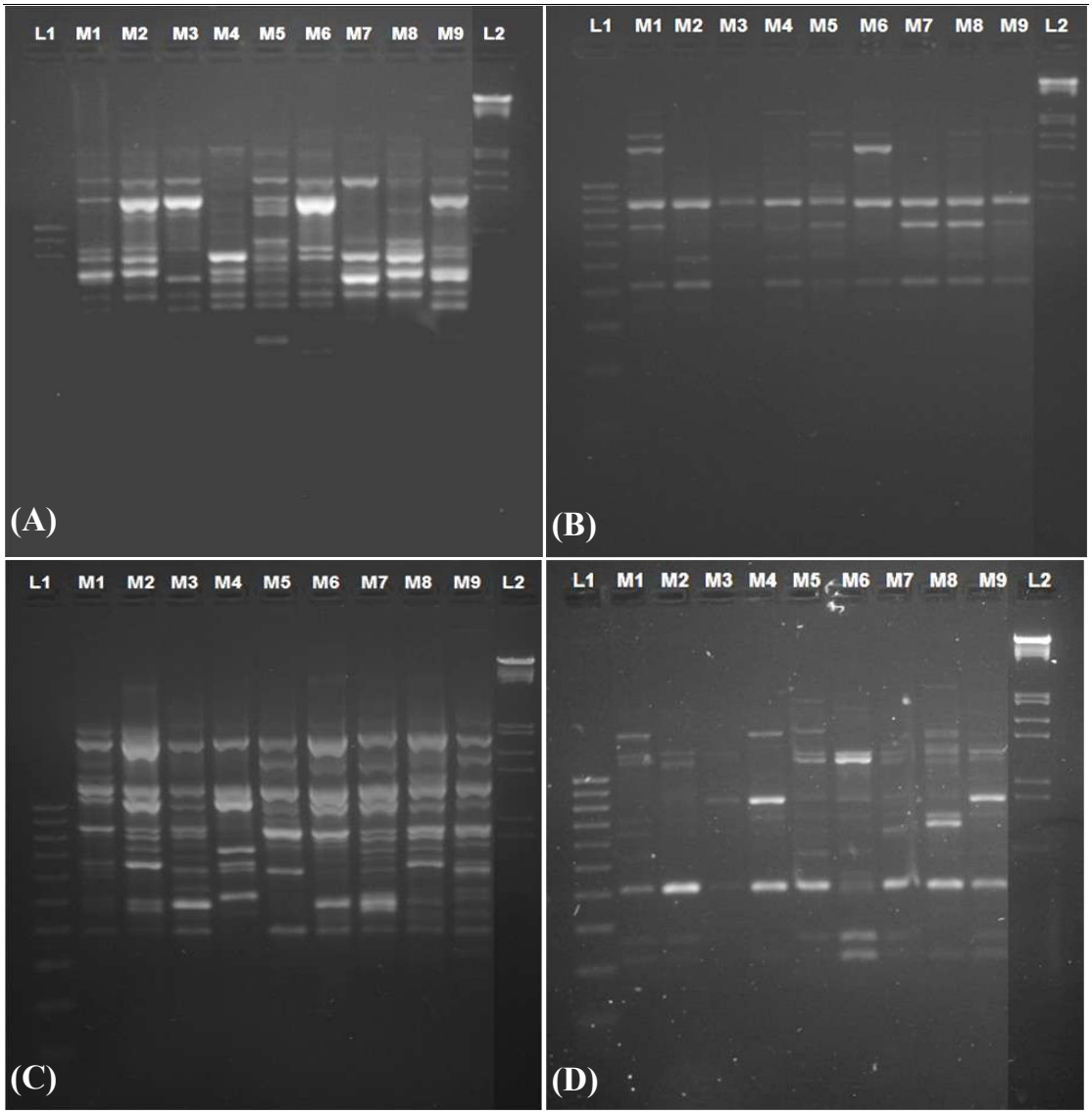


Fig. 4.32. Representatives of RAPD profiling of 9 accessions of Mimosoids amplified with **(A)** OPA01, **(B)** OPA 16, **(C)** OPB 13 and **(D)** OPN 05 primers. Lane L1: 100 bp molecular marker; Lane M1-M9 different accessions of Mimosoids under study (refer table 3.2); Lane L2: λ DNA/EcoRI/HindIII double digest DNA ladder.

The lowest similarity was observed between *Mimosa pudica* and *Albizia lebbeck*, while the highest value was recorded between *Acacia catechu* (*Senegalia catechu*) and *A. nilotica*. A dendrogram was constructed on the basis of the data obtained from RAPD analysis using NTSYSpc (Fig. 4.33).

In fact, RAPD markers are the most suitable ones to analyze the genetic variation of both intra and inter-population (Li *et al.*, 2008). The dendrogram prepared from RAPD analysis revealed that the members of *Mimosa*, *Acacia*, *Albizia* and *Samanea* formed a group in which members of *Acacia* were found to be form a loose sub-group. *Acacia nilotica* and *A. catechu* shared a node at 86.7% whereas *A. concinna* exhibited a cluster with *A. nilotica* and *A. catechu* sharing a node at 84.9% and 85.8% respectively. In addition, *Albizia lebbeck* appeared as a distinct

Table 4.21. The similarity matrix obtained using Dice coefficient of similarity among the 9 species of Mimosoideae based on RAPD profiling.

	M1	M2	M3	M4	M5	M6	M7	M8	M9
M1	1								
M2	0.616	1							
M3	0.622	0.651	1						
M4	0.584	0.654	0.684	1					
M5	0.648	0.643	**0.867**	0.687	1				
M6	0.589	0.613	0.849	0.634	0.858	1			
M7	**0.528**	0.545	0.587	0.595	0.613	0.572	1		
M8	0.625	0.631	0.66	0.634	0.663	0.628	0.648	1	
M9	0.631	0.648	0.672	0.669	0.699	0.657	0.637	0.7	1

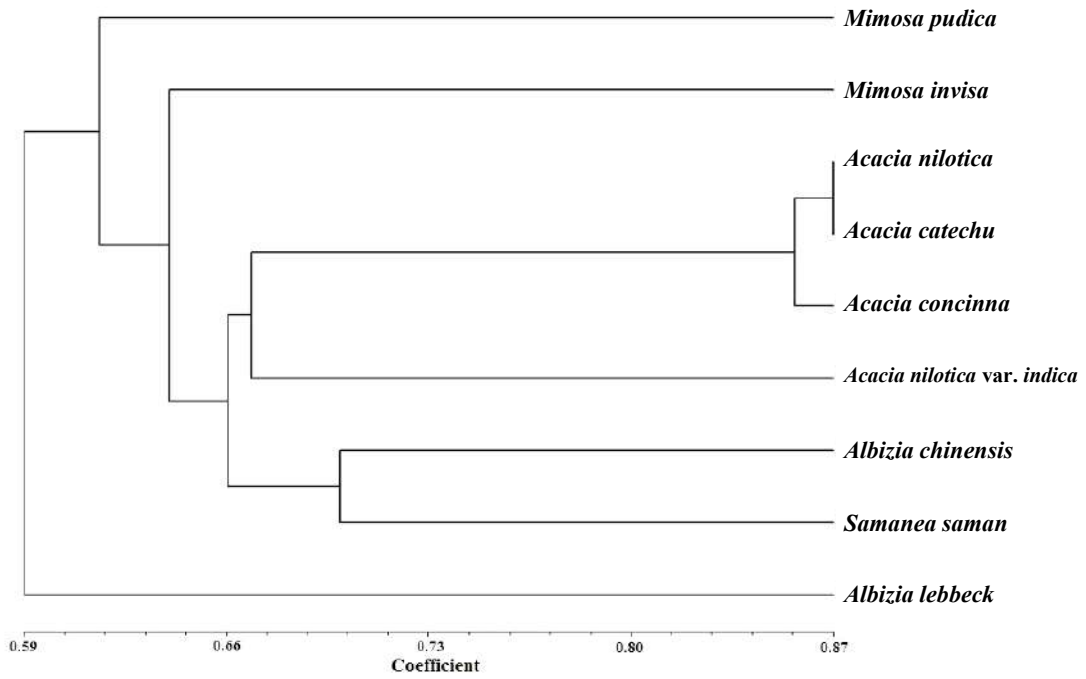


Fig. 4.33. Dendrogram obtained from UPGMA cluster analysis of RAPD markers illustrating the genetic relationships among the 9 accessions of Mimosoids.

outgroup in the dendrogram. The correspondence analysis of both 2D (Fig. 4.34) and 3D (Fig. 4.35) plotting and corroborated the cluster analysis result.

Hence, it might be inferred that RAPD markers are praiseworthy for analyzing genetic variations among the species and could be utilized as molecular taxonomic characters to analyze the genetic relationships among the species of Mimosoideae. Similar results were also documented by Sulain *et al*. (2013) using RAPD analysis where *M. pudica* was found to be closely related to *M. pigra* and *M. invisa*. Nanda *et al*. (2004) also reported the genetic relationships of six Acacia species using RAPD in which *A. farnesiana* and *A. catechu* were the closest

member sharing with 30% similarity whereas *A. auriculiformis* and *A. concinna* shared about 28% and 18% similarity, respectively, with the cluster formed by *A. farnesiana* and *A. catechu*. Hence, the RAPD markers exhibited the potentiality to conserve the identified clones as well as

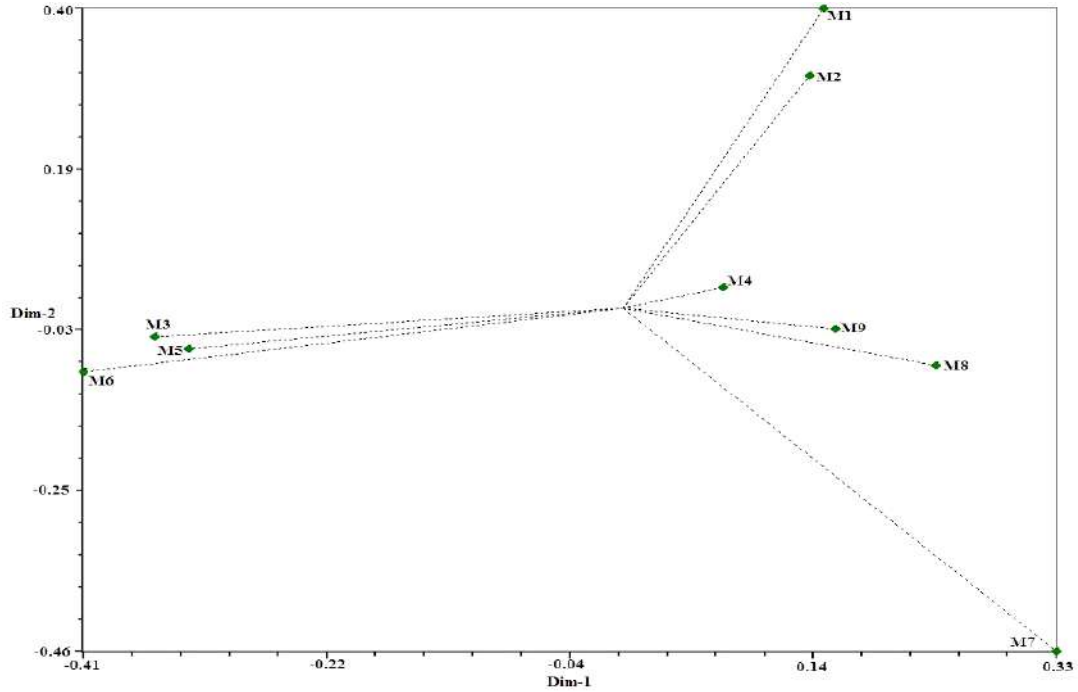


Fig. 4.34. Principal co-ordinate analysis of 9 species of Mimosoids based on RAPD analysis data representing 2-dimensional plot.

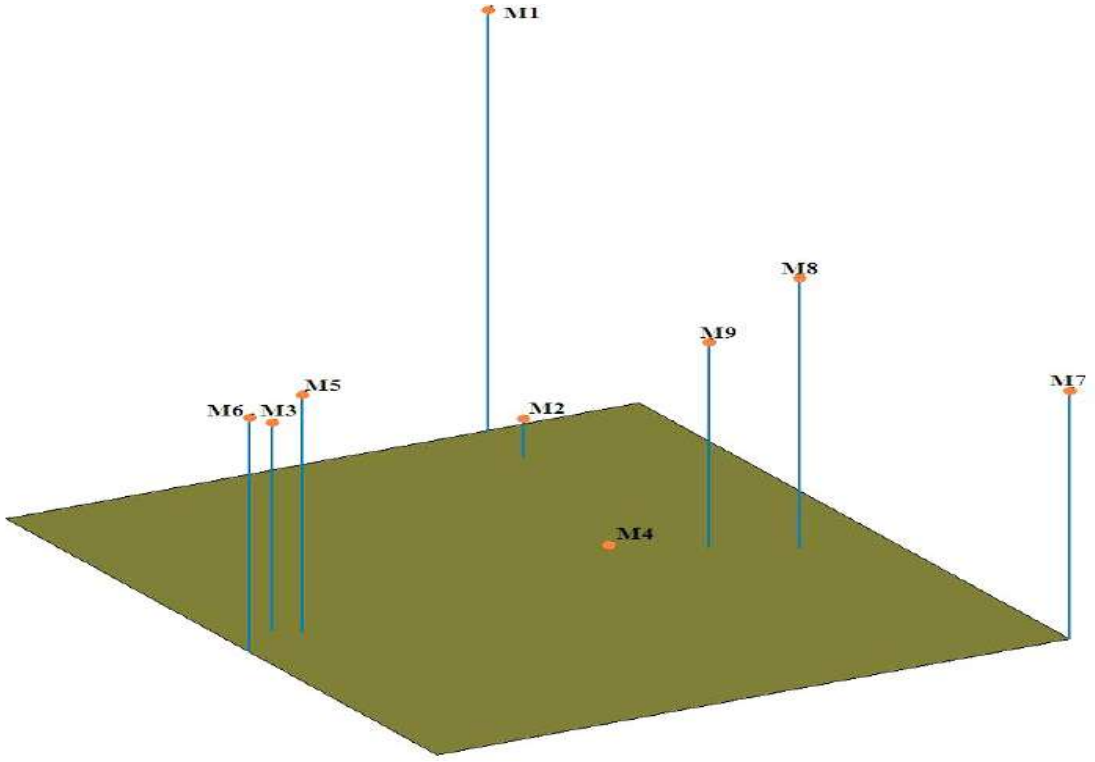


Fig. 4.35. Principal co-ordinate analysis of 9 species of Mimosoids based on RAPD analysis data representing 3-dimensional plot.

to characterize the genetic relatedness among the species of Mimosoids.

4.9.3. PCR-RFLP analysis of the trnL-trnF region of Mimosoids chloroplast genome

PCR-RFLP is a simple and cheap method playing a vital role in accessing the genetic diversity of different plant species. Therefore, it is applied to study the fingerprinting of selected species under the subfamily Mimosoideae found in Northern provinces of Bengal.

4.9.3.1. PCR amplification

In the present study, 9 species from the subfamily Mimosoideae were employed to PCR amplification with locus specific primer pair Tab c 5′-CGAAATCGGTAGACGCTACG-3′and Tab f 5′-ATTTGAACTGGTGACACGAG -3′ developed based on the Tab c-f in "Taberlet" (TrnL-TrnF) region of the chloroplast genome of different Mimosoids for which the nucleotide information was available with respect to other plant species in the public domain. The primer pair effectively amplified the Tab c-f in "Taberlet" (TrnL-TrnF) region of the Inter-Generic Spacer (IGS) region of the species of Mimosoideae. The amplified product is shown in Fig. 4.36.

4.9.3.2. PCR product restriction digestion and agarose gel analysis

The PCR product obtained from the prime pair Tab c-f were subjected to restriction digestion using 5 different restriction enzymes like *Eco*RI, *Taq*I, *Hinf*I, *Hae*III and *Hpa*I to short out the degree of genetic variation among different species under Mimosoideae. Amongst 8 enzymes studied, 3 restriction enzymes (*Alu*I, *Mbo*I and *Msp*I) were found to be unsuccessful to digest the PCR products while the other five restriction enzymes produced a total of 20 polymorphic bands (Table 4.22).

Digestion with *Eco*RI resulted in producing three bands except *Albizia lebbeck* and *Samanea saman* where only one band was generated. The total percentage of polymorphism was found to be 86.96%. Among the five restriction enzymes, *Hae*III, *Hpa*I and *Hinf*I produced four bands whereas *Taq*I and *EcoR*I reproduced six and two bands respectively. The percentage of polymorphism was found to be 100% in case of *Hae*III, *Hpa*I and *Taq*I while *Hinf*I and *Eco*RI showed 66.66% polymorphism (Fig. 4.37.A-D).

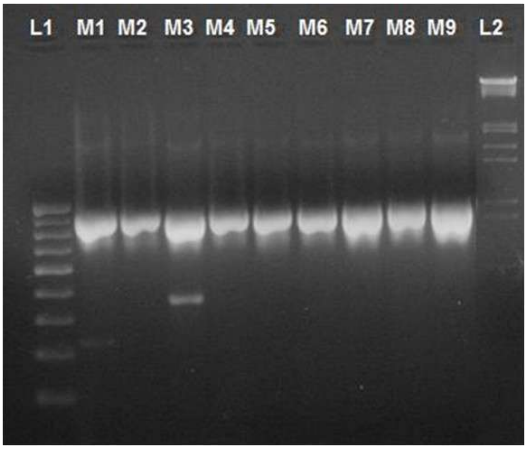


Fig. 4.36. Amplification of 9 species of Mimosoids with primer Tab c-f (TrnL-TrnF). Lane L1: 100bp DNA ladder; Lane M1-M9: Different species of Mimosoids as listed in **Table 3.2** and L2: λ DNA/ EcoRI/HindIII double digest DNA ladder.

Table 4.22. Total fragments, number of polymorphic bands generated by using different restriction enzymes.

Restriction enzyme	Optimum temperature	No. of cuts	No. of polymorphic bands	Percentage of polymorphism
Eco RI	37ºC	3	2	66.66%
Hin fI	37ºC	6	4	66.66%
Hae III	37ºC	4	4	100%
Hpa I	37ºC	4	4	100%
Taq I	65ºC	6	6	100%
		23	**20**	**86.96%**

4.9.3.3. PCR-RFLP data analysis

A total of 23 scorable bands were produced by the various restriction digestion enzymes. Of the 23 cuts 20 were polymorphic. The number of polymorphic bands ranged from two in *Eco*RI and six in *Taq*I. These clear and distinct bands were scored and used for further analysis (Table 4.22).

A dendrogram was prepared (Fig. 4.38) on the basis of similarity estimates using the unweighted pair group method with arithmetic average (UPGMA) using NTSYSpc version (2.0) (Rohlf, 1998b).

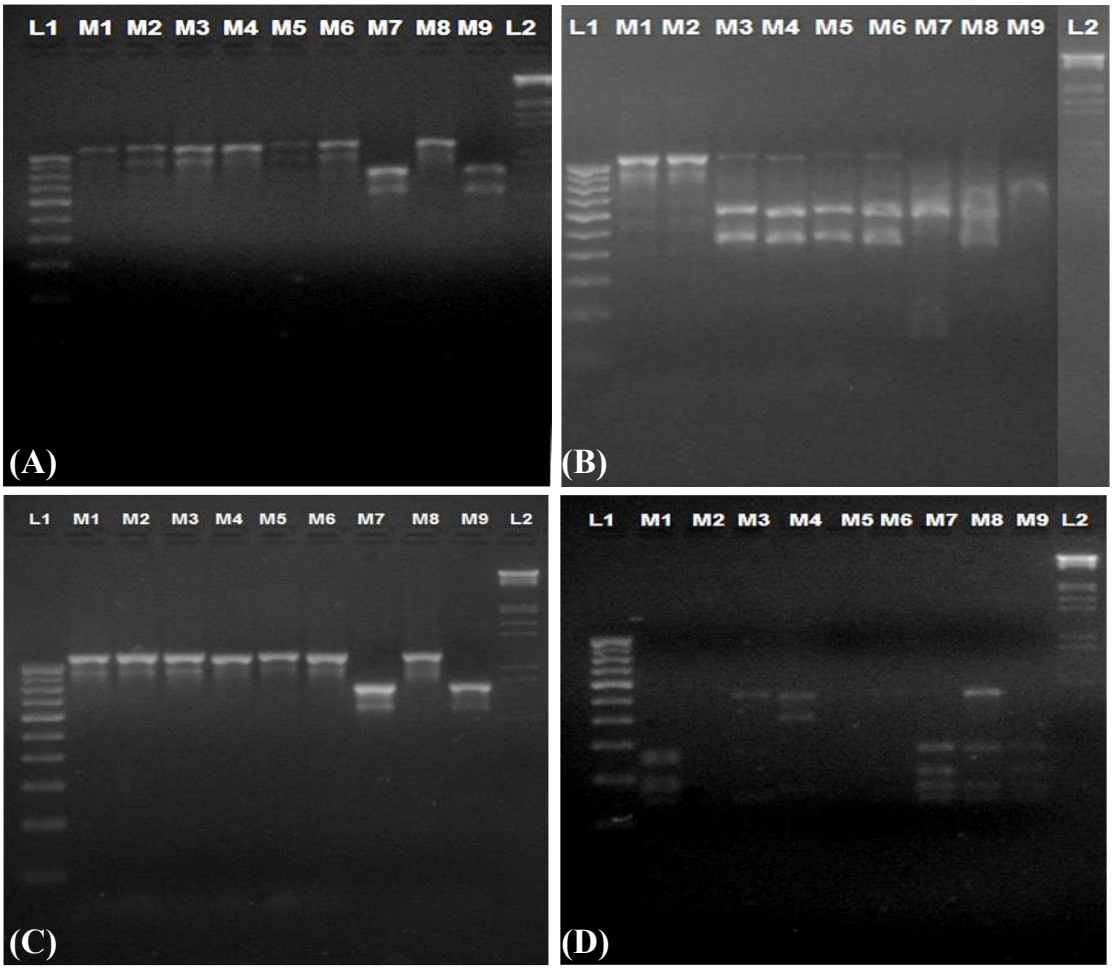


Fig. 4.37. Restriction digestion products of TrnL-TrnF region of chloroplast genome by **(A)** *Eco*RI , **(B)** *Hae*III , **(C)** *Hinf* I and **(D)** *Taq*I. Lane L1: 100 bp molecular marker; Lane M1-M9 different accessions of Mimosoids under study (refer table 3.2); Lane L2: λ DNA/EcoRI/HindIII double digest DNA ladder.

The dendrogram revealed two clusters; one of which consisted of 7 species sub-dividing into two groups. The first group comprises with 3 species (*Mimosa pudica*, *M. invisa* and *Albizia chinensis*) with a high level of genetic similarity (77%) between *M. pudica* and *M. invisa* (Table 4.23). The second group consisted of 4 species (*Acacia nilotica*, *Acacia nilotica var indica*, *Acacia catechu*, *Acacia concinna*). Interestingly, similar type of closeness among the four species of *Acacia* was also observed in the RAPD-dendrogram.

The second cluster is made up of two species i.e. *Albizia lebbeck* and *Samanea saman* with a genetic similarity of 100%. Both the 2D and 3D plot (Fig. 4.39.A-B) of the correspondence analysis of the RFLP data corroborated the dendrogram. Henceforth, from the above analysis it could be inferred that in the present study notable polymorphism (86.96%) has been found among the selected species due to their polyphyletic nature of the different genera under Mimosoideae.

4.9.4 DNA barcoding analysis

DNA barcoding is a novel and innovative technique which can be used to explore the evolution, identification and genetic relatedness of unknown plants and animal species by using a short stretch of DNA sequence (Hebert and Gregory, 2005). Chloroplast and mitochondrial genes are being recently used to study the sequence variation at generic and species level. The chloroplast genes such as matK and TrnL-F have been utilized by various workers to study the plant evolutionary pattern as well as to resolve various anomalies in the taxonomic levels.

4.9.4.1. Sequencing of PCR-product and Submission to GenBank

Since sequencing of matK and TrnL-F region of *Acacia catechu*, *A. nilotica*, *A. nilotica* var *indica*, *Albizia lebbeck* and *Samanea saman* were found in gene bank, PCR amplification of only 4 species were

Table 4.23. The similarity matrix obtained using Dice coefficient of similarity among the 9 species of Mimosoideae based on PCR-RFLP profiling.

	M1	M2	M3	M4	M5	M6	M7	M8	M9
M1	1.00								
M2	0.77	1.00							
M3	0.59	0.66	1.00						
M4	0.59	0.66	0.92	1.00					
M5	0.59	0.81	0.85	0.85	1.00				
M6	0.59	0.81	0.85	0.85	1.00	1.00			
M7	0.48	0.25	0.37	0.37	0.37	0.37	1.00		
M8	0.77	0.55	0.74	0.66	0.59	0.59	0.48	1.00	
M9	0.48	0.25	0.37	0.37	0.37	0.37	1.00	0.48	1.00

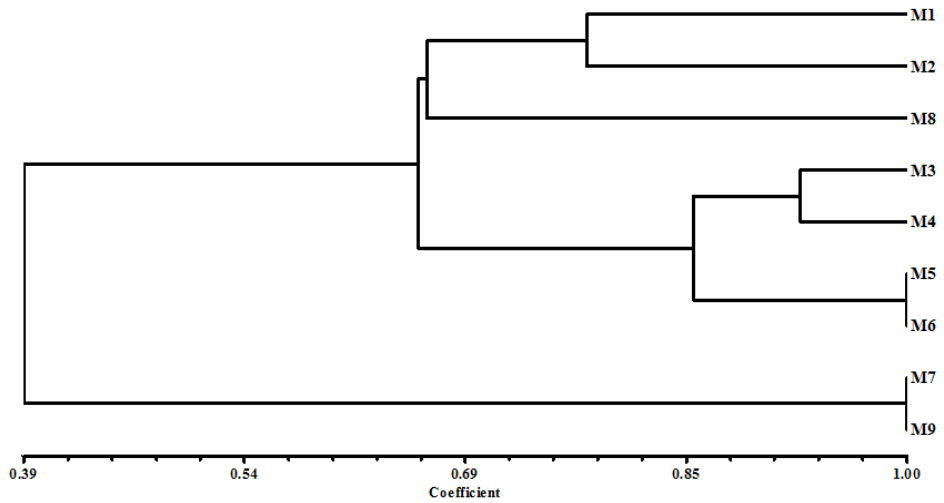


Fig. 4.38. A dendrogram based on the restriction digestion products data of the TrnL-TrnF region of 9 species under Mimosoideae.

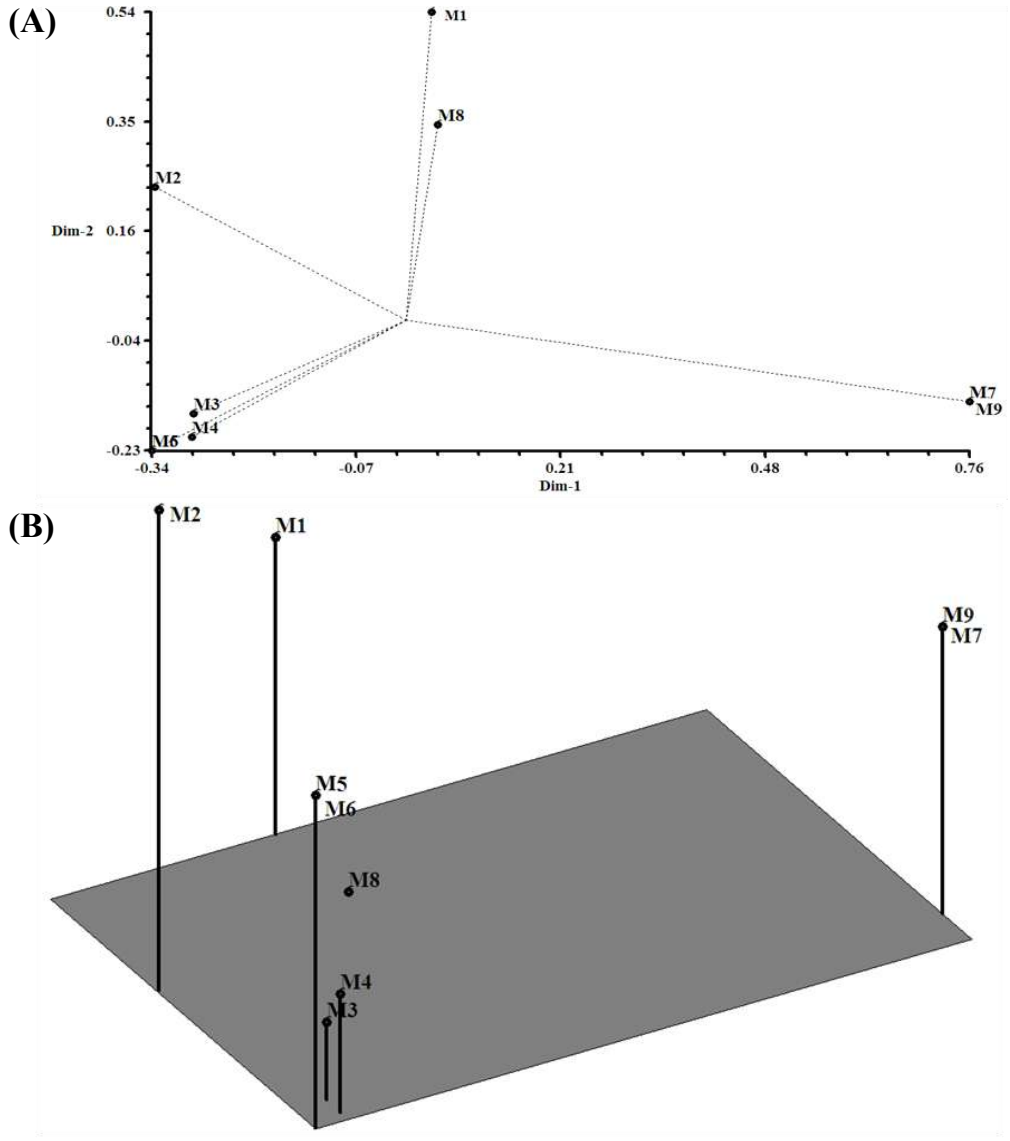


Fig. 4.39. Principal coordinates analysis of 9 species of Fabaceae based on restriction digestion products of TrnL-TrnF region of chloroplast genome. **(A)** 2-dimensional plot and **(B)** 3-dimensional plot.

performed including *Mimosa pudica*, *M. invisa*, *Acacia concinna* and *Albizia chinensis* (Table 4.24). A total of 6 samples (3 matK and 3 TrnL-F) of above mentioned species were sequenced from Chromous Biotech Pvt. Ltd, Bangalore for both the forward and reverse primers individually. The sequence analysis resulted in an average of 790 bp for each reaction. Nucleotide BLAST was further performed for each of the obtained sequence to find out the homology with the sequences already present in the GenBank. The nucleotide BLAST showed 95-100% identity with the *Mimosa, Acacia* and *Albizia* sequences already available in the GenBank. After authentication of the sequences, they were submitted to the GenBank (Fig. 4.40.A-B). The list of different species of Mimosoids along with their GenBank accession number is given in Table 4.24.

4.9.5. Data analysis

Apart from RAPD analysis, DNA barcoding is another kind of taxonomic method that has become a rational approach for identifying million species of animals and plants, based on the analysis of short, standardized and universal DNA regions. Molecular documentation of different taxa and their validated systematic position in the respective family of plant kingdom had always been a challenging task. Chloroplast gene like matK and IGS region like TrnL-F could be pivotal to resolve this problem. In the present study, a few selected species under family Fabaceae (please refer Table 3.6) were employed to explore inter-generic and intra-generic differences using matK and TrnL-F locus. The phylogenetic analysis (Fig. 4.41.A-B) of the matK and TrnL-F region region revealed a close relationship among the selected taxa. Interestingly, Fig. 4.41.A discloses that *M. pudica* and *M. invisa* share 99% similarity while *Senegalia catechu* (Syn: *Acacia catechu*) and *Acacia concinna* share 96% similarity reflecting their close genetic relatedness as found in traditional classification (Cronquist, 1981).

Result also exhibited that a total of 2 major clades were formed; one of which consisted of Mimosoideae and Caesalpinioideae while other single one is Papilionoideae. Therefore, it can be attributed to the fact that the all the selected taxa under the subfamily Mimosoideae and Caesalpinioideae were clubbed together and shared more similarities with each other (94%) than the subfamily Papilionoideae (Fig. 4.41.A). What's more a similar trend was also observed in Fig. 4.41.B that the seven species from Mimosoideae and one species from Caesalpinioideae grouped together forming the first clade whereas the remaining six genera of Papilionoideae clubbed together to make the second clade demonstrating their different place within the family. The present genetical approach through matK and TrnL-F clearly reflected

Table 4.24. List of species with the submitted GenBank accession numbers for TrnL-TrnF and matK.

Sl. No.	Taxa	matK accession number	TrnL-F accession number
1.	*Mimosa invisa*	LM643807	LM643811
2.	*Mimosa pudica*	----	LM643810
3.	*Acacia concinna*	LM643808	----
4.	*Albizia chinensis*	LM643809	LM643812

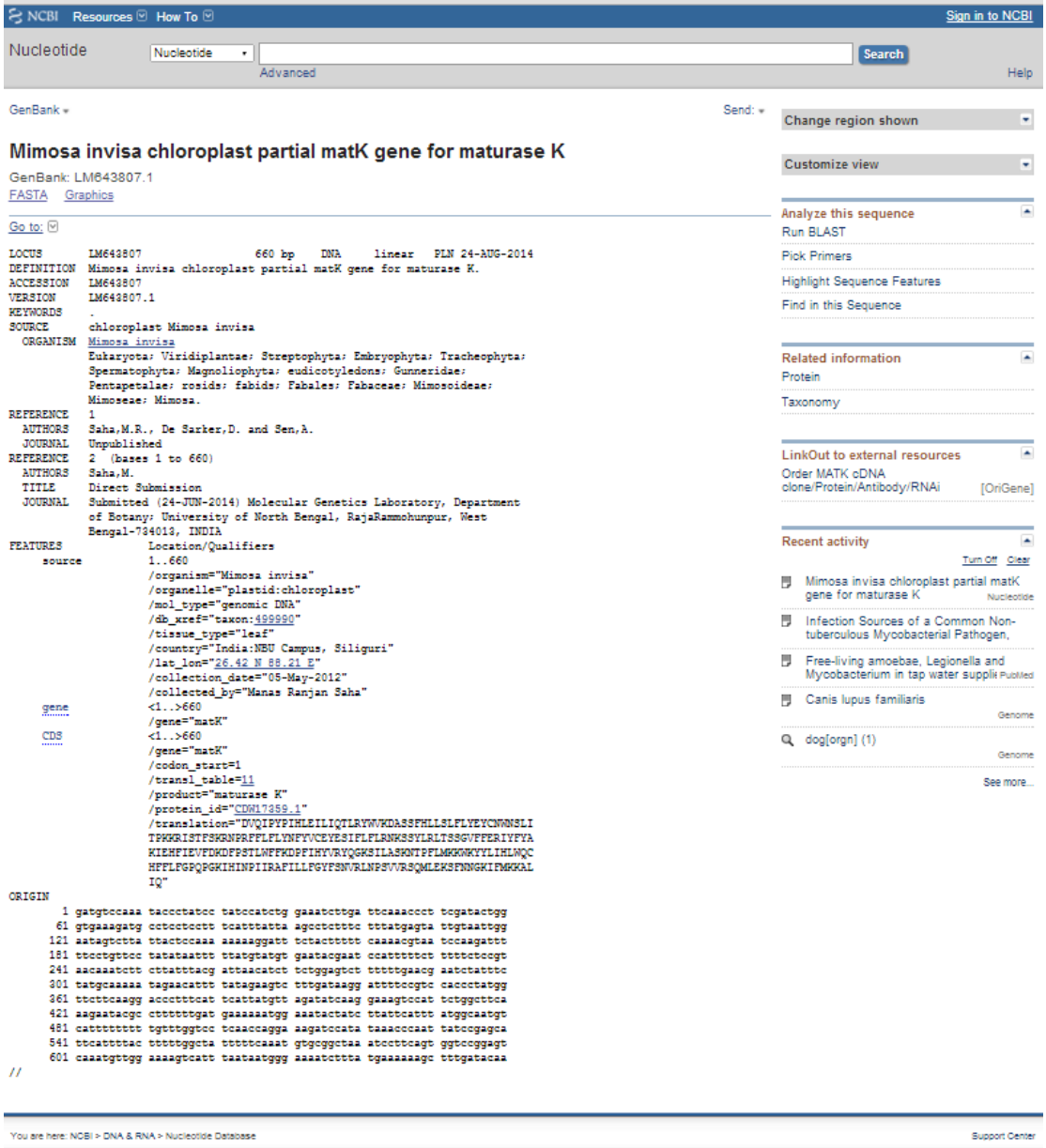


Fig. 4.40.A. Snapshot of partial matK gene sequence of *Mimosa invisa* submitted to GenBank (NCBI).

that the members of Mimosoideae and Caesalpinioideae are closer than the members from Papilionoideae validating the traditional classification (Cronquist, 1981). Hence, from the above illustration, it may conclude that DNA barcode serve a reliable genetical approach to place the morphologically similar or dissimilar or

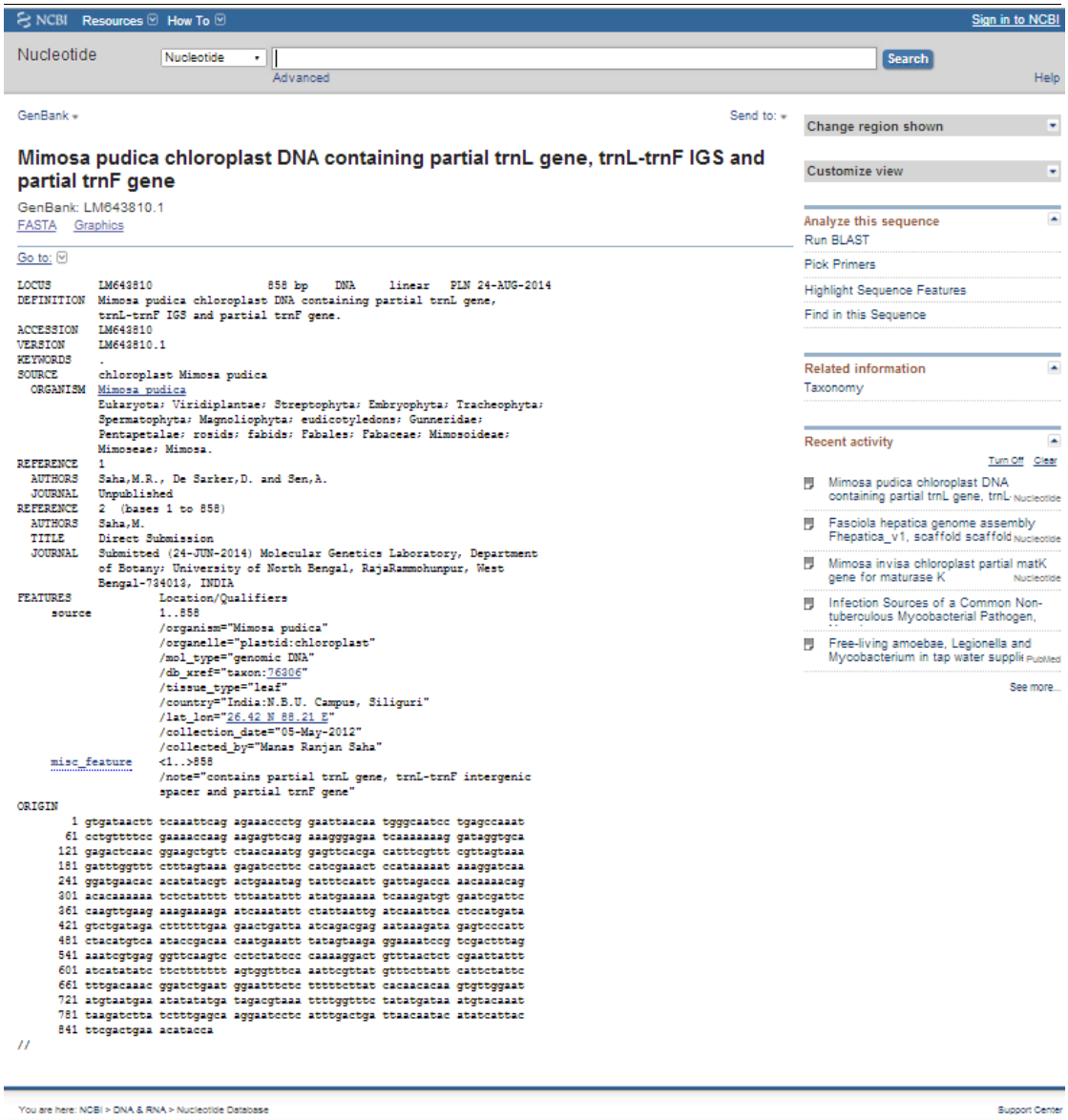


Fig. 4.40.B. Snapshot of partial trnL-trnF Intergenic Spacer (IGS) sequence of *Mimosa pudica* submitted to GenBank (NCBI).

disputed taxa into its appropriate systematic position.

In summary, the present study of DNA profiling in selected Mimosoideae clearly showed that it was possible to analyze the RAPD patterns for correlating their similarity and distance between species by which one could predict the origin of the species to a great extent. Further, the application of DNA barcode like matK and TrnL-F became more practical for defining the uniqueness as well as helpful in validation of systematic position of species and taxa identification (Schaferhoff *et al.*, 2010; Selvaraj *et al.*, 2008).

4.10. Exploration of medicinal and diversity of microsymbionts

4.10.1. Medicinal aspect

4.10.1.1. In-vitro antioxidative assay

In-vitro free radical scavenging activity between root and root-nodule collected

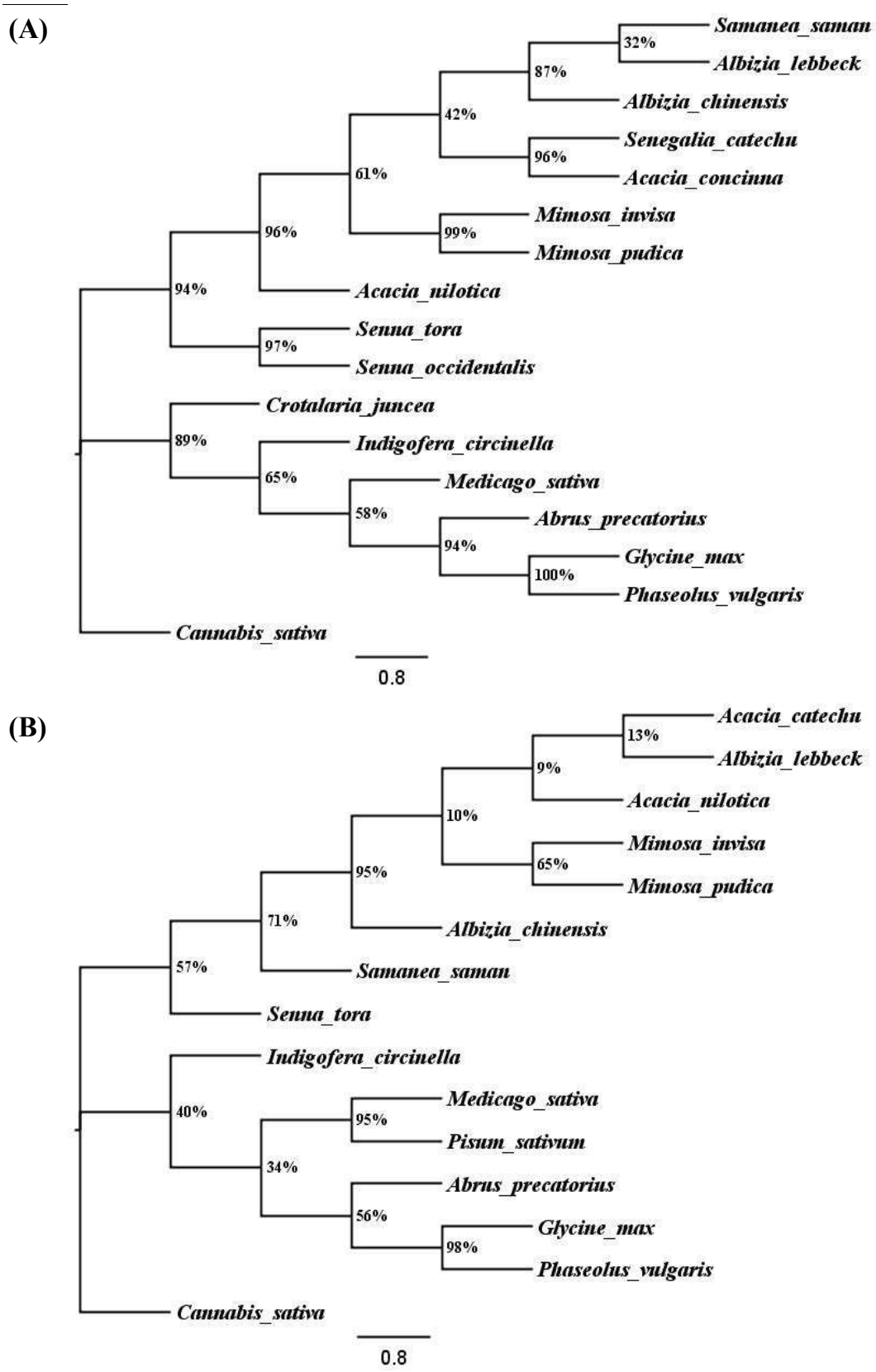


Fig. 4.41. Most parsimonious tree (neighbour joining method) showing the relationship of matK region of 16 different taxa **(A)** and TrnL-F region of 15 different taxa **(B)** belonging from subfamilies, Mimosoideae, Papilionoideae and Caesalpinioideae within the family Fabaceae and one outgroup taxa from Cannabaceae. The tree indicates the phylogenetic evolution of different subfamilies Mimosoideae; Papilionoideae; Caesalpinioideae. Numbers at nodes indicate the bootstrap values.

from *M. pudica*, *M. invisa* and *A. nilotica* were evaluated separately through DPPH, hydroxyl radical and nitric oxide scavenging assay. Result revealed lower percentage of inhibitory activity in all cases (< 30% at 200µg/ml) in comparison with leaf extract. Between root and root-nodule extract, we found hardly a little difference (Table 4.25) suggesting that the presence of *Rhizobium* in the root-nodule failed to change the antioxidant properties of the plant part. Therefore, no further initiative was carried out.

4.10.2. Molecular documentation

Since no difference was obtained in antioxidative profiling of roots and root nodules, it was further perceived whether there is any genetical variation among micro-symbionts. Therefore, root nodules of *M. pudica*, *M. invisa* and *A. nilotica* were collected from two different geographical locations of Bengal; one is from Malda and another one from Sibmandir and subsequently *Rhizobium* was collected from pure culture. Afterward, isolation of genomic DNA was subjected.

4.10.2.1. DNA isolation, purification and quantification

4.10.2.1.1. DNA isolation

Rhizobium genomic DNA was isolated using the standard protocol of William and Feil (2012) with minor modifications. Prior to isolation, an efficient lysis is a prerequisite for good yield of nucleic acid for further downstream processing. The agarose gel analysis of the DNA thus obtained showed distinct and clear bands.

4.10.2.1.2. DNA purification

Crude DNA is basically mixed with many contaminants including RNA, protein, polysaccharides etc. which lead to enzymatic reaction with DNA. Therefore, DNA purification is prerequisite step before performing downstream analysis like PCR amplification, DNA restriction and gene cloning. Inclusion of CTAB method in DNA extraction process helps to eliminate polysaccharides from DNA precipitations to a large extent. Subsequently, extraction with phenol:chloroform:isoamyl alcohol (25:24:1) indicates the removal of protein impurities from the DNA samples. Further, RNAase enzyme is used to remove RNAs from samples.

4.10.2.1.3. DNA quantification

In the present study, 2 different types of quantification methods were followed to analyze the quality of DNA. First one is spectrophotometric method and the other one is agarose gel electrophoresis (Fig. 4.42). In spectrophotometric method, the DNAs were quantified in a UV spectrophotometer with 260 nm and 280 nm filters. The results were calculated as the ratio of A_{260}/A_{280} after performing of six replicates and the samples considered only showing a ratio of around 1.8 (Table 26).

Table 4.25. Antioxidant activities of root and root-nodule collected from different host.

Host	Root			Root-nodule		
	DPPH#	Hydroxyl#	NO#	DPPH#	Hydroxyl#	NO#
M. pudica	22.17±0.35	19.04±0.60	24.52±0.93	21.05±1.51	19.27±1.25	22.31±0.93
M. invisa	24.74±1.35	18.37±0.34	23.87±1.43	23.87±0.46	20.85±2.46	24.40±1.78
A. nilotica	21.57±2.62	20.26±0.84	23.21±0.89	22.81±0.67	21.46±0.78	22.98±1.34

inhibition percentage at 200µg/ml.

4.10.2.2. RAPD analysis

RAPD fingerprinting was used for studying the genetic diversity among indigenous *Rhizobium* population isolated from two distinct regions from northern parts of Bengal province in India (Please refer Table 3.7). In RAPD analysis, 12 different primers each of 10-mers in length have been used for the six isolated strains.

Table 4.26. List of *Rhizobium* samples showing their purity.

Sample ID	A_{260}/A_{280} ratio for purity
I	1.77
II	1.79
III	1.74
IV	1.76
V	1.81
VI	1.83

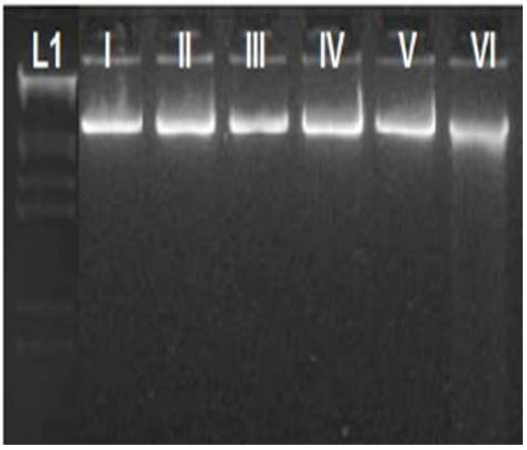


Fig. 4.42. Crude DNA of *Rhizobium*. Lane L1: λ DNA/ EcoRI/ Hind III double digest DNA ladder; Lane I-VI: different *Rhizobium* strains under study (please refer **Table 3.7** for the strain's name).

Out of the 12 primers screened, 7 revealed distinct and scorable bands of various intensities and the size of the bands was ranged from 134 bp to 2175 bp (Table 4.27).

The amplification patterns revealed 97.39% polymorphism and the number of polymorphic bands generated by each decamer primers ranged in between 12 (OPD-03) and 21 (OPQ-01). After analysis of the sequences of the primers, it was found that all the primers which failed to amplify were of AT-rich. It sounds reasonable now since the *Rhizobium* is largely a GC rich genus. A representative of RAPD profile of the 6 *Rhizobium* strains generated using OPA 18 and OPY 04 has been showed in Fig. 4.43.A-B.

A similarity matrix was further drawn using Dice coefficient of similarity (Nei and Li, 1979) ranging from 0.59 to 1.00 (Table 4.28). The lowest similarity was observed between isolate-I and isolate-III while the highest value was recorded between isolate-V and isolate-VI. A dendrogram was constructed on the basis of the data obtained from RAPD analysis using NTSYSpc (Fig. 4.44).

Herein, the dendrogram was divided into

Table 4.27. Total number and size of amplified bands, number of monomorphic and polymorphic bands generated and percentage of polymorphism generated by the RAPD primers.

Primer ID	Sequence (5'-3')	Band No	MB	PB	Pol %	Band size (bp)
OPA 02	TGCCGAGCTG	19	0	19	100%	385-**2175**
OPQ 01	GGGACGATGG	**21**	0	21	100%	165-1137
OPA18	AGGTGACCGT	17	0	17	100%	145-1875
CRL 7	GCCCGCCGCC	14	2	12	85.71%	230-1900
OPY 04	AAGGCTCGAC	20	0	20	100%	275-1813
DAF 9	CCGACGCGGC	12	0	12	100%	**134**-1024
OPD 03	GTCGCCGTCA	**12**	1	11	91.66%	338-1800
Total		115	3	112	97.39%	

MB= Monomorphic bands; PB= Polymorphic bands; Pol %= Polymorphism %

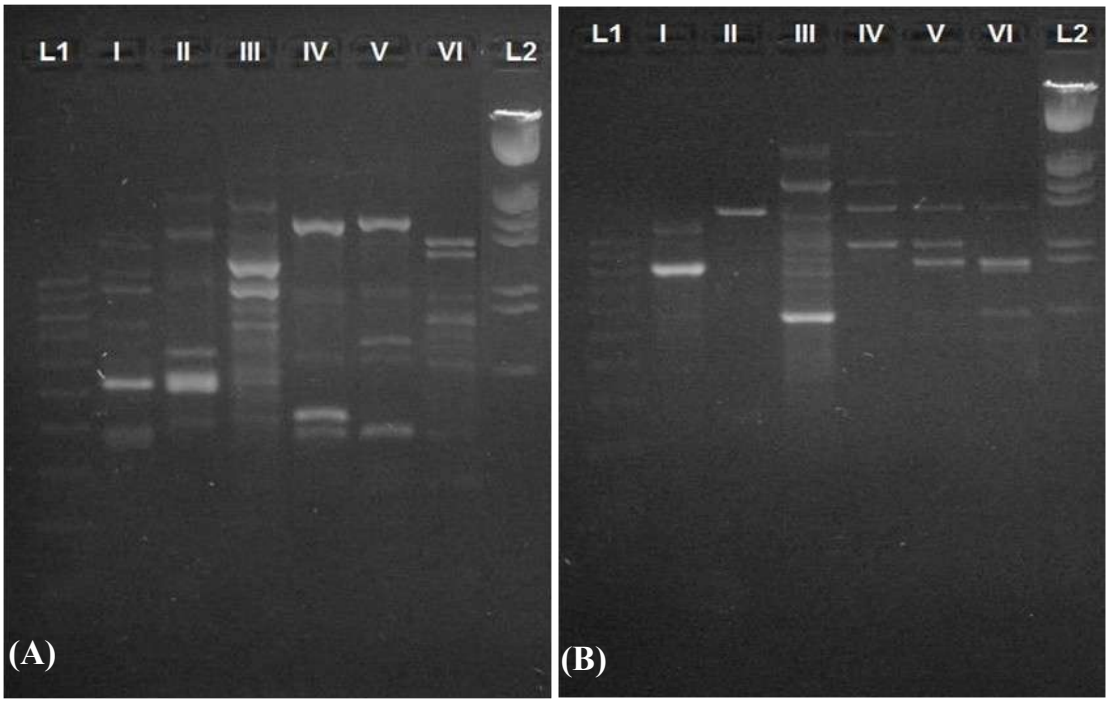


Fig. 4.43. DNA Gel showing the DNA bands of six *Rhizobium* strains amplified by the RAPD primers **(A)** OPA 18 and **(B)** OPY 04. Lane L1: 100 bp DNA ladder; Lane I-VI: different *Rhizobium* strains under study (**Please refer Table 3.7** for the strain's name); Lane L2: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder.

Table 4.28. The similarity matrix obtained using Dice coefficient of similarity among the 6 isolates of *Rhizobium* from 2 regions of northern parts of Bengal province in India based on RAPD profiling.

	I	II	III	IV	V	VI
I	1.00					
II	0.76	1.00				
III	**0.59**	0.68	1.00			
IV	0.59	0.68	0.92	1.00		
V	0.59	0.82	0.85	0.85	1.00	
VI	0.59	0.82	0.85	0.85	**1.00**	1.00

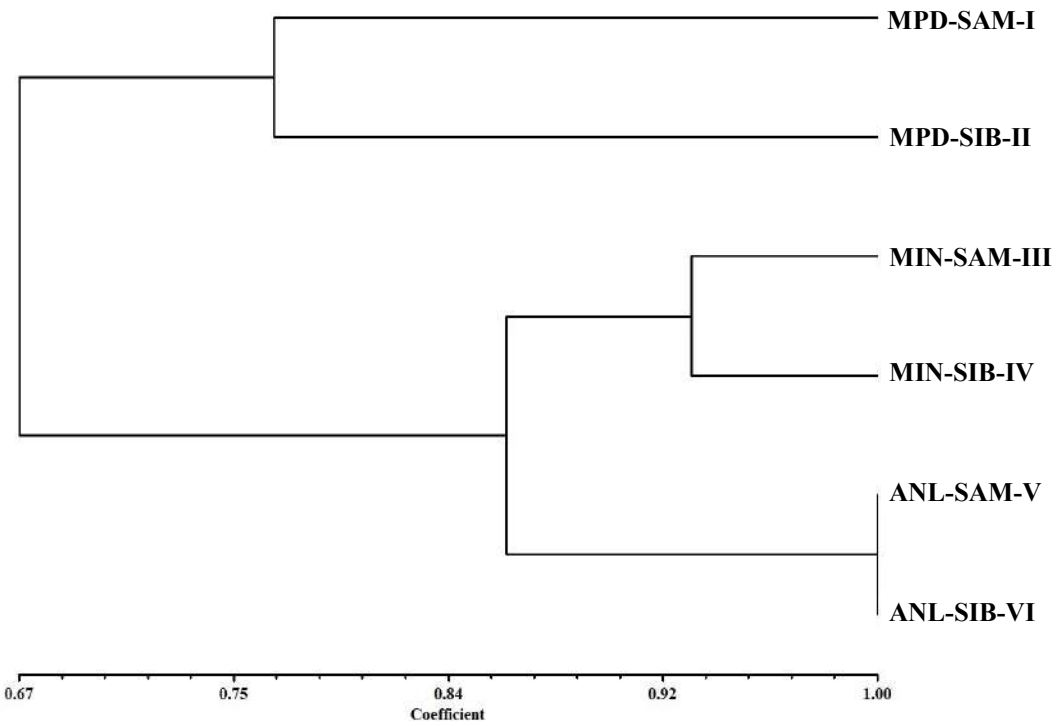


Fig. 4.44. Dendrogram derived from UPGMA cluster analysis of RAPD markers illustrating the genetic relationships among the 9 *Rhizobium* strains.

three clusters, the first cluster contained isolate-I and isolate-II share a node at 76%, whereas, the second cluster revealed high level of genetic similarity (92%) i.e. within isolate-II and isolate-III and 100% of genetic similarity was observed in case of isolate-V and VI comprising the third clade. The only variation found is from host species to host species.

Hence, it can be inferred that there is a major difference present between the micro-symbionts of various species; for instances, three major clades were observed in the phylogenetic tree which were separated as per their host specificity. This resut once again reiterated the host specificity of *Rhizobium*.

Conclusion

"Striving for success without hard work is like trying to harvest where you haven't planted".
-Dabid Bly

Being a village-dweller, I was much interested about the tribal people, especially their food and life-style. In my early age itself, I noticed that most of the tribal peoples are thriving entirely on local produce and usually formulate their own medicine from the locally available herbs and shrubs. Being a student of Botany at later stage, I have developed a strong intension to work on medicinal herbs and shrubs used by the tribal.

Subsequently, I initiated the present endeavor in spring of 2012 with a view to explore different ethnic knowledge as well as pharmacomedicinal properties of selected Mimosoids. In addition, a comprehensive molecular appraisal of those Mimosoid species and their microsymbionts from Northern part of Bengal was subjected to investigate for the first time. Wide lists of plants have been reported locally to be used in treating several local ailments. Since my focus is on the utilization of Mimosaceae, I found different Mimosoids having therapeutic effects; for instance, *Mimosa pudica* and *M. invisa* were reported to heal leucorrhoea, breast cancer or tumor. Further, tender leaves of *Acacia nilotica* were found to be used to treat diabetes. The bark of *A. catechu* was reported to be effective against bone crack, ankle sprain and leucorrhea while *A. concinna* is used to promote hair growth. Furthermore, the bark of *Albizia lebbeck* is used as anthelmintic and also in case of eczema, leucoderma and other skin disorders. Interestingly, *A. catechu* was identified to be used as ethnoveterinarian purposes.

Initiative was further taken to justify local ethnomedicinal claims. In-depth antioxidant and cytotoxic profiling through different standard methods reflected that *A. catechu* (ACL), *A. nilotica* (ANL), *M. pudica* (MPD) and *M. invisa* (MIN) could be used as potent antioxidative as well as non-toxic stuffs which in turn can ameliorate different ROS-mediated disorders including diabetes and neurodegenerative ailments leading to a healthy and hassle-free life. Based on the information obtained from native *Oraon* community, the first idea came to mind is to validate of the use of *A. nilotica* (ANL)

as anti-diabetic. It was observed that ANL significantly modulated blood glucose level, insulin level and glycogen level in alloxan-induced diabetic mice along with restoring of different biochemical and metabolic aspects responsible for the progression of diabetes. Thus the potentiality of ANL is well-justified and holds a new hope for the discovery of new anti-diabetic drug. In addition, findings of enhanced cholinesterase (AChE) inhibitory approach (*in-vitro*) in different extracts provoked me to explore the probable memory-restoration capabilities in rodent model (*in-vivo*) after the administration of extract. Interestingly, *M. pudica* and *M. invisa* were found to be effective to ameliorate the brain-impairment by means of endorsing brain enzymatic function against oxidative stress. Thus, most importantly, it is the first evidence of neurotherapeutic role of MPD and MIN in neutralizing ROS-mediated neuronal damage thereby demands utmost attention for developing CNS drug.

Advanced phytochemical screening involving FT-IR, GC-MS and NMR analysis explored wide array of phytochemicals in ACL, ANL, MPD and MIN extracts. Amongest these, galiic acid, squalene, catechin, epicatechin, isoquercetrin, β-sitosterol, campesterol, stigmasterol, pyrogallol, catechol, α-tocopherol, γ-tocopherol etc. were the main active bio-molecules contributing pivotal role in the management of several diseases and disorders. Moreover, it may easily be postulated that these phytochemicals were the main contributors of exerting free-radical scavenging activity, anti-diabetic activity and anti-neurodegenerative activity. More surprisingly, finding of dopamine (a neurotransmitter) in *A. catechu* extract and its probable biosynthetic pathway is the first report that enriched this endeavor to a great extent.

I, further, aimed to obtain the underlying mechanism that how does a phytocompound act with selective proteins as agonist or antagonist in drug discovery, thereby I designed *in-silico* drugability predictions of different phytocompounds and selected proteins. The ROS-generating proteins including FAS Ligand protein, Toll like receptors and NADPH oxidase effectively bind with targeted ligands such as, β-sitosterol, campesterol and stigmasterol (identified in MPD and MIN extract) signifying their probable role in hindering ROS generation while prominent binding of the same ligands with human brain membrane protein (dopamine receptor D3 protein) suggests their role in the management of neurodegeneration like AD, PD etc. Further pharmacokinetic study supports this view and confirms that MPD and MIN extracts could be treated as future CNS drug. Effective binding patterns of selected phytochemicals (α-tocopherol, γ-tocopherol; identified in ANL extract) with Nrf2 protein (5FNQ) could represent a useful strategy to prevent ROS-mediated

diabetes and several diabetic complications including kidney, liver damage after various toxic insults. Hence, ANL may play pivotal role in the management of diabetes.

While considering molecular study, a good deal of genetic diversity was observed among 9 important Mimosoids including *Mimosa pudica*, *M. invisa*, *Acacia nilotica*, *A. nilitica* var. *indica*, *A. catechu*, *A. concinna*, *Albizia lebbeck*, *A. chinensis* and *Samanea saman* (*Albizia saman*) using different DNA fingerprinting techniques. A total of 330 polymorphic bands were generated using several RAPD primers while PCR-RFLP analysis explored a total of 20 polymorphic bands with 86.96% of polymorphism due to their polyphyletic nature of the different genera under Mimosoideae. DNA barcode analysis through matK and TrnL-F clearly reflected that the members of Mimosoideae and Caesalpinioideae are closer than the members from Papilionoideae validating the traditional classification. During molecular documentation of micro-symbionts of selected Mimosoids, it was observed that the six isolates of *Rhizobium* strains produced 97.39% of polymorphism.

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Index

Appendix-A

List of Publication till May, 2017

* **Article**

Saha MR, Kar P & Sen A. **2017.** Assessment of phytochemical, antioxidant and genetic diversities among selected ethnomedicinal members of Mimosoideae (Mimosaceae). ***Indian Journal of Traditional Knowledge.*** (Accepted, in press). **(Impact factor- 0.371**)

Saha MR, Dey P, Sarkar I, Kar P, Sarker DD, Das S, Haldar B, Chaudhuri TK and Sen A. **2017**. *Acacia nilotica* (L.) Delile could be a potential drug combating diabetes: an evidence-based and in-silico approach. ***Diabetes Technology and Therapeutics***, 19 (Supplement 1): A -128. **(Impact factor-2.198)**

Saha MR, Dey P, Begum S, De B, Chaudhuri TK., Sarker DD, Das AP, Sen A. **2016**. Effect of *Acacia catechu* (L.f.) Willd. on Oxidative Stress with Possible Implications in Alleviating Selected Cognitive Disorders. ***PLoS ONE*****,** 11(3): e0150574. doi:10.1371/ journal.pone.0150574. **(Impact factor- 3.057**)

Saha MR, Dey P, Chaudhuri TK, Goyal AK, Sarker D De & Sen A. **2016.** Assessment of haemolytic, cytotoxic and free radical scavenging activities of an underutilized fruit, *Baccaurea ramiflora* Lour. (Roxb.) Muell. Arg. ***Indian Journal of Experimental Biology***, 54: 115-125. **(Impact factor- 1.165**)

Dey P, **Saha MR,** Chowdhuri SR, Sen A, Sarkar MP, Haldar B & Chaudhuri TK. **2015.** Assesment of anti-diabetic activity of an ethnopharmacological plant *Nerium oleander* through alloxan induced diabetes in mice. ***Journal of Ethnopharmacology***, 161: 128-137. **(Impact factor- 3.055**)

Saha MR, Rai R, Kar P, Sen A & Sarker D De. **2015**. Ethnobotany, traditional knowledge and socioeconomic importance of native drink among the *Oraon* tribe of Malda district in India. ***Journal of Intercultural Ethnopharmacology***, 4(1): 34-39.

Saha MR, Kar P, Sen A & Sarker D De. **2014.** Ethnobotany of Chanchal block of Malda district (West Bengal, India): plants used in local healthcare. ***Pleione,*** 8(2): 381-390.

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Saha MR, Sarker D De & Sen A. **2014.** Ethnoveterinary practices among the tribal community of Malda district of West Bengal, India. ***Indian Journal of Traditional Knowledge***, 13(2): 359-367. **(Impact factor- 0.371**)

Dey P, **Saha MR** & Sen A. **2013.** Hepatotoxicity and the present herbal hepatoprotective scenario. ***International Journal of Green Pharmacy***, 7: 265-73.

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Saha MR & Sarker D De. **2013.** Medicinal properties of ethnobotanically important plants of

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Das D, **Saha MR**, Biswas A & Chaudhri S. **2011.** *Allelopathic effects of three weed plants on Mycorrhizal association of Zea mays root*. ***Journal of Mycopathological Research***, 49(2): 345-348.

* **Book**

De Sarker D., **Saha MR.** and Saha S. **2015.** Perspective of Dietetic and Antioxidant Medicinal Plants. Notion Press, Chennai, India. [ISBN- 978-93-84878-95-5]

De Sarker D., Chowdhury T. and **Saha MR**. **2011**. Biodiversity and Medicinal Plants of West Dinajpur and Malda (Vol.-I). Raiganj College (University College) Press, Raiganj, Uttar Dinajpur, West Bengal, India.

* **Sequence Submission (NCBI)**

1. LM643807- **Saha MR**, De Sarker D. & Sen A. 2014. *Mimosa invisa* chloroplast partial matK gene for maturase K
2. LM643808- **Saha MR**, De Sarker D. & Sen A. 2014. *Acacia concinna* chloroplast partial matK gene for maturase K.
3. LM643809- **Saha MR**, De Sarker D. & Sen A. 2014. *Albizia chinensis* chloroplast partial matK gene for maturase K.
4. LM643810- **Saha MR**, De Sarker D. & Sen A. 2014. *Mimosa pudica* chloroplast DNA containing partial trnL gene, trnL-trnF IGS and partial trnF gene
5. LM643811- **Saha MR**, De Sarker D. & Sen A. 2014. *Mimosa invisa* chloroplast DNA containing partial trnL gene, trnL-trnF IGS and partial trnF gene.
6. LM643812- **Saha MR**, De Sarker D. & Sen A. 2014. *Albizia chinensis* chloroplast DNA containing partial trnL gene, trnL-trnF IGS and partial trnF gene.

Appendix-B

Buffers and chemicals used for DNA fingerprinting studies

*** CTAB- buffer**

100mM Trizma Base (Sigma, Cat# T1503) (pH-8.0)

20mM EDTA (Merck India, Cat# 60841801001730) (pH-8.0)

1.4 M NaCl (Merck India, Cat#60640405001730)

2% (w/v) CTAB (Hexadecyl cetyl trimethyl ammonium bromide) (Sigma, Cat# H6269)

12.11g of molecular grade Trizma base was dissolved in 400 ml double distilled water, pH was adjusted to 8.0 and was divided into two parts of equal volume. To one part 7.44g EDTA was added and to the other part 81.8g NaCl and 20g CTAB. Both the parts were than mixed and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

Note: Add 1% PVP (Polyvinylpyrrolidone) (Sigma, Cat #P5288) and 0.3% β-mercaptoethanol (Sigma, Cat# M3148) just before use.

*** CTAB/NaCl**

CTAB (Sigma, Cat# H6269)=10gm

NaCl (Merck India, Cat#60640405001730)= 4.1gm

Final volume=100ml

In 80ml double distilled water, 4.1gm of NaCl was dissolved. Slowly 10gm of CTAB was added with continuous heating and stirring until the solution becomes clear. The final volume of the solution was adjusted to 100ml by adding ddH_2O. The solution was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

*** 5X TBE (Tris-borate-EDTA) buffer**

Trizma base (Sigma, Cat# T1503) = 27 gm

Boric acid (Sigma, Cat# 15663)= 13.75 gm

0.5M EDTA (pH 8.0)=1.86 gm

All the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

*** 1X TE:**

Tris- Cl (pH 8.0) (i.e. 10Mm) =0.6055gm

EDTA (pH 8.0) (i.e. 1mM) =0.186 gm

Both the reagents were dissolved separately and finally mixed together and the final volume was

made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

*** 3M Sodium Acetate (Sigma, Cat# S9513):**

The required amount of sodium acetate i.e.12.31 g was dissolved in 50ml double distilled water prior to autoclaving. The solution was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

*** 6X gel loading buffer:**

TYPE 3:

0.25% Bromophenol blue (Sigma, Cat# B0126)

0.25% Xylene cyanol FF (Sigma, Cat# X4126)

30% Glycerol (Merck India, Cat#61756005001730) in water

Store at 4ºC.

*** RNase A:**

The RNase A enzyme (Sigma, Cat# R4875) was dissolved at a concentration of 10mg/ml in 0.01M sodium acetate (Sigma, Cat# S9513) (pH 5.2). The solution was heated at 100ºC for 15 minutes in a water bath and allowed to cool slowly to room temperature. The pH was adjusted by adding 1/10 volume of 1M Tris- Cl (pH 7.4) and stored at -20ºC for further use.

Note: Both 0.01M sodium acetate and 1M Tris-Cl were prepared and autoclaved at 121ºC and 15 psi for 20 mins prior to use.

*** SDS(10%)**

SDS (Sigma, USA. Cat #L4390)=5gm

Final volume=50ml

In 20ml of ddH_2O, 5gm of SDS was added and heated to dissolve. The final volume was made upto 50ml and autoclaved for future use.

PLOS | ONE

RESEARCH ARTICLE

Effect of *Acacia catechu* (L.f.) Willd. on Oxidative Stress with Possible Implications in Alleviating Selected Cognitive Disorders

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Abstract

In human body, several categories of degenerative processes are largely determined by free radicals originating in cell. Free radicals are also known to have correlated with a variety of cognitive disorders (CDs) resulting in neuronal injury and eventually to death. Alzheimer's disease (AD) and Parkinson's disease (PD) are such kind of killer CDs that occur due to dysfunction of cholinergic and dopaminergic neurons. Plant parts of *Ginkgo biloba*, *Bacopa monnieri* etc. are being used for the treatment of cognitive disorders in several countries. The present study was aimed to explore the detailed antioxidant and anti-cholinesterase activity of *Acaciacatechu* leaf (ACL) over CDs. Gas chromatography-Mass spectroscopy (GC-MS) analysis and Nuclear Magnetic Resonance (NMR) were employed to identify the bioactive components present in ACL. Furthermore, the extract was evaluated to check the cytotoxic effects of ACL on normal cells. Amongst several antioxidant assays, DPPH assay, hydroxyl radical, nitric oxide radical and hypochlorous acid inhibitory activities were found to be greater in ACL than that of the respective standards while other assays exhibited a moderate or at per inhibitory activity with standards. Total phenolic and flavonoid content were also found to be present in decent amount. In addition, we found, a greater acetylcholinesterase (AChE) inhibitory activity of ACL when compared to other medicinally important plants, indicating its positive effect over CDs. Forty one bioactive components were explored through GC-MS. Of these, gallic acid, epicatechin, catechin, isoquercitrin etc. were found, which are potent antioxidant and a few of them have anti-neurodegenerative properties. Eventually, ACL was found to be nontoxic and safer to consume. Further studies with animal or human model however, would determine its efficacy as a potential anti-schizophrenic drug.

Introduction

Generation of highly Reactive Oxygen Species (ROS) is an integral feature of a normal cell [1]. Of late, knowledge of free radicals and ROS has brought about a revolution in the domain of health and disease management [2]. The free radicals, including superoxide anion, hydrogen peroxide, hydroxyl radicals, nitric oxide, peroxynitrite etc. have potential to initiate degenerative processes in human body and react with important cellular components like DNA, cell membranes and proteins [3]. In fact, there is a proper balance between ROS and enzymatic defense system i.e. oxidant, antioxidant balance in a normal cell. This balance is shifted when antioxidant level is reduced, which is known as oxidative stress causing serious cell damage leading to atherosclerosis, arthritis, cancer and a variety of cognitive disorders (CDs) like Alzheimer's disease (AD) and Parkinson's disease (PD) etc [4].

Cognitive disorders are those disorders that affect the brain's ability to remember and process information [5]. Beside AD and PD, cognitive disorders include dementia, amnesia and hallucination. AD is characterized by loss or decline of memory, language deterioration, poor judgment and cognitive impairment and mainly found in elderly persons [6]. AD is believed to be linked to a deficiency in the brain neurotransmitter, acetylcholine. Inhibition of acetylcholinesterase (AChE) is important for the systemic treatment of AD [6]. PD, the second most common neurodegenerative disorder after AD, is characterized by resting tremor, bradykinesia, muscular rigidity, and postural imbalance occurring due to progressive death of substantia nigral cells leading to dysfunction of dopaminergic neurons [7]. Moreover, ROS are continuously generated in brain leading to a progressive accumulation of cellular damage which is correlated with AD and PD [7].

Donepezil, tacrine, rivastigmine, galanthamine, levodopa, apomorphine etc. are some of the commercial drugs used for the treatment of cognitive disorders including AD and PD. However, they show several adverse effects in human including insomnia, anorexia, diarrhea, fatigue, nausea, gastrointestinal disorders and cardiovascular disorders [6]. The plant products on the other hand are long been used in curing several cognitive disorders without having much side effects. *Ginkgo biloba*, *Catharanthus roseus*, *Bacopa monnieri*, *Acorus calamus*, *Centella asiatica* etc. are some of the plants whose extracts are routinely used in herbal formulation as remedy for AD [8–10]. What's more, herbal products have antioxidant properties which may help fighting against reactive oxygen species (ROS) [2, 3, 11, 12].

Acacia catechu (L.f.) Willd. or Khair (AC), belonging to the family Mimosaceae is used in most of the herbal preparations of ayurveda in India. Traditionally, Khair is beneficial against several gastrointestinal and stomach related ailments, and leprosy [13, 14]. Some antioxidative properties of AC heartwood extract have also been reported [15, 16]. However, no significant work has been done with *Acacia catechu* leaf (ACL) extract; especially so far there is no report of anti-acetylcholinesterase, anti-schizophrenic or anti-cognitive disorder properties of ACL extract. A preliminary study exhibited that *A. catechu*-catechin helps to improve behavioral patterns in animals [17]. However, the mechanism is still unknown. Therefore, in the present study, we attempted probably for the first time, to work out the possible mechanism of activity of ACL extract for neuroprotection and prevention of CDs. To evaluate the free radical scavenging level of ACL extract, a detailed antioxidant profiling has been done which is directly associated with CDs like Parkinson's, Alzheimer's, trauma, seizures etc [6]. Besides, we also evaluated the cytotoxicity of ACL to access the safety of its consumption. Emphasis was also given on those chemical compounds which might have potent role in counteracting oxidative damage.

Materials and Method

Plant material

The plant, *A*. *catechu* (L.f.) Willd. was authenticated by the Taxonomists of Department of Botany, University of North Bengal, India and a voucher specimen (No.- NBU/UD/1039) was deposited at the herbaria of the same Department. The fresh leaves of the *Acacia catechu* were collected during the month of September 2013.

Ethics statement

The plant sample was collected from the medicinal plant garden of Department of Botany, University of North Bengal, West Bengal, India. The garden is not within a National Park/ Reserve Forest/Govt. protected area, therefore, only verbal but formal permission from the respective Department was obtained before collection and the study did not involve any endangered or protected species.

All the experiments using animals were reviewed and approved by the Animal Ethical Committee of the University of North Bengal (Permit No. 840/ac/04/CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

Chemicals and reagents

Chemicals and reagents in the present study were of analytical grade and purchased either from HiMedia Laboratories Pvt. Ltd., Mumbai, India or Merck, Mumbai, India, or Sigma-Aldrich, USA. The EZcount™ MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Assay Kit was procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

Preparation of extract

Fresh leaves of AC were air-dried for 2–3 weeks and then pulverized to fine powder by electric grinder. Exhaustive extraction was performed in Soxhlet apparatus for 11 h using ethanol as a solvent. The extract was then concentrated under reduced vacuum pressure at 40°C in a rotary vacuum evaporator (Buchi Rotavapor R-3, Switzerland). The concentrated extracts were further lyophilized using Eyela Freeze Dryer (FDU-506, USA). Finally, the lyophilized extract was stored in sterile container and placed in -20°C until further use.

In-vitro antioxidant assays

In order to determine the ability of ACL extract to serve as antioxidant against free radicals or ROS, a series of antioxidant assays were performed first.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay. Free radical scavenging activity through DPPH assay was performed as per Chewet al. [18] with a brief modification. Various concentrations of plant extracts (0–100 μg/ml) were prepared and mixed properly with freshly prepared DPPH solution (1mM; diluted in 95% methanol) and kept in dark. Optical density (OD) was measured after 30 minutes of reaction at 517 nm using UV-Vis Spectrophotometer (Thermo UV1, Thermo Electron Corporation, England, UK). Ascorbic acid was taken as standard. The percent radical scavenging activity was calculated using Equation I:

$$\text{Percentage of scavenging DPPH} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 = absorbance of the control and A_1 = absorbance in the presence of samples and standard.

Reducing power assay. The Fe^{3+}-reducing power of ACL extract was evaluated by the method of Oyaizu et al. [19] with slight alterations. Different concentrations (0–64 μg/ml) of plant extract (0.5 ml) were mixed with 0.2 M of phosphate buffer (0.5 ml, pH 6.6) and 0.1%of potassium hexacyanoferrate (0.5 ml), followed by incubation at 50°C for 20 min. in a water bath. After incubation, 0.5 ml of TCA (10%) was added to the mixture to terminate the reaction. The upper portion of the reaction mixture (1 ml) was then transferred to another tube and mixed with 1 ml of distilled water followed by 0.1 ml of $FeCl_3$ solution (0.01%). The mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. Butylated hydroxytoluene (BHT) was used as standard.

Hydroxyl radical scavenging assay. Hydroxyl radical scavenging assay of ACL was carried out on the basis of Fenton reaction [20] with a few changes. A reaction mixture was prepared containing 2-deoxy-2-ribose (2.8 mM), monopotassium phosphate-potassium hydroxide buffer (KH_2PO_4-KOH; 20 mM; pH 7.4), ferric chloride ($FeCl_3$; 100 μM), ethylenediaminetetraacetic acid (EDTA; 100 μM), hydrogen peroxide (H_2O_2; 1.0 mM), ascorbic acid (100 μM) and various concentrations of extracts (0–200 μg/ml) up to a final volume of 1 ml and the reaction mixture was left for 1 h incubation at 37°C. Following incubation, 0.5 ml of incubated mixture was taken into another tube and mixed with 1 ml of tricarboxylic acid (TCA; 2.8%) and 1 ml of aqueous thiobarbituric acid (TBA; 1%). The final mixture was incubated at 90°C for 15 min then cooled down to room temperature and the absorbance was measured at 532 nm against a blank solution. Mannitol was used as positive control. Percentage of inhibition was evaluated following Eq I.

Superoxide radical scavenging assay. This assay was performed by the reduction of nitro blue tetrazolium (NBT) as described by Fontana et al. [21] with brief modifications. Generally, the nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce NBT to a purple formazan. The reaction mixture (1 ml) contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations (0–100 μg/ml) of plant extract. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. Quercetin was used as standard.

Singlet oxygen scavenging assay. The production of singlet oxygen ($_1O^2$) was determined by monitoring the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO) using the method of Pedraza-Chaverrí et al. [22] with minor modifications. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2, 50 mM L-histidine, 10 μM RNO and various concentrations (0–200 μg/ml) of plant extract to make final volume of 2 ml. The mixture was then incubated for 40 min at 30°C and decrease in the absorbance of RNO was measured at 440 nm. Lipoic acid was used as a reference compound. Singlet oxygen scavenging activity was calculated using the Eq I.

Nitric oxide radical scavenging assay. The nitric oxide radical quenching activity was performed following the GriessI-llosvoy reaction [23] with minor modifications. Briefly, phosphate buffered saline (pH 7.4), sodium nitroprusside (SNP; 10 mM) and various concentrations of ACL (0–100μg/ml) were mixed to make final volume of 3 ml. After incubation for 150 minutes at 25°C, 1 ml of sulfanilamide (0.33%; diluted in 20% of glacial acetic acid) was added to 0.5 ml of the pre-incubated reaction mixture and left for 5 min. Following the incubation, 1 ml of N-(1-Naphthyl)ethylenediamine dihydrochloride (NED; 0.1%) was added and incubated for 30 min at 25°C to develop the color. The absorbance was measured spectrophotometrically at 540 nm against blank sample. Curcumin was used as standard. The percentage inhibition was calculated using Eq I.

Peroxynitrite scavenging assay. Peroxynitrite ($ONOO^-$) was prepared following the method of Beckman et al. [24]. Briefly, an acidic solution (0.6M HCl) was prepared mixing with 5 ml of H_2O_2 (0.7 M) and 5 ml of KNO_2 (0.6 M) on an ice bath for 1min. Then 5 ml of ice-cold NaOH (1.2 M) was added to the mixture. Excess H_2O_2 was removed by the treatment with granular MnO_2 prewashed with NaOH (1.2 M) and the reaction mixture was left overnight at -20°C. Finally, peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm (ε = 1670 M-1 cm-1).

Peroxynitrite scavenging activity was measured by Evans Blue bleaching assay. The assay was carried out as per the method of Bailly et al. [25] with a slight modification. A reaction mixture was prepared containing phosphate buffer (50 mM; pH 7.4), DTPA (0.1 mM), NaCl (90 mM), KCl (5 mM), 12.5 μM of Evans Blue, various doses of plant extract (0–200 μg/ml) and 1 mM of peroxynitrite in a final volume of 1 ml. After incubation at 25°C for 30 min the absorbance was measured at 611 nm. The scavenging percentage of $ONOO^-$ was calculated by comparing the results of the test and blank samples. Gallic acid was used as the reference compound.

Hypochlorous acid scavenging assay. Hypochlorous acid (HOCl) was prepared freshly by mixing 10% (v/v) solution of NaOCl to 6.2 (pH) with 0.6 M H_2SO_4 and the concentration was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 M-1 cm-1 as per Aruoma et al. [26] with few changes. A reaction mixture was prepared containing 50 mM phosphate buffer (pH 6.8), catalase (7.2 μM), HOCl (8.4 mM) and plant extract of different concentrations (0–100 μg/ml) into a final volume of 1 ml. The mixture was incubated for 20 min at 25°C and absorbance was measured against an appropriate blank. The quenching activity was accessed by measuring the decrease in absorbance of catalase at 404 nm. Ascorbic acid was used as standard.

Iron chelation assay. The ferrous ion chelating activity was carried out as per the method of Haro-Vicenteet al. [27] with slight changes. Various concentrations of ACL (0–200μg/ml) were mixed properly with ferrous sulfate solutions (12.5 μM) in HEPES buffer (20 mM; pH 7.2) followed by the addition of ferrozine (75 μM) to initiate reaction. The reaction mixture was shaken vigorously and incubated for 20 min at room temperature. The absorbance was measured at 562 nm. EDTA was used as positive control.

Hydrogen peroxide scavenging assay. The scavenging activity was determined by the method of Long et al. [28] with minor modifications. A mixture was prepared with H_2O_2 (50 mM) and various concentrations of plant samples (0–2000μg/ml) and left for 30 min of incubation at room temperature followed by the addition of 90 μl H_2O_2, 10 μl of Methanol (HPLC grade) and 0.9 ml of FOX reagent (prepared by mixing 9 volumes of 4.4 mM BHT in HPLC grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The whole mixture was then vortexed and left for incubation for 30 min. The absorbance was measured at 560 nm. Sodiun pyruvate was used as positive control.

Lipid peroxidation inhibition assay. Lipid peroxidation assay was followed by the method of Kizilet al. [29] with a few modifications. Brain homogenate was prepared by centrifuging of Swiss albino mice brain (20 ± 2 g) with phosphate buffer (50 mM; pH 7.4) and potassium chloride (KCl; 120 mM) at 3000 rpm for 10 min. Various concentrations of ACL extracts (0–25 μg/ml) were mixed with the homogenate (100 μl) followed by addition of ferrous sulfate ($FeSO_4$; 0.1 mM) and ascorbic acid (0.1 mM) and incubated for 1 h at 37°C. Following incubation, TCA (500 μl; 28%) and TBA (380 μl; 2%) were added in the reaction mixture and then heated at 95°C in water bath for 30 min. Then the mixtures were cooled down to room temperature and centrifuged at 8000 rpm for 2 min. The absorbance of the supernatant was measured at 532 nm. Trolox was used as positive control.

Quantification of total phenolic content. The total phenolic content (TPC) was determined using Folin-Ciocalteu reagent [30] with slight changes. Briefly, ACL extract (100 μl) was

mixed with 0.75 ml of Folin—Ciocalteu reagent (previously diluted 1000-fold with distilled water) and left for 5 min at room temperature followed by the addition of sodium carbonate (Na_2CO_3; 0.06%) to the mixture. After incubation of 90 min at room temperature, the absorbance was measured at 725 nm. The phenolic content was measured against a gallic acid standard curve.

Quantification of total flavonoid content. Total flavonoid content was measured using aluminum chloride ($AlCl_3$) method [31] with few modifications. Briefly, ACL extract (100μl) was added to 0.3 ml of distilled water followed by addition of $NaNO_2$ (5%; 0.03 ml). After 5 min of incubation at room temperature, $AlCl_3$ (10%; 0.03 ml) was added and left for 5 min. The reaction mixture was then treated with 0.2 ml of sodium hydroxide (NaOH; 1 mM) and diluted to 1 ml with water. The absorbance was measured at 510 nm. The flavonoid content was determined from a quercetin standard curve.

Acetylcholinesterase (AChE) inhibition assay

AChE inhibiting activity of ACL was carried out based on Ellmanet al. [32] method with brief modification. Reaction mixture was prepared containing sodium phosphate buffer (0.1 mM), 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB; 0.1mM), various concentrations of plant extracts (0–200 μg/ml) and acetylcholinesterase (2 U/ml) in a 96 well micro plate and incubated for 15 min at 25°C. After incubation, acetylthiocholin iodide (0.05mM) was added as substrate in the reaction mixture and the enzyme activity was measured immediately after 3 min in a Bio-Rad iMark™ microplate absorbance reader at 412 nm. Eserine was used as positive control. The percentage of inhibition was calculated in terms of percentage by dividing the difference of sample absorbance from control with control absorbance × 100.

MTT cell viability assay

The assay was performed to evaluate the cytotoxic properties of ACL extract on murine splenocytes and macrophages. The splenocyte and macrophage cells were collected from Swiss albino mice [33]. Swiss albino mouse was sacrificed under mild ether anesthesia and the spleen was aseptically removed from the body and washed thrice (1000 rpm) with RPMI-1640 and splenocytes suspension was prepared and resuspended in 0.16 M NH_4Cl (in 0.17 M Tris; pH 7.2) to remove any trace of erythrocytes. After 5 min, the reaction was stopped using chilled RPMI-1640 and the cells were washed as previous. Peritoneal exudate macrophages were collected by washing the mouse peritoneal region with RPMI-1640. Cell suspension (2×10^6 cells/ml) was prepared with penicillin (50 U/ml), streptomycin (50 U/ml), nystatin (50 U/ml) and fetal bovine serum (FBS, 10%) in RPMI-1640 medium. The cell suspension (100 μl) was added with 100 μl of different concentrations (0–200 μg/ml) of ACL (dissolved in RPMI-1640) to the wells of 96-well plate. The plates were then covered and incubated under 5% CO2 and humidified atmosphere of 90% air at 37°C temperature for 48 h. The cytotoxicity assay was performed according to the manufacturer's instructions of EZcount™ MTT Cell Assay Kit (HiMedia).

GC-MS analysis

Ethanolic leaf extract of AC was derivatized with 10 μl of methoxyamine hydrochloride (20 mg/ml in Pyridine) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) 2 μl FAME (Fatty Acid Methyl Esters) markers [a mixture of internal Retention Index (RI) markers was prepared using fatty acid methyl esters of C8, C10, C12, C14, C16, C18, C20, C22, C24 and C26 linear chain length, dissolved in chloroform (HPLC) at a concentration of 0.8 mg/ml (C8-C16) and 0.4 mg/ml (C18-C30)] was added [34]. GC-MS analysis was performed following the method of Kind et al. [34] after little modification.

HP-5MS capillary column (Agilent J & W; GC Columns (USA) (length 30 m plus Duraguard 10 m, diameter 0.25 mm narrowbore, film 0.25 μm) was used. The analysis was performed under the following oven temperature programme: Injection in sandwich mode with fast plunger speed without viscosity delay or dwell time, oven ramp 60°C (1 minute hold) to 325°C at 10°C/minute, 10 minute hold before cool-down, 37.5 minute run time. The injection temperature was set at 250°C; the MS transfer line at 290°C and the ion source at 230°C. Prior to analysis the method was calibrated with the FAME standards available with the Fiehn GC/MS Metabolomics library (2008) (Agilent ChemStation, Agilent Technologies Inc., Wilmington, USA). Helium was used as the carrier gas at a constant flow rate of 0.723ml / min (carrier linear velocity 31.141 cm/sec). Samples (1 μl) were injected via the split mode (Split ratio 1:5) onto the GC column. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute GC-MS results and to identify chromatographic peaks. Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra, retention times and retention indices with entries of those in Agilent Fiehn Metabolomics library using Agilent retention time locking (RTL) method. Response ratio was calculated as peak area ratios of metabolite and ribitol as internal standard. Relative response ratio (RRR) is normalized response ratio per g crude extract.

NMR spectroscopic analysis

^{1}H and ^{13}C NMR spectra of ACL extract were recorded on a 300 MHz Bruker FT-NMR (Avance AV-300) spectrometer. Sample was dissolved in DMSO-d_6 and the chemical shifts were reported in δ values [35].

Statistical methods

For the reproducibility, all data were prepared as the mean ± SD of six measurements. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's test using KyPlot version 5.0 beta 15 (32 bit) for windows where $p < 0.05$ was considered as significant. The graphs were prepared using KyPlot (version 5.0 for windows).

Results and Discussion

The endogenous free radical forming pathway demonstrates a cascade of diverse free radicals originating from molecular oxygen. Since oxidative stress causes extensive lipid peroxidation and increases the risk of neurodegeneration and subsequent cognitive disorders, exogenous dietary antioxidants proves to be the foremost choice for prevention of such conditions [7].

In the present antioxidant profiling, ACL extract exhibited higher free radical scavenging activity (86.30±0.18 at 100μg/ml) than the respective standard (ascorbic acid) as per DPPH assay (Fig 1A). This was evident from the discoloration of DPPH and low IC_{50} value of ACL extract (15.52±0.46μg/ml). Our result proved that the DPPH scavenging activity in ACL was higher or same in comparison to many other recognized medicinal plants [36]. The elevated radical scavenging activity in ACL extract was probably due to the presence of its electron or hydrogen donating capacity [3]. *A. catechu* extract has higher reducing capacity than the standard BHT indicating its superior protective ability (Fig 1B). The formation of extremely reactive hydroxyl radical (OH^{-}) by way of Haber-Weiss and Fenton reaction in the presence of hydrogen peroxide and excess iron leads to apoptotic cell death through some intermediate pathways [37]. Free hydroxyl radicals are also known for its ability of damaging purine and pyrimidine bases and also affect the deoxyribose backbone [37]. Our experiment with ACL showed that it can significantly scavenge hydroxyl radicals in dose dependant manner (54.91 ±1.76 at 200 μg/ml) than the mannitol standard (Fig 1C) and many other species [38, 39].

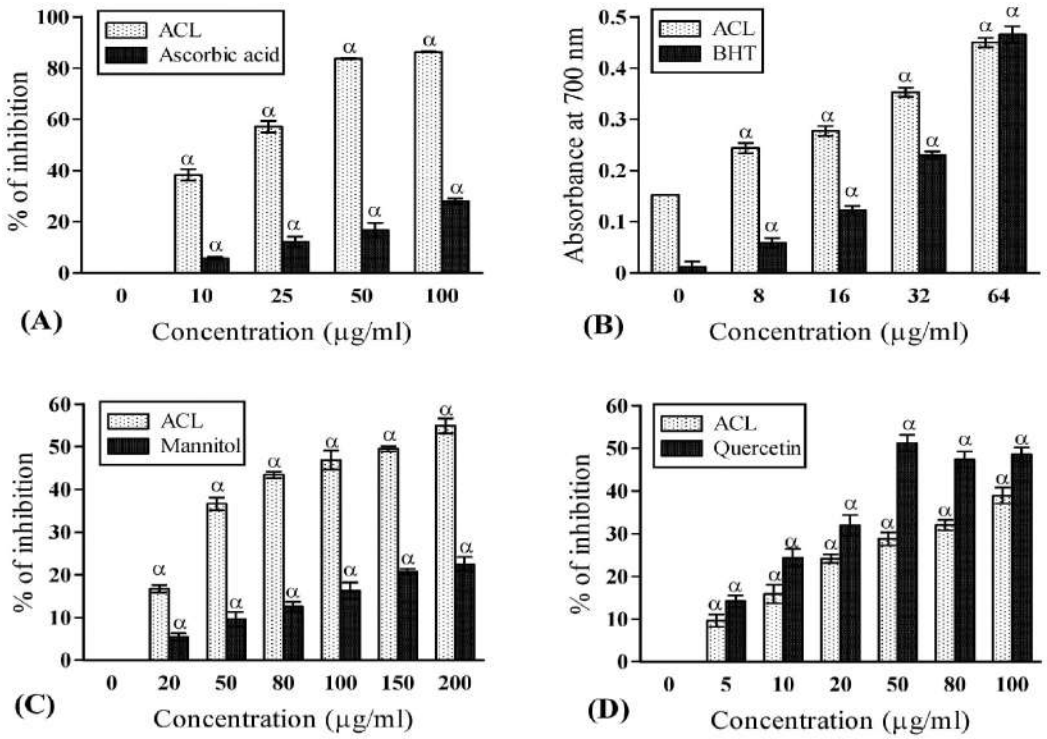

Fig 1. Antioxidant and free-radical scavenging activities of ACL extract. (A) DPPH radical scavenging activities of ACL extract and standard ascorbic acid (IC_{50} value: ACL = 15.52±0.46µg/ml and ascorbic acid = 240.10±28.35 µg/ml; $p<0.001$). **(B)** Total reductive abilities of ACL extract and standard butylated hydroxytoluene (BHT). The absorbance (A_{700}) was plotted against concentration of sample; higher absorbance value signified greater reducing capacity. **(C)** Hydroxyl radical scavenging capacities of ACL extract and standard mannitol (IC_{50} value: ACL = 121.20±1.22µg/ml and mannitol = 589.06 ±46.57µg/ml; $p<0.01$). **(D)** Superoxide radical scavenging activities of ACL extract and standard quercetin (IC_{50} value: ACL = 131.900±4.40µg/ml and quercetin = 63.93±4.16µg/ml; $p<0.01$). [Each value represents mean ±SD (n = 6); Where, α = $p<0.001$ Vs 0 µg/ml].

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Mitochondria are endogenous reservoir of ROS due to its high O_2 consumption rate and any dysfunction in mitochondria may result several neurodegenerative diseases [7]. The highly toxic superoxide anion ($O^{\bullet 2-}$) originated in mitochondria undergoes spontaneous dismutation generating singlet oxygen ($_1O^2$) which is one of the primary causative agent of lipid peroxidation. Present study exhibited significant O^{2-} (Fig 1D) and $_1O^2$ (Fig 2A) scavenging capacity ultimately providing protection to cellular lipid components by prevention of peroxide formation which is beneficial to the brain function in persons with psychiatric disorders such as AD and PD [1]. Nitric oxide (NO) is a potent mediator of pro-inflammatory cellular activation resulting subsequent inflammatory cellular injury. Moreover, spontaneous coupling of NO with superoxide radicals give rise to highly reactive peroxynitrite ($ONOO^-$), which is responsible for causing inflammation in cognitive disorders [40]. Besides, hypochlorous acid (HOCl) is produced at the sites of inflammation due to oxidation of Cl^- ions by the neutrophil enzyme, myeloperoxidase and induces target cell lysis [41]. ACL extract not only possess higher capacity to scavenge NO, $ONOO^-$ and HOCl, the quenching activities (63.04±0.37 at 100 µg/ml; 17.52 ±0.56 at 200 µg/ml; 41.37±2.56 at 100 µg/ml respectively) are also better (Fig 2B–2D) than the respective standards such as curcumin, gallic acid and ascorbic acid. Therefore, ACL extract might help in prevention of inflammation.

Free iron is a potential enhancer of ROS formation as it leads to reduction of H_2O_2 and generation of the highly aggressive hydroxyl radical. In the present study, ACL was found to fade the color of ferrozine-complex, indicating its iron chelating activity due to presence of certain

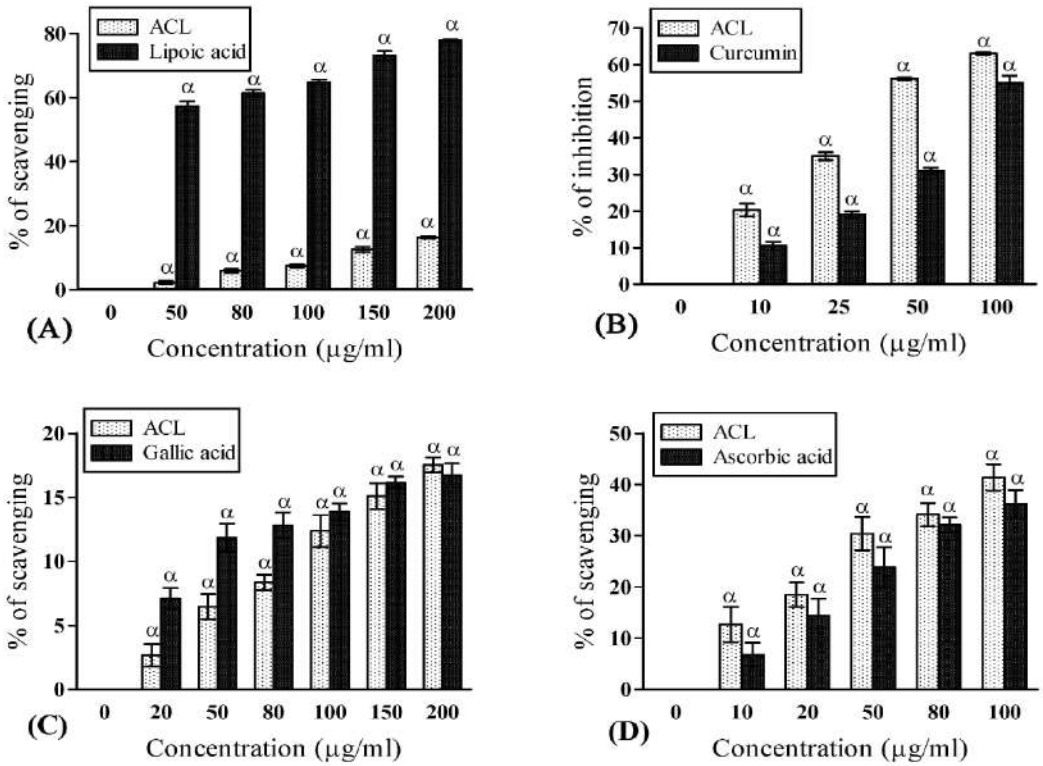

Fig 2. Free-radical scavenging potentials of ACL extract. (A) Singlet oxygen scavenging capacities of ACL extract and standard lipoic acid (IC_{50} value: ACL = 1103.79±24.69µg/ml and lipoic acid = 48.40±2.02µg/ml; $p<0.001$). **(B)** Nitric oxide (NO) scavenging activities of ACL extract and standard Curcumin (IC_{50} value: ACL = 45.57±1.33µg/ml and curcumin = 96.88±5.09µg/ml; $p<0.01$). **(C)** Peroxynitrite scavenging activities of ACL extract and standard gallic acid (IC_{50} value: ACL = 854.05±59.96 µg/ml and gallic acid = 734.81±28.30 µg/ml; $p>0.05$). **(D)** Hypochlorous acid (HOCL) scavenging activities of ACL extract and standard ascorbic acid (IC_{50} value: ACL = 130.675±4.78 µg/ml and ascorbic acid = 165.91±16.31µg/ml; $p<0.01$). [Each value represents mean ±SD (n = 6); Where, α = $p<0.001$ Vs 0 µg/ml].

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components. Moreover, the iron chelating activity (35.14±0.55 at 200 µg/ml) of ACL was found to be higher (Fig 3A and 3B) than many other studied plants [42, 43]. However, the hydrogen peroxide scavenging activity of plant extract was lower (Fig 4A) than the standard (sodium pyruvate), it is still enough to establish its positive role in protecting our body. Lipid peroxidation is a natural metabolic process under normal aerobic conditions and it is one of the most investigated consequences of ROS action [44]. As mentioned earlier, hydroxyl radical and singlet oxygen are the main causative agents of this peroxidation. In presence of antioxidants, lipid peroxidation becomes minimal [44]. Our study revealed significant lipid peroxidation inhibitory activity (Fig 4B). However, the quenching activity was found to be lower (50.28 ±0.51 at 25 µg/ml) than the respective standard Trolox (77.58±1.0 at 25 µg/ml). Nevertheless, it is still enough to induce a protective effect to establish its positive role in oxidative degradation. The phenolic compounds having redox potentiality play an important role in absorbing and neutralizing the free radicals, scavenging singlet and triplet oxygen, or decomposing peroxidase [45]. Similarly, flavonoids have also been reported to be responsible for its antioxidant activity, as they act on enzymes and pathways involved in anti-inflammatory processes [45]. In the present study, ACL showed decent amount of flavonoid and phenol content exhibiting 13.92 ±1.60 mg quercetin equivalent per 100 mg of plant extract and 89.59±2.71 mg gallic acid equivalent per 100 mg of plant extract respectively. These contents are the major determinant for such antioxidant activities. Therefore, *A. catechu* can well be considered as a plant of medicinal

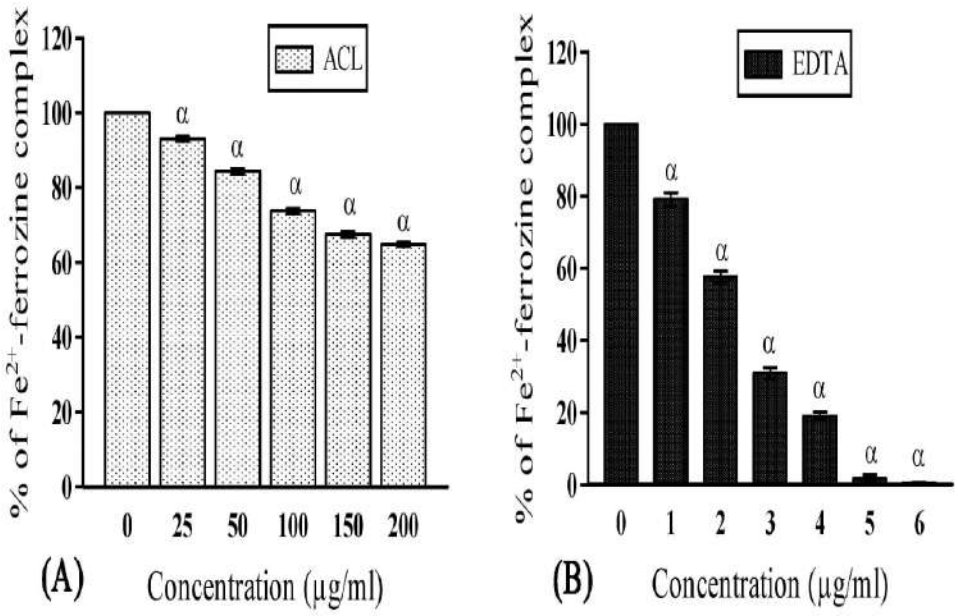

Fig 3. Iron (Fe^{2+})-chelation activities of ACL extract and the reference compound. (A) ACL extract and **(B)** standard Ethylenediaminetetraacetic acid (EDTA), represented as % of Fe^{2+}-ferrozine complex (IC_{50} value: ACL = 320.63±10.82µg/ml and EDTA = 1.45±0.01µg/ml; p<0.001). [Each value represents mean ±SD (n = 6); Where, α = *p*<0.001 Vs 0 µg/ml].

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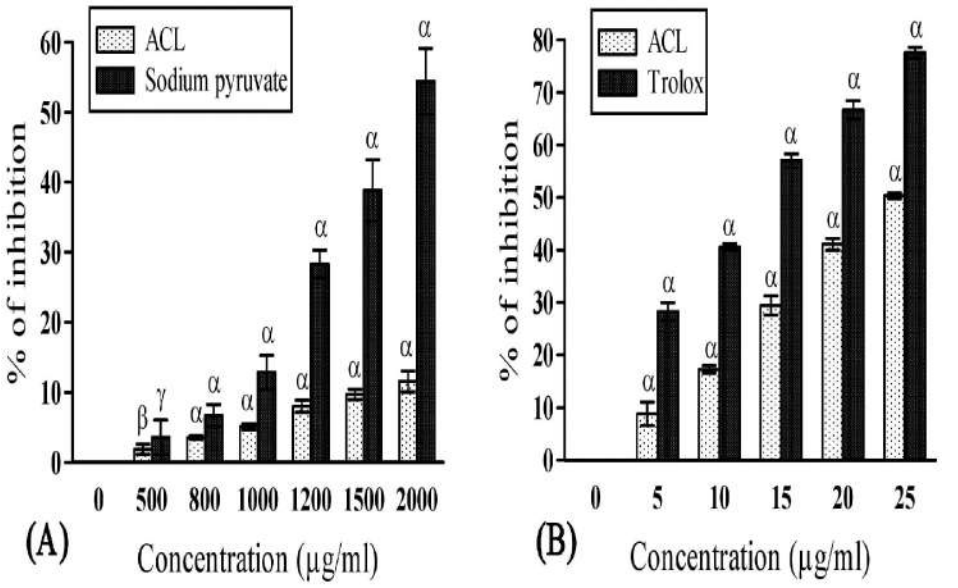

Fig 4. Hydrogen peroxide scavenging and lipid peroxidation inhibitory activity of ACL extract and the reference compound. (A) Hydrogen peroxide (H_2O_2) scavenging activities of ACL extract and standard sodium pyruvate (IC_{50} value: ACL = 15604.93±613.81µg/ml and sodium pyruvate = 3176.40±140.22µg/ml; *p*<0.001). **(B)** Inhibition of lipid peroxidation by ACL extract and standard trolox (IC_{50} value: ACL = 32.13 ±0.99µg/ml and trolox = 11.11±0.22µg/ml; *p*<0.001). [Each value represents mean ±SD (n = 6); Where, α = *p*<0.001, β = *p*<0.01 and γ = *p*<0.05 Vs 0 µg/ml.].

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importance and the leaf extract of AC might play a central role in prevention of various neurodegenerative disorders in terms of oxidative stress and free radical generation [46].

Since a large number of evidence exhibit that oxidative stress is closely involved in age-related neurodegenerative diseases, there have been a great number of studies which have examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders. We therefore wanted to perceive whether the ACL extract has any acetylcholinesterase inhibitory activity over CDs. Acetylcholinesterase enzyme hydrolyses the substrate (we used acetylthiocholine iodide) and produces thiocholine which in turn reacts with Ellamn's reagent (5, 5'-dithiobis-2-nitrobenzoic acid) and 5-thio-2-nitrobenzoic acid is thus produced which is a yellow color compound [47]. The inhibition of AChE enzyme activity is evident by fading the yellow color of the product [47]. Our result exhibited better AChE enzyme inhibition activity (73.47±0.303 at 200μg/ml) with low IC_{50} value of 75.91 ±2.28μg/ml (IC_{50} of standard eserine = 0.023±0.0005 μg/ml) compared to other medicinal plants including *Andrographis paniculata*, *Cetella asiatica*, *Nelumbo nucifera*, *Nardostachys jatamansi*, *Myristica fragrans* [8,48] suggesting ACL extract as an effective cholinesterase inhibitor for the first time and beneficial against several neurodegenerative disorders or CDs such as dementia, AD, PD.

The enhanced inhibitory effects of ACL against AChE over other medicinal plants prompted us to study the probable compounds present in AC leaf. We have chosen GC-MS analysis and NMR spectroscopy in this regard. GC-MS analysis of ACL (Fig 5) was employed to identify the presence of various bioactive compounds and neurotransmitters, if any. A total of 41 different bioactive metabolites (Fig 6) have been identified by GC-MS analysis (Table 1). Out of 41 compounds we found 5 different compounds which have proven as potent

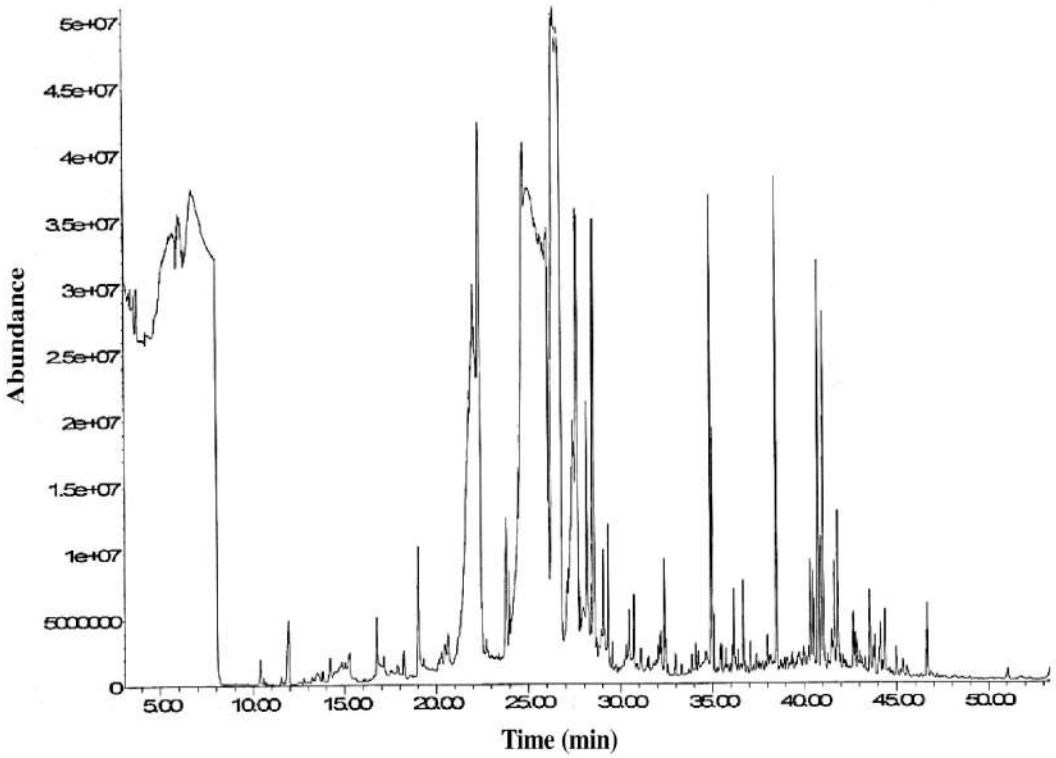

Fig 5. Gas chromatogram-Mass spectroscopy of *A. catechu* leaf extract.

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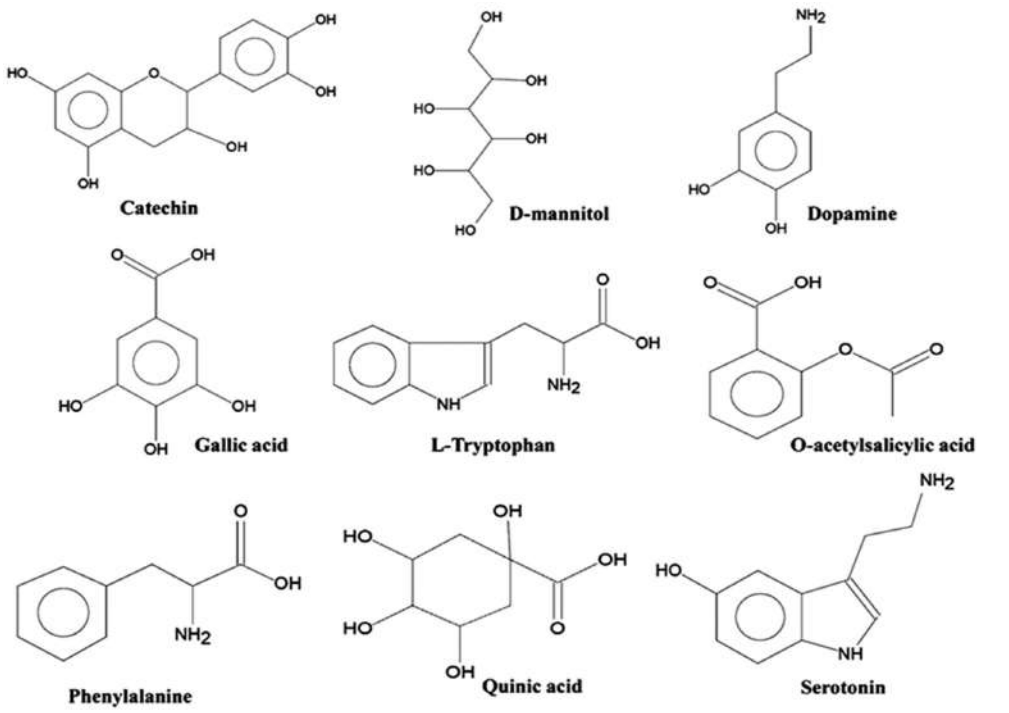

Fig 6. Chemical structures of some essential bioactive metabolites identified in ACL extract by GC-MS.

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antioxidant activities. These are D-mannitol, gallic acid, epicatechin, catechin and isoquercitrin. Amongst these compounds, gallic acid is known as strong antioxidative agent [49] while D-mannitol, catechin, epicatechin and isoquercitrin are good scavenger of hydroxyl, peroxyl, superoxide and DPPH radicals and exhibit remarkable anti-cancer effects [50–52]. Besides, catechin and epicatechin also show monosamine oxidase inhibitory activity which is partly responsible for PD, AD and others cognitive disorders [53].

We have also found a few bioactive compounds which have either anti-neurodegenerative properties or are known as precursor of compounds which are anti-CDs. These compounds were found to be tyramine, dopamine, and serotonin. Out of these bio-active compounds, dopamine and norepinephrine are collectively called catecholamine [54]. Generally, the catecholamines are synthesised following two equally active routes [55]. In one of the routes, phenylalanine converts into tyrosine with the help of amino acid hydroxylase. Tyrosine then decarboxylated to tyramine using tyrosine decarboxylase and subsequently produces dopamine and norepinephrine (noradrenaline) catalysed by enzyme monophenol hydroxylase and dopamine beta- hydroxylase respectively. In our present study, the GC-MS analysis of ACL extract revealed the presence of neurotransmitters like, phenylalanine, tyramine, dopamine, serotonin etc. Therefore, we may conclude that the synthesis of catecholamines in ACL probably follows the described route (Fig 7). In addition, it may conclude that the serotonin probably synthesised from its immediate precursor L-tryptophan. Several studies and reviews [55–57] exhibited that catecholamines and other neurotransmitters have distinct role to combat against cognitive disorders like AD, PD and dementia.

Furthermore, NMR spectroscopy of ACL extract was employed in the present study. From the ^{13}C NMR spectra (Fig 8A), several peaks in the aliphatic (δ = 19.0–84.2) and aromatic region (δ = 115.8–156.6) have been identified confirming the presence of both aliphatic and

Table 1. List of metabolites detected in *A. catechu* leaf extract by GC-MS analysis.

Sl. No.	Name of the Metabolites	Log of RRR*	
		Average	SD
1.	L-(+) lactic acid	1.07	0.32
2.	L- alanine	0.55	0.19
3.	L- valine	0.94	0.12
4.	Urea	0.23	0.33
5.	Pipecolic acid	1.28	0.42
6.	Glycerol	2.32	0.02
7.	Phosphoric acid	1.30	0.02
8.	L-threonine	1.10	0.17
9.	Glycine	0.56	0.09
10.	Succinic acid	0.77	0.19
11.	Glyceric acid	1.22	0.09
12.	Beta-alanine	0.90	0.10
13.	D- malic acid	1.53	0.03
14.	O-acetylsalisylic acid	0.40	0.21
15.	L- glutamic acid 3 (dehydrated)	1.64	0.13
16.	4-guanidinobutyric acid	1.57	0.25
17.	Phenylalanine	0.59	0.17
18.	Phenylethylamine	1.09	0.08
19.	Meleamic acid	0.48	0.17
20.	L-glutamic acid	0.16	0.05
21.	Lauric acid	1.33	0.50
22.	L- asparagine	1.77	0.05
23.	Xylitol	2.14	0.25
24.	Arabitol	0.79	0.05
25.	Putrescine	1.04	0.09
26.	Methyl-beta-D-galactopyranoside	1.25	0.05
27.	Quinic acid	1.41	0.48
28.	Allantoin	1.69	0.06
29.	Tyramine	3.22	0.11
30.	D-sorbitol	1.19	0.07
31.	D-mannitol	1.13	0.09
32.	Gallic acid	3.19	0.02
33.	Palmitic acid	1.80	0.08
34.	Dopamine (hydroxytyramine)	1.02	0.07
35.	L-tryptophan	1.52	0.02
36.	Stearic acid	1.10	0.22
37.	Serotonin	1.01	0.04
38.	Sucrose	2.28	0.00
39.	(-)- epicatechin	2.14	0.02
40.	Catechin	2.05	0.07
41.	Isoquercitrin	1.69	0.03

*RRR: Relative Response Ratio; SD: Standard Deviation.

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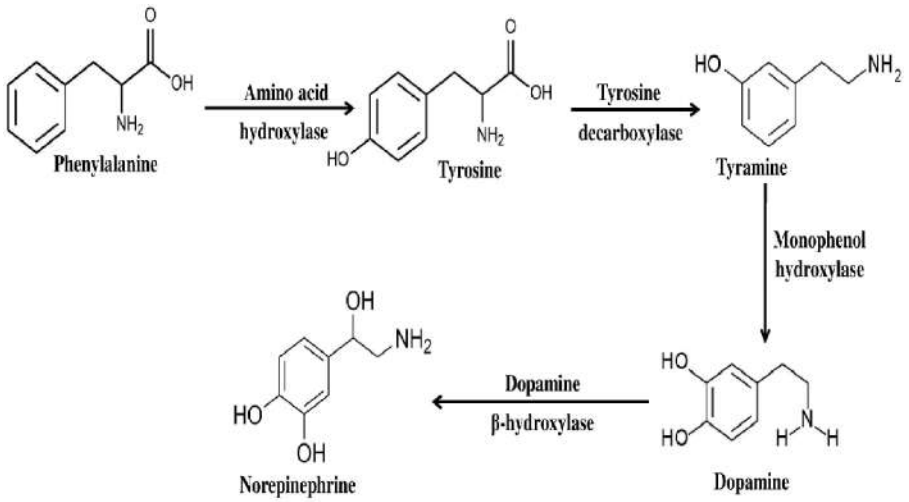

Fig 7. Schematic representation of possible biosynthetic pathway of catecholamines identified in ACL extract.

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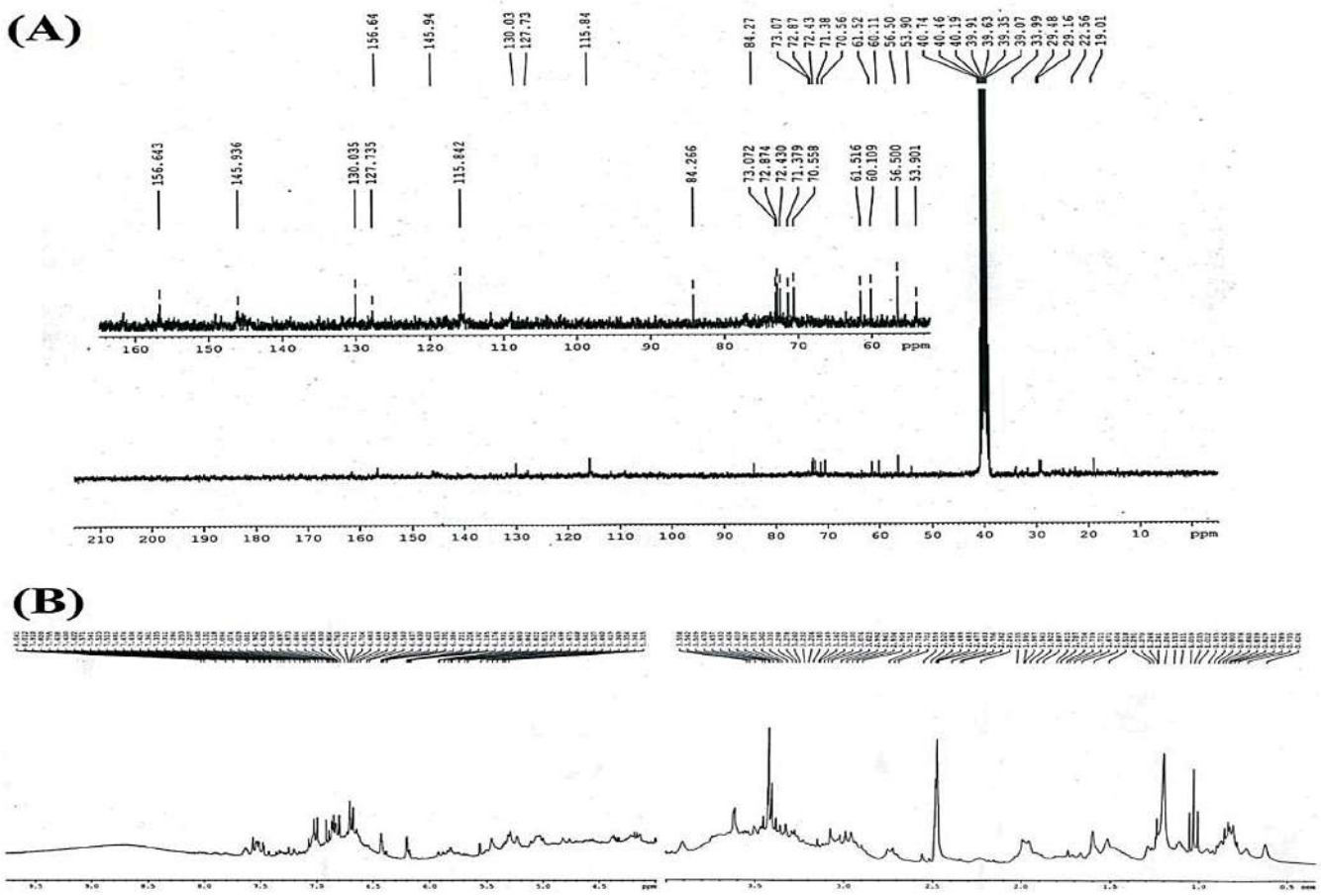

Fig 8. (A) ^{13}C NMR spectra and (B) ^{1}H NMR spectra of ACL extract.

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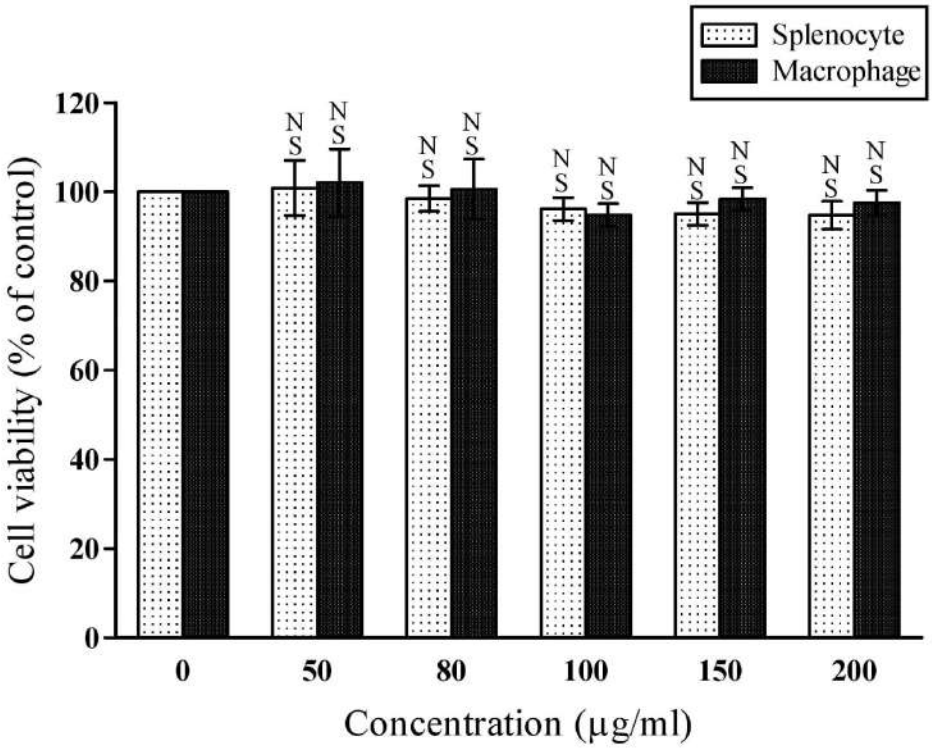

Fig 9. The effect of ACL extract on the viability of murine splenocytes and peritonealexudate macrophages, evaluated by MTT method. Each value represents mean ±SD (n = 6); Where, $^{NS}p = 0>0.05$.

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aromatic carbons [35]. Similarly, ^{1}H NMR spectra (Fig 8B) exhibited peaks related to aliphatic, aromatic and olefinic protons. In addition, a hump near $\delta = 8.75$ indicates the presence of amine functional group supporting the result found in GC-MS analysis which might be responsible for the bioactive properties of the ACL extract.

The cytotoxic effect of ACL was further assessed on mice splenocyte and macrophage cells whether the extract has any deleterious effects on normal body cells. The effect of the ACL on the cell viability was non-significant ($P>0.05$) relative to control. At highest dose (200 µg/ml) of extract, the magnitude of cell viability compared to the control was 94.77±3.09% and 97.48 ±2.85% for cultured splenocytes and macrophages respectively (Fig 9) displaying no cytotoxic effect on either of splenocyte or macrophage cells. Hence, ACL extract represents as safety stuff to consume.

Conclusion

The occurrence of dementia along with other cognitive disorder is increasing. Survey exhibited that near about 35.6 million people lived with dementia worldwide in 2010 and the number is expected to be doubled in the next 20 years [58]. However, the prevalence of dementia is more in North and South America and Europe [58–60] in comparison to Sub-Saharan Africa and India [61]. Besides genetic factors, relatively low occurrence of dementia in India may be attributed to socio-cultural activities, lifestyle, dietary habits etc [62]. From the lifestyle and dietary point of view, one of the major differences between the population of Indian sub-continent and rest of the world is the chewing of betel leaf (*Piper betel*) and betel nut (*Areca catechu*) along with lime and Khair (*A. catechu*). Several workers [63,64] have been studied the medicinal

properties of betel leaf. In a recent study, Sullivanet al. [65] showed that betel nut chewing is positively associated with less severe symptoms of schizophrenia.

We, therefore, wanted to perceive whether *Acacia catechu* leaf has effect over CDs (beneficial or harmful). Result exhibited that ACL extract had significant antioxidant activity against various free radicals. The pronounced antioxidant activity of ACL extract was found to be potent due to its phenolic and flavonoid content. Presence of dopamine in ACL extract made it more significant and potential for use in the preparation of anti-CD drugs. Moreover, ACL extract was found to be nontoxic and safer through MTT cell viability assay. The chemical characterization of ACL using NMR and GC-MS revealed presence of several antioxidative compounds with potential benefit in psychiatric disorders such as AD and PD. However, extensive research with human models with those bioactive compounds, found in ACL will justify its use in novel drug delivery systems.

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Author Contributions

Conceived and designed the experiments: AS TKC MRS DDS. Performed the experiments: MRS PD SB BD AS. Analyzed the data: MRS PD SB BD TKC DDS AS. Contributed reagents/materials/analysis tools: APD DDS BD TKC AS. Wrote the paper: AS MRS PD BD.

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Assessment of anti-diabetic activity of an ethnopharmacological plant *Nerium oleander* through alloxan induced diabetes in mice

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ABSTRACT

Ethnopharmacological relevance: *Nerium oleander* L. (syn. *Nerium indicum* Mill. and *Nerium odorum* Aiton.) is used for its anti-diabetic properties in Pakistan, Algeria, Morocco and is also recognized in Ayurveda. The present study was undertaken to investigate the anti-diabetic capacity of a standardized hydromethanolic extract of *Nerium oleander* in alloxan induced diabetes in mice.

Materials and methods: *Nerium oleander* leaf extract (NOLE) was orally administered at 50 and 200 mg/kg body weight (BW) dose to alloxanized mice (blood glucose >200 mg/dl). After 20 consecutive days of treatment, various diabetic parameters were studied and compared with untreated mice. Furthermore, gas chromatography–mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) analysis was employed to reveal the phytochemical composition of the plant extract.

Results: NOLE demonstrated antihyperglycaemic activity by reducing 73.79% blood glucose level after 20 days of treatment. Oral glucose tolerance test (OGTT) revealed increase in glucose tolerance as evident by 65.72% decrease in blood glucose in 3 h post treatment. Percentage decrease in different liver marker enzymes were significant along with decrease in triglyceride and cholesterol levels, displaying potent antihyperlipidemic activity. Peroxidase and catalase activity in liver, kidney and skeletal muscle were significantly restored besides marked reduction in lipid peroxidation and normalization of hepatic glycogen level in the NOLE treated alloxanized mice. Different bioactive phytocompounds with potent anti-diabetic activity were identified by GC–MS and HPLC analysis.

Conclusion: The present investigation revealed that *Nerium oleander* possess potent anti-diabetic activity as claimed in different ethnopharmacological practices.

1. Introduction

Diabetes mellitus is a collective form of various metabolic disorders affecting different organs of the body. Glucose metabolism may get severely hindered due to improper insulin production from the pancreatic β-cells (Jarald et al., 2008). Glycogen catabolism in liver increases due to low insulin level resulting in low hepatic glycogen content in diabetes. Hepatic damage induced in such condition may demonstrate elevation of the liver marker enzymes such as transaminases and phosphatases (Amarapurkar and Das, 2002). Diabetic nephropathy results in further increase of urea, uric acid and creatinine level in serum. Besides, hyperglycemia induces oxidative stress exacerbates the pathogenesis of diabetic complications (Johansen et al., 2005).

It is estimated that 1200 species of plants are used for the treatment of diabetes in traditional and ethnopharmacological practices around the world (Hsu et al., 2009). One such plant is *Nerium oleander* L. (Apocynaceae) which is known as 'Karabi' by the local Bengali people of West Bengal, India and in Bangladesh, 'Kaner' in most part of India and commonly called 'Oleander' in English. *Nerium oleander* is used for the treatment of diabetes in different

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ethnopharmacological and indigenous medicinal systems around the world such as in Morocco (Jouad et al., 2001; Bnouham et al., 2002; Tahraoui et al., 2007), Pakistan (Hussain et al., 2013), Algeria (Rachid et al., 2012) and is also mentioned in Ayurveda (Sudha et al., 2011). Interestingly, during a recent survey in Malda district of West Bengal, India, we found that *Nerium oleander* leaves are used in a tribal anti-diabetic formulation (Saha et al., 2014). Previous study of the anti-diabetic potentials of *Nerium indicum* was confined only within the study of glucose load and body weight measurement of diabetic rats (Sikarwar et al., 2009). Chloroform and ethanolic extracts of oleander leaves were shown to down-regulate blood glucose level more than petroleum ether extract in diabetic animals. Besides the extracts prevented body weight loss in addition to improvement in oral glucose tolerance. Bas et al. (2012) further studied the effect of the shoot distillate of *Nerium oleander* on streptozotocin induced diabetes showing increased insulin sensitivity in addition to normalization of homeostasis model assessment-insulin resistance (HOMA-IR) and leptin levels in distillate treated groups without any gastrointestinal damage. The distillate administration also ameliorated β-cell function and elevated peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ mRNA levels.

In this study, we have investigated *in vivo* anti-diabetic activity of *Nerium oleander* leaf extract (NOLE) on alloxan induced insulin dependent type 1 diabetes mellitus in murine model to establish the ethnopharmacological claim of its anti-diabetic capacity. We have studied various aspects of diabetic complications in a more comprehensive way by measuring blood glucose level, serum insulin level, various enzymatic parameters, lipid peroxidation, endogenous antioxidative levels etc. In addition, we have attempted to chemically characterize NOLE by GC–MS and HPLC to identify the bioactive components responsible for its anti-diabetic activity as well as to assess the secondary effect due to the presence of various antioxidants present in NOLE in order to understand its *in vivo* physiological implications.

2. Materials and methods

2.1. Chemicals

All the reagents were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), unless otherwise indicated. Acid phosphatase (ACP), alkaline phosphatase (ALP), cholesterol, creatinine, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid and urea kits were purchased from Crest Biosystem, Goa, India. Thiobarbituric acid reactive substances (TBARS) assay kit was procured from Cayman chemical company (USA) and insulin level was estimated using AccuBind insulin ELISA kit from Monobind Inc. (USA).

2.2. Preparation of plant extract

The leaves of white flowered oleander was collected from the garden of University of North Bengal (26.71°N, 88.35°S), India, during the month of August. A voucher specimen was stored at the herbarium of Department of Botany, University of North Bengal (Accession no. 09618).

The leaves were washed properly with double distilled water and shade dried at room temperature for 2 weeks followed by grinding to powder. The powder (100 g) was mixed with 1000 ml 7:1 methanol: water (v/v) and kept at 37 °C in a shaking incubator (160 rpm) for 18 h. The mixture was centrifuged (2850 x *g*) for 15 min. The pellet was mixed with 70% methanol (1000 ml) and kept in a shaking incubator as previously described. The supernatant was collected from both the phases, filtered and the filtrate was concentrated under reduced pressure in a rotary evaporator. The resultant was lyophilized and stored at −20 °C until further use.

2.3. Alpha-amylase inhibitory activity

The assay was carried out according to the standard method of Hansawasdi et al. (2000) with some modifications. Briefly, 1% starch azure was suspended in 0.5 M Tris–HCl (pH 6.9) containing 0.01 M $CaCl_2$ and boiled on water bath for 5 min. The tubes were cooled down to room temperature and different concentrations (0–200 μg/ml) of NOLE and amylase (2 U/ml) in Tris–HCl buffer was added to it. The solution was mixed properly and incubated for 5 min at 37 °C. The reaction was stopped by addition of 250 μl of 1 M HCl followed by centrifuge at 3000 rpm for 10 min. Supernatant was collected and absorbance was read at 595 nm. The percentage inhibition was calculated from the following formula:

$$\%\ \text{of inhibition}\left[\alpha-\text{amylase}\right]=\frac{A0-A1}{A0}\times 100$$

where *A*0 was the absorbance of the blank and *A*1 was the absorbance in the presence of the varying concentrations of NOLE.

2.4. Animal maintenance

Swiss albino mice (either sex) of 6–8 weeks old were maintained under standard laboratory conditions in the animal house of the Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12 h photoperiod (temperature 25 ± 2 °C). The experiments were approved by the ethical committee of University of North Bengal (No. 840/ac/04/CPCSEA; date: 15/09/10) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

2.5. Acute toxicity study

OECD guidelines (test 423: Acute oral toxicity – Acute toxic class method; 2002) were followed to study the acute toxicity of NOLE (OECDiLibrary, 2002). Animals were divided into four groups ($n=6$) and fasted overnight prior to experiment. NOLE was administered orally in an increasing dose upto 2000 mg/kg BW. Following the dose all the groups were carefully observed for the development of any clinical or toxicological symptoms at 30 min and then 2, 4, 8, 24 and 48 h.

2.6. Induction of experimental diabetes

Diabetes was induced in experimental mice by a single intraperitoneal injection (0.2 ml) of freshly dissolved alloxan monohydrate in saline (154 mM NaCl) with a dose of 150 mg/kg BW. Mice with 12 h fasting blood glucose level > 200 mg/dl on the third day of alloxan administration were considered diabetic and selected for the following treatments.

2.7. Drug administration in diabetic animals

Total 30 Swiss albino mice of either sex were randomly divided into 5 groups ($n=6$). Group I (control; non-diabetic mice) received normal saline. Group II received (diabetic mice; T1D) received normal saline. Group III (diabetic mice; glibenclamide) received glibenclamide at 5 mg/kg BW per day. Group IV (diabetic mice; NOLE low) received NOLE (in distilled water) at 50 mg/kg BW per day. Group V (diabetic mice; NOLE high) received NOLE (in distilled water) at 200 mg/kg BW per day. The treatments were done for 20 consecutive days.

2.8. Estimation of body weight and blood glucose level

Total body weight of all the experimental animals were recorded on day 1, day 10 and day 20 of the treatments and 12 h fasting blood glucose levels were estimated using Bayer glucometer (contour TS meter) from the tail vein on the aforementioned days.

2.9. Collection of serum and tissue

On day 21, *i.e.* 24 h after the last treatment, (12 h fasting) mice were sacrificed under mild ether anesthesia and blood was collected by puncturing the heart. 50 μl whole blood was used to estimated glycated hemoglobin (HbA1c) level by ion-exchange high-performance liquid chromatography (HPLC) using Bio-Rad D-10™ Dual HbA1c program 220-0201 according to manufactures instructions. The remaining blood was allowed to clot for 60 min at room temperature and then serum was separated by centrifuging at 1000 rpm for 10 min. Separated serum was kept at −20 °C until further use. Skeletal muscle collected from the thigh muscle, liver and kidney was separated and washed thoroughly with phosphate buffer saline (PBS) and stored at −20 °C for future use.

2.10. Study of serum biochemical parameters

Serum insulin was estimated by the ELISA method using Accu-Bind Universal ELISA kit (Monobind Inc., USA) according to manufactures instructions. The serum samples from all the groups were used to study ACP, ALP, cholesterol, creatinine, triglyceride, AST, ALT, uric acid, urea and urea N_2 levels using commercially available kits of Crest Biosystems (India).

2.11. Measurement of lipid peroxidation

The extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) content in serum. The assay was performed in six sets by the thiobarbituric acid reactive substances (TBARS) assay kit according to the manufacturer's (Cayman, USA) instructions.

2.12. Estimation of hepatic glycogen

Glycogen level was estimated in liver samples by the standard anthrone reagent method previously described by Carroll et al. (1956). Glycogen content was measured from a glucose standard curve prepared in parallel to the sample.

2.13. Estimation of peroxidase activity and catalase activity

Peroxidase activity in liver, kidney and skeletal muscle of the experimental mice was estimated by measuring the oxidation of guiacol according to a standard method (Sadasivam and Manickam, 2008). Catalase activity was measured by degradation of substrate H_2O_2 by catalase in the tissue samples following the standard method described by Luck (1963).

2.14. Oral glucose tolerance test (OGTT)

A different set of diabetic and non-diabetic mice was divided into 6 groups ($n=6$) as previously described (Section 2.7) and treated with glibenclamide and NOLE for 20 days. After the last dose, the mice were fasted for 12 h and subsequently, a dose of glucose (2.5 g/kg body weight) was orally administered. Blood glucose levels were estimated from the blood samples collected from the tail vein just prior to glucose administration and 60, 120 and 180 min post-glucose administration.

2.15. GC–MS analysis

NOLE was dissolved in *n*-hexane and the mixture was centrifuged thrice at 12,000 rpm for 15 min. The clear supernatant was used for GC–MS analysis. Agilent 5975C GCMS system (Agilent Technologies, USA) attached with HP-5ms Capillary Column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) and equipped with inert MSD triple axis mass detector conditioned at ion trap 200 °C, transfer line 280 °C, electron energy 70 eV (vacuum pressure- 2.21e-0.5 Torr) was used for analysis. The carrier gas was helium at a flow rate of 1 ml/min. 2 μl sample was injected in a splitless mode. The column temperature was set at 60 °C for 1 min followed by 5 °C/ min upto 250 °C. The major and essential compounds in NOLE were identified by their retention times and mass fragmentation patterns using Agilent Chem Station integrator and the database of National Institute Standard and Technology (NIST) with a MS library version 2010.

2.16. HPLC analysis

NOLE was subjected to Bligh and Dyer method (Bligh and Dyer, 1959) to remove the lipid contents. The methanolic fraction was separated and mixed with 4 volumes of chilled acetone and incubated for 60 min at −20 °C. The solution was then centrifuged at 15,000 x *g* for 15 min at 4 °C. The pellet containing protein was discarded and the supernatant was subjected to thin layer chromatography (TLC) on a silica gel plate using 10% acetic acid in chloroform as solvent. The corresponding bands of secondary metabolites were eluted by acetonitrile after detection with 20% (w/v) Na_2CO_3 and diluted Folin–Ciocaltaeu reagent (1:3). The solution was then analyzed using HPLC (Agilent, USA) having Zorbax SB-C18 column (4.6 × 150 mm^2, 3.5 μm) and equipped with a Diod Array Detector. Gradient concentrations of mobile phase A – methanol (M) and B – water (W) with 0.02% H_3PO_4 were 25% A+75% B for 5 min, 30% A+70% for 10 min, 45% A+55% for 30 min and 80% A+20% B for 45 min. The injection volume was 20 μl and the flow rate was kept at 0.4 ml/min. Analytes were scanned in four wave length of 254 nm, 275 nm, 280 nm and 320 nm. The peaks were identified by comparing the relative retention time (RRT) against standard phenolic acids (Sigma, USA; ChromaDex, USA), flavonoids and methylphenols (ChromaDex, USA); co-chromatography with the authentic compounds and considering their respective spectral patterns. Identified compounds were estimated using an external method after calibration with response factor of authentic compounds with specific concentration, considering proper validation criteria by the following formula:

$$\text{Response factor} = \frac{\text{area of the standard}}{\text{amount of the standard}}$$

$$\text{Amount of the analyte} = \frac{\text{peak area of the analyte}}{\text{response factor}}$$

The concentration of the analytes were expressed in μg/mg as calculated from the dry weight (dw) of NOLE initially measured for the extraction process.

2.17. Statistical analysis

Statistical analysis was performed and graphs were prepared using KyPlot version 2.0 beta 15 (32 bit). Data were statistically analyzed using one-way analysis of variance (ANOVA) with Dunnett's test. Data of OGTT was analyzed using two-way ANOVA $P<0.05$ was considered significant. The IC_{50} value was calculated by the formula: $Y = 100 \times A1/X \times A1$ where $A1 = IC_{50}$, Y=response (Y=100% when X=0), X=inhibitory concentration. All data are reported as the mean ± SD of six measurements.

3. Results

3.1. Alpha-amylase inhibitory activity

The α-amylase inhibitory activity of NOLE is demonstrated in Fig. 1. At 200 μg/ml, the extent of inhibition was 22.63 ± 1.69% ($P < 0.001$) with an IC_{50} value of 703.01 ± 56.47 μg/ml.

3.2. Acute toxicity study

No mortality was observed in the experimental animals at an optimum dose of NOLE (2000 mg/kg). Consequently, 1/40th and 1/10th of the maximum dose was considered for the anti-diabetic study.

3.3. Body weight and blood glucose level

Increase in body weight on day 20 for control, diabetic control, glibenclamide treated and low and high dose of NOLE treated groups was 10.31%, 20.65%, 9.26%, 12.50% and 10.64% respectively, compared to weight on day 0 of the respective groups (Table 1). Measurement of blood glucose level from the tail vein was performed on the same days. The diabetic control group displayed 263.66 ± 8.02 mg/dl glucose on day 0, which was lowered to 245 ± 3.00 mg/dl on day 20, accounting for a mere 7.07% decrease (Fig. 2). Whereas, glucose level was significantly ($P < 0.001$) lowered 67.13% on day 20 in the glibenclamide group when compared to day 0. Glucose level was lowered from 255.66 ± 1.52 mg/dl on day 0 to 67.00 ± 6.24 mg/dl in day 20 in NOLE high group, accounting for a significant ($P < 0.001$) 73.79% decrease.

3.4. Study of serum biochemical parameters

Normalization of glycated hemoglobin (HbA1c) and insulin level due to NOLE treatment are demonstrated in Fig. 3. The effects of glibenclamide and NOLE on various serum enzymatic and biochemical parameters of diabetic experimental mice are shown in the Table 2. All the elevated parameters in the diabetic mice were decreased with subsequent administration of glibenclamide or NOLE for 20 days. The percentage change of these parameters are demonstrated in Fig. 5.

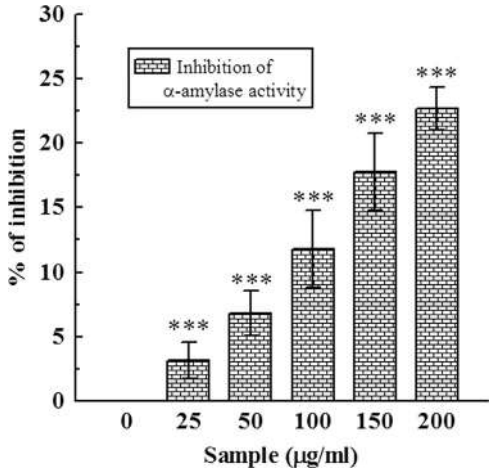

Fig. 1. *In vitro* α-amylase inhibitory activity of NOLE represented as percentage of inhibition. ***$P < 0.001$ vs 0 μg/ml.

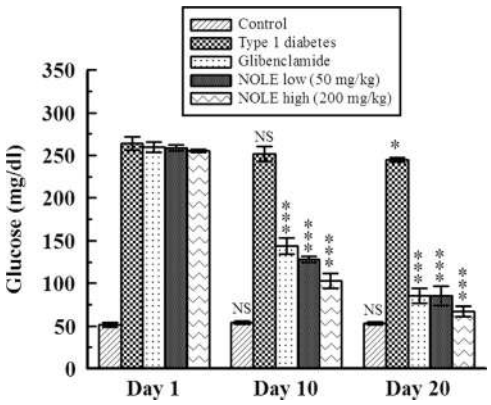

Fig. 2. Represents change in glucose level of alloxan induced diabetic mice on 20 days of treatment. $^{NS}P > 0.05$, *$P < 0.01$ and ***$P < 0.001$ vs day 1 of corresponding group.

3.5. Measurement of lipid peroxidation

The level of serum MDA was increased significantly ($P < 0.001$) in the diabetic control mice compared to the normal control. Consequently, administration of glibenclamide as well as 50 mg/kg and 200 mg/kg BW doses of NOLE resulted in decrease in serum MDA content, signifying lowering of the extent of lipid peroxidation (Fig. 4A). The percentage decrease in the MDA contents were 26.25%, 13.65% and 30.30% respectively for glibenclamide, NOLE low and NOLE high groups compared to diabetic control (Fig. 5A).

3.6. Estimation of hepatic glycogen

The level of hepatic glycogen was decreased dramatically ($P < 0.001$) in the diabetic control compared to normal control (Fig. 4B). Though, neither glibenclamide ($P < 0.001$) nor NOLE ($P < 0.001$) was capable of restoring the hepatic glycogen level close its normal value (control), but NOLE at 200 mg/kg dose demonstrated slight higher activity than glibenclamide. Increase in MDA content in glibenclamide, NOLE low and NOLE high groups were 28.76%, 20.4% and 32.46%, compared to diabetic control (Fig. 5B).

3.7. Estimation of peroxidase and catalase activity

Activity of peroxidase in liver ($P < 0.01$; 32.87%), kidney ($P < 0.001$; 28.81%) and skeletal muscle ($P < 0.01$; 27.53%) was deceased in the alloxan induced experimental diabetic condition (Fig. 4C) and ameliorated in glibenclamide, NOLE low and NOLE high groups. Increase in peroxidase activity in liver, kidney and muscle for NOLE high group was 26.72%, 24.03% and 26.03%, respectively (Fig. 5B) compared to diabetic control. Catalase activity was also identically decreased in

Table 1
Effect of NOLE on the body weight (g) of diabetic animals.

	Control	Diabetic control	Glibenclamide	NOLE low	NOLE high
Day 1	24.53 ± 1.64	26.92 ± 0.89	28.06 ± 3.02	27.67 ± 2.61	27.81 ± 2.74
Day 10	25.94 ± 1.37^{NS}	28.32 ± 3.04^{NS}	29.22 ± 2.03^{NS}	28.57 ± 2.64^{NS}	29.41 ± 2.34^{NS}
Day 20	27.06 ± 1.35^{NS}	32.48 ± 1.46**	30. 66 ± 2.72^{NS}	31.13 ± 1.67^{NS}	30.77 ± 1.17^{NS}

^{NS} $P > 0.05$.
** $P < 0.01$ vs day 1 of corresponding group.

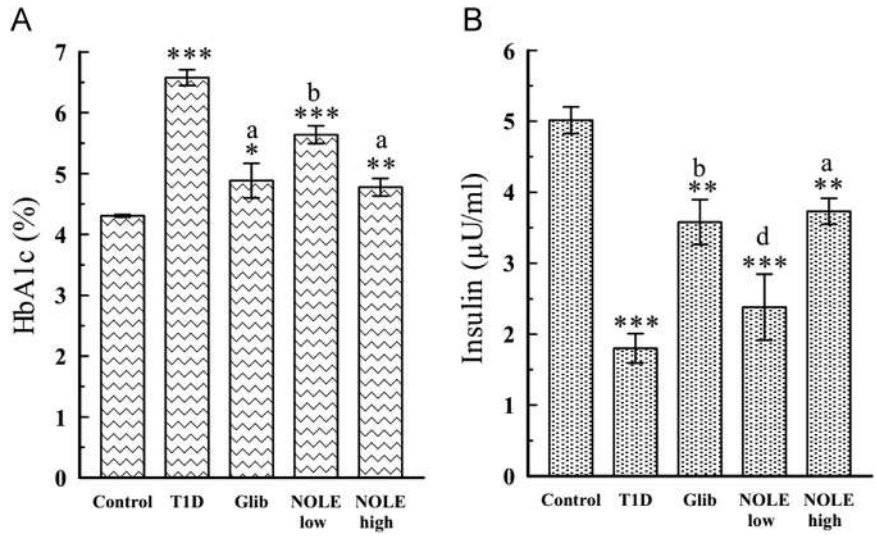

Fig. 3. Represents (A) HbA1c level and (B) Insulin level after 20 days of treatment. *$P < 0.01$, **$P < 0.01$ and ***$P < 0.001$ vs control. T1D= type 1 diabetes and Glib=glibenclamide treated.

Table 2
Levels of various enzymatic and biochemical parameters of the serum of five. experimental groups. The data are represented as mean ± SD.

Parameters (units)	Control	Diabetic control	Glibenclamide	NOLE low	NOLE high
ACP (K.A.)	3.61 ± 0.31	8.28 ± 0.69***	5.64 ± 0.47** b	7.15 ± 0.60* b	4.72 ± 0.47** d
ALP (K.A.)	10.86 ± 1.29	21.7 ± 1.41***	11.55 ± 1.05NS a	16.51 ± 1.09** b	10.73 ± 0.85NS a
AST (U/ml)	65.88 ± 3.74	98.71 ± 5.75**	76.63 ± 2.72* b	82.68 ± 3.63** c	74.79 ± 2.51* b
ALT (U/ml)	42.63 ± 1.4	90.77 ± 5.31***	54.49 ± 2.71** a	70.97 ± 2.62*** a	50.45 ± 2.96*** a
Creatinine (mg/dl)	0.19 ± 0.00	0.35 ± 0.01***	0.19 ± 0.01NS a	0.26 ± 0.00*** b	0.21 ± 0.00* a
Triglyceride (mg/dl)	94.33 ± 4.21	138.36 ± 2.66***	96.53 ± 1.90NS a	122.12 ± 4.31** b	100.73 ± 3.51NS a
Cholesterol (mg/dl)	78.86 ± 3.49	118.39 ± 3.04***	78.43 ± 1.35NS a	99.19 ± 2.59** b	85.75 ± 2.33* a
Urea (mg/dl)	20.58 ± 2.39	57.42 ± 2.06***	26.54 ± 1.95NS a	44.43 ± 0.36*** a	28.47 ± 0.70** a
Urea N_2 (mg/dl)	9.60 ± 1.12	26.81 ± 0.96***	12.39 ± 0.91* a	20.61 ± 0.31*** a	13.29 ± 0.32** a
Uric acid (mg/dl)	1.68 ± 0.13	2.43 ± 0.12**	1.94 ± 0.07* b	2.22 ± 0.07** d	1.83 ± 0.06NS b

NS P=non-significant ($p > 0.05$).
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ vs group control.
a $P < 0.001$ vs diabetic control.
b $P < 0.01$.
c $P < 0.05$.
d P=non-significant ($p > 0.05$).

diabetic control liver liver ($P < 0.01$; 40.11%), kidney ($P < 0.01$; 38.44%) and muscle ($P < 0.01$; 64.28%) samples (Fig. 4D). Compared to diabetic control, significant changes ($P < 0.01$) in catalase activity accounted for 37.65%, 37.55% and 60% increase in liver, kidney and skeletal muscle respectively for NOLE high group (Fig. 5B).

3.8. Oral glucose tolerance test

OGTT was performed on a different set of mice so that the administration of glucose do not influence the serum enzymatic and biochemical parameters. After 20 days of treatment, mice treated with NOLE demonstrated significantly ($P > 0.05$) low glucose level in comparison with diabetic control (Fig. 6). After 3 h of glucose administration, the blood glucose level of NOLE high group was similar ($P > 0.05$) to that of control. The decrease of blood glucose at 3 h post-glucose intake was 25.13%, 55.42% and 65.72% respectively for diabetic control, glibenclamide and NOLE high groups, compared to glucose level on 30 min.

3.9. GC–MS analysis

GC–MS analysis (Supplementary data 1) of NOLE revealed presence of several (Table 3) compounds of diverse chemical natures, many of them possess distinct and definitive pharmacological activities.

3.10. HPLC analysis

HPLC profile of secondary metabolites (Supplementary data 2) at 270 nm revealed the presence of phenolic acids, flavonoids and methylphenols present in NOLE. Gallic acid (GA), 4-Hydroxy benzoic acid (4-HBA), Vanillic acid (VA), p-Coumaric acid (PCA) and Jasmonic acid (JA) were the major identified phenolic acids along with a flavonoid, Rutin (RU) and two metylphenols o-Cresol (oCR) and 3,4-Xylenol (XYL). Jasmonic acid was found to be in highest content (31.357 μg/mg dw) followed by 4-HBA- 0.362 μg/mg dw, GA- 0.297 μg/mg dw, XYL– 0.212 μg/mg dw. The concentration of VA, PCA, RU and o-Cresol in NOLE was 4.254×10^{-3} μg/mg dw, 0.011 μg/mg dw, 0.069 μg/mg dw and 0.076 μg/mg dw respectively. The spectral patterns of the identified compounds are given in Supplementary data 3.

4. Discussion

The initial breakdown of starch to maltose, maltotriose, various α-(1–6) and α-(1–4) oligoglucans is mediated by α-amylase enzyme

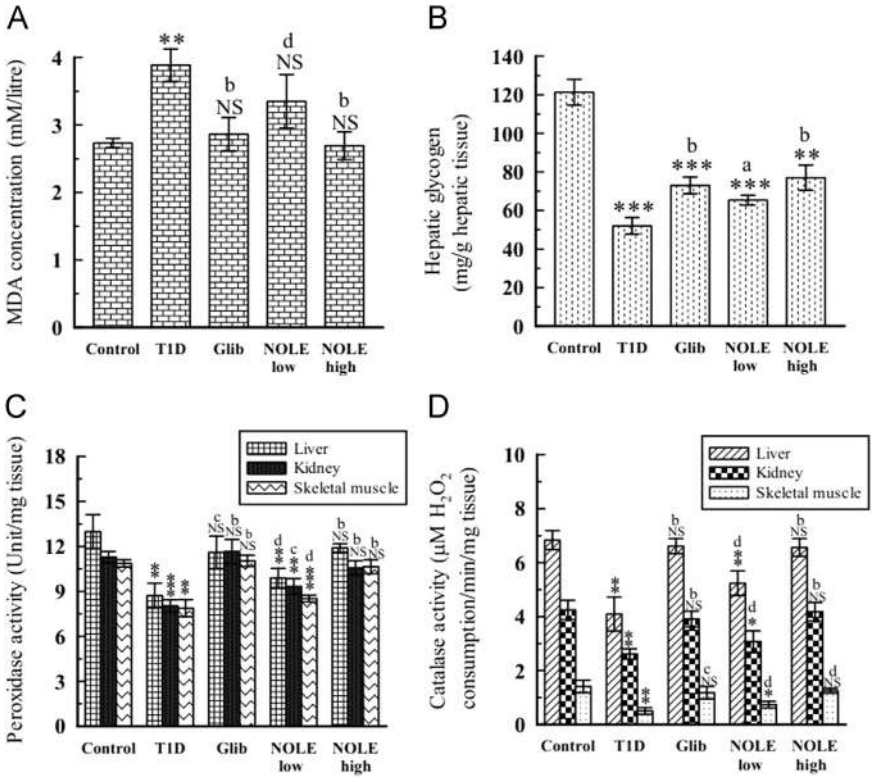

Fig. 4. Anti-diabetic activity of NOLE. (A) MDA concentration. (B) Hepatic glycogen level. (C) Peroxidase activity. (D) Catalase activity. T1D= type 1 diabetes and Glib=glibenclamide treated, NOLE low=50 mg/kg NOLE treated and NOLE high=200 mg/kg NOLE treated. ^{NS}P= non-significant ($P>0.05$), $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs control. ^{d}P= non-significant ($P>0.05$), $^{c}P<0.05$, $^{b}P<0.01$ and $^{a}P<0.001$ vs diabetic control.

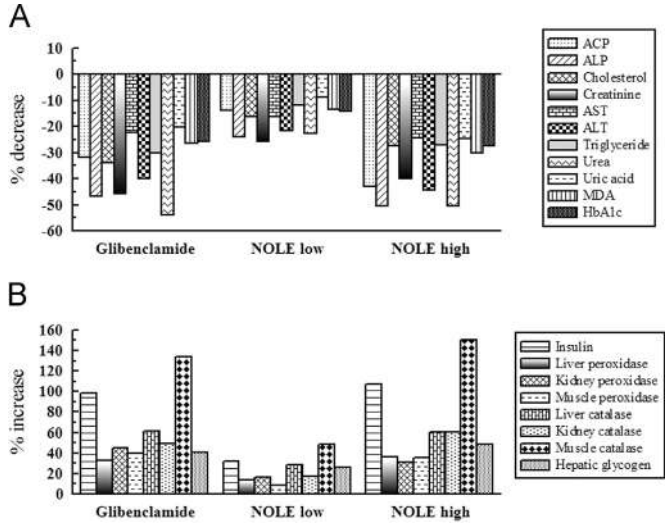

Fig. 5. Describes the percentage change in different enzymatic and biochemical parameters of glibenclamide, NOLE low and NOLE high groups compared to diabetic control. (A) Percentage decrease in ACP, ALP, cholesterol, creatinine, AST, ALT, triglyceride, urea, uric acid, MDA and HbA1c (B) Percentage increase in insulin, peroxidase, catalase and hepatic glycogen.

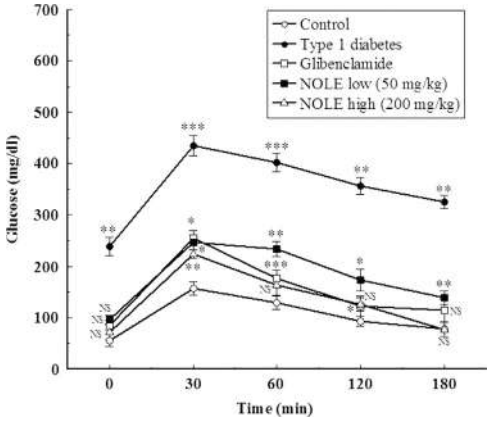

Fig. 6. Blood glucose level at 0, 30, 60, 120 and 180 min in oral glucose tolerance test. ^{NS}P= non-significant ($P>0.05$), $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs corresponding time of control.

and followed by subsequent α-glucosidase activity to finally yield glucose (Sudha et al., 2011). Ishikawa et al. (2007) have already demonstrated α-glucosidases inhibitory activity of *Nerium indicum* and its isolate chlorogenic acid. However, the activity of α-amylase remained unexamined. In this study, dose dependent α-amylase inhibitory activity of NOLE concludes that *Nerium oleander* could be utilized as an anti-nutritional supplement to block the breakdown of carbohydrates to delay the intestinal glucose absorption. Therefore, *Nerium oleander* possess inhibitory effect on both the primary enzymes of complex carbohydrate catabolism and thereby cause delay in the primary breakdown of carbohydrates to monosaccharides. This leads to slower glucose absorption and subsequently lower postprandial blood glucose level.

Direct free radical mediated cytotoxicity to β-cells cause lower insulin level, leading to abnormal glucose metabolism. Alloxan inhibits glucokinase enzyme causing inhibition of glucose induced insulin secretion. Besides, it cause reduction in glucose oxidation and ATP formation that further leads to the inhibition of insulin secretion (Lenzen, 2007). These events result in downfall of insulin level and eventually lead to persistent hyperglycaemic condition. A previous anti-hyperglycaemic study with *Nerium indicum* aqueous extract showed no sub-acute glucose reduction at 500 mg/kg dose (Sikarwar et al., 2009). On the contrary, Ishikawa et al. (2007) showed that *Nerium indicum* aqueous extract lowers the blood glucose level in

Table 3
Chemical fingerprint of NOLE revealed by GC–MS analysis corresponding to Supplementary data 1.

Peak no.	Compounds	Retention time
1	1-Hexanol, 2-ethyl-	8.935
2	Nonanoic acid, 9-oxo-, methyl ester	20.251
3	2-Tridecen-1-ol, (E)-	26.726
4	Tridecanoic acid, 12-methyl-, methyl ester	26.926
5	Tetradecanoic acid	28.077
6	Pentadecanoic acid, methyl ester	29.052
7	2-Pentadecanone, 6,10,14-trimethyl	29.459
8	6-Octen-1-ol, 3,7-dimethyl-, propanoate	29.816
9	7,10,13-Hexadecatrienoic acid, methyl ester	30.454
10	7-Hexadecenoic acid, methyl ester, (Z)-	30.654
11	Hexadecanoic acid, methyl ester	31.279
12	Isophytol	31.555
13	n-Hexadecanoic acid	33.000
14	Heptadecanoic acid, methyl ester	33.050
15	Octadecanoic acid	34.176
16	8,11-Octadecadienoic acid, methyl ester	34.370
17	9-Octadecenoic acid, methyl ester, (E)-	34.570
18	Phytol	34.857
19	Methyl stearate	34.989
20	Isopropyl palmitate	35.039
21	Oleic Acid	35.977
22	cis-Vaccenic acid	36.159
23	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	36.684
24	7,10,13-Hexadecatrienoic acid, methyl ester	37.691
25	Tricosane	37.847
26	Methyl 18-methylnonadecanoate	38.354
27	Vitamin E	47.418
28	dl-α-Tocopherol	47.524
29	Squalene	48.338

maltose and sucrose loaded rats at very high dose of 16 g/kg. They further reported that the blood glucose lowering capacity of *Nerium indicum* aqueous extract was only specific towards disaccharide and had no effect on glucose loading. In the present study, after 20 days of treatment blood glucose level became significantly ($P < 0.001$) lower in the diabetic mice. The anti-hyperglycaemic activity of NOLE at 200 mg/kg dose was recorded to be greater than glibenclamide. Alloxan induced insulin level was attenuated in NOLE treated animals with 32.22% (NOLE low) and 107.22% (NOLE high) increase in serum insulin level compared to diabetic control. In fact, NOLE high group demonstrated 8.34% higher insulin level than glibenclamide treated group. Improvement of insulin level accompanied with the α-amylase inhibitory activity would aid in slower release of glucose and its faster absorption in blood, thereby ensuring a better regulation of hyperglycaemic condition. Additionally, NOLE increased the glucose tolerance in the diabetic mice compared to diabetic control. Blood glucose level at 3 h post-glucose intake was 79.00 ± 14.93 mg/dl, 325.66 ± 12.34 mg/dl and 76.66 ± 13.42 mg/dl respectively, for normal control, diabetic control and NOLE high groups. This suggests increase in peripheral glucose utilization after 20 consecutive days of NOLE treatment. This resulted due to the amelioration of impaired glucose metabolism and insulin secretion, which further increased the peripheral insulin-sensitivity and the rate of glucose clearance from the blood in the NOLE treated animals. Furthermore, higher glycated hemoglobin (HbA1c) level was seen in diabetic control animals, which eventually decreased on NOLE administration. This implicates lower glucose carriage by the erythrocytes which may resulted due to better absorption of glucose by improved level of insulin. The result also corroborated with previous finding of Bas et al. (2012) who demonstrated the HbA1c lowering capacity of oleander shoot distillate. NOLE may thus, cut the risk of microvascular complications such as retinopathy, nephropathy, and neuropathy which arise from higher HbA1c level.

Decrease in body weight is often found to be associated with diabetic conditions. Interestingly the present study demonstrated better weight gain in the alloxanized animals compared to control. This might be due to the elevation of triglyceride level in the untreated diabetic animals. Higher blood triglyceride is associated with higher body weight (Despres et al., 1989; Bray, 2004). High level of uric acid as seen in diabetic control, is also a positive signal for elevated body mass index (Oyamada et al., 2006) and in the pathogenesis of diabetes (Johnson et al., 2013). In NOLE treated groups, there was no drastic significant increase in body weight as demonstrated in Table 1. In this context, Gayathri et al. (2011a) demonstrated the body weight lowering capacity of *Nerium oleander* in experimental rats through hypolipidemic activity. In addition, leptin, which trends to be higher in obese individuals (Considine et al., 1996), was found to be much lower in *Nerium oleander* distillate treated animals compared to untreated diabetic animals (Bas et al., 2012).

The present investigation showed significant ($P < 0.001$) lowering of hepatic glycogen, which was parallel with decrease in insulin level in diabetic control. The activity and amount of glucokinase and phosphofructokinase are influenced by insulin causing hepatic glycolysis (Dodamani et al., 2012). Elevated glycogenesis and/or decreased glycogenolysis cause increase in hepatic glycogen content. Insulin promotes glycogen deposition by regulating glycogen synthase and by inhibiting glycogen phosphorylase. Significant ($P < 0.01$) restoration of hepatic glycogen was observed in case of NOLE treated animals. NOLE administration may have restored the alloxan toxified β-cells by protecting through free radical scavenging activity which eventually resulted in gradual increase in insulin level and influence the restoration of hepatic glycogen. Besides, the increased glucose absorption as demonstrated by OGTT, contributed in the conversion of blood glucose to glycogen and its further restoration in hepatic tissue.

Elevated blood glucose causes overproduction of superoxide radicals ($O_2\bullet^-$) and H_2O_2 (Ceriello et al., 2002) which are further held responsible for microvascular complications in hyperglycemia (Scott and King, 2004). Autoxidation of alloxan derived dialuric acid generates $O_2\bullet^-$, H_2O_2 and hydroxyl radical ($OH\bullet$) (Lenzen, 2007). Superoxide dismutase (SOD) converts superoxide radical ($O_2\bullet^-$) to H_2O_2, which in turn is converted to O_2 and H_2O by

CAT and PX activity. Diabetes mellitus is recognized to be associated with free radical mediated lipid peroxidation leading to atherosclerotic and cardiovascular mortality. In this autocatalytic process, polyunsaturated fatty acids in the plasma membrane undergo degradation forming lipid hydroperoxides such as MDA (Tangvarasittichai et al., 2009). In the present study, ROS mediated oxidative stress was confirmed through the increase of MDA in the alloxanized mice. NOLE has demonstrated stabilization of CAT and PX activity in liver, kidney and skeletal muscle in addition to protection from ROS mediated lipid peroxidation in terms of decrease in MDA content in systemic circulation. Besides, NOLE has previously demonstrated the capacity of direct *in vitro* scavenging activity against some of the major ROS generated in alloxan diabetes such as $O_2\bullet^-$, H_2O_2, OH● as well as inhibition of lipid peroxidation activity (Dey et al., 2012). These findings were supported by the previous investigations of Gayathri et al. (2011b) who demonstrated that a similar hydromethanolic extract of *Nerium oleander* inhibits the lipid peroxidation by maintenance of antioxidant enzymes and by the inhibition of free radicals. In addition, Singhal and Gupta (2012) reported normalization of hepatic SOD level by oleander flower methanolic extract.

Hepatotoxicity and nephropathy are accentuated results of diabetes mellitus. Present observation has documented that AST and ALT levels were significantly elevated in the diabetic control animals and therefore, corroborates the previous findings that chronic elevation of hepatic transaminases are common with diabetic patients (Ahn et al., 2014). Substantial decrease in liver marker enzymes such as ACP, ALP, AST, ALT due to NOLE treatment in alloxanized animals correlates with the findings of Singhal and Gupta (2012) who formerly demonstrated under hepatoprotective evaluation that the oleander flower normalized AST, ALT and ALP levels possibly through antioxidative mechanism. Amelioration of hepatic transaminase and phosphatase took place as a result of decreased free fatty-acids and their peroxides in serum, reduced oxidation, phosphorylation and hepatic inflammation (Drabkin and Marsh, 1947; Harris, 2005) due to NOLE administration. Moreover, alloxan induced free radical mediated hepatic inflammation was lowered in the diabetic animals due to increase in antioxidant enzymes as well as hepatoprotective and antioxidant effects of NOLE (Dey et al., 2012; Singhal and Gupta, 2012). Furthermore, renal morbidity and mortality resulting due to diabetic nephropathic symptoms such as renal atherosclerosis, urinary tract infections, papillary necrosis and glomerular lesions cause chronic kidney failure. NOLE treatment significantly lowered the elevated urea, uric acid and creatinine levels in the alloxanized animals. In fact low serum insulin level in diabetic condition results in inadequate carbohydrate derived energy leading to increased protein catabolism which cause glomerulo-dysfunction. Therefore, NOLE mediated increase in insulin level and improvement in carbohydrate metabolism contributed in amelioration of diabetic nephropathy. The antioxidative protection rendered by NOLE may likewise functioned in safeguarding the renal system as free radical facilitated oxidative stress possess serious implications on kidney disease in diabetes (Forbes et al., 2008).

Elevation of cholesterol and triglyceride levels in diabetic condition are the hallmarks of dyslipidaemia. Marked increase in liver triglyceride occurs in non-alcoholic fatty liver disease in diabetic patients (Harris, 2005). In fact, malfunction of β-cells in insulin production or insulin resistant condition may give rise to hyperlipidemia since insulin possess inhibitory effect on 3-hydroxy-3-methyl-methylglutaryl coenzyme-A, a key enzyme of cholesterol biosynthesis (Ghoul et al., 2012). Severe retinopathy leading to complete blindness due to blockage of blood vessels of eye may result in diabetes due to the high cholesterol. The present study demonstrated 16.21% and 40% decrease in cholesterol level and 11.73% and 27.19% decrease in triglyceride levels respectively, for NOLE low and NOLE high groups compared to diabetic control. The present findings are comparable with two previous interconnected studies which demonstrated anti-hyperlipidemic activity of oleander flower by studying cholesterol, triglyceride, very low-density lipoprotein (VLDL) and lipolytic enzymes (Gayathri et al., 2011a, 2013). Diabetes associated cardiovascular diseases such as angina, myocardial infarction, stroke, peripheral artery disease, and congestive heart failure, which are the most prevalent cause of mortality among diabetic patients, may thus be improved due to NOLE treatment attributed to its anti-hyperglycaemic and anti-hyperlipidemic activity (International Diabetes Federation, 2013).

In the current set of experiments, GC–MS analysis was included to highlight the phytochemical composition of NOLE. The results revealed presence of several comparatively low molecular weight and moderately low boiling bioactive ingredients possessing significant anti-diabetic activity. Vaccenic acid displayed potent hypotriglyceridemic effect when evaluated on diabetic condition (Wang et al., 2008). Besides vaccenic acid mediated lowering of serum IL-10 may contribute in the inflammatory regulation of diabetic complications (Wang et al., 2008). Elmazar et al. (2013) through biochemical modulation and docking stimulation demonstrated that anti-diabetic efficiency of phytol is mediated by activation of nuclear receptor X (RXR) in addition to heterodimerization of RXR with PPAR-γ. Oleic acid not only down-regulates TNF-α expression, a substantial risk factor for diabetes, but also improves insulin production and lowers glucose level on experimental animals (Vassiliou et al., 2009). A tocopherol fraction from *Cucurbita pepo* down-regulated glucose, HbA1c, insulin HOMA-IR, cholesterol and triglyceride levels in diabetic animals (Bharti et al., 2013). In fact in diabetic animals, increase in catalase, SOD and glutathione-S-transferase levels and reduction in lipid peroxidation reflects enhanced anti-oxidant protection due to tocopherol treatment (Bharti et al., 2013). Furthermore, it is a well-known fact that antioxidants of plant extract have great potential in ameliorating diabetes mellitus, chronic hyperglycemia and protect body against increased oxidative stress in diabetic state (Logani and Davies, 1980; Collier et al., 1990). Secondary metabolites for example, phenolics and phenolic derived compounds are the major determinants for free radical scavenging capabilities (Harborne, 1968; Heim et al., 2002). The phenolic compounds identified by HPLC analysis therefore, had serious implications on the anti-diabetic activity of NOLE. 4-hydroxybenzoic acid demonstrated dose-dependent increase in peripheral glucose absorption in addition to normalization of serum insulin and hepatic glycogen *in vivo* (Peungvicha et al., 1998a, 1998b). Patel and Goyal (2011) studied the effect of gallic acid on diabetes-induced myocardial dysfunction in rat model. The results indicated that gallic acid possess hypoglycaemic effect and resulted significant lowering of serum cholesterol, triglyceride, lactate dehydrogenase in diabetic animals. Furthermore, antioxidative protection in diabetic animals attributed to gallic acid administration was confirmed due to elevation in SOD, catalase, reduced glutathione levels and lowering in MDA content in serum. The polyphenolic flavonoid rutin displayed hyperglycaemic activity as evaluated by normalization of plasma glucose, insulin and HbA1c levels (Kamalakkannan and Prince, 2006). Lipid hydroperoxides and TBARS contents were reduced contributing to the anti-diabetic efficiency of rutin. Besides, literature survey reveals that many of the identified phytocompounds are also active constituents of other herbal formulations possessing anti-diabetic capacity.

5. Conclusion

It is noteworthy that oleander extracts are used to treat diabetic complications in different parts of the world and we believe that the protection excreted by NOLE is due to the synergistic activities of bioactive metabolites. The present investigation provides evidence that *Nerium oleander* possess hypoglycaemic potential as well as ameliorates diabetes associated hyperlipidemic and nephropathic

complications in a dual way *i.e.* by improving carbohydrate metabolism and providing antioxidative protection.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.12.012.

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Assessment of haemolytic, cytotoxic and free radical scavenging activities of an underutilized fruit, *Baccaurea ramiflora* Lour. (Roxb.) Muell. Arg.

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Baccaurea ramiflora Lour. (Roxb.) Muell. Arg. is an underutilized juicy fruit bearing plant found in sub-Himalayan area, South China, Indo-Burma region, etc. The fruit is considered to be nutritive, and in this study, we evaluated its antioxidant, haemolytic and cytotoxic properties. The juice was examined for the quenching activity of hydroxyl radical, nitric oxide, singlet oxygen, peroxynitrite, total antioxidant activity (TAA), erythrocyte membrane stabilizing activity (EMSA) along with quantification of phenolic and flavonoid contents and also tested for its potential activity as iron chelator, inhibitor of lipid peroxidation and total reducing power. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were also performed to correlate antioxidant capacities with the phenolic and flavonoid content. Haemolytic activity on murine erythrocyte and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxic test was performed on murine splenocytes, thymocytes, hepatocytes and peritoneal exudates macrophage to examine the cytotoxic effect of its juice. The result exhibited its potent free radical scavenging activity. In case of TAA, DPPH (2, 2-diphenyl-1-picrylhydrazyl), EMSA and lipid peroxidation, the fruit juice was found to have significant (P <0.001) antioxidant capacity, which is evident from low IC_{50} (half maximal inhibitory concentration) value. Results obtained from haemolytic inhibition assay and MTT cytotoxic test confirms that the juice does not contain any cytotoxic effect and the fruit is safe for consumption. Fourier transform infrared (FTIR) spectra analysis exhibited high possibility of presence of flavonoid compounds in the juice.

Keywords: Antioxidant, Burmese grape, EMSA, HCA, FTIR, MTT assay, PCA, ROS, TAA.

Fruits are the most common sources of essential micronutrients such as vitamin C, tocopherol, carotenoids, polyphenolics, flavonoids, etc. which provide health reimbursement along with their nutritional value and also protect the body from oxidative damage[1-6]. Due to the presence of natural antioxidant, fruits play a defensive role against free radical mediated cellular damage and thus, reduce the risk of several diseases such as cancer, cardiovascular problems and other age-related disorders[1,3,7,8]. Due to redox properties, polyphenols in fruits perform multiple roles, for instance, hydrogen donation, singlet oxygen quenching, metal chelation and as reducing agents[1,2,4,9]. Nevertheless a combination of phenolic compounds always exhibit better antioxidant activity in comparison to a single phenol, as they are responsible for cooperative and modified biological activity[10].

Baccaurea ramiflora Lour. (Roxb.) Muell. Arg. (synonym *Baccaurea sapida* (Roxb.) Muell. Arg.), locally known as *Latkan*, is a semi-evergreen tree which grows up to 10 m in height. The fruits (Fig. 1) are yellowish pink to purple in colour with leathery pericarp, glabrous, 2-4 cm in diameter with 3-4 seeds, embedded in pale-rose coloured delicious pulp. This species of Phyllanthaceae family is distributed in the

Fig. 1 — Fruits of *Baccaurea ramiflora* (Insert: a single fruit after peeling)

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sub-Himalayan tract, mainly on the eastern side from Nepal to Sikkim, Darjeeling hills and Arunachal Pradesh to Assam, Tripura, Bhutan, Burma, South China, Malaya peninsula and Andamans[11]. The fruits are largely exploited as a good source of vitamin C and several nutrients[12] and also used for preparing wine[13]. In Chinese Dai medicine, the plant is used as an antiphligistic and anodyne against rheumatoid arthritis, cellulites and abscesses[14]. Previously, Yang *et al*. have isolated two new phenols, 6'-O-vanilloylisotachioside and 6'-O-vanilloyltachioside from the leaves[15] of *B. ramiflora* and further, three new vanilloid derivatives from its stems[16]. Ullah *et al*.[17] investigated the hypoglycaemic, hypolipidemic and antioxidant activity of the methanolic leaf extract of *B. ramiflora*, whereas Prakash *et al*.[18] highlighted the DNA protective activity, lipid peroxidation and superoxide radical scavenging activity of dried fruit extract of *B. ramiflora*.

In the present study, we determined the detailed antioxidant and free radical scavenging profiling of *B. ramiflora* fruit juice (BRJ); and correlation patterns between several antioxidant traits and various functional groups like phenols, alkenes, aromatic moiety etc. The haemolytic and cytotoxic activity was also performed to study the toxic effect of BRJ, if any.

Materials and Methods

Chemicals

Chemicals and reagents used, were of analytic grade, and procured either from Merck, Mumbai, India or HiMedia Laboratories Pvt. Ltd., Mumbai, India including the EZcount™ MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Assay Kit.

Preparation of *B. ramiflora* fruit juice (BRJ)

Latkan fruits were procured from the market and identified by the plant taxonomist, and submitted under voucher specimen no: BS/NBU/1503 at the University of North Bengal. The fruits were washed thoroughly in water thrice, and the juice was extracted by squeezing the pulp after removing the outer leathery skin. The collected fresh juice was filtered through sterile 4-fold muslin cloth and filtrate was stored in sterile plastic containers for further analysis.

Since a person consumes fruit or fruit juices directly, we performed all the bioassays with untreated fresh fruit juice, not in any solvent-extracted form.

Antioxidant assays

Twelve different biochemical assays were performed in the present study to evaluate the antioxidant properties of BRJ. All the standards were prepared as 1 mg/mL of stock and suitably diluted as per requirements.

DPPH (*2, 2-diphenyl-1-picrylhydrazyl*) *radical scavenging assay*

Free radical scavenging activity of BRJ was measured using the method of Chew *et al*.[19] with slight modification. The freshly prepared DPPH (1 mM; diluted in 95% methanol) and various concentrations of juice (0-100 µL/mL) was mixed to make final volume of 1 mL. The solutions were mixed properly. Immediately after 2 min, optical density (OD) was measured at 517 nm using UV-Vis Spectrophotometer (Thermo UV1, Thermo Electron Corporation, England, UK). Ascorbic acid was taken as reference. The percent radical scavenging activity was calculated using equation I:

$$\% \text{ of scavenging DPPH} = [(A_0 - A_1) / A_0] \times 100 \quad (I)$$

where, A_0= the absorbance of the control and A_1= the absorbance in the presence of the samples and standard.

Hydroxyl radical scavenging assay

The assay was done based on the method of Elizabeth *et al*.[20] with a few changes. The reaction mixture consisted of 2-deoxy-2-ribose (2.8 mM), monopotassium phosphate-potassium hydroxide buffer (KH_2PO_4-KOH; 20 mM, pH 7.4), $FeCl_3$ (100 µM), ethylenediaminetetraacetic acid (EDTA; 100 µM), hydrogen peroxide (H_2O_2; 1.0 mM), ascorbic acid (100 µM) and various concentrations of juice (0–200 µL/mL) to make the final volume of 1 mL. The mixture was incubated for 1 h at 37°C. About 200 µL of reaction mixture was taken into another tube and mixed with 400 µL of tricarboxylic acid (TCA; 2.8%) and 400 µL of aqueous thiobarbituric acid (TBA; 1%). After 15 min of incubation at 90°C, solutions were brought to room temperature (22°C) and the absorbance was measured at 532 nm against an appropriate blank solution. Mannitol, a classical OH scavenger was used as the positive control. Percentage of inhibition was evaluated using equation I.

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging assay was performed following GriessI-llosvoy reaction[21] with minor modifications. Sodium nitroprusside (SNP;

10 mM), phosphate buffered saline (pH 7.4) and various concentration (0–200 µL/mL) of juice were mixed to make final volume of 1 mL. After incubation at 25°C for 150 min, .0.4 mL of sulfanilamide (0.33%; diluted in 20% of glacial acetic acid) was added to 0.2 mL of the incubated reaction mixture and allowed to stand for 5 min. Finally, 0.4 mL of N-(1-Naphthyl) ethylenediamine dihydrochloride (NED; 0.1%) was added and incubated for 30 min at 25°C. The changing of colour was measured spectrophotometrically at 540 nm against blank sample. Curcumin was used as standard. The percentage inhibition was calculated using equation I.

Peroxynitrite scavenging activity

To synthesize the peroxynitrite ($ONOO^-$)[22], 5 mL of KNO_2 (0.6 M) was mixed with an acidic solution (0.6 M hydrochloric acid) of H_2O_2 (5 mL; 0.7 M) in an ice bath for 1 min followed by adding of ice cold sodium hydroxide (NaOH; 5 mL; 1.2 M). Excess amount of H_2O_2 was removed by the treatment of granular manganese oxide (MnO_2; prewashed with NaOH; 1.2 M) and left for overnight at −20°C to prepare peroxynitrite solution. The solution was collected from the top of the frozen mixture and measured spectrophotometrically at 302 nm.

Then Evans Blue bleaching assay[23] was performed to measure the peroxynitrite scavenging activity with slight changes. A reaction mixture was prepared containing phosphate buffer (50 mM; pH 7.4), Diethylenetriaminepentaacetic acid (DTPA; 0.1 mM), sodium chloride (90 mM), potassium chloride (5 mM), Evans Blue (12.5 µM), various concentrations of juice (0–200 µL/mL) and peroxynitrite solution (1 mM) into a final volume of 1 mL. The final absorbance was measured at 611 nm after incubation at 25°C for 30 min. The scavenging percentage was calculated by comparing the results of the test and blank samples. Gallic acid was used as reference. The scavenging percentage was calculated using equation I.

Singlet oxygen scavenging assay

Chakraborty *et al*.[24] with minor modifications was followed to determine singlet oxygen scavenging activity. The reaction between sodium hypochlorite (NaOCl), H_2O_2 and bleaching of N,N-dimethyl-4-nitrosoaniline (RNO) generated the singlet oxygen ($^1O_2$). Various concentrations of juice (0–200 µL/mL), phosphate buffer (45 mM; pH 7.1), NaOCl (50 mM), H_2O_2 (50 mM), L-histidine (50 mM), and RNO (10 µM) were mixed together and made the final volume up to 1 mL. The final mixture was incubated at 30°C for 40 min and absorbance of RNO was measured at 440 nm. Lipoic acid was taken as positive control. The scavenging activity was determined using equation I.

Iron chelation

The ferrous ion chelating activity was followed as per the method of Haro-Vicente *et al*.[25] with minor changes. Various concentrations of juice (0–200 µL/mL) were mixed with 12.5 µM ferrous sulfate solutions in HEPES buffer (20 mM; pH 7.2) followed by the addition of ferrozine (75 µM) to initiate the reaction. The mixture was mixed properly and left for 20 min at room temperature. The absorbance was measured at 562 nm. EDTA was used as a positive control.

Measurement of reducing power

The method of Oyaizu[26] was followed with slight modification to determine the Fe^{3+} reducing power of the juice. A reaction mixture of 1 mL was prepared with phosphate buffer (pH 6.6) and potassium hexacyanoferrate (0.1%) and different concentrations (0–60 µL/mL) of juice and incubated in water bath at 50°C for 20 min. Following incubation, TCA (10%; 0.5 mL) was added to the mixture. The upper portion of the solution was taken into another tube and mixed with equal volume of distilled water and ferric chloride solution ($FeCl_3$; 0.01%) and left for 10 min at room temperature. The absorbance was measured at 700 nm. Butylated hydroxytoluene (BHT) was used as a positive control.

Lipid peroxidation inhibition assay

Lipid peroxidation assay was followed by the method of Kizil *et al*.[27] with a few changes. Brain homogenate of Swiss albino mice (20 ± 2 g) was prepared by centrifuging with phosphate buffer (50 mM; pH 7.4) and 120 mM of potassium chloride (KCl) at 3000 rpm for 10 min. The homogenate (100 µL) was mixed with the juice of various concentrations (0-25 µL/mL), followed by addition of ferrous sulfate ($FeSO_4$; 0.1 mM) and ascorbic acid (0.1 mM) to make final volume of 1 mL and left for 1 h incubation at 37°C. After incubation, TCA (500 µL; 28%) and TBA (380 µL; 2%) were added in the reaction mixture and then heated at 95°C in water bath for 30 min. Then the samples were brought to the room temperature and centrifuged at 8000 rpm for 2 min. The absorbance of the supernatant was measured at 532 nm. Trolox was used as positive control.

Total antioxidant activity

Slightly modified method of Prieto *et al*.[28] was followed to study the total antioxidant activity (TAA) of BRJ. Different concentrations of juice (0-150 µL/mL) were mixed with 1 mL of reaction mixture containing 0.6 M of sulfuric acid (H_2SO_4), sodium phosphate (28 mM) and ammonium molybdate (1%). The mixture was kept on a water bath at 95°C for one h. Then the mixture was cooled down to room temperature and the absorbance was read at 695 nm against a suitable blank. Ascorbic acid was used as a standard. The antioxidant activity was measured by the capacity of the yam extract to reduce molybdenum (VI) to molybdenum (V) using equation II:

$$\% \text{ of TAA} = [(OD_0 - OD_1) / OD_0] \times 100 \quad \text{(II)}$$

where OD_0 was the absorbance of the blank and OD_1 was the absorbance in the presence of the samples and standard (ascorbic acid).

Erythrocyte membrane stabilizing activity (*EMSA*)

The protocol developed by Concepcion Navarro *et al*.[29] was followed to estimate the erythrocyte membrane stabilizing activity of juice. A reaction mixture of 1 mL was prepared containing 50 mM of phosphate buffer (0.2 mL; pH 7.2), 0.4 mL of distilled water, 0.1 mL of RBC suspension (10%; diluted in PBS), EDTA (40 µL; 12 mM), 60 µL of nitro blue tetrazolium (NBT; 1%), 40 µL of riboflavin and varying concentrations of juice (0-200 µL/mL). The reaction mixture was kept under bright light for 30 s and incubated for 30 min at 50ºC. Following incubation, the mixture was centrifuged at 1000 rpm for 10 min and absorbance of the supernatant was measured at 562 nm. Quercetin was taken as reference standard. The erythrocyte membrane stabilizing activity was measured using equation III:

$$\% \text{ of protection} = (A_s / A_b) \times 100 \quad \text{(III)}$$

where, A_s and A_b are the absorbance value of sample and blank, respectively.

Quantification of total phenolic and total flavonoid content

We followed Singleton *et al*.[30] with slight modifications for quantification of total phenolic content. Briefly, the juice (100 µL) was mixed with 0.75 mL of Folin–Ciocalteu reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at room temperature (RT), then sodium carbonate (Na_2CO_3; 0.06%) solution was added to the mixture. After incubation at RT for 90 min, the absorbance was measured at 725 nm. The phenolic content was measured against a gallic acid standard curve.

Aluminum Chloride ($AlCl_3$) method[31] was used to assess total flavonoid content. The absorbance was measured at 510 nm. One hundred µL of juice was added to 0.3 mL distilled water followed by 0.03 mL of $NaNO_2$ (5%). After 5 min at RT, $AlCl_3$ (10%; 0.03 mL) was added followed by further incubation of 5 min at RT. The reaction mixture was then treated with 0.2 mL of NaOH (1 mM) and diluted to 1 mL with water. The flavonoid content was determined from a quercetin standard curve.

***In vitro* haemolytic assay**

Haemolytic effect of BRJ was evaluated according to the standardized method of Malagoli[32] with few modifications. Briefly, human blood was collected in citrated tubes and the erythrocytes were then washed (150 × g for 5 min) three times with 20 mM Tris–HCl containing 144 mM NaCl (pH 7.4). The erythrocyte suspension (100 µL) was prepared with the same solution and plated into each well of the 96-well plate. NaCl solution (0.85%) containing 10 mM $CaCl_2$ was added (100 µL) to each well. The first lane served as the negative control without plant extract. From the second lane, various concentrations (0-200 µL/mL) of *latkan* juice were added into different lanes. In another set of lanes, 100 µL of Triton X-100 (0-200 µL/mL) in 0.85% saline were plated and used as standard. The plate was then incubated for 30 min at 37°C. After incubation, the suspension was centrifuged (604 × g for 5 min) and the supernatant was taken to a fresh 96-well plate and measured the absorbance of liberated haemoglobin at 540 nm. The percentage of haemolytic activity was calculated using the following equation IV:

$$\% \text{ of haemolytic activity} = [(H_0 - H_1) / H_0] \times 100 \quad \text{(IV)}$$

where H_0 was the absorbance of the blank and H_1 was the absorbance in the presence of the samples and standard (Triton X-100).

MTT cell cytotoxicity assay

To evaluate the cytotoxic effect, Swiss albino mice were sacrificed and the spleen, liver and thymus were separated. Peritoneal exudates macrophages were collected by washing the mouse peritoneal region with RPMI-1640. Cell suspension (2×10^6 cells/mL) was prepared in RPMI-1640 medium supplemented with 50 U/mL penicillin, 50 U/mL streptomycin, 50 U/mL nystatin and 10% fetal bovine serum (FBS).

One hundred µL of the cell suspension was added with 100 µL of different concentrations (0-200 µL/mL) of juice (diluted in RPMI-1640) to 96-well plate. The cytotoxicity assay was performed according to the manufacturer's instructions of Ezcount™ MTT Cell Assay Kit (HiMedia).

All the experiments were approved by the Animal Ethical Committee of the University of North Bengal (Permit No. 840/ac/04/CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

FTIR spectrophotometric analysis

Juice (10 µL) was taken in calcium fluoride (CaF_2) vessel and placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Shimadzu 8300 FTIR spectrophotometer at ambient temperature (25ºC). Background correction was made by taking IR spectrum of de-ionized water as the reference in identical condition. The sample was scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal to noise ratio. The functional groups were identified from the IR peaks[33].

Statistical analysis

For reproducibility, all the data were prepared as the mean ± SD of 6 measurements. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's test using SPSS statistics software package (version 20.0 for windows) where $P < 0.05$ was considered as significant. The graphs were prepared using KyPlot (version 2.0 for windows).

In order to analyze the relationship between the antioxidant traits and the quantified phytochemicals, principal component analysis (PCA) based on the correlation matrix was drawn. Two factors were extracted under varimax method. The data obtained from the antioxidant profile were analyzed by multivariate statistical approach, employing hierarchical cluster analysis (HCA). The method employed was 'proximity' matrix using between group linkages. The differences between the measured variables were calculated by square Euclidean distances. Transform values of variables (average zero and SD 1) called Z scores was carried out as a pre-treatment of the data. Horizontal dendrogram with all clusters icicle chart was carried out to elucidate the similarity or nearness of the various measured variables.

Results

B. ramiflora fruit juice was subjected to concentration-dependent free radical scavenging activity using different assays and expressed in terms of inhibition percentage and IC_{50} values (Table 1). Out of the different bioassays, juice was found better in DPPH activity, TAA, hydroxyl, EMSA and in peroxynitrite scavenging activity as compared to standard. The low IC_{50} values (Table 1) of different biochemical assays suggest the BRJ to be a potent antioxidant agent. TPC of the BRJ was 78 ± 0.009 mg/mL, gallic acid equivalent (GAE) per 100 mL plant juice whereas TFC of the juice was 64 ± 0.005 mg/mL quercetin equivalent (QE) per 100 mL plant juice. Figure 2a-d exhibit significant ($P < 0.001$) scavenging activity at every studied dose of four different antioxidant assays whereas Fig. 3a-d of BRJ shows the scavenging percentage of singlet oxygen assay, iron chelating, lipid peroxidation and TAA.

Table 1 — IC_{50} values of different antioxidant assays and their percentage of inhibition

Name of the assays	IC_{50} values[$]		% of inhibition	
	BRJ (µL/mL)	Standard (µL/mL)	BRJ (µL/mL)	Standard (µL/mL)
DPPH	14.01±0.88**	Ascorbic acid 240.12±28.35	95.69±0.17 at 100	27.93±1.10 at 100
Hydroxyl radical (OH^-) scavenging	410.66±23.13**	Mannitol 589.06±46.57	30.48±1.89 at 200	22.48±1.68 at 200
Nitric oxide (NO) radical scavenging	162.87±8.22**	Curcumin 86.84±3.29	61.11±0.87 at 200	86.95±1.39 at 200
Peroxynitrite ($ONOO^-$) scavenging	901.87±25.79*	Gallic acid 734.81±28.30	16.01±0.48 at 200	16.72±0.91 at 200
Singlet oxygen ($^1O_2$) scavenging	795.50±11.68***	Lipoic acid 48.40±2.02	20.56±0.35 at 200	77.97±0.30 at 200
Iron chelating activity	677.43±28.17***	EDTA 1.45±0.01	25.28±1.52 at 200	99.55±0.17 at 6
Lipid peroxidation	105.14±2.46***	Trolox 11.11±0.22	19.70±0.77 at 25	77.58±1.03 at 25
Total antioxidant activity	7.72±0.19***	Ascorbic acid 118.16±5.87	96.61±0.04 at 150	52.17±3.52 at 150
EMSA	149.48±5.46*	Ascorbic acid 77.50±17.18	67.20±1.55 at 200	68.50±3.45 at 200

[$]Data are expressed as mean ± S.D; *P <0.05, **P <0.01 and ***P <0.001 *vs.* Standard.

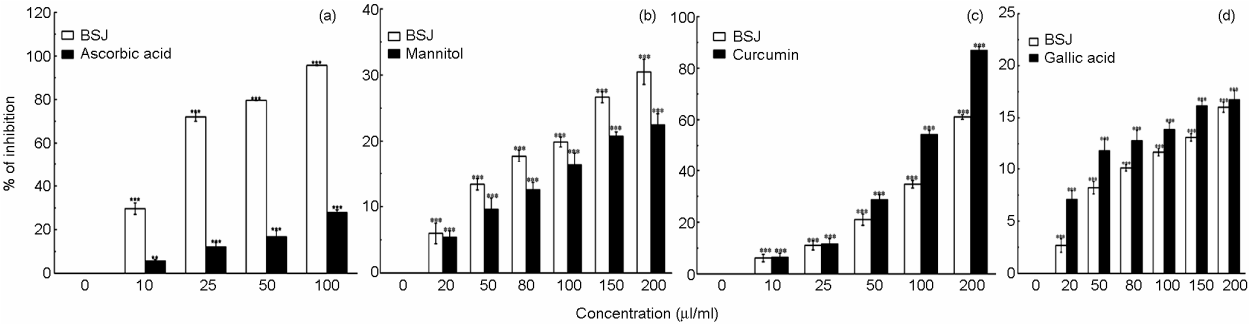

Fig. 2 — (a) DPPH radical scavenging activity of BRJ and ascorbic acid (as standard); (b) Hydroxyl radical scavenging assay of BRJ and standard mannitol; (c) Nitric oxide radical scavenging activity of BRJ and curcumin; and (d) Peroxynitrite anion scavenging assay of BRJ and gallic acid. [Each value represents mean ± SD (n = 6). ***P* <0 .01 and ****P* <0.001 *vs*. 0 µL/mL]

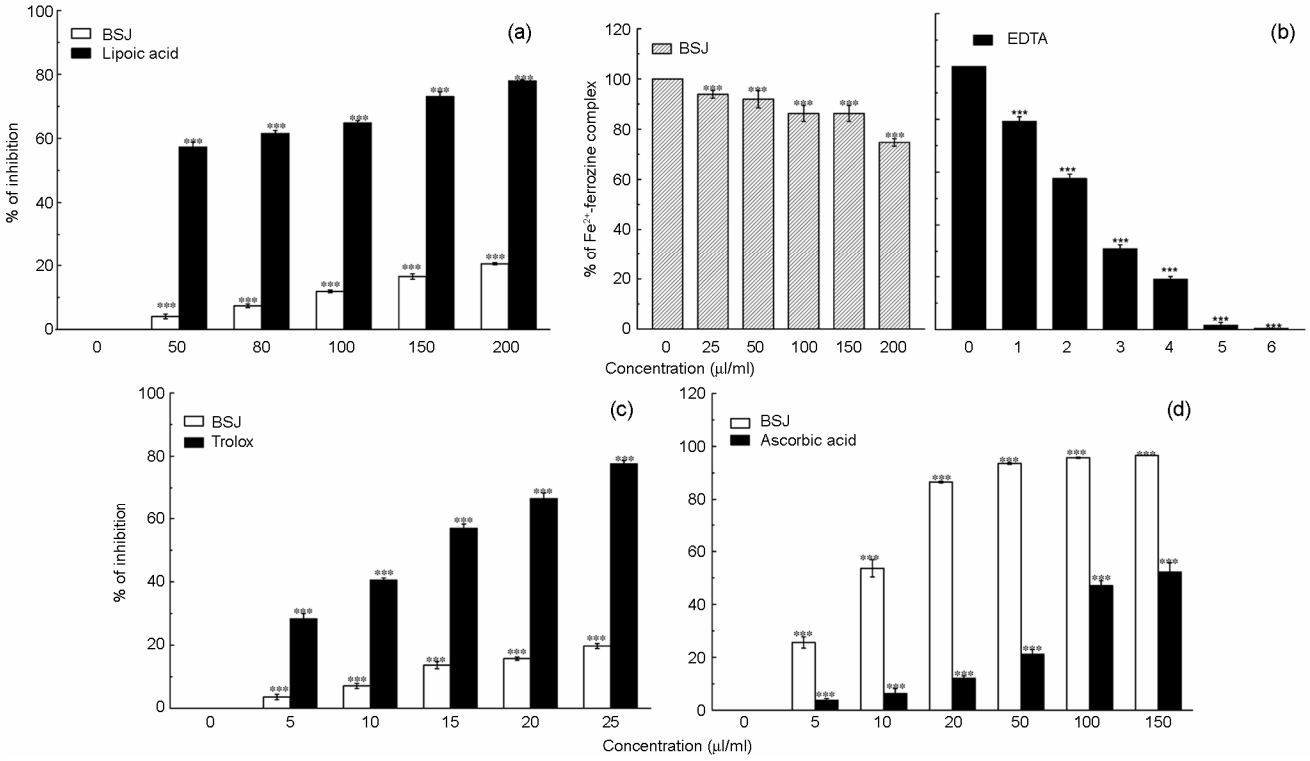

Fig. 3 — (a) Singlet oxygen scavenging assay of BRJ and lipoic acid; (b) Iron chelation assay of BRJ and standard EDTA on Fe^{2+}-ferrozine complex formation. The data were expressed as percentage inhibition of chromogen formation; (c) Inhibition of Lipid peroxidation by BRJ and trolox (the data were expressed as the percentage of lipid peroxidation inhibition of brain homogenate, induced by Fe^{2+} ascorbic acid); and (d) Total antioxidant activity (TAA) of BRJ and standard ascorbic acid. [The results are mean ± SD ***P* <0.01 and ****P* <0.001 *vs*. 0 µL/mL]

Similarly, Fig. 4a display the significant (*P* <0.001) percentage of erythrocyte membrane protection in all the doses. In case of reducing power assay, BRJ showed greater reducing capacity of 0.475 ± 0.004 at highest studied concentration (60 µL/mL) while the standard, ascorbic acid showed 0.465 ± 0.016 at 60 µg/mL concentration (Fig. 4b). Further, BRJ displayed negligible haemolytic activity as evident from its low haemolytic value compared to the positive control Triton X-100 which displayed very high haemolytic activity. At 100 µL/mL, percentage of haemolysis for BRJ and Triton X-100 was 5.69 ± 2.62 and 60.28 ± 2.34, respectively. For each of the concentrations, the haemolytic activity of BRJ were significantly (*P* <0.001) less than that of the Triton X-100 (Fig. 4c). Figure 4d reveals negligible cytotoxic activity of BRJ. At the highest concentration

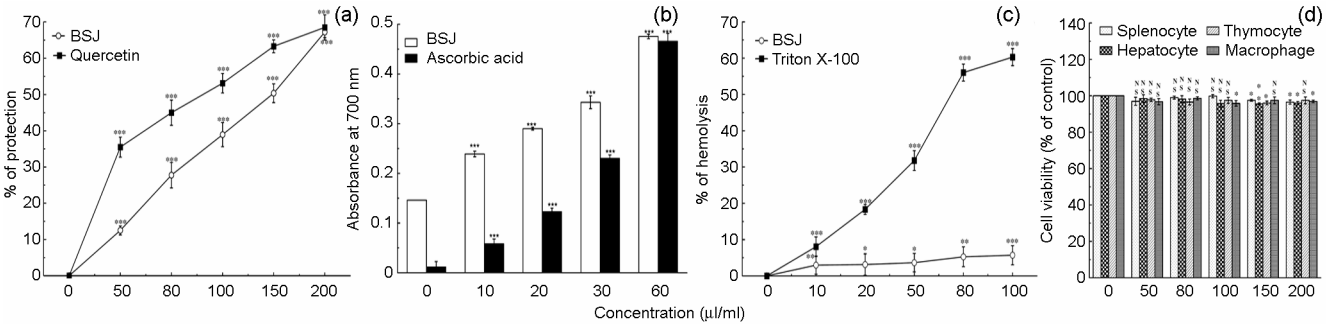

Fig. 4 — (a) Erythrocyte membrane stabilizing activity of BRJ and standard quercetin; and (b) Reducing power assay of BRJ and standard Ascorbic acid. [Each value represents mean ± SD (n = 6). ****P* <0.001 *vs.* 0 µL/mL]; (c) Haemolytic activity of BRJ and standard Triton X-100. **P* <0.05, ***P* <0.01 and ****P* < 0.001 *vs*. 0 µL/mL; and (d) MTT cytotoxic effect of BRJ on murine splenocytes, hepatocytes, thymocytes and peritoneal macrophages. [The value represents mean ± SD (n = 6). NS= non-significant and **P* <0.05, ***P* <0.01 *vs*. 0 µL/mL.]

Table 2 — Correlation Matrix of different antioxidant assays

	DPPH	EMSA	Hydro	Iron	Lipid	NO	Peroxy	Singlet	TAA	Phenol	Flavo
DPPH	1.00										
EMSA	-0.094^{NS}	1.00									
Hydro	-0.966^{NS}	-0.169^{NS}	1.00								
Iron	-0.683^{NS}	-0.663^{NS}	0.850^{NS}	1.00							
Lipid	-0.710^{NS}	0.768^{NS}	0.502^{NS}	-0.029^{NS}	1.00						
NO	-0.998*	0.024^{NS}	0.981*	0.733^{NS}	0.659^{NS}	1.00					
Peroxy	-0.943^{NS}	-0.243^{NS}	0.997*	0.888^{NS}	0.435^{NS}	0.964^{NS}	1.00				
Singlet	0.341^{NS}	-0.968^{NS}	-0.084^{NS}	0.454^{NS}	-0.904^{NS}	-0.274^{NS}	-0.008^{NS}	1.00			
TAA	-0.805^{NS}	-0.516^{NS}	0.932^{NS}	0.983*	0.153^{NS}	0.844^{NS}	0.957^{NS}	0.284^{NS}	1.00		
Phenol	0.827^{NS}	-0.638^{NS}	-0.652^{NS}	-0.154^{NS}	-0.983*	-0.785^{NS}	-0.592^{NS}	0.810^{NS}	-0.332^{NS}	1.00	
Flavo	-0.074^{NS}	-0.986*	0.331^{NS}	0.778^{NS}	-0.650^{NS}	0.143^{NS}	0.402^{NS}	0.913^{NS}	0.651^{NS}	0.500^{NS}	1.00

[The matrix is based on the PCA performed by SPSS statistics. NS= Correlation is not-significant (1-tailed) and *= Correlation is significant at the 0.05 level (1-tailed)]

(200 µL/mL), the percentage of cell viability for murine splenocytes, hepatocyte, thymocyte and macrophages were 96.54 ± 1.10, 95.98 ± 0.90, 97.46 ± 1.85 and 96.84 ± 0.72%, respectively.

In the present investigation, principal component analysis (PCA) was performed to determine how the eleven parameters namely DPPH, EMSA, hydroxyl radical, iron chelation, lipid peroxidation, NO, peroxynitrite, singlet oxygen, TAA, total phenolic and flavonoid content contributed to the overall antioxidant capacity of BRJ. The loading plot (Fig. 5a) was used to draw an overview of the correlation among the various ROS scavenging potential of juice and the correlation matrix described how intricately the correlation between the various antioxidant capacities exist (Table 2).

The loading of the first and second principal component (PC1 and PC2) accounted for 57.35 and 42.64% of the variance, respectively (Fig. 5a). The loading plot demonstrated that hydroxyl radical, iron chelation, lipid peroxidation, NO, peroxynitrite and TAA were firmly located positively on the PC1 with squared cosine value of 0.998, 0.81, 0.561, 0.992, 0.989 and 0.909, respectively, whereas, the singlet oxygen, total phenolic and flavonoid content displayed high quantum of positive loading on PC2 with squared cosine value of 0.988, 0.712 and 0.964, respectively.

Dendrogram (Fig. 5b) prepared through hierarchical cluster analysis (HCA) grouping the antioxidant capacities of BRJ into various statistically significant clusters established the findings of PCA. Like PCA, there were three distinct groups in HCA. In one group, there was cluster between hydroxyl, peroxynitrite, nitric oxide, iron chelating and TAA whereas in other group, singlet oxygen, flavonoid,

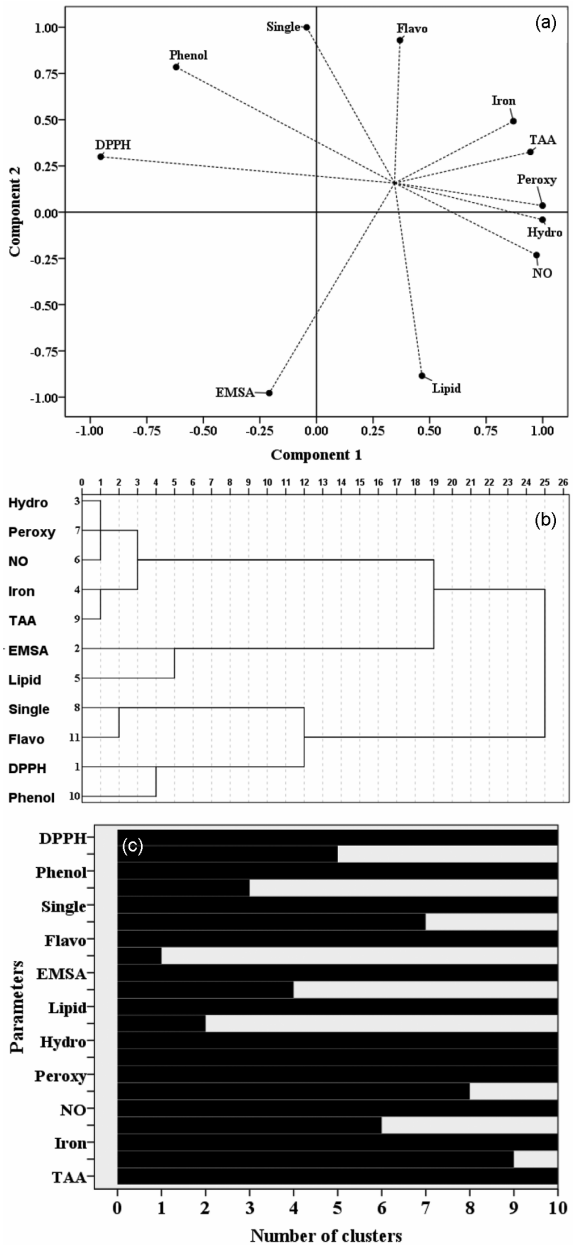

Fig. 5 — (a) Loading plot of principal component analysis of the various antioxidant capacities of BRJ; (b) The dendrogram describes the hierarchical clustering of the different antioxidant capacities of BRJ. HCA was performed using Euclidian distance as distance measured and between group linkages as clustering method; and (c) Icicle diagram of the different antioxidant capacities of BRJ which corresponds the dendrogram.

DPPH and phenol clustered among themselves while EMSA and lipid peroxydation remained in between. The data of HCA virtually corroborated with the results of PCA. In the present dendrogram, the hydroxyl radical scavenging activity was found to be closely associated with the peroxynitrite scavenging activity bearing a coefficient value of 0.012, when average linkage was performed between groups. On the other hand, DPPH and EMSA were merged at the highest distance of 5.452 among all the variables. The icicle diagram (Fig. 5c) for the HCA indicated at which steps various antioxidant capacities were merged to the cluster and gave the visual display of the agglomeration schedule table.

Table 3 — FTIR absorption values and functional groups of Baccaurea ramiflora juice (BRJ)

Wave no. (cm^{-1}) of BRJ	Types of Bond	Functional groups
3163	O-H	Phenols
2113.8	Unknown	Unknown
1645.2	C=C	Alkene
1535.2	Unknown	Unknown
1456.2	C-C=C	Aromatics
1137.9	Unknown	Unknown
1058.8	C–N stretch	Aliphatic amines
675	C–H "oop"	Aromatics
613.3	Unknown	Unknown
555.5	C–Cl stretch	Alkyl halides
515.0	C–Br stretch	Alkyl halides
478.3	Unknown	Unknown
445.5	Unknown	Unknown

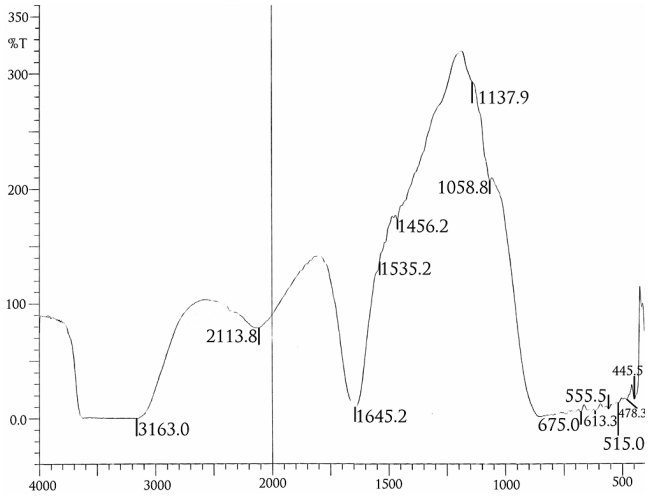

Fig. 6 — FTIR Spectroscopic analysis of *B. ramiflora*.

The juice was further subjected for FTIR spectrophotometric analysis and the functional groups (Table 3) of the components were separated based on its peak ratio. The IR spectrum of BRJ indicated the presence of phenols, alkene, aliphatic amine, alkyl halides, aromatics, alkyl halides which gave the major peaks at 3163 cm^{-1}, 1645.2 cm^{-1}, 1456.2 cm^{-1}, 1058.8 cm^{-1}, 555.5 cm^{-1}, 675 cm^{-1}, 515 cm^{-1}, respectively (Fig. 6).

Discussion

Oxidative stress which is the predominant effect of reactive oxygen species (ROS) mainly occurs due to disturbance in the prooxidant-antioxidant balance in the body. Fruits are potential natural source of antioxidants which protects us from various ROS mediated chronic diseases either by inhibiting or blocking the activation of ROS[34].

In the present study, free radical scavenging capacity (DPPH method) of BRJ was much higher than the standard ascorbic acid indicating higher reducing capacity probably due to its electron or hydrogen donating[35] ability (Fig. 2a). Hydroxyl radical, a highly reactive free radical has capability to damage almost every type of biomolecule in living system[36]. Hydroxyl radical scavenging capacity was found to be much higher (Fig. 2b) in comparison to mannitol suggesting its positive role in protection against hydroxyl radicals. In NO scavenging assay, BRJ exhibited impressive inhibition against the standard, curcumin (Fig. 2c). The NO scavenging activity was significant (P <0.001) at each dose since the antioxidant compounds present in BRJ, directly competed with the reactive oxygen, leading to the decrease in nitrite ions production[37-39]. Spontaneous coupling of NO with superoxide radicals give rise to highly reactive peroxynitrite ($ONOO^-$). In our experiment, we found equivalent scavenging activity of BRJ with the standard gallic acid (Fig. 2d). The singlet oxygen ($^1O_2$) is normally generated in presence of light and photosensitizers, present in the human body. This $^1O_2$ can be quenched either by physical means such as energy excitation to another molecule or by a few antioxidants. When $^1O_2$ activity was performed with BRJ, we found statistically significant (P <0.001) quenching activity virtually in every dose (0-200 µL/mL concentration) studied against blank. Though, the quenching activity of BRJ was relatively low against lipoic acid (Fig. 3a).

Fe^{+2} ion can form a complex with ferrozine. In the presence of chelating agents, if the ferrozine-complex formation is disrupted, it results in decreased red colour intensity of the complex[40]. In the present study, BRJ showed fading of ferrozine-complex colour (Fig. 3b), suggesting that it had chelating activity and captured ferrous ion before ferrozine. This underlines the importance of *B. ramiflora* juice as a chelating agent and as an effective secondary antioxidant. In lipid peroxidation also, BRJ exhibited certain degree of scavenging in a dose dependent manner (Fig. 3c). Total antioxidant activity (TAA) was significantly high (P <0.001) at all the studied concentrations (0-150 µL/mL) which is higher than that of the standard ascorbic acid (Fig. 3d). It signifies BRJ as an effective antioxidative agent.

Erythrocyte membrane stabilizing activity (EMSA) is another type assay which indirectly evaluates the antioxidant capacity of test sample against the superoxide radical mediated destruction of the erythrocyte membrane. Erythrocytes are packed with haemoglobin and their membranes are composed of highly unsaturated fatty acids. The auto-oxidation of riboflavin takes place in presence of light, generating superoxide radicals which induce the haemolysis of RBC[41]. In this study, Fig. 4a depicts significant (P <0.001) percentage of erythrocyte membrane protection in all the doses implying BRJ as a natural dietary supplement. As evident from Fig. 4b, the reducing capacity of BRJ increased with the increased concentration with highly significant value (P <0.001). The result showed greater reducing aptitude as compared to the standard ascorbic acid suggesting BRJ as a potent antioxidant.

The fruit of *B. ramiflora* is an edible one and widely consumed. We, therefore, checked the cytotoxicity of fresh juice. The result showed no cytotoxicity as any drastic change in metabolic activity of cells (murine splenocytes, hepatocyte, thymocyte and macrophages) was not found. So far the haemolytic activity due to haemoglobinopathies or oxidative stress[42] is concerned, BRJ revealed negligible activity up to certain consumable doses, and therefore, can be safely used as bio-safety nutrient supplement.

High amount of phenolic and flavonoid content observed in BRJ could have played major role in free radical scavenging activities. It was very interesting to note that both phenolic and flavonoid compounds had shown high positive index on the PC2. It was also observed that the antioxidant capacity of BRJ mainly resides in the major cluster bearing iron chelation, TAA, peroxynitrite, hydroxyl radical and NO as per the result of PCA (Fig. 5a). This cluster is the major determinant for the antioxidant capacity of the juice. These parameters may act together in a close correlation with the flavonoid content which directly works in scavenging the free radicals. Furthermore, Hierarchical Cluster Analysis (HCA) was performed to confirm the results of PCA by distinguishing similar kind of assay among various antioxidant

capacities. The results of PCA and HCA gave a distinct picture of how phenolic and flavonoid contents were associated with the antioxidant properties of BRJ.

FTIR analysis is one of the widely used methods to identify chemical constituents based on the peak value in the region of infrared radiation and a requisite method to identify medicines in Pharmacopoeia of many countries[43]. In the present study, the peak characteristics and the colour of juice[33] revealed that BRJ might be a probable source of many natural flavonoids including quercetin, kaempferol, myricetin, isorhamnetin, etc. Such results might be due to either by inhibiting initiation and breaking of chain reaction or suppressing formation of free radicals by binding to the metal ions or quenching singlet oxygen[44, 45].

Conclusion

Baccaurea ramiflora is a juicy fruit bearing plant whose fruits and juice are widely consumed in the Indo-Burma region. In the present study, we demonstrated that *B. ramiflora* fruit juice is not only a delicious drink but also has immense medicinal values. Its antioxidative properties from various aspects showed its potentiality as an economically effective health drink. Besides, BRJ is devoid of any adverse effect as it contains no significant haemolytic and cytotoxic activity.

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Ethnoveterinary practices among the tribal community of Malda district of West Bengal, India

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Present study aimed at documenting and analyzing the ethnoveterinary practices of domesticated animals and birds of Malda district of West Bengal in India. Successive field surveys were conducted to assemble data from tribal practitioners by means of semi-structured individual interviews, open ended questionnaires, informal interviews, group discussion, etc. Present study recorded 70 phytotherapeutic practices involving 60 plants which were used to treat 34 types of disease and disorders of livestock. Out of 34 livestock disease and disorders, mostly treated ailment was agalactia with 7 different therapeutic uses followed by fever, treated with 5 different preparations. Leaves were most frequently used plant parts in preparing medicinal formulations. However, five new ethnoveterinary formulations were documented during this survey for the first time. The informants' consensus factor (F_{IC}) was found very high (0.88 - 1) establishing the study more authentic. This is the first detailed documentation of ethnoveterinary knowledge as well as first report of determining F_{IC} among the tribal communities of this region.

Keywords: Ethnoveterinary practice, Livestock, Medicinal plants, Malda, India

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Use of medicinal plants for the treatment of diseases is a recent vogue developed in the western countries. This trend is not only restricted to the treatment of human diseases but also in the disease management of domesticated animals and birds. This branch of science, known as Ethnoveterinary practices or EVPs[1] is gradually pronouncing its impact in the field of ethnobotany in the West. In the 80's and 90's of last century, a few researchers reported the use of medicinal plants as herbal veterinary drugs. Morgan[2] stated the use of plants treating ailments of livestock in Kenya. McCorkle[3] documented a vast introductory research work of ethnoveterinary practices of Latin America whereas Perezgrovas[4] reported the healthcare management practices of sheep by the women of Tzotzil region of southern Mexico. Farah *et al*.[5] described the ethnoveterinary practices of *Maasai* ethnic group of Kenya. In the first decade of present century, several researchers[1,6-13] from west reported ethoveterinary or livestock healthcare management practices.

However, in India, ethnoveterinary practices were common since time immemorial. A few oldest existing book of ancient era such as *Asvayurvedasiddhanta* (Ayurvedic practices for horses), *Asvacikitsita* (therapeutics of horses), *Asvavaidyaka* (medicines of horses), *Hastyayurveda* (Ayurveda of elephants) are the assets or repository of livestock healthcare practices in India[14]. Recently, several workers have also enriched this repository by documenting the knowledge of ethnoveterinariy practices of different provinces of India. These include: Tiwari & Pande[14]; Katewa & Chaudhary[15]; Mistry *et al*.[16]; Takhar & Chaudhary[17]; Gaur *et al*.[18]; Galav *et al*.[19]; Phondani *et al*.[20]; Deshmukh *et al*.[21]; Rajakumar & Shivanna[22], Sharma[23], etc.

Besides, in West Bengal, the eastern most provinces of India possess variety of ethnic communities having versatile knowledge about ethnoveterinary uses of plants. A few workers, tried to gather information which are as follows: Bandyopadhyay & Mukherje[24] reported 25 ethnoveterinary preparations for treating various ailments like swelling of abdomen, constipation, intestinal worm, etc. using around 23 plants by ethnic communities of Koch Bihar district. Dey & De[25] reported 25 species used by tribal like *Santhali, Bhumijs, Mundas*, etc. of Purulia district for the preparations of same. Mitra & Mukherjee[26] also did the similar kind of work on the tribal of Uttar

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and Dakshin Dinajpur districts and reported 23 medicinal plants of ethnoveterinary interest whereas Mukherjee & Namhata[27] and Ghosh[28] reported the ethnoveterinary use of plants used by tribal people of Bankura district while Das & Tripathi[29] documented the ethnoveterinary knowledge of plants for Sunderbans of West Bengal.

However, despite being rich in biodiversity and with wide array of ethnic communities (*Santala*, *Oraon*, *Rajbanshi*, *Namasudre*, *Polia*, *Mundas*, *Malpaharias,* etc.), Malda district of West Bengal, India still remains elusive for ethnobotanists. The district located between the latitude and longitude of 24°40'20"N to 25°32'08"N and 88°28'10"E to 87°45'50"E, respectively. Malda covers an area of 3455.66 sq km. having population density of 1,071 inhabitants per square km (Fig. 1). Eighty seven per cent of the total population live in villages and the rest are in towns[30]. Majority of the tribes are directly or indirectly depend on agriculture, rearing of animals and practices of medicinal plants (both human and veterinary purposes). They remain busy throughout the year with their practice of earning from these sectors. Hence, they rarely manage to visit the veterinary hospitals for livestock diseases. Concurrently, high costs of modern medicines and communication problem encourage them to avail old traditional systems to heal their livestock. This is why, the ethnoveterinary practice means a lot to the rural people in this province as because of its accessibility, ease of preparation, low cost and ecofriendly nature.

Therefore, authors selected this district as study area and present study is the first effort to survey the livestock healthcare management practices in this territory. Informants consensus factor (F_{IC}) of tribal community was also determined to validate these practices and it is the second report from India next to Kumar *et al*.[31], as per authors' best knowledge. Furthermore, analysis of different plant parts used, growth forms of those plant species and a few new findings have also been discussed in the present paper.

Methodology

The data had been collected during 2010-2011 from the local tribal healers known as *go-kaviraj* or *go-baidya* or *Ojhas* and also from local knowledgeable person belonging to different ethnic communities of villages namely, Nalagola, Bamongola, Pakuahut, Lakhitur, Aiho, Bulbulchandi, Nityanandapur, Rishipur, Olandar, Kenpukur, Gazole, Old Malda, Habibpur, Valuka, Suvasganj, Vabuk,

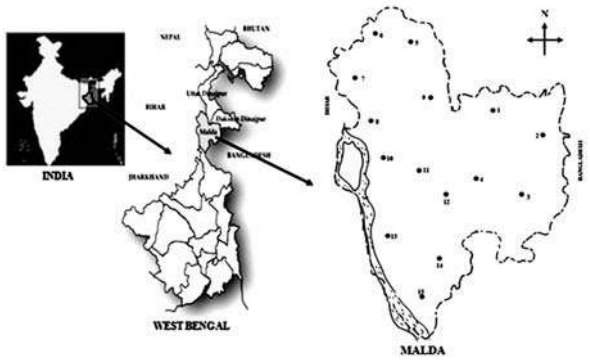

Fig. 1—Location of the study area

Manikchak, Samsi, Krisnapur, Malatipur, Milki, Amriti, Lakkhipur, Enayetpur, Jadupur, etc. with the help of semi-structured individual interviews, open ended questionnaires, informal interviews and group discussion. Cow, buffalo, goat, sheep, horse, duck and hen are the domesticated livestock that the healers treated. The traditional veterinary practitioners were very much cognizant about the surroundings of the villages with experience of at least 10-15 yrs regarding animal healthcare management practices. Hundreds of informants were interviewed and after cross verification from different sources, authors retained only 91 informants confirming the authenticity of their information. Out of ninety one, 47 (33 men, 14 women) were of herbal veterinary practitioners, 5 herbalists (all of men), 28 livestock owners (19 men, 9 women) and 11 (8 men, 3 women) local knowledgeable elderly person. During interview prior informed consent was also obtained from the informants such as, Subed Ali (42 yrs old) of Malatipur village, Nujjum Shekh (68 yrs) of Jadupur village, Kisori Barman (71 yrs) of Nalagola, Jharna Mandal (59 yrs) of Bulbulchandi, Doman Mandal (45 yrs) of Piyasbari, Faeshed Ali (58 yrs) of Kaliyachak, Ratan Mandal (38 yrs) of Jadupur, Lakhsmi Mandal (54 yrs) of Kotuali, Jagadish Murmu (52 yrs) and Vaben Barman (75 yrs) of Habibpur, Jhumadebi Sarkar (41 yrs) of Gazole, Sultana Begam (53 yrs) of Lakkhipur, etc. as per the ethical guidelines of the International Society of Ethnobiology[32]. More emphasis was given to the trained herbal veterinary practitioners as because of their vast experience in management of animal healthcare.

Plants prescribed for the treatment of animal diseases and disorders were collected from various places of study area as per the informants and in presence of them. The plants were properly photographed and herbarium was prepared for each specimen. The collected

specimens were identified with the help of Central National Herbarium (CNH), Kolkata, India. The voucher specimens of each species were deposited at Raiganj University College, Raiganj, India. The method of collection of voucher specimens, preservation and herborization was done as per Jain and Rao[33].

A database has been recorded with parameters like, names of the taxon, family, voucher numbers, vernacular names, plants parts, diseases and disorders, mode of application with the name of informant, etc. The informant consensus factor was determined as per the method of Trotter and Logan[34].

Results and discussion

The present study revealed that a total of 60 medicinal plants belonging to 38 families with 70 different formulations were used for the treatment of 34 veterinary diseases and disorders. Out of 70 formulations, 57% were orally administered, while the rest were for external use only. In most cases, more than one plant was used for the preparation of many formulations. The authors interviewed several tribal people and ultimately kept information of only 91 informants based on their efficiency in veterinary treatment and knowledge of medicinal plants (Table 1). The result from F_{IC} value shows the greater agreement (F_{IC}= 0.88 - 1) of selecting plant taxa among tribal informants.

Out of the 60 medicinal plants, 18 species were shrub followed by 16 species of herbs, 14 climbers, 11 trees and 1 parasite (Fig. 2) belonging to 38 plant families. Amongst 60 plant species, 8 species were recorded in curing various diseases and disorders. *Oroxylum indicum* and *Persicaria glabra* were used for three different treatments; *Alstonia scholaris*, *Amaranthus spinosus*, *Andrographis paniculata*, *Azadirachta indica, Calotropis gigantea* and *Physalis minima* were used for the treatment of two different types of disease and disorder each including drowsiness, nipple crack, tonsillitis, paralysis, intestinal worm, anorexia, flatulence, eczema, agalactia, appetizer, lice and itching problem, etc.

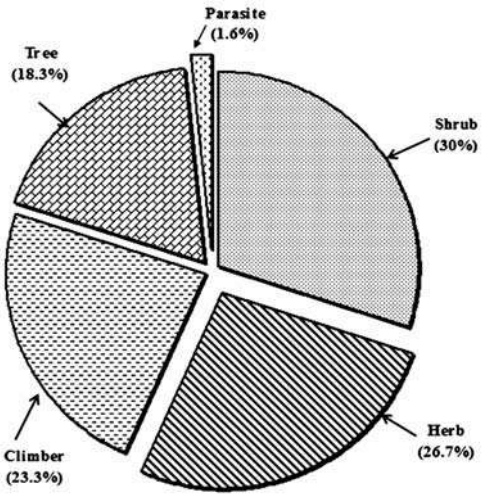

Fig. 2—Distribution of growth form of studied plant species

Agalactia was the most treated ailment with 7 different preparations followed by fever, treated with 5 different preparations whereas loose motion and lice problems were treated with 5 different preparations each (Fig. 3). In most of the cases, traditional healers prepared a veterinary drug using several plant parts along with their own secret ingredients which they did not disclose and apply those formulations according to their own understanding. Sometimes, Veterinarists use a particular formulation for a particular group of animal which had also been discussed separately in Table 1. Leaves were used in 18 occasions whereas in 15 cases roots were found to be used; seeds and flowering branches were used in 5 and 4 cases respectively whereas bark was used in 3 cases and whole plant parts were used for 6 cases to make herbal veterinary preparations (Fig. 4).

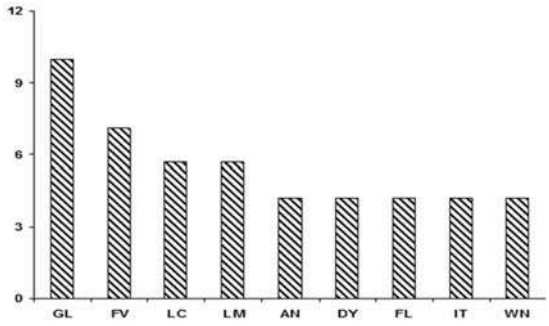

Fig. 3—Frequency of different types of diseases treated in Malda (GL= Agalactia; FV= Fever; LC= Lice; LM= Loose Motion; AN= Anorexia; DY= Dysentery; FL= Flies; IT= Itching; WN= Wound)

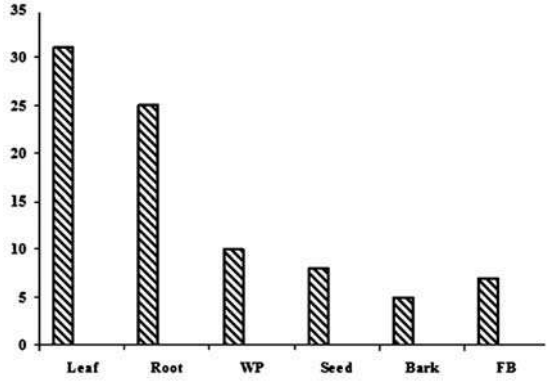

Fig. 4—Frequency of floral parts, used in veterinary treatment (WP= Whole Plants, FB= Flowering Branch)

Table 1—Animal healthcare management practices in Malda district (*contd*.)

Diseases and disorders	Plants species used with their family		Mode of application
Abdominal pain	*Vernonia anthelminctica* (L.) Willd. (Asteraceae)		The seeds (50-100 gm) of *Vernonia anthelminctica* (*somraji*) are crushed with slight water and juice is fed orally.
Agalactia	*Holarrhena antidysentrica* (L.) Wall. (Apocynaceae) *Amaranthus spinosus* L. (Amaranthaceae) *Ampelocissus latifolia* (Roxb.) Planch. (Vitaceae) *Asparagus racemosus* Willd. (Asparagaceae) *Centella asiatica* (L.) Urban (Apiaceae) *Glycosmis pentaphylla* Retz. (Rutaceae) *Tinospora cordifolia* (Willd.) Hook. f. (Menispermaceae)	1 2 3 4 5 6 7	A juice prepared from the bark powder of *Holarrhena antidysenterica* (*kurchi*) and 6-10 slices of *Carica papaya* fruits (*pepe*) with water, is fed orally thrice a day to induce lactation. A paste prepared from the whole plant (2-5 plants) of *Amaranthus spinosus* (*kantakhure*) along with the roots (50 gm) of *Glycosmis pentaphyla* (*atiswar*) is fed orally. Whole plants of (100-200 gm) *Ampelocissus latifolia* (*goaliarlata/ goal lata*) are chopped into pieces and fed with the straw. Fresh roots (80-150 gm) of *Asparagus racemosus* (*satamul*) are fed orally to induce lactation. Whole plant (300-400 gm) of *Centella asiatica* (*thankuni*) fed orally. The roots (50-60 gm) of *Glycosmis pentaphyla* (*atiswar*), whole plant (2-4 plants) of *Amaranthus spinosus* (*kantakhure*) and 500 gm of tender grass are fed together to induce lactation. Whole plant (200-400 gm) of *Tinospora cordifolia* (*goronchlata/ gulancha*) is also used as galactagogue.
Anorexia	*Cardiospermum halicacabum* Linn. (Sapindaceae) *Zingiber cassumunar* Roxb. (Zingiberaceae) *Alstonia scholaris* R. Br. (Apocynaceae)	1 2 3	The leaf decoction (250 ml) of *Cardiospermum halicacabum* (*latafatki*) is fed orally. The rhizome (50-70 gm) of *Zingiber cassumunar* (*banada*) are cut into pieces and fed orally. A paste prepared from the roots (100-200 gm) of *Alstonia scholaris* (*chatim*) and zinger is fed orally.
Anthelmintic	*Allium sativum* L. (Liliaceae)		The bulbs (200-400 gm) of *Allium sativum* (*peyaj*) are crushed along with fodder and fed thrice in a day.
Appetizer	*Andrographis paniculata* (Burm.f.) Wall. ex Nees (Acanthaceae) *Nyctanthes arbor-tristis* L. (Oleaceae)	1 2	The leaves (200-400 gm) of *Andrographis paniculata* (*kalmegh*) are crushed along with *Nyctanthes arbor-tristis* (*siuli*) leaf and the juice is mixed with husk and fed as appetizer. The leaves (250 gm) of *Nyctanthes arbor-tristis* (*siuli*) are chopped into pieces and fed along with tender grasses.
Black quarter	*Oroxylum indicum* (L) Vent. (Bignoniaceae)		A paste is prepared from the bark powder (75-100 gm) of *Oroxylum indicum* (*krishnadinga/noukadinga*) and fed thrice a day.
Bloat	*Trigonella foenum-graecum* L. (Fabaceae)		A decoction prepared from the seed (50-75 gm) of *Trigonella foenum-graecum* (*methi*) and fed orally.
Body Pus	*Chrysopogon gryllus* L. (Poaceae) *Curcuma longa* L. (Zingiberaceae)	1 2	A paste is prepared from the roots (25-40 gm) of *Chrysopogon gryllus* (*chorkanta*) along with 1 gm *Piper nigrum* seeds (*golmorich*) and given under the tongue of cattle. A paste is prepared from the rhizomes of *Curcuma longa* (*halud*) and applies as emollient on tongue at every morning and evening for 5 days to cure from pusses.
Bodyache	*Cuscuta reflexa* Roxb. (Convolvulaceae)		A paste is prepared from the whole plant (300-500gm) of *cuscuta reflexa* (*swarnalata*) along with the roots (75 gm) of *Achyranthes aspera* (*baro chirchiri/apang*) and applies externally on body.

(*contd*.)

Table 1—Animal healthcare management practices in Malda district (*contd*.)

Diseases and disorders	Plants species used with their family		Mode of application
Boil	*Urena lobata* L. (Malvaceae)		The leaves (100 gm) of *Urena lobata* (*hegra*) are crushed to make paste and applied externally on affected area.
Bone fracture	*Cissus quadrangularis* L. (Vitaceae) *Litsea glutinosa* L. (Lauraceae)	1	300-400 gm of stem of *Cissus quadrangularis* (*harjora*) are crushed to make paste and applied on broken leg of cattle tying up with bamboo stick.
		2	A paste is made from the roots (200-250 gm) of *Litsea glutinosa* (*darodmoyda*), leaves (70 gm) of tamarind and rhizome (10-20 gm) of zinger and applied on bone cracked area, tying up with bamboo stick.
Constipation	*Acacia catechu* Willd. (Mimosaceae) *Fumaria officinalis* L. (Fumariaceae)	1	A juice is prepared from the roots (200 gm) of *Acacia catechu* (*khayer*) and given to prevent constipation.
		2	Whole plants (400 gm) of *Fumaria officinalis* (*khetpapra*) are chopped and fed orally to cattle.
Diarrhoea	*Cocculus hirsutus* (L.) Diels (Menispermaceae)	1	The tender leaves (500 gm) of *Cocculus hirsutus* (*joljomani*) are chopped with straw and fed orally.
	Rauvolfia serpentina (L.) Benth. ex Kurz (Apocynaceae)	2	A paste is made The roots (200 gm) of *Rauvolfia serpentina* (sarpagandha) along with 200 gm of *Azadirachta indica* leaves (neem) and fed orally.
Drowsiness	*Eleusine indica* (L.) Gaertn. (Poaceae) *Oroxylum indicum* (L) Vent. (Bignoniaceae)	1	The roots (70-100 gm) of *Eleusine indica* (*jabra ghas*/ *katilaghas*) are crushed along with slight zinger to make paste and fed orally.
		2	A paste is prepared from the bark powder (100 gm) of *Oroxylum indicum* (*krishnadinga*) along with whole plant (100 gm) of *Tinospora cordifolia* (*goronchlata*) and given against drowsiness.
Dysentery	*Allium sativum* Lin. (Liliaceae) *Cannabis sativa* L. (Urticaceae) *Ficus glomerata* Roxb. (Moraceae)	1	The bulbs (200-400 gm) of *Allium sativum* (*peyaj*) are crushed along with fodder and fed orally thrice in a day.
		2	The chopped leaves (250 gm) of *Cannabis sativa* (*bhang*) along with fruits (250 gm) of *Ficus glomerata* (*dumur*) are fed together orally.
Dyspepsia	*Aristolochia indica* L. (Aristolochiaceae)		The tender leaves and buds (150 gm) of *Aristolochia indica* (*iswarnath*/*iswarmul*) are crushed with 25 gm of black pepper and slight salt to make paste and fed to cattle.
Ear Pus	*Eclipta prostrata* L. (Asteraceae)		A paste is prepared from the leaves (25-50 gm) of *Eclipta prostrata* (*kesut*) and applies on ears.
Eczema	*Andrographis paniculata* (Burm.f.) Wall. ex Nees (Acanthaceae) *Datura metel* L. (Solanaceae)	1	A leaf paste of *Andrographis paniculata* (*kalmegh*) and *Azadirachta indica* (neem) is prepared (1:1 ratio) and applied on the body of the cattle.
		2	A paste is prepared from the root (50-75 gm) of *Datura metel* (*kalo dhtura*) along with 2 teaspoon of mustered oil and applies on body.
Eye cataract	*Pergularia daemia* (Forssk.) Chiov. (Asclepiadaceae)	1	The leaves (50 gm) of *Pergularia daemia* (*ajashringi*) are crushed to make juice and apply externally on the eyes of cattle.
	Stephania japonica (Thunb.) Miers. (Menispermaceae)	2	The young leaves (50 gm) of *Stephania japonica* (*gorochlata*/ *ghapata*/*aknadi*) are crushed to make juice and pour in the ear of goat externally for 12-15 days. [This preparation is applied only on goat.]

(*contd*.)

Table 1—Animal healthcare management practices in Malda district (*contd*.)

Diseases and disorders	Plants species used with their family		Mode of application
Fever	*Achyranthes aspera* L. (Amaranthaceae) *Costus speciosus* (J.Konig) Sm. (Costaceae) *Erythrina indica* Lam. (Papilionaceae) *Heliotropium indicum* L. (Boraginaceae) *Leonurus japonicas* Houtt. (Lamiaceae)	1	The roots (100-200 gm) of *Achyranthes aspera* (*baro chirchiri/apang*) are crushed with cumin seeds (10-15 gm) and water to make paste and the pate is fed orally.
		2	A paste is prepared from the root of *Costus speciosus* (*kuttus*), 3-5 pieces of carrot and stem (50 gm) of *Tinospora cordifolia* and given thrice a day for 3-4 days.
		3	Young tender branches (200-300 gm) of *Erythrina indica* (*madar*) are feed at early morning before taking water for 3-4 days.
		4	A paste is prepared from the flowering inflorescences (250 gm) of *Heliotropium indicum* (*hatisur*) along with 5 gm of black pepper seeds (*golmorich*) and fed thrice a day.
		5	A juice from *Leonurus japonicas* (*raktodron*) root (150 gm) along with the roots (150 gm) of *Achyranthes aspera* is prepared and fed orally.
Flatulence	*Calotropis gigantea* (L.) W. T. Aiton (Asclepiadaceae) *Physalis minima* L. (Solanaceae)	1	The apical twigs (30-50 gm) of *Calotropis gigantea* (*akanda*) are crushed along with slight amount of salt to make paste against flatulence.
		2	A paste is prepared from the roots (50 gm) of *Physalis minima* (*pokapaia/tepari*) and cumin seeds with slight water and given to cattle.
Flies problem	*Azadirachta indica* A. Juss. (Meliaceae) *Hyptis suaveolens* (L.) Poit (Lamiaceae) *Tragia involucrata* L. (Euphorbiaceae)	1	The branches of *Azadirachta indica* (*neem*) are burnt in the cowshed at evening as repellent.
		2	The branches of *Hyptis suaveolens* (*bontulsi*) are burnt in the cowshed to prevent mosquitoes or flies.
		3	The roots (50-100 gm) of *Tragia involucrata* (*bichuti/bichatu*) are crushed with mustered oil and the paste is applied externally on horns of cattle.
Intestinal worm	*Acacia auriculiformis* A. Cunn. ex Benth. (Fabaceae) *Alstonia scholaris* R. Br. (Apocynaceae)	1	The seeds (50 gm) of *Acacia auriculiformis* (*akashmoni*) are crushed and mixed with cattle-food and given orally.
		2	The roots (50-100 gm) of *Alstonia scholaris* (*chatim*) are crushed with water and the juice is given to cattle.
Itching	*Azadirachta indica* A. Juss. (Meliaceae) *Jatropha gossypifolia* L. (Euphorbiaceae) *Phyla nodiflora* (L.) Greene (Verbenaceae)	1	The leaves (50-100 gm) of *Azadirachta indica* (neem) are crushed with mustered oil and the paste is applied on the body of cattle.
		2	The leaves of (100 gm) *Jatropha gossypifolia* (*varenda*) are rubbed on the whole body.
		3	The leaves (100 gm) of *Phyla nodiflora* (*koi okhra*) are rubbed on the body to cure from itching.
Lice problem	*Clerodendrum viscosum* Vent. (Verbenaceae) *Mikania micrantha* Kunth. (Asteraceae) *Ocimum kilimandscharium* Guerke (Lamiaceae) *Persicaria glabra* (Willd.) M.Gomez (Polygonaceae)	1	The apical twigs (75 gm) of *Clerodendrum viscosum* (*ghetu*) are crushed and the juice is macerated over the head of cattle.
		2	The flowering branches (8-10) of *Mikania micrantha* (*bantulsi*) are kept in poultry house to keep away lice from the body of hen. [This procedure is applicable only for poultry birds.]
		3	The flowering branches of *Ocimum kilimandscharium* (*dulalbabu*) are kept in poultry house to keep away lice from the body of hen. [This procedure is applicable only for poultry birds.]
		4	The leaves (350 gm) of *Persicaria glabra* (*bis-kantal/laltota*) are rubbed on the body of cattle.

(*contd*.)

Table 1—Animal healthcare management practices in Malda district

Diseases and disorders	Plants species used with their family		Mode of application
Loose motion	*Alocasia macrorrhiza* Schott. (Araceae) *Eleusine indica* (L.) Gaertn. (Poaceae) *Lawsonia inermis* L. (Lythraceae) *Physalis minima* L. (Solanaceae)	1	A paste is prepared from the rhizomes (10-15 gm) of *Alocasia macrorrhiza* (*mankachu*) with slight lemon juice and given to the poultry birds. [This preparation is applied only for poultry birds.]
		2	The roots (50-100 gm) of *Eleusine indica* (*jabra ghas*) are crushed along with unripe banana (10-12 pieces) and the paste is fed to cattle.
		3	The chopped leaves (400-600 gm) of *Lawsonia inermis* (*mehandi*) are fed to cattle at empty stomach for 3-4 days.
		4	The root (60-100 gm) of *Physalis minima* (*pokapaia*) is crushed along with black pepper seed (5 gm) and the paste is fed orally for 3 days.
Nipple crack	*Oroxylum indicum* (L) Vent. (Bignoniaceae)		A paste is prepared from the bark (100 gm) of *Oroxylum indicum* (*krishnadinga*) along with mustered oil and applied on cracked nipple of cattle.
Paralysis	*Persicaria glabra* (Willd.) M.Gomez (Polygonaceae)		The leaves (40-50 gm) of *Persicaria glabra* (*biskantal*) are crushed and the paste is taken in a cloth and tied on legs of poultry till they can walk well. [This preparation is applied only for poultry birds.]
Sex stimulant	*Mucuna pruriens* (L.) DC. (Fabaceae)		The seed (250-500 gm) of *Mucuna pruriens* (*alkushi*) is crushed to make paste and fed to cattle.
Stomachache	*Premna corymbosa* (Burm.f.) Rottl. &Willd. (Verbenaceae)		A paste is made from the leaves (250 gm) of *Premna corymbosa* (*gonal*) along with the seeds (70-100 gm) of *Trigonella foenum-graecum* (*methi*) and given to cattle.
Swelling of neck	*Calotropis gigantea* (L.) W. T. Aiton (Asclepiadaceae)		The leaves of *Calotropis gigantea* (akanda) are warmed with ghee (made from milk) and applied on neck.
Tonsillitis	*Argyreia nervosa* (Burm. f.) Bojer (Convolvulaceae) *Persicaria glabra* (Willd.) M.Gomez (Polygonaceae)	1	The leaves (70-100 gm) of *Argyreia nervosa* (*briddhadarak*) are crushed along with 40 gm of *Azadirachta indica* (*neem*) leaves and slightly warmed mustered oil (2-4 teaspoon) to make paste and keep this paste in a cloth and tied up on neck of cattle.
		2	The leaves (50-100 gm) of *Persicaria glabra* (biskantal) are crushed along with zinger (20 gm) and the paste is kept in a cloth and tied on the neck of cattle.
Post natal disorder	*Dendrophthoe falcata* (L.F) Etting. (Loranthaceae)		The stem bark (40-50 gm) of *Dendrophthoe falcata* (*dhara*) is crushed along with 35 gm of *Azadirachta indica* (*neem*) leaves and zinger (3 gm) to make paste and applied on vagina of cattle to reduce its enlargement after delivery.
Wound	*Ricinus communis* L. (Euphorbiaceae) *Saccharum munja* Roxb. (Poaceae)	1	The seed (60-100 gm) of *Ricinus communis* (*reri*) are crushed to make oil and applied externally until it cures the wounds.
		2	The root (80-100 gm) of *Saccharum munja* (*biyanal/ sikighas*) along with small amount of horn scales of cattle are crushed and applied externally on the affected area, occurred by sharp ends of plough.

Present study showed greater agreement among the tribal informants on selection of plant species for a particular livestock healthcare management category. The informants' consensus ratio, i.e. F_{IC} value ranges from 0.88-1 (Table 2), with an average of 0.96 indicating high level of agreement factor and confirmed the homogeneity among informants knowledge and reliability of the traditional knowledge of this district. Hence, *Andrographis paniculata*, *Amaranthus spinosus*, *Asparagus racemosus*, *Trigonella foenum-graecum*, *Heliotropium indicum*, *Mucuna pruriens*, *Oroxylum indicum,* etc. plant species, having high F_{IC} value may be used for the development of future eco-friendly veterinary drugs.

The authors found 5 species with new reports of treatment which had not been reported previously in any literature till date. These include- *Cardiospermum halicacabum* for anorexia; *Tragia involucrata*

for the prevention of flies; *Dendrophthoe falcata* for post natal disorders; *Alocasia macrorrhiza* for loose motion and *Heliotropium indicum* for fever. Interestingly one species namely, *Heliotropium indicum* had not been previously documented to be used as medicinal plant in any ethnoveterinary practices of India. Thus, present study provides the first report of the use of this taxon in livestock healthcare management.

Table 2—Consensus ratio of the ethnoveterinary practices among the tribes of Malda district

Animal healthcare management category	N_{TAXA}	N_{UR}	F_{IC}
Abdominal pain	1	4	1
Agalactia	7	120	0.95
Anorexia	3	30	0.93
Anthelmintic	1	9	1
Appetizer	2	39	0.97
Black quarter	1	4	1
Bloat	1	7	1
Bodyache	1	9	1
Body pus	2	42	0.98
Boil	1	3	1
Bone fracture	2	56	0.98
Constipation	2	20	0.95
Diarrhoea	2	15	0.93
Drowsiness	2	10	0.89
Dysentery	3	35	0.94
Dyspepsia	1	20	1
Ear Pus	1	6	1
Eczema	2	31	0.97
Eye cataract	2	9	0.88
Fever	5	50	0.92
Flatulence	2	15	0.93
Flies problem	3	21	0.90
Intestinal worm	2	22	0.95
Itching	3	53	0.96
Lice problem	4	33	0.91
Loose motion	4	45	0.93
Nipple crack	1	4	1
Paralysis	1	3	1
Sex stimulant	1	10	1
Stomachache	1	7	1
Swelling of neck	1	22	1
Tonsillitis	2	12	0.91
Post natal disorder	1	7	1
Wound	3	28	0.93

F_{IC}= N_{UR}-N_{TAXA}/ (N_{UR}-1); Where, N_{NUR}= Total number of use reports found for the treatment of a particular illness category; N_{TAXA}= Total number of taxa used for treatment of that particular category by informants.

Conclusion

Since time immemorial, the tribal healers of this region had acquired their own traditional system of treatment from their ancestors, which is unique and varies from community to community and even from village to village. Present study also observed that some of healers uproot the whole plant and virtually throw them out after collecting the required portion of the plant parts like roots, rhizomes, etc. This is causing a great deal of erosion of medicinal plants from the study area. Thus increasing demand of medicinal plants and their improper uses may result in disappearance of important plant species in near future. The problem is compounded by massive deforestation and urbanization of the region. The study also revealed that almost all the studied species, showing high informant consensus factor may be regarded as the resource of future veterinary eco-friendly drug. So, a comprehensive phytochemical investigation with those studied plants would be a handy work to prove the efficacy and validation of herbal veterinary medicine. Therefore, the need of hour is to cultivate the medicinal plants along with their conservation, proper documentation and phytochemical investigation. This is very much required for the sustainability of the ethnoveterinary practices of this region.

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Original Research

Ethnobotany, traditional knowledge and socioeconomic importance of native drink among the Oraon tribe of Malda district in India

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ABSTRACT

Aim: Preparation of daily traditional drink by the indigenous tribes is a common phenomenon in India. Oraon tribes in Malda district of West Bengal, India are very much practiced in making of their own native brew, known as Chullu. Therefore, the aim of this study was to explore the whole Chullu procedure technology of the region and its socioeconomic effect on Oraon. Ethnomedicinal investigation of local plants involved in Chullu preparation was another aspect of this study. **Materials and Methods:** The present study was conducted from April 2012 to June 2013. Consecutive field surveys were performed to collect information from Chullu producers to focus the procedure technology of local brew by means of semi-structured individual interviews, informal interviews and group discussion. A semi-structured questionnaire process was also performed to obtain the information regarding the ethnic use of plant species involved in Chullu preparation. **Results:** The present study revealed that four medicinal plant species along with rice having strong local ethnomedicinal value were used to prepare this indigenous drink. Oraon prepare the brew using their unique home-made distillation process. Commercialization of this local brew represents an alternative income to develop their economic condition, especially for poor households. The index of importance value was considered to evaluate the importance, usage, and knowledge of the five studied species. **Conclusion:** It could be concluded that practices of Chullu preparation represent a bonding between ethnic knowledge and Oraon people of the province. Commercialization of Chullu may be considered as a source of alternative way of income for poor households in the region.

KEYWORDS: Alternative income, Chullu, ethnobotany, Oraon, Malda district

INTRODUCTION

Traditional liquor preparation is a common household practice among different tribal communities in India. They consume native drink (e.g., sajpani, kiad etc.) virtually in every occasion of life such as various festivals, marriage ceremony and even in funeral [1]. Almost all tribes prepare and consume traditional drink but their names, ingredients, and mode of preparation differ greatly from community to community and region to region. Rice is the main ingredient preparing these native drinks or liquors. However, the tribals use local medicinal plant parts along with rice to prepare their native liquor and believe that the beverage prepared in this process prevents headache, insomnia, body ache, urinary troubles and cholera [2-4]. The North-eastern provinces of India, colonized by native Bodo, Garo, Rabha, Karbi, Ahom, Deori, Dimasa, Kachari, etc. communities are best known for the production of household liquors [4,5]. However, there is virtually no data available regarding the traditional alcoholic beverage preparation in the sub-Himalayan non-hilly region of Bengal.

Malda, district of West Bengal in India, is characterized by its diversified historical dynasty, mango production, rich wetland, natural vegetation and its diversified ethnic groups including Santala, Oraon, Rajbanshi, Namasudre, Polia, Mundas, Malpaharias etc. [6] Amongst these tribes, Oraon community are famous in preparing quality local drink or known as Chullu from rice. In fact Chullu production is a part of their culture, identity, myths, and spiritual practices. Their own traditional way of preparing the brew with medicinally important plants distinguished them from rest of the tribal communities of the region. The recipe of brew preparation is however a secret and passed on generation after generation orally. Besides, they also make a good living by selling this native drink.

So far there is no authentic documentation of Chullu preparation technology used by Oraons' of Malda district of West Bengal, India. The plants those were used in preparing Chullu, either known medicinally or have ethnic use among Oraons'. Therefore, a semi-structured questionnaire process was also intended to focus the local use of the plants, their parts involved in medicinal practices and preparation of drugs from those plant parts. Another aspect which was considered in this study was the economic significance of Chullu production and sale. Hence, it may be a pioneer study to explore the Chullu procedure technology and its economic impact among Oraon community of the district along with ethnomedicinal value of used plant species.

MATERIALS AND METHODS

Study Area

Malda (latitude and longitude of 24°40′20″N to 25°32′08″N and 88°28′10″E to 87°45′50″E respectively), a district of West Bengal, India with a total land area of 3455.66 km^2. [6] is known as '*mango district*' for its wide array of mango variety and production. It consists of two municipalities, 15 blocks or sub-divisions and 3701 villages with a population of more than forty lakh. The district is also characterized by its diversified wetland and forest vegetation. The Adh soi wetland (beel), located at Harischandrapur-II block of the region, is one of the largest among the wetlands of the state comprising rich vegetation due to its macrophytic diversity [7]. Adina and Bhalluka forests are the two most important forest areas of the district. A few small forest areas are also scattered in Old Malda, Habibpur, Harishchandrapur and in Gajol blocks [8].

Most of ethnic communities live mainly in the four blocks of this province namely, Gazole, Bamongola, Habibpur and Old Malda comprising more than 85% of total tribal population. Hence we considered these four blocks as our study area [Figure 1] due to its noticeable Oraon population. The climate of this region is extreme because of its geographical position. The district has a hot summer (35-42°C) from March to September and a very cold winter (6-12°C) from November to February. The monsoon starts from June and continues to the mid-September and the average rainfall is approximately 1453.1 mm.

Oraon Community

Oraon community is one of the largest tribal groups in India, possessing a unique tradition and culture. In Malda district, a sizeable portion of the tribal communities are the Oraons who mostly inhabits in the remote villages [9]. They have distinctive lifestyle and are fond of festivals of various kinds like Jatrapala (one kind of play portraying colorful stories), Gambhira (a kind of play portraying the social satire, political circumstances or the life story of god and goddesses), folk songs and dances, traditional musical instruments etc. Consumption of Chullu prepared mainly from rice is common for Oraons during these occasions. Besides, they regularly consume this drink during marriage ceremony, birth of a child or even in funeral. In fact,

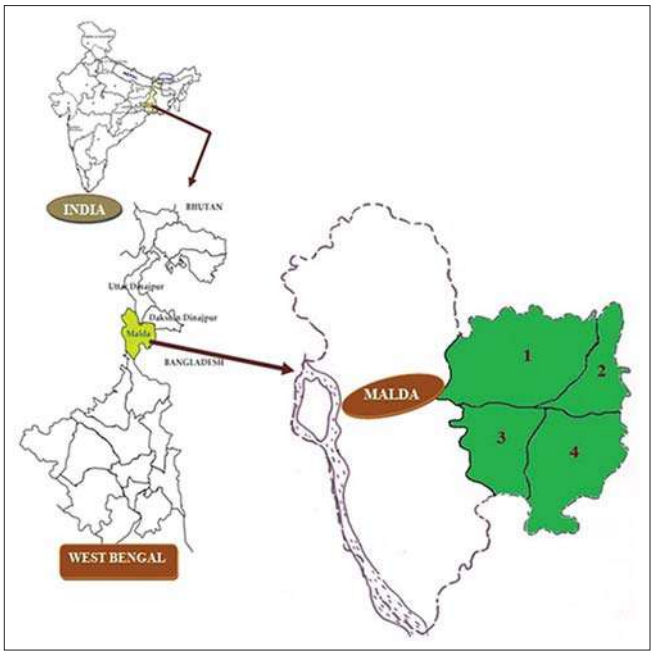


Figure 1: The map of study area (Malda district) showing four main Oraon populated zones (1: Gazole block; 2: Bamongola block; 3: Old Maldah block; 4: Habibpur block)

a section of the community makes a good living by preparing Chullu.

Data Collection

Prior to survey, several meetings were held with the community members to explain the purposes of the study being conducted and to obtain their prior informed consent (PIC). The survey was carried out among the 19 villages (Chaknagar, Haspukur, Rishipur, Bhabuk, Parameshpur, Dhumpur, Nityanandapur, Aktail, Dangapara, Lakhitur, Habibpur, Jhinjhinipukur, Pakuahut, Jagdala, Kanturka, Kenpukur, Salaidanga, Majhra, Baidyapur) of above mentioned four blocks (Gazole, Bamongola, Habibpur and Old Malda) during last year (April 2012 to June 2013). Hundreds of informants were interviewed to get the information regarding the Chullu preparation and plants and plant-parts involved therein. The sociocultural and marketing value of this drink has also been considered. The whole survey procedure involved several levels of interviewing such as semi-structured individual interviews, informal interviews, open-ended questionnaires, and group discussion with the local informants.

Total Key Informants

Hundreds of villagers of the study area were interviewed, but the information given by professional Chullu producers and persons with proven knowledge on plants involved in Chullu production were only recorded. After cross verification, the information obtained only from 201 Chullu producers based on their experiences (174 female, 27 male) of 19 villages were recorded. Among the ethnomedicinal practitioners, we found 27 healers who were involved in traditional healing practices,

gave information regarding the ethnic use of the same plants involved in Chullu production for treating ailments. To survey the economic aspect of the Chullu production, authors also interviewed 56 vendors of 19 villages.

Data Analysis

In order to evaluate the importance of the medicinal plants as per the local informants of the villages, the value of importance (IVs) index was determined [10]. IVs index measures the importance of a plant species based on how many informants cite one species as the most important one among the total number of informants (Value varies from 0 to 1). IVs= nis/n; where, nis= number of informants who consider the species to be the most important, and n= total number of informants.

RESULTS

After interviewing with 201 Chullu producers, the local beverage procedure technology by Oraon people was summarized under two main sub-legends: preparation of Chullu -starter and preparation of Chullu. It was observed that Chullu production which has great impact on Oraon people, indirectly help to uplift the village economy. Virtually, commercialization of Chullu is an alternative way of their daily livelihood. A total of four medicinal plants including *Holarrhena pubescens*, *Wattakaka volubilis*, *Ichnocarpus frutescens* and *Clerodendrum viscosum* along with rice (*Oryza sativa*) which were used in the preparation of this drink had great ethnomedicinal value in the locality.

Chullu Procedure Technology

Preparation of Chullu-starter

To prepare Chullu-starter (locally known as *modguli*), rice grains and 4 different plant parts are mixed together in a 2:1 ratios and dusted. Briefly, rice grains are taken in earthen pot and cleaned in water, followed by drying under sunlight for 1-2 days. Different plant parts like bark and leaves of *H.* pubescens, fruit and bark of *W. volubilis*, leaves of *I. frutescens* and *C. viscosum* are also cleaned well to remove dust particles and dried. Then, the plant parts along with rice grains are powdered by *dheki* (a wooden mortar with a large wooden handle). The powdered material is then sieved. The sieved material is locally known as *modgura*. Water (1/3rd of the total powder) is added to modgura to make dough and thick tablet like structures of 5-8 cm. in diameter are prepared [Figure 2]. These tablets are called modguli. The *modgulis* are kept in between two layers of straw for 4-5 days or until the pungent smell comes (the process is known as jag-dewa). Now, the starters are prepared for sundry. These modgulis are kept on clean cloth under sunlight for another 7-10 days. Finally, the sundried Chullu-starters are packaged depending upon their sizes for marketing [Figure 2].

Preparation of Chullu

To prepare Chullu, cooked rice is the main ingredient. Briefly, starters (four to five pieces for 1 kg of cooked rice) are dusted

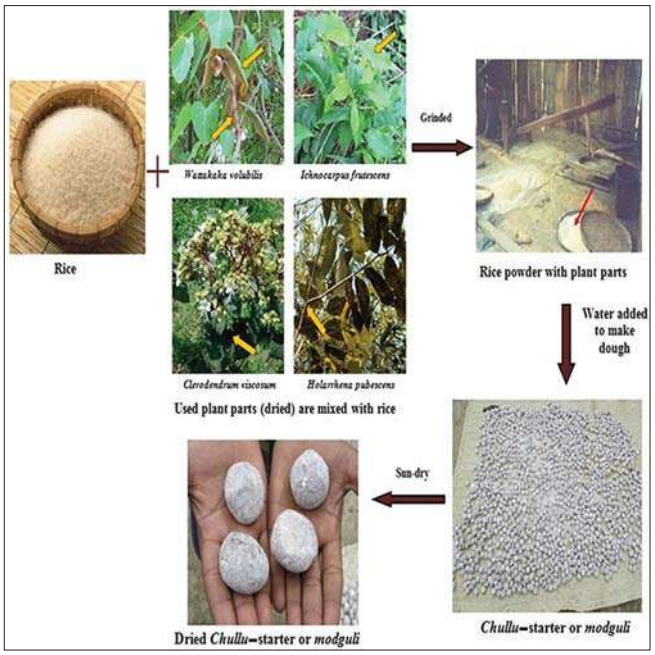


Figure 2: Preparation of Chullu-starter

and mixed properly with cooked rice and taken in an earthen pot (handi). Water (400-500 ml) is added into the pot covering the mouth with a banana leaf, followed by an earthen lid and left for fermentation. A yellowish watery juice with a strong alcoholic pungent smell comes out after 3-4 days, which is filtered with a clean cloth into another pot. This first fermented yellowish beverage is known as *hanria*. Fresh water (2-2.5 lit) is added in the same earthen pot containing fermented rice and *hanria* and left for 12-18 h [Figure 3].

Now to prepare typical Chullu, Oraon follow their unique distillation process. In this preparation, three pots are piled one above the other. The lowest pot containing the fermented rice with hanria, the middle one being an empty earthen pot with several pores (known as *jhanjhi*) at the bottom and the topmost aluminum pot filled with cold water. The air gaps between each pot are sealed with mud. A pipe is inserted and sealed with mud at the side bottom of the middle earthen pot. The entire preparatory set is then placed over earthen oven starting the heating process [Figure 3]. After heating, the vapor goes up from the lower-most pot passes through the pores of the middle pot and comes in contact with upper-most pot containing cold water. Due to cooling, the vapor condenses into water which comes down and is collected in the bottle through the pipe of the middle pot. Thus, the prepared beverage is watery in color possessing alcoholic odor and is known as Chullu or mod. Finally, this alcoholic beverage is packaged in glass-bottle for selling in the market.

Ethnomedicinal Uses of Plants Involved in Chullu

During survey, we found that the plants used in Chullu preparation had great local medicinal values. Therefore, a semi-structured questionnaire and individual interview [Figure 4] among the healers had been carried out among 19

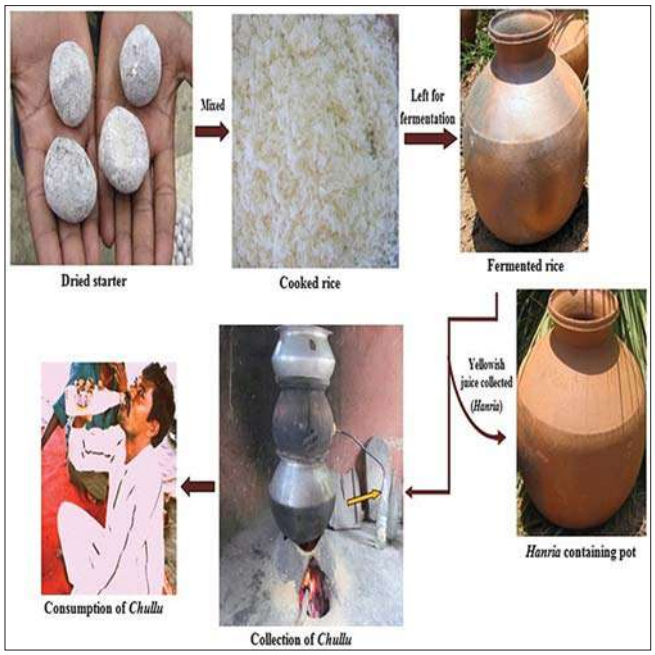
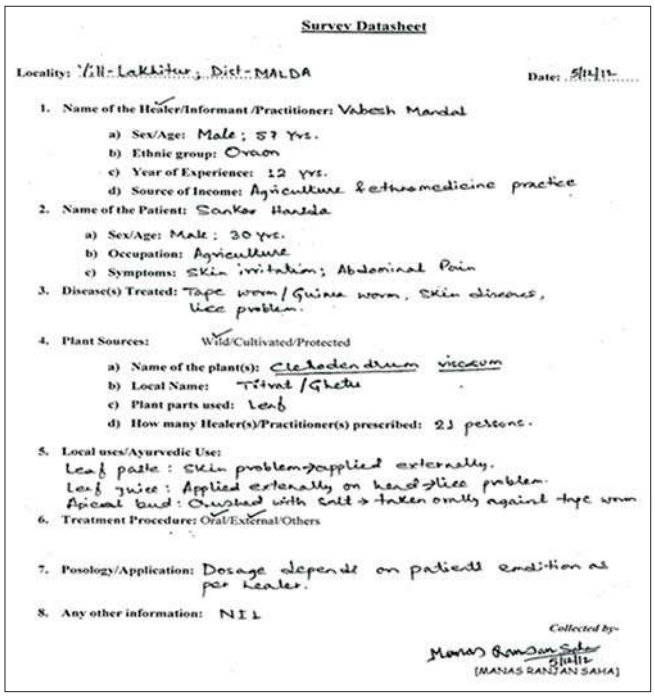


Figure 3: Preparation of Chullu



Survey Datasheet

Locality: Vill - Lakhitur ; Dist - MALDA Date: 5/11/12

1. Name of the Healer/Informant /Practitioner: Vabesh Mandal
   a) Sex/Age: Male ; 57 Yrs.
   b) Ethnic group: Oraon
   c) Year of Experience: 12 Yrs.
   d) Source of Income: Agriculture & ethnomedicine practice
2. Name of the Patient: Sankar Hansda
   a) Sex/Age: Male ; 30 Yrs.
   b) Occupation: Agriculture
   c) Symptoms: Skin irritation; Abdominal Pain
3. Disease(s) Treated: Tape worm / Guinea worm, Skin disease, lice problem.
4. Plant Sources: Wild/Cultivated/Protected
   a) Name of the plant(s): Clerodendrum viscosum
   b) Local Name: Tirial / Ghetu
   c) Plant parts used: Leaf
   d) How many Healer(s)/Practitioner(s) prescribed: 23 persons.
5. Local uses/Ayurvedic Use:
   Leaf paste : Skin problem-applied externally.
   Leaf juice : Applied externally on head-lice problem.
   Apical bud : Crushed with salt & taken orally against tape worm
6. Treatment Procedure: Oral/External/Others
7. Posology/Application: Dosage depends on patient's condition as per healer.
8. Any other information: NIL

Collected by-
(MANAS RANJAN SAHA)

Figure 4: Questionnaire datasheet of ethnomedicinal use of plants in studied region

villages to obtain the information. After interviewing with some experienced traditional healers authors found a massive ethnomedicinal value in the region. However, as per our title concerned, we focused only on the above mentioned five plant species [Table 1] as those are used in Chullu preparation. A total of 27 traditional experienced healers were chosen after cross verification for obtaining the information regarding ethnic use of those species only.

It was observed that the herbal formulation from the bark of *H. pubescens* was prescribed by 22 healers out of 27 to treat chronic diarrhoea, chronic dysentery, urinary troubles, bleeding of piles etc. whereas *C. viscosum* was prescribed by 21 healers to treat several disorders. Similarly, *W. volubilis*, *I. frutescens*, *O. sativa* were also used in various purposes as shown in Table 1. The IVs result exhibited high IV for all the species [Table 1] establishing greater ethnic knowledge regarding plant resources in the studied area. However, the IVs value of *O. sativa* is low (0.49) in comparison to others and prescribed only by only 13 healers. The study attempts to highlight that the above mentioned five plants are most valued species in the studied region and if sustainably harvested, they could be used as an alternative livelihood strategy for poor people.

DISCUSSION

Chullu and Village Economy

The local traditional liquor, Chullu occupies a sizable portion of village economy, especially the economy of poor tribal people. The Oraon community is actively involved in production and marketing of Chullu. Though the Oraons are involved in Chullu production, sometimes non-tribal agents provide funds to the tribals to produce liquor in a large scale and collect from them to be sold in the urban areas. The starters are processed into two different packets depending upon their sizes and sold at market. Similarly, traditional Chullu is packaged in bottles to sell at beer-shop. It was found that the large packets (5-7 cm diameter each) of starter are sold @ 15 or USD 0.24 of per packet containing 8 pieces whereas small packets (2-3 cm diameter each) are sold @ 8 or USD 0.13 of per packet with 8 pieces. Hanria, the first alcoholic product during preparation of Chullu is also sold @ 5-7 or USD 0.08 to 0.11 per glass of 100 ml whereas the typical Chullu is sold @ 30 or USD 0.48 per bottle of 550 ml.

During survey, we found that though the tribals consume Chullu throughout the year, production of this drink usually at its peak in dry season like summer mainly because drying of starter is relatively easy in dry season. It was also observed that commercialization of Chullu occurred when a groups of villagers from different parts gathered in local fairs, ritual ceremonies, Jatrapalas, folk songs or dance programs or in other social activities. The other means of selling occurred when someone or a group of villagers go to the urban areas due to their personal purposes carry the native drink and sell those during their stay in urban areas. Sales also occur through the agents who directly purchase the indigenous brew. As evident from Table 2, inhabitants of Haspukur, Baidyapur, Kanturka, Dhumpur, Lakhitur, Pakuahat, Bhabuk and Habibpur villages amongst 19 are more interested than others in preparing the local brew which indirectly helps to boost up their economic condition. However, the frequency of alcohol preparation in the villages was more than 65 percent suggesting high concern to prepare the traditional liquor. Excess amount of local brew are

Table 1: List of medicinal plants investigated for Chullu preparation and their respective ethnic uses

Plant species/family/Vn	Used parts	Ethnomedicinal use	Formulation prescribed by no. of healers	IVs
H. pubescens (Buch.-Ham.) Wall. ex DC. (Apocynaceae)/ Vn Koriya/Indrajab	Bark	The bark is grinded to make powder and taken orally with milk to cure from chronic diarrhea, chronic dysentery, urinary troubles and bleeding of piles. The seeds are used as anthelmintic	22	0.81
W. volubilis (Linn.f.) Stapf. (Asclepiadaceae)/Vn Muniraj/ Barka Dabai	Leaves and roots	i) Leaves are crushed to make paste and used externally on eczema, boils and abscesses ii) Leaves are grinded to make powder and taken orally along with cow's milk in body weakness iii) Leaves are used as sex stimulant along with the healers' own ingredients iv) Roots are utilized in case of fever and jaundice	18	0.66
O. sativa L. (Poaceae)/Vn Dhan	Grain	The grains (*chal*) are soaked in water for whole night and next morning the decoction is taken orally to treat gastric problems	13	0.49
I. frutescens (L.) W.T. Aiton. (Apocynaceae)/Vn Kathmol	Root	i) Roots are used in leucorrhoea, skin diseases ii) Root decoction is used in fever and cough	19	0.70
C. viscosum Vent. (Verbenaceae)/Vn Titvat	Leaf	i) The apical bud are crushed with salt and take to prevent worm (tapeworm or guinea worm) infection and also used in liver disorders ii) Leaf paste is applied externally to prevent skin problems The leaf juice is applied on head to prevent lice	21	0.78

Vn: Vernacular name, *C. viscosum: Clerodendrum viscosum, I. frutescens: Ichnocarpus frutescens, O. sativa: Oryza sativa, W. volubilis: Wattakaka volubilis*, IV: Importance value

Table 2: Frequency of Chullu preparation in studied villages

Name of studied villages	Total no. of population studied[#]	Total number of persons preparing alcohol in village	Frequency of Chullu preparation by local people (%)
Chaknagar	345	269	77.97
Haspukur	460	375	81.52
Parameshpur	439	349	79.49
Baidyapur	521	439	84.26
Jhinjhinipukur	296	203	68.58
Aktail	345	268	77.68
Nityanandapur	211	153	72.51
Kanturka	362	301	83.14
Rishipur	209	164	78.46
Srirampur	358	277	77.37
Dhumpur	421	355	84.32
Lakhitur	379	330	87.07
Jagdala	265	211	79.62
Dangapara	119	86	72.26
Pakuahat	451	368	81.59
Salaidanga	223	162	72.64
Majhra	195	142	72.82
Bhabuk	385	324	84.15
Habibpur	535	450	84.11

[#]Total population studied=(Number of Chullu producing person+No. of non-Chullu producing person)

also produced in those areas as per the demand of agents. Hence, commercialization of local drink would be a good alternative way to the ethnic people if properly manufactured.

In selling local traditional brew, the most critical factors are the lack of proper infrastructure, proper management, communication, transportation, local market or beer-shops etc. It has been observed that some of the villages are in such remote areas that the transportation facilities are inaccessible. Therefore it becomes difficult to reach to the desired places or sometimes become detached due to some natural calamities. As a result the transportation charges become more than the production cost of Chullu. Seasonality, especially rainy season is also a great factor. Due to the presence of heavy moisture in the environment and/or inadequate sunlight, the starters don't get dried up well leading to fungal contamination. As a result, the drink prepared with those half-dried starters becomes toxicated or sometimes fatal.

Transmission of Knowledge

The knowledge of the wild medicinal plants used in various purposes are based on regular practices, oral transmission and are also influenced by several factors such as age, gender, relationship and other sociocultural factors generating variability in a particular zone [11]. The privacy of traditional medical practices in the indigenous people is a common phenomenon [12]. The use of selected plant species in Chullu preparation distinguishes the Oraon people from other communities in the studied region and it confirms that the knowledge is confined within this community. The informants reported that they always keep their medicinal plant knowledge secret. The open transfer of indigenous knowledge could only take place verbally along the family line, usually from older knowledgeable person to younger ones. The transfer of knowledge takes place hardly to the people outside the family and passed only on substantial cash payment.

CONCLUSION

Through the present survey we intended to have a detailed account of local drink production in selected regions of rural Bengal. Rural Malda district to be precise, we have also given special emphasis to the tribal community and their way of preparing local drink, Chullu. Oraon economy involved typical method of Chullu production which is indigenous to their community. We found that Chullu production, trade and marketing are a popular occupation among Oraon communities.

Any kind of traditional alcoholic drink is popular among the tribal communities and the non-tribals because of its cheap cost and high alcohol content. The tribal communities use native brew virtually in every occasion, from birth to funeral. They used several herbs and shrubs for the preparation of local drink. Some of which are ethno-botanically important. Oraons have a popular belief that the plant parts used in Chullu actually help them in combating against various ailments such as headache, insomnia etc.

Overall, it is apparent that the present survey is some of the most comprehensive one on Chullu production and marketing in Bengal and certainly the most exhaustive for rural Malda district of West Bengal.

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Original Research

GESDAV

Indigenous knowledge of plants in local healthcare management practices by tribal people of Malda district, India

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ABSTRACT

Aim: The present study was aimed at exploring the indigenous knowledge of native tribes on the utilization of wild plant species for local healthcare management in Malda district of West Bengal. **Materials and Methods:** Successive field surveys were carried out from July 2012 to August 2013 in search of traditional healers or practitioners who ceaselessly use their worthy knowledge to treat several ailments for human purposes. The information was collected by means of open-ended conversations, semi-structured questionnaire, group discussion, etc. Information obtained from the informants was also cross verified to check the authenticity. **Results:** The present study revealed that a total of 53 medicinal plants belonging to the 37 families are frequently used to treat 44 types of ailments with 88 herbal preparations. Of 53 plants, herbs possess the highest growth forms (32%) that were used in making traditional preparation, followed by shrubs (24%), trees (23%), climbers (17%), and parasites (4%). Roots comprised the major plant parts used (25%), followed by leaves (21%), seeds (17%), bark (13%), whole plant (8%) and fruits (6%) to prepare the medicinal formulations. The chief ailments treated in this province were azoospermia, diabetes, menstrual disorder, dysentery, rheumatism, etc. **Conclusion:** It can be concluded that the documentation of the ethnobotanical knowledge in management of local healthcare is the first step, which will open new door for the researchers in the field of modern drug development.

KEY WORDS: Ethnobotany, healthcare management, India, Malda district, tribal, West Bengal

INTRODUCTION

The knowledge of medicinal plants in India has been accumulated in course of many centuries based on several ancient medicinal systems, including ayurveda, unani and siddha [1]. According to the survey report of World Health Organization [2], 80% people of the developing world use plant remedies for several therapeutic purposes. India, one of the richest floristic regions of the world has diverse socio-economic, ethnic, linguistic and cultural areas. Therefore, the indigenous knowledge of medicinal plants and their use in treating several ailments might reasonably be expected in this country. Chandel *et al*. [3] have reported that nearly about 70% of tribal and rural inhabitants of India are to a large extent depended on medicinal plants for their primary healthcare management due to either insufficient or inaccessible or less availability of modern healthcare system. The information regarding the medicinal properties of plants came down traditionally generation after generation through traditional healers. Apart from the tribal groups, many other forest dwellers and rural people also possess unique knowledge regarding plant utilization.

Malda district of West Bengal, India [Figure 1] is situated between the latitude and longitude of 24°40′20″N to 25°32′08″N and 88°28′10″E to 87°45′50″E respectively with a total geographical area of 3455.66 sq km [4]. The district is characterised by its great archaeological relics such as Mourya Empire, Gupta Dynasty and Pala Dynasty. The region is covered with plentiful natural vegetation, which makes it verdant. River beds, ponds, marshy land etc. are good habitats for the wetland undergrowth. Most of the remote villages are covered by jungles, which consist chiefly of thorny scrub bush and large trees showing wide distribution of flora. The soil of the western region of the district is particularly suited to the growth of mulberry and mango, for which Malda has become famous. Various ethnic communities, including Santala, Rajbanshi, Namasudre, Polia, Oraon, Mundas, Malpaharias etc. are the inhabitants of this region. Of these Santala, Oraon is different from others due to their unique culture and tradition. They are quite popular to treat several types of local ailments of human and

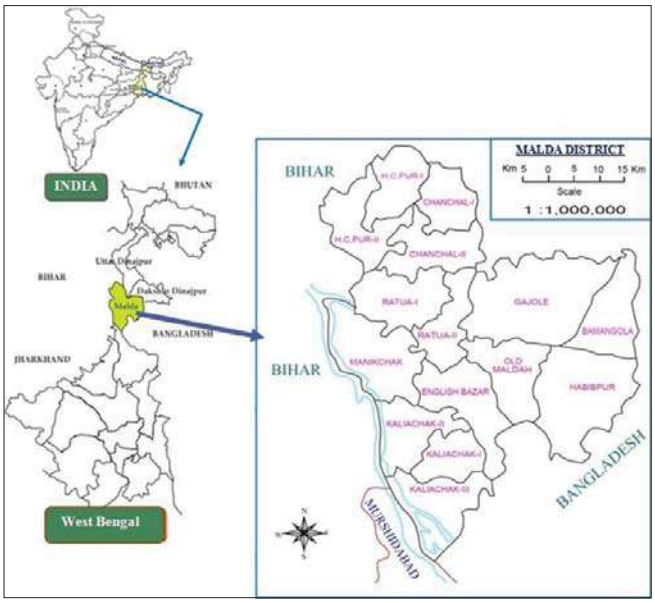

Figure 1: Map of study area (Malda district)

veterinary purposes [4]. Agriculture is the main source of income in the territory. Besides rearing of cattle, sheep, goats, fowls, etc. are the common practices among the tribal communities in this district. They also earn their livelihoods by selling milk, egg, flesh, etc., which plays a significant role in the rural economy of this district.

Preliminary floristic survey and a few numbers of folk usages of local plants had been studied for Malda district by Sur *et al.* [5,6], Pal and Das [7] and Chowdhury and Das [8], whereas Saha *et al.* [4] demonstrated a detailed picture regarding the ethnoveterinarian uses of plants. However, no detailed ethnomedicinal practices by local tribal communities had been done so far for this province. Hence, this is the first hand information on the ethnomedicinal usage by the ethnic people of Malda district as per author's best knowledge.

Now-a-days the traditional knowledge is in the way of erosion due to environmental degradation, deforestation, agricultural expansion and population pressure. Traditional knowledge of medicinal plants and their use by indigenous cultures are not only useful for conservation of cultural traditions and biodiversity but also for community healthcare and drug development at present and in the future. Therefore, recording of indigenous knowledge of medicinal plants is an urgent task. The objective of this study was to interact with local traditional healers and to document their knowledge on utilization of medicinal plants, their usage and the types of diseases treated, etc.

MATERIALS AND METHODS

Ethno Botanical Survey

The practice of medicinal plants is widespread among the tribal people of Malda district, and it is deeply rooted in their socioeconomic culture. However, the documentation of local medicinal practices is distinctly absent for the region. Considering the great cultural and ethnolinguistic diversity of the tribal people of the province, several field interviews were designed to cover as broad an area of the region as possible, in order to maximize the diversity of knowledge and the plant species employed in traditional remedy. The present survey was conducted during July 2012 to August 2013 in the district. Different interviewing procedures, including direct interview, group discussion, open-ended conversations, semi-structured questionnaire etc. were followed to get the information from the local traditional healers, known as Kavirajs, Baidyas or Ojhas and aged knowledgeable persons regarding the use of different medicinal plants curing several ailments. The purpose of this survey was explained to them in details, and prior informed consent was taken as per ethical guidelines of the International Society of Ethnobiology [9]. The villages were visited in different seasons to get the plant in its flowering condition. Plants were pointed out by the informants and their local names, used plant parts, formulation and dosages were also recorded.

Plant Collection and Identification

The plants were properly photographed, and herbarium was prepared for each specimen and deposited at Raiganj University College, Raiganj, India. The collected specimens were identified with the help of Central National Herbarium, Kolkata, India. The survey method followed in this study was that of the guided field-walk method as described by Jain [10] and the collection of voucher specimen, preservation, herbaria technique was followed as per Jain and Rao [11].

Total Key Informants

During the survey, we interacted with more than 100 informants and retained the information only from 74 informants. Among these, 55 were male (74.33%), and 19 were female (25.66%). More emphasis was given to the aged knowledgeable healers due to their vast experience in treating the local diseases and disorders. Kishori Barman (71 years), Uttam Kr. Mandal (53 years), Nargis Bibi (48 years), Farshed Ali (58 years), Fatema Begum (68 years), Basudeb Rajbanshi (55 years) Md. Subed Ali (44 years) etc. were the healers in the study area that we found.

Data Analysis

To analysis the data more clearly, obtaining from the informants, we set up our own database using Microsoft Access version 2007 and the parameters were name of the taxon, family name, voucher number, vernacular names, parts used, diseases treated, mode of administration or medicinal uses. We also analyzed the percentage between the used parts of plant species, growth forms of the species by putting them in the graph.

RESULTS

Plants Used

The present study revealed that a total of 53 medicinal plants belonging to 37 families were frequently used in the treatment

of 44 types of local ailments with 88 phytotherapeutic uses in the territory. The number of species most frequently used in the treatment of several disorders by each family was mentioned as Euphorbiaceae-6 species, Fabaceae-5 species, whereas Acanthaceae, Amaranthaceae, Vitaceae, Malvaceae, Solanaceae, Mimosaceae, and Zingiberaceae contributed 2 species to each family. The rest of 28 families were represented by 1 species in each. The scientific names of recorded species, their families, vernacular names, voucher number, used parts, mode of administration and local ethnic uses were illustrated in Table 1. Our study also exhibited that herbs were the most dominant growth forms with 17 species (32%), followed by 13 shrubs (24%), 12 trees (23%), 9 climbers (17%) and only 2 parasitic species (4%) treating different ailments as shown in Figure 2. *Andrographis paniculata*, *Amaranthus spinosus*, *Alstonia scholaris*, *Cuscuta reflexa*, *Jatropha gossypiifolia*, *Caesalpinia crista*, *Tamarindus indica*, *Sida rhombifolia* etc. were the most important plant used in the treatment of several diseases.

Parts of the Plant Used and Mode of Preparation

Various preparations of roots were used most number of occasions with 18 times (25%), followed by leaves with 15 times (21%), seeds with 12 times (17%), barks with 8 times (13%), whole plants with 6 times (8%), fruits with 4 times (6%), latex and gum with 3 times (4%) etc. as shown in Figure 3 in the treatment of several human disorders. A total of 88 types of formulations was being administrated to heal 44 types of ailments including azoospermia, diabetes, bone crack or ankle sprain, several types of pain, menstrual disorders, rheumatism, dysentery, etc. It had been observed that 20 types of diseases were healed by leaves, whereas 26 types of ailments cured by roots [Table 1]. A single plant part of same plant species was involved in treating different ailments and vice-versa.

The majority of remedies were prepared from fresh plant material in the form of a decoction, infusion or a paste. The most frequently used mode of remedy administration is oral ingestion, followed by external use. Most of the diseases and pains were usually treated either with a single plant or a mixture of plant parts. In some cases, ointments like mustered oil, ghee (a remedy from milk) etc. and other ingredients such as black peeper, ginger, curcuma, milk etc. were also used to make ethnic formulations along with the parts of plant species.

Diseases Treated and Medical Applications

A total of 44 types of diseases were reported to be cured in the present study. Azoospermia with 8 times was mostly healed disease in the study area, followed by different types of pains with 6 times, ankle sprain and diabetes with five occasions each whereas dysentery, inflammation, menstrual disorder, rheumatism, skin disorders, leucorrhea with 4 times each. Further, it can be concluded from Table 1 that the most of the preparations were oral except a few of external use. Various methods of preparation like crushing, grinding, direct use and homogenizing in water or with other plant extracts were used to prepare the traditional remedy. Mustered oil or ghee (a remedy from milk) was being utilized as an ointment at the time of external use such as itching, eczema, inflammation, pus, etc.

DISCUSSION

The prevalent diseases identified in the study area were azoospermia, ankle sprain, pain, diabetes, menstrual disorders, rheumatism, dysentery, skin disorders, etc. To expel ankle sprain or bone crack of local people, different plant parts like whole plant of *Cissus quadrangularis*, roots of *Tragia involucrata*, bark of *Litsea glutinosa*, bark of *Acacia catechu*, rhizome of *Alocasia macrorrhiza*, fruits of *Terminalia chebula* were administrated whereas eight plant species namely roots of *Bombax ceiba*, seeds of *C. reflexa*, *Ocimum kilimandscharicum* and *Abrus precatorius*, roots of *Curculigo orchioides* etc. were administrated to treat azoospermia [Table 1]. Diabetes was cured by means of leaf of *A. paniculata*, seeds of *Trigonella foenum-graecum*, seeds of *Syzygium cumini*, fruit of *Alpinia zerumbet* and whole parts of *Oxalis corniculata*. To treat menstrual disorders several plants had been utilized by the local traditional healers as explained in Table 1. There were few species used more than one occasion to prepare medicinal preparations curing different ailments, viz. *C. quadrangularis* known as harjora was used in bone crack and ankle sprain;

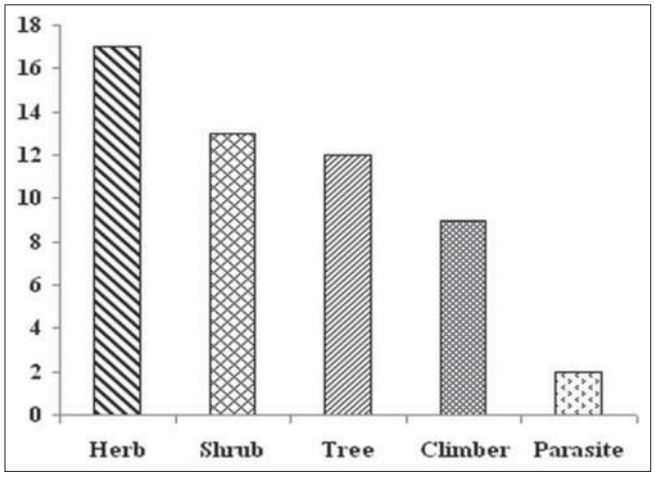

Figure 2: Growth forms of utilized species

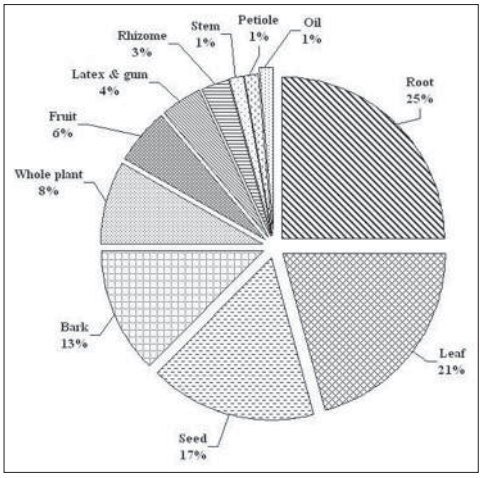

Figure 3: Pie chart of used plant parts

Table 1: List of medicinal plants investigated for local healthcare management with their ethnic use

Name of the plants/ voucher specimen number	Family	Local names	Parts used	Disease/formulation/administration
A. paniculata (Burm. f.) Wall. Ex Nees RUC/MLD-255	Acanthaceae	Kalmegh/ Mahatita/ Iswarnath	Leaf	Fever/Dysentry: The leaf is crushed and the juice is used to treat fever and chronic dysentery at early morning in empty stomach Diabetes: The leaf is grinded along with leaf of *S. chirata* to make a paste against diabetes. The formulation is taken twice a day for 2 months
H. hirta T. Ander. RUC/MLD-294	Acanthaceae	[Not Known]	Root	Bleeding piles: The root is crushed along with *Mentha* leaves and the paste is applied on rectum to stop bleeding piles for 2-3 weeks
A. spinosus L. RUC/MLD-251	Amaranthaceae	Kanta Khuria/ Kanta note	Root	Menstrual problem: The root is grinded and the decoction is mixed with milk and sugar to make a paste and used to treat irregular menstruation. The paste is taken twice in a day for 1 month Rheumatism: The root is grinded with sugar (slight), goat milk and mustered oil to make a paste and applied on affected area to treat rheumatism Cuts and wounds: The grinded root paste is used as an emollient on all types of cuts and wounds
A. aspera L. RUC/MLD-287	Amaranthaceae	Apang/Baro chirchiri	Root	Inflammation: The root is grinded with black pepper and the decoction is taken to treat inflammations in abdominal areas Pain: The roots are chewed with betel and lime to treat liver pain
F. vulgare Mill. RUC/MLD-286	Apiaceae	Mouri	Seed	Menstrual problem: The seeds with roots of *Ageratum conyzoides* and ginger are crushed and the juice is eaten to stop excessive blood discharge during menstruation Inflammation: The seeds along with the roots of *Sida rhombifolia* are crushed and the paste is applied to relief from inflammation of breast
A. scholaris (L.) R. Br. RUC/MLD-275	Apocynaceae	Chatim	Bark, leaf, latex	Anorexia: The bark decoction along with ginger is used to treat anorexia Pregnancy: The leaf decoction is feed to pregnant women to enhance delivery Pain: Latex is externally used in gum pain
A. macrorrhiza Schott. RUC/MLD-310	Araceae	Mankachu	Rhizome	Ankle sprain: The rhizomes are crushed along with ginger and mustered oil and slightly warmed. Finally, the paste is applied externally on ankle pain
C. rotang L. RUC/MLD-330	Aracaceae	Bet	Seed	Bronchitis: The seeds are dusted and mixed with cow-milk to treat bronchitis or cold and cough Skin disorders: Leaf paste along with seeds with *A. maxicana* is used externally in skin disorders
A. indica L. RUC/MLD-288	Aristolochiaceae	Iswarmul	Root	Impotency: The root is washed clearly and burned with the roots of *S. ovalifolia*. The ash is mixed with banana and taken in impotency of female
B. ceiba L. RUC/MLD-283	Bombacaceae	Simul (Beng.)	Root, gum	Azoospermia: The tender root (2-3-years-old tree) is crushed along with the roots of *C. orchioides* and used to induce sex and sperm production Laxative: Gum is used as an ingredient of laxative preparation
H. indicum L. RUC/MLD-277	Boraginaceae	Hatisur	Leaf	Dysentery: Leaf juice is used for curing dysentery and cough. Fresh leaf decoction is applied to wounds, boils and pruritus Conjunctivitis: The leaf juice is applied on eyes to cure eye disorders like inflammation, conjunctivitis etc.
T. chebula Retz. RUC/MLD-341	Combretaceae	Haritaki	Fruit	Bone crack: The *Cissus* stem (Harjora), *Litsea* stem (Daradmayda), *T. bellirica* fruit (Bahera) and an egg (white part) is crushed along with the Haritaki fruit, then the paste is applied externally on bone crack
C. reflexa Roxb. RUC/MLD-296	Convolvulaceae	Swarnalata/ Aloklata	Whole plant, seed	Nervous disorder: The plant is crushed with goat milk and the juice is feed to treat nervous disorder Azoospermia: The seed infusion is used to enhance sperm health and motility. The formulation is taken at night before sleep for 1-2 month
D. bulbifera L. RUC/MLD-411	Dioscoreaceae	Kham alu	Rhizome	Skin disorders: The rhizome is crushed along with leaf of *T. indica* and golmorich and the paste is applied as emollient in herpes, pusses and other skin diseases
D. montana Roxb. RUC/MLD-399	Ebenaceae	Choto gab/Ban gab	Bark, leaf	Leucoderma: The bark and leaves are together crushed and applied externally against leucoderma Diarrhoea: The decoction of bark is used against diarrhoea
J. gossypiifolia L. RUC/MLD-265	Euphorbiaceae	Varenda/ Jamalkota.	Leaf	Toothache: The leaves with salt and golmorich (2-3 pieces of seeds) are crushed and the paste used in toothache Abscesses: Leaf paste and latex are used as emollient on boils and abscesses Vomiting: Leaves juices are used to induce vomiting
P. reticulatus Poir. RUC/MLD-263	Euphorbiaceae	Panichitki	Root	Tumor: The root bark along with fruits of *Tamarindus* and zinger are crushed and slightly wormed then the paste is used as emollient on tumor
T. involucrata L. RUC/MLD-269	Euphorbiaceae	Bichatu/Bichuti	Root	Ankle sprain: The roots are crushed along with stem of harjora, curcuma and ginger to make paste and applied externally on broken leg and ankle sprain
E. tirucalli L. RUC/MLD-318	Euphorbiaceae	Shibjota	Stem	Galatogouge: The stem portion with leaf is crushed and the paste is taken orally to enhance milk production of women Pain: Stem is crushed along with zinger and the paste is applied as emollient on affected area to relief from pain

Contd...

Table 1: Contd.

Name of the plants/ voucher specimen number	Family	Local names	Parts used	Disease/formulation/administration
E. neriifolia L. RUC/MLD-305	Euphorbiaceae	Patsaij	Bark	Leucorrhea: Bark is crushed along with *P. betel* (3-5 pieces), lime and khoir (*A. catechu*) then the paste is taken orally to cure from leucorrhea
T. nudiflora L. RUC/MLD-343	Euphorbiaceae	Pithalu	Root	Enlargement of uterus: The roots are crushed and slightly warmed then it is applied externally until it cures
C. sophera L. RUC/MLD-279	Fabaceae	Kalkasunda/ Jhanjhane.	Root	Rheumatism: The root with ginger, garlic and black pepper are crushed and the paste is eaten to treat rheumatism
A. precatorius L. RUC/MLD-304	Fabaceae	Kunch (Beng.)	Seed	Pain: Seeds are crushed and paste is applied in stiffness of shoulder joint pain Azoospermia: Seeds are used to enhance sperm production
C. crista L. RUC/MLD-319	Fabaceae	Nata (Beng.)	Leaf, seed	Hydrocele: 3-4 pieces of apical leaf part are crushed with black pepper and taken to cure from hydrocele for 1 month Inflammation: Seed oil is applied externally against burning sensation of body
T. indica L. RUC/MLD-322	Fabaceae	Tetul (Beng.)	Fruit	Abdominal fat: Fresh fruits (1 kg) are boiled in water along with sugar (michri), and taken the juice twice to minimize abdominal fat Dysentery: Young fresh leaves are crushed along with sugar (michri), and the decoction is taken to treat dysentery
T. foenum-graecum L. RUC/MLD-333	Fabaceae	Methi	Seed	Kidney stone: The seeds are kept in a bowl of water then the decoction (1 glass) is taken at next morning in case of kidney stone for 15-20 days Diabetes: The seed powder is mixed with milk and taken at bed-time for 30 days against diabetes Dandruff: The seed paste is applied on head to prevent dandruff
C. orchioides Gaertn. RUC/MLD-335	Hypoxidaceae	Talmuli	Root	Azoospermia: The root (1-2 pieces) is chewed at every morning for 15-20 days to improve sperm production and motility
O. kilimandscharicum Guerke RUC/MLD-347	Lamiaceae	Dulal babu	Seed	Azoospermia: The seeds are taken in a bowl of water and left for whole night; then next morning it is crushed along with that water and taken to induce sperm production. The formulation is taken for 1 month
L. glutinosa (Lour.) C. B. Rob. RUC/MLD-259	Lauraceae	Daradmoyda	Bark, leaf	Bone crack: The stem bark is crushed along with harjora, curcuma to make a paste and applied as emollient on bone crack, ankle pain etc. Loose motion: Leaves are crushed and the juice is taken in case of loose motion
B. acutangula (L.) Gaertn. RUC/MLD-313	Lecythidaceae	Hizal (Beng.)	Bark, seed	Azoospermia: Bark is taken in a bowl of water and at the next morning the infusion is taken to condense watery semen for 30 days Sinus problem: The seeds are dusted and mixed with warm milk and then eaten at every evening for 1 month which effectively cure sinus problem
D. falcata (L.f.) Etting. RUC/MLD-340	Loranthaceae	Dharua	Bark	Menstrual problem: The bark is crushed along with bark of *S. indica*, fennel seeds and ginger and the juice is taken in case of irregular menstruation
S. rhombifolia L. RUC/MLD-310	Malvaceae	Peet Berala/ Bariala	Root	Abscesses: The roots are crushed with black pepper and areca nut and applied externally to cure from abscesses Inflammation: The roots and fennel seeds are crushed and the paste is used to relief from inflammation of breast
A. moschatus Medik. RUC/MLD-280	Malvaceae	Latakasturi (Beng.)	Seed, whole plant	Sex stimulant: Seeds are kept in a bowl for whole night. On the very next morning seeds are crushed along with roots of *C. orchioides* to make paste which act as sex stimulant and it enhances semen production
C. hirsutus (L.) Diels RUC/MLD-261	Menispermaceae	Jalkasha (Beng.)	Leaf	Azoospermia/late ejaculation: The leaf is crushed with water in a bowl and left for whole night and next morning the decoction is taken to induce semen production. It is also effective against late ejaculation
A. catechu Willd. RUC/MLD-326	Mimosaceae	Khoir	Bark	Ankle sprain: The bark is crushed along with harjora, curcuma, an egg and zinger to make a paste and applied externally on bone crack and ankle sprain Leucorrhea: The bark is crushed along with *P. betel*, lime and bark of *Euphorbia neriifolia* and then the paste is taken orally to cure from leucorrhea for 15-30 days
M. pudica L. RUC/MLD-307	Mimosaceae	Lajjabati (Beng.)	Root, leaf	Leucorrhea: Root decoction is used to treat leucorrhea for 20 days Breast Cancer: Leaves decoction is effectively used in breast cancer
F. benghalensis L. RUC/MLD-257	Moraceae	Bot	Latex, root	Nervous or body weakness: The latex mixed with sugar (batasa) are fed to induce semen production and in nervous or body weakness Rheumatism: The crushed apical prop root mixing with goat milk and sugar (batasa) are used to treat rheumatism
S. cumini (L.) Skeels RUC/MLD-337	Myrtaceae	Jam	Leaf, seed	Dysentery: Leaf is crushed along the leaf of *Tamarindus* sp. (tetul), michri (a type of sugar) and the roots of *Cephalandra* sp. (telakucha) and the paste is taken at empty stomach to prevent dysentery Diabetes: Seed powder is mixed with milk and taken twice a day in diabetes
O. corniculata L. RUC/MLD-320	Oxalidaceae	Amrul	Whole plant, root	Diabetes: The whole plant is crushed and juice is taken at early morning to prevent diabetes for 1-2 months Acidity/vomiting: The roots (3-4 pieces) are crushed with salt and taken to cure from acidity and vomiting

Contd...

Table 1: Contd.

Name of the plants/ voucher specimen number	Family	Local names	Parts used	Disease/formulation/administration
A. mexicana L. RUC/MLD-311	Papaveraceae	Siyal kata/ Gandhila	Seed, leaf	Skin disorders: The seeds are fried and crushed and then this seed-dust are mixed with coconut oil and applied on body to prevent skin disorders like eczema, pus etc Conjunctivitis: The leaf juice is applied on eyes to cure eye disorders like inflammation, conjunctivitis etc
P. emblica L. RUC/MLD-270	Phyllanthaceae	Amlaki (Beng.)	Fruit	Late ejaculation: Dried fruits are dusted and eaten at morning and night after meal, which is very useful to prevent late ejaculation. The formulation is taken for 1-2 months Stungury: Boiled fresh fruits with slight salt are taken for 20-25 days to treat stungury
P. betel L. RUC/MLD-317	Piperaceae	Pan	Leaf	Leucorrhea: Leaf is crushed along with stem bark of *E. nerifolia*, lime and fruit of *A. catechu* (khoir), the paste is taken orally to cure from leucorrhea
P. zeylanica L. RUC/MLD-250	Plumbaginaceae	Sadachita/ Agrochita	Root	Appetizer/blood enhancer: The root is crushed and the decoction used as an appetizer and also acts as blood enhancer
S. munja Roxb. RUC/MLD-289	Poaceae	Siki ghas/ Biyana	Root, oil	Allergy/pain: The roots are crushed with curcuma and zinger and the paste is applied externally to cure from allergy and body pain Lumbago: The oil extracted from leaves, are used to treat from lumbago
H. cordata Thunb. RUC/MLD-335	Saururaceae	Anstagach	Leaf	Vomiting: The leaves are crushed along with zinger and golmorich to induce vomiting
S. ovalifolia Roxb. RUC/ MLD-271	Smilaceae	Bagnocha/ Kumarilata	Whole plant, root	Impotency: The root is washed clearly and burned with the roots of *A. indica*. The ash is mixed with banana and taken in impotency of female Rheumatism: The whole plants are crushed with the bark of *C. religiosa* and the juice is taken to treat rheumatism for 2 months
S. xanthocarpum Sch. and Weldl. RUC/MLD-293	Solanaceae	Kantikari (Beng.)	Whole plant, root, seed	Conjunctivitis: Whole plant is burned along with peyaj and used as emollient on eyes to cure from conjunctivitis Pain: Roots and seeds are crushed along with the stem of *E. tirucalli* to make paste and applied externally to treat chest pain
D. metel L. RUC/MLD-328	Solanaceae	Kalo Dhutura	Root, leaf	Paralysis: The roots are crushed along with mustered oil, ghee (a remedy from milk), black pepper, curcuma and sindur, and then used as an emollient on paralyzed area until it cures Hair growth: The leaves are crushed and applied on head for over night, and washed off by tea-liquor to promote new hair growth. It is applied for 10-12 days
A. augusta (L.) L. f RUC/MLD-300	Sterculiaceae	Ulatkambal	Petiole, bark	Azoospermia: The petiole is crushed and kept in a bowl of water for a whole night, then the infusion is taken at early morning at empty stomach as semen and sperm enhancer
C. quadrangularis L. RUC/MLD-312	Vitaceae	Harjora (Beng.)	Whole plant	Bone fracture/ankle sprain: The plant is crushed along with roots of *D. metel* (kalo dhutura), *Glycosmis* sp. (atiswar) leaves of *Tamarindus* sp. (tetul), ginger, salt and the pest is applied as emollient on bone fracture, ankle sprain (5-12 days)
C. trifolia (L.) Domin RUC/MLD-315	Vitaceae	Choto goaliarlata	Leaf	Menstrual disorder: The leaves are crushed along with roots of *A. aspera* (apang) and *Areca* fruit and the juice is taken on empty stomach at early morning to prevent irregular menstruation (20-30 days)
Z. cassumunar Roxb. RUC/MLD-323	Zingiberaceae	Ban ada/Bau ada	Rhizome	Ankle sprain: The rhizome is crushed along with ginger and roots of bichuti (*Tragia* sp.) and a paste is made which is used as emollient on broken bone and ankle sprain
A. zerumbet (Pers.) Burtt & Smith RUC/MLD-314	Zingiberaceae	Elach	Fruit	Diabetes: Fruit (10-12 pieces) is crushed along with *Musa* stem, (3-4 pieces; 10 cm each) *I. aquatica* (kalmi sag), leaf of *N. indicum* and pinch of michri (remedy of sugar) and then the extract juice is taken orally to treat diabetes (30-45 days)

A. paniculata: Andrographis paniculata, H. hirta: Hemigraphis hirta, A. spinosus: Amaranthus spinosus, A. aspera: Achyranthes aspera, F. vulgare: Foeniculum vulgare, A. scholaris: Alstonia scholaris, A. macrorrhiza: Alocasia macrorrhiza, C. rotang: Calamus rotang, A. indica: Aristolochia indica, B. ceiba: Bombax ceiba, H. indicum: Heliotropium indicum, T. chebula: Terminalia chebula, C. reflexa: Cuscuta reflexa, D. bulbifera: Dioscorea bulbifera, D. montana: Diospyros montana, J. gossypiifolia: Jatropha gossypiifolia, P. reticulatus: Phyllanthus reticulatus, T. involucrata: Tragia involucrata, E. tirucalli: Euphorbia tirucalli, E. neriifolia: Euphorbia neriifolia, T. nudiflora: Trewia nudiflora, C. sophera: Cassia sophera, A. precatorius: Abrus precatorius, C. crista: Caesalpinia crista, T. indica: Tamarindus indica, T. foenum-graecum: Trigonella foenum-graecum, C. orchioides: Curculigo orchioides, O. kilimandscharicum: Ocimum kilimandscharicum, L. glutinosa: Litsea glutinosa, B. acutangula: Barringtonia acutangula, D. falcata: Dendrophthoe falcata, S. rhombifolia: Sida rhombifolia, A. moschatus: Abelmoschus moschatus, C. hirsutus: Cocculus hirsutus, A. catechu: Acacia catechu, M. pudica: Mimosa pudica, F. benghalensis: Ficus benghalensis, S. cumini: Syzygium cumini, O. corniculata: Oxalis corniculata, A. mexicana: Argemone mexicana, P. emblica: Phyllanthus emblica, P. betel: Piper betel, P. zeylanica: Plumbago zeylanica, S. munja: Saccharum munja, H. cordata: Houttuynia cordata, S. ovalifolia: Smilax ovalifolia, S. xanthocarpum: Solanum xanthocarpum, D. metel: Datura metel, A. augusta: Abroma augusta, C. quadrangularis: Cissus quadrangularis, C. trifolia: Cayratia trifolia, Z. cassumunar: Zingiber cassumunar, A. zerumbet: Alpinia zerumbet, S. chirata: Swertia chirata, A. conyzoides: Ageratum conyzoides, A. maxicana: Argemone maxicana, T. bellirica: Terminalia bellirica, S. indica: Saraca indica, C. religiosa: Crateva religiosa, A. aspera: Achranthus aspera, I. aquatica: Ipomoea aquatica, N. indicum: Nerium indicum

A. spinosus was used to treat menstrual disorders, rheumatism, cuts and wounds; *T. foenum-graecum* was used against kidney stone, diabetes and dandruff problems.

As the tribal people remain busy throughout the year with their practice of livelihood from the agricultural sector, they rarely visit the hospitals in towns. Simultaneously, they cannot afford the cost of modern medicines. It has also been observed that some of the villages are in such remote areas where transportation facilities are inaccessible or sometimes become detached due to some natural calamities. Hence, the villagers cannot reach the nearby hospital. As a result, the ethnomedicinal practices are popular in the study area as it is more accessible, easy to prepare, low costs, and eco-friendly. Besides, the practice of medicinal plants treating the patients is an alternative source of income for the healers.

CONCLUSION

The present study exhibited that how different interviewing procedures helped to gather the information regarding the name of the diseases treated, plant resources and their usage, including their mode of administration. A total of 44 types of local ailments was treated with 88 phytotherapeutic uses in this district. The making procedure of herbal preparation is yet a secret and passed on generation after generation verbally. Proper analysis of herbal formulations and phytoconstituents of used plants can open new door for the researchers. However, ethnobotanical data is the basis of further validation of practices and plant uses in the context of a professional approach to develop new herbal drug [12].

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Ethnobotany of Chanchal Block of Malda District of West Bengal (India): plants used in local healthcare

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Abstract

The ethnobotanical survey in Chanchal block of Malda district recorded a total of 49 species belonging to 29 families were used to treat 25 local ailments. Root was the most frequently used plant part in preparing herbal remedies followed by leaf. The most common diseases treated in the study area were bone fracture, gastritis, diabetes, rheumatism, gastritis, asthma, bronchitis, skin disorders etc. In most of the cases, more than one species has been utilized to treat one disease. For instance, five plant species namely *Acacia nilotica* (Linnaeus) Delile, *Musa x paradisiaca* Linnaeus, *Bombax ceiba* Linnaeus, *Catharanthus roseus* (Linnaeus) G. Don, *Syzygium cumini* (Linnaeus) Skeels were used to treat diabetes whereas six different plant species were utilized to alleviate gastritis problems. Now-a-days, the indigenous knowledge is on the way of erosion due to several extrinsic factors. Therefore, the documentation of plant resources and their sustainable utilization is a necessary step towards the goal of raising awareness in local people about the importance of these plants and their further conservation.

***Key words*:** Ethnobotany, Medicinal plants, Healthcare, Tribe, Chanchal, Malda, West Bengal.

INTRODUCTION

Man has been dependent on nature for their survival and medicine since time immemorial. This dependency led the indigenous people living in harmony with nature to evolve a unique system of medicinal plant practices (Ghosh *et al.* 2004; Teron & Borthakur 2014). This knowledge and description of the use of a variety of plant-derived medications has been passed orally generation to generation. In India, there are about 54 million aboriginal people of different ethnic groups inhabiting various regions. The aboriginal groups have their own distinctive culture, religious rites, food habit and a rich knowledge of plant utilization (Parinitha *et al.* 2005; Boro & Sarma 2013). The indigenous knowledge of medicinal plants and their use in treating several ailments might reasonably be expected in India due to its rich floristic vegetation (Gupta *et al.* 2004; Shil *et al.* 2014). Virtually, ethnobotanical survey may be regarded as one of the most reliable approaches to new drug discovery and it is a prerequisite for any developmental planning concerned with the welfare of tribal and their environment (Lokho & Narasimhan 2013).

However despite being rich in biodiversity and with diversified ethnic communities, Malda district (Fig. 1) of West Bengal in India is still remained elusive for ethnobotanists (Saha *et al.*

2014). The district is characterized by its rich wetland and forest vegetation which consist chiefly of herbs, shrubs, thorny scrub bushes and large trees. *Santals*, *Rajbanshi*, *Oraon*, *Namasudra*, *Polia*, *Mundas*, *Malpaharias* etc. are the main aboriginal communities living throughout the district. Practices of Traditional Medicines using plant species is a common phenomenon to them apart from agriculture. Even today, the ethnic people of the district practice herbal medicine to treat a variety of ailments using wild medicinal plants due to its efficiency, ease and low cost.

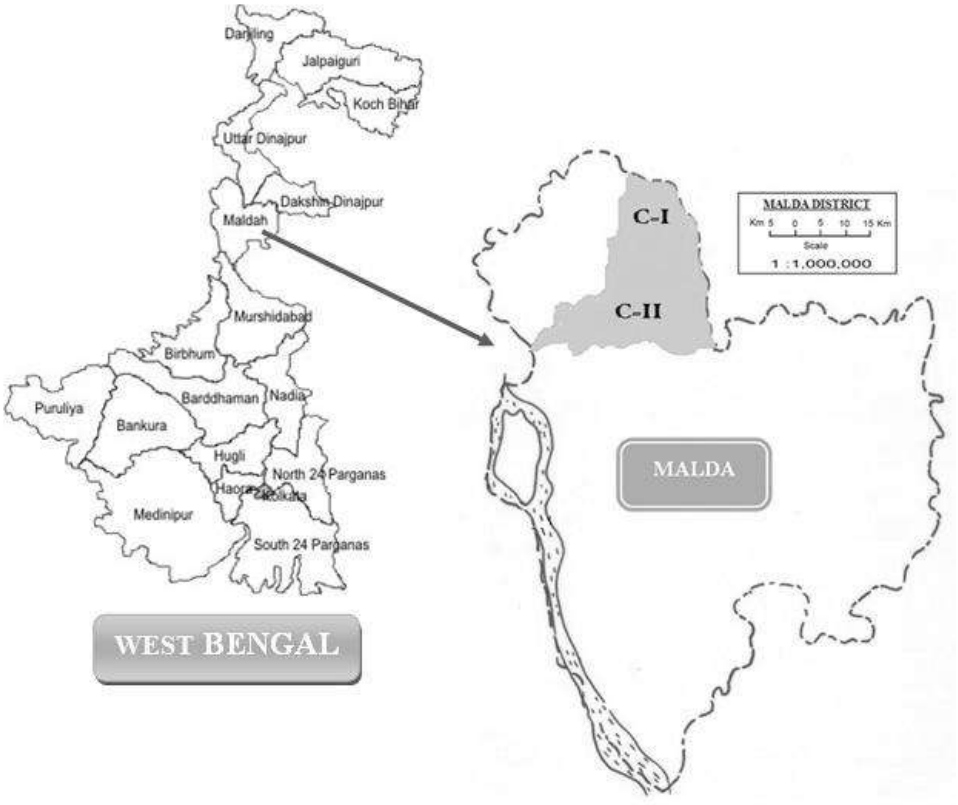

Fig. 1. Location map of study area [C-I= Chanchal block I; C-II= Chanchal block II]

Chanchal block I & II are such areas of the district where medicinal plants are utilized vigorously for local healthcare management by tribal community and located between the latitude and longitude of 25°23´ N and 88°0´ E respectively. The practice of such traditional knowledge is so popular in those provinces that it is deeply rooted in their culture. However, no such documentation has so far been conducted in Chanchal block I & II. The purpose of the current study was, therefore, to gather information on the exploitation of local medicinal plants by ethnic people in Chanchal block I and II of the district to manage diseases.

MATERIALS AND METHODS

Extensive field survey has been conducted in ten villages (Malatipur, Samsi, Ratanpur, Sripur, Chanchal, Mahanandapur, Jalalpur, Kandaron, Vado and Bhakri) of Chanchal blocks I & II, Malda, India was conducted in the year June 2011 to August 2012. The information regarding the traditional knowledge, local uses of plants, parts used (e.g. leaf, root, bark, fruit etc.), purposes, name of diseases, modes of administration, and curative properties were recorded

through intensive interviews and group discussions with traditional healers and elderly knowledgeable persons using semi-structured questionnaire process. Prior Informed Consent (PIC) was also obtained from them. Many practitioners were interviewed in the study area and retained information only from 25 informants. The survey method followed in the present study was that of guided field-walk method as suggested by Jain (1995). The plants used for various purposes were identified using different available literatures including Hooker (1875 – 1897); Prain (1903); Kanjilal *et al* (1934 – 1940); Balakrishnan (1981 & 1983) and further updated from websites including www.theplantlist.org. For family delimitation APG III system (Chase & Reveal 2009). Voucher specimens were processed into mounted herbarium sheets following Jain & Rao (1977) and stored in the Herbarium of Department of Botany, Raiganj University College.

RESULT AND DISCUSSION

Present study recorded that a total of 49 species belonging to 29 families had been utilized to treat local ailments by tribal people of Chanchal block I & II. Of these, six species, namely *Rauvolfia serpentina*, *Ervatamia coronaria*, *Carissa carandas*, *Catharanthus roseus*, *Hemidesmus indicus* and *Holarrhena pubescens* belonging to Apocynaceae were considered to have ethnobotanical uses, followed by Malvaceae with 4 species including *Abroma augusta*, *Bombax ceiba*, *Hibiscus rosa-sinensis* and *Sida rhombifolia*. Moraceae and Fabaceae with 3 species each whereas Acanthaceae, Apiaceae, Combretaceae, Arecaceae, Rutaceae, Lamiaceae, Vitaceae and Solanaceae contributed 2 species to each and the rest of 17 families were represented by one species in each (Table 1). The present study revealed that trees were the most dominant growth forms with 18 species, followed by herbs with 15 species, shrubs with 13 species and climbers with 3 species.

Root was the most commonly used plant part to prepare herbal formulations accounting for 31 % of the recorded plants, followed by leaves with 24 %, fruits and stem with 11 % each etc (Fig. 2). It was noticed that most of the formulations prepared were of fresh plant materials and processed by crushing, boiling or frying. A single species was involved more than one occasion to prepare remedies. It had been observed that the mostly used form of herbal preparations were paste, juice or infusion of either single or mixture of plant parts. Honey, sugar-candy (*michri* or *tal-michri*), salt, ginger, fennel seeds, black pepper, milk etc. were often used as additives (Table 1). Result also exhibited that most of the preparations were administrated orally whereas other mode of application was external by directly or tying the preparation with the affected part of the body.

A total of 25 types of local diseases were reported to be cured in the study area. The most common diseases were body pain, bone fracture, rheumatism, asthma, bronchitis, diabetes, gastritis, skin disorders etc. The study revealed that a single species like *Vitex negundo*, *Mimosa pudica*, *Phyllanthus emblica*, *Terminalia chebula* etc. have been utilized more than one occasion to treat several disorders. For instance, *Vitex negundo* was utilized in case of pain, paralysis and tumor while *Mimosa pudica* was used in leucorrhoea and dysentery.

However, Dey & De (2010) and Chakraborty & Bhattacharjee (2006) reported a few plants (*Ampelocissus tomentosa*, *Glossogyne bidens* and *Ichnocarpus frutescens*) which were used against bone fracture at Ajodhya hill region of Purulia district, India whereas in the present study four plants including *Cissus quadrangularis* (stem), *Datura metel* (root), *Ampelocissus latifolia* (stem), *Tamarindus indica* (leaf) were used to prepare traditional remedies and applied on afftected area to alleviate bone fracture. Application of 3 species namely *Alstonia scholaris*, *Holarrhena antidysenterica* and *Centella asiatica* were found

Table 1. Name of local diseases and their management practices in Chanchal block of Malda district

Disease	Plant name [Family]; Vernacilar name; Voucher no.	Parts used	Ethnic use/Administration
Bone fracture	***Cissus quadrangularis*** Linnaeus [Vitaceae]; *Harjora*; *RUC/MLD-312*	Stem	All plant parts are crushed along with leaves of *Tamarindus*, ginger, salt to make paste and applied as emollient on affected area of fracture
	Datura metel Linnaeus [Solanaceae]; *Kalo dhutura; RUC/MLD-328*	Root	
	Ampelocissus latifolia (Roxburgh) Planchon [Vitaceae]; *Goaliar lota*; *RUC/MLD-563*	Stem	
	Tamarindus indica Linnaeus [Fabaceae]; *Tnetul*; *RUC/MLD-322*	Leaf	
Pain	***Glycosmis pentaphylla*** A.J. Retzius [Rutaceae]; *Aatiswar*; *RUC/MLD-422*	Root	Plant parts are crushed together to make paste and applied as emollient on affected area to relieve the pain
	Datura metel Linnaeus [Solanaceae]; *Kalo dhutura*; *RUC/MLD-328*	Root	
	Vitex negundo Linnaeus [Lamiaceae]; *Nisinda*; *RUC/MLD-444*	Leaf & stem	Leaves are crushed along with ginger, salt, Black pepper and the paste is applied as an emollient
	Litsea glutinosa (Loureiro) C.B. Robinson [Lauraceae]; *Daradmoida*; *RUC/MLD-259*	Stem	Stem bark is crushed to make paste and applied as emollient on affected area
Paralysis	***Aegle marmelous*** (Linnaeus) J.F. Correa [Rutaceae]; *Bel*; *RUC/MLD-449*	Leaf	Leaves of both the plants are fried red in mastered oil and the mixture is sieved and mixed with tarpin oil (4 teaspoon), pipermint and camphor and massaged on paralyzed area twice in a day
	Vitex negundo Linnaeus [Lamiaceae]; *Nisinda*; *RUC/MLD-444*	Leaf	
Rheumatism	***Justicia gendarussa*** N.L. Burman [Acanthaceae]; *Jagatmadan*; *RUC/MLD-467*	Root	Root paste is applied in rheumatism
	Coccinia grandis (Linnaeus) J.O. Voigt [Cucurbitaceae]; *Telakucha*; *RUC/MLD-473*	Root & Fruit pills	Root decoction mixed with sugar, goat milk is used to treat rheumatism for 3-4 months (twice a week) Concurrently, the paste of fruit pills are applied on affected area
Tumor	***Vitex negundo*** Linnaeus [Lamiaceae]; *Nisinda*; *RUC/MLD-444*	Leaf	Leaves and bark of both the plants are crushed along with turmeric and ginger to make paste, warmed slightly and applied on tumor
	Neolamarkia kadamba (Roxburgh) J.M. Bosser [Rubiaceae]; *Kadam*; *RUC/MLD-509*	Bark	
Body weakness	***Asparagus racemosus*** Wildenow [Asparagaceae]; *Satomul*; *RUC/MLD-499*		Roots of all plants are crushed together and dried to make powder and taken with milk twice a day to reduce body weakness
	Hemidesmus indicus R. Brown [Apocynaceae]; *Anantamul*; *RUC/MLD-512*		

Disease	Plant name [Family]; Vernacilar name; Voucher no.	Parts used	Ethnic use/Administration
Body weakness (contd.)		Root	
	Rauvolfia serpentina (Linnaeus) Bentham *ex* Kurz [Apocynaceae]; *Chandramul*; *RUC/MLD-145*		
	Aristolochia indica Linnaeus [Aristolochiaceae]; *Iswarmul*; *RUC/MLD-177*		
	Bombax ceiba Linnaeus [Malvaceae]; *Simul*; *RUC/MLD-283*		
Asthma	***Ficus glomerata*** Roxburgh [Moraceae]; *Dumur*; *RUC/MLD-256*	Fruit	Fruits boiled in water, mixed with little amount of pipermint and sieved; the decoction is taken to treat asthma
	Solanum xanthocarpum M.L. Schrad & J.C. Wendland [Solanaceae]; *Kontikari*; *RUC/MLD-293*	Root	Roots are crushed to powder along with joan, clove, black pepper, dry ginger and littje salt and taken two table spoons twice a day after meals
Bleeding on the body	***Ageratum conyzoides*** Linnaeus [Asteraceae]; *Chikasunga*; *RUC/MLD-92*	Root	Roots are crushed along with turmeric and the extract is applied twice a day to reduce bleeding from nose, ear etc.
Gastritis	***Phyllanthus emblica*** Linnaeus [Phyllanthaceae]; *Amloki*; *RUC/MLD-270*	Fruit	Fruits are dried and powdered with salt and taken twice a day in empty stomach
	Terminalia bellirica (Gaertner) Roxburgh [Combretaceae]; *Boahera*; *RUC/MLD-126*		
	Terminalia chebula A.J. Retzius [Combretaceae]; *Haritaki*; *RUC/MLD-341*		
	Trachyspermum copticum T.A. Sprague [Apiaceae]; *Ajoan*; *RUC/MLD-510*		
	Ficus hispida Lineaus *f* . [Moraceae]; *Khoska*; *RUC/MLD-452*	Hypanthodium /Fruit	Extract of its fruits and seeds of *Sesamum indicum* Linnaeus is applied on head to lower the gas from head
	Ficus benghalensis Linnaeus [Moraceae]; *Bot*; RUC/MLD-257	Prop root	Paste of slender prop roots is taken with batasa (a product made from sugar) to treat gastritis
Azoospermia	***Curculigo orchioides*** J. Gaertner [Hypoxidaceae]; *Talmuli*; *RUC/MLD-335*	Root	1-2 pieces of root is chewed in the morning for 15-20 days to improve sperm production and density
Kidney stone	***Phyllanthus emblica*** Linnaeus [Phyllanthaceae]; *Amloki*; *RUC/MLD-270*	Fruit	Powder of dried fruits are taken twice a day to dissolve kidney stone
	Terminalia bellirica (Gaertner) Roxburgh [Combretaceae]; *Boahera*; *RUC/MLD-126*		
	Terminalia chebula A.J. Retzius [Combretaceae]; *Haritaki*; *RUC/MLD-341*		
	Kalanchoe pinnata (Lamarck) Persoon [Crassulaceae]; *Patharkuchi*; *RUC/MLD-69*	Leaf	Boiled fresh leaves are very much effective to dissolve stone in kidney

Disease	Plant name [Family]; Vernacilar name; Voucher no.	Parts used	Ethnic use/Administration
Kidney stone (contd.)	***Cocos nucifera*** Linnaeus [Arecaceae]; *Narkel*; *RUC/MLD-364*	Fruit	The tender fruit water along with seeds of *Trigonella foenum-graecum* Linnaeus juice is taken as a stone dissolver
Leucoderma	***Borassus flabellifer*** Linnaeus [Arecaceae]; *Taal*; *RUC/MLD-374*	Leaf	Tender leaves are crushed along with seeds of *Argemone mexicana* Linnaeus shell of *Pila* and the paste is applied as emollient on affected region with coconut oil
Allergy	***Argemone mexicana*** R. Sweet [Papaveraceae]; *Sialkata*; *RUC/MLD-311*	Seed	Seeds are crushed along with tender leaves of Taal, shell of *Pila globasa* (apple-samuk) to make paste and applied as emollient on affected region with coconut oil before bath
Inflammation	***Sida rhombifolia*** Linnaeus [Malvaceae]; *Peet Berala*; *RUC/MLD-310*	Root	Paste of roots and fennel seeds is applied to get relief from inflammation in breast
	Argemone mexicana R. Sweet [Papaveraceae]; *Sialkata*; *RUC/MLD-311*	Root	Paste of root is applied on affected area to prevent inflammation
Menstruation	***Hibiscus rosa-sinensis*** Linnaeus [Malvaceae]; *Joba*; *RUC/MLD-178*	Flower	Decoction of crushed flower in water is feed to women to induce menstruation
	Abroma augusta (Linnaeus) Linnaeus *f.* [Malvaceae]; *Ulatkambal*; *RUC/MLD-300*	Petiole	Petioles are crushed and kept in a bowl of water for whole night, and next morning the decoction is taken against irregular menstruation
Diabetes	***Musa x paradisiaca*** Linnaeus [Musaceae]; *Kola*; *RUC/MLD-439*	Stem	Tender stem (3-5 pieces; 10 – 15 cm long) is crushed along with the root of *Alpinia* sp. (*elach*), whole parts of *Ipomoea aquatica* and sugar candy then the extract is taken orally to treat diabetes
	Acacia nilotica (Linnaeus) Delile [Fabaceae]; *Babla*; *RUC/MLD-433*	Leaf	Tender leaves are crushed along with the stem of *Tinospora sinensis* (Loureiro) Merrill in 2:1 ratio and the juice is taken at early morning for 3-4 months to treat diabetes
	Bombax ceiba Linnaeus [Malvaceae]; *Simul*; *RUC/MLD-283*	Root	Crushed tender root (2-3 years old tree) is used to cure diabetes
	Catharanthus roseus (Linnaeus) G. Don [Apocynaceae]; *Nayantara*; *RUC/MLD-76*	Leaf	Extract of its leaves along with leaves of *Andrographis paniculata* is taken in empty stomach at the morning
	Syzygium cumini (Linnaeus) Skeels [Myrtaceae]; *Jam*; *RUC/MLD-337*	Seed	Seed powder is mixed with goat milk and taken at early morning in diabetes
Leucorrhoea	***Amaranthus spinosus*** Linnaeus [Amaranthaceae]; *Katanote*; *RUC/MLD-251*	Root	Root-decoction is mixed with sugar and milk and taken to treat leucorrhoea
	Punica granatum Linnaeus [Lythraceae]; *Dalim*; *RUC/MLD-179*	Leaf	Leaf extract is taken orally at the morning with in empty stomach to reduce leucorrhoea
	Mimosa pudica Linnaeus [Fabaceae]; *Lajjabati*; *RUC/MLD-307*	Root	Root decoction is used for leucorrhea

Disease	Plant name [Family]; Vernacilar name; Voucher no.	Parts used	Ethnic use/Administration
Bronchitis	***Andrographis paniculata*** (Burman *f.*) Wallich *ex* Nees [Acanthaceae]; *Kalmegh*; *RUC/MLD-255*	Seed	Paste of its leaves with *tulsi, basak* and honey is taken with warm milk twice a day until it cures
Conjunctivi-tis	***Ervatamia coronaria*** (Jacquin) Stapf [Apocynaceae]; *Tagar*; *RUC/MLD-533*	Flower	Juice from crushed flowers is applied on eyes to relive from various type of conjunctivitis
	Barringtonia acutangula (Linnaeus) Gaertner [Lecythidaceae]; *Hizal*; *RUC/MLD-442*	Seed	Seed powder is used as emollient in conjunctivitis
Hypertension	***Aristolochia indica*** Linnaeus [Aristolochiaceae]; *Iswarmul*; *RUC/MLD-177*	Root	Its roots along with the roots of *Rauvolfia serpentina* are crushed and the juice is taken in hypertension twice a day
	Clerodendrum colebrookianum W.G. Walpers [Lamiaceae]; *RUC/MLD-392*	Leaf	Leaf decoction is used in hypertension
Jaundice	***Andrographis paniculata*** (Burman *f.*) Wallich ex Nees [Acanthaceae]; *Kalmegh*; *RUC/MLD-255*	Leaf	Leaf decoction is taken orally
Skin disorder	***Rauvolfia serpentina*** (Linnaeus) Bentham *ex* Kurtz [Apocynaceae]; *Sarpagandha*; *RUC/MLD-145*	Root	Root paste is applied in several kinds of skin diseases
	Holarrhena antidysenterica (Linnaeus) Wallich [Apocynaceae]; *Kurci*; *RUC/MLD-35*	Bark	The bark along with roots of *Hemidesmus indicus* are crushed to make paste and applied externally to treat skin disorders *viz.* eczema, puses etc.
Anorexia	***Carissa carandas*** J. Loureiro [Apocynaceae]; *Karamcha*; *RUC/MLD-475*	Fruit	The ripe fruit is used to treat anorexia.
Dysentery	***Tinospora sinensis*** (Loureiro) Merill [Menispermaceae]; *Goronch lata*; *RUC/MLD-120*	Stem	Extract of its stem along with bark of *Alstonia scholaris* is taken twice a day
	Mimosa pudica Linnaeus [Fabaceae]; *Lajjabati*; *RUC/MLD-307*	Root	The root and kalmegh leaves are crushed to make juice and used in dysentery
Vomiting	***Coriandrum sativum*** Linnaeus [Apiaceae]; *Dhane pata*; *RUC/MLD-365*	Whole plant	The whole plant along with zinger and black pepper is crushed to make juice and used against vomiting
	Houttuynia cordata C.P. Thunberg [Saururaceae]; *Ansta gaach*; *RUC/MLD-451*	Leaf	The leaves are crushed to make juice and taken to induce vomiting

to be effective on dysentery in Narsinghdi District of Bangladesh (Rahmatullah *et al* 2010) while the authors ascertained new treatment procedures in the studied area by local tribal people for the same purposes using the species, *Tinospora sinensis* (stem) and *Mimosa pudica* (leaf). Chakraborty & Bhattacharjee (2006) reported the use of *Curculigo orchioides* for treating leucorrhoea and nasal bleeding problem in Purulia district of West Bengal in India, however, our findings suggested enhancement of sperm production on consumption of its roots. Five plant species namely, *Musa x paradisiaca, Acacia nilotica, Bombax ceiba, Catharanthus roseus, Syzygium cumini* were found to be effective to treat diabetes in the study area while Biswas *et al* (2011) reported the use of *Acacia nilotica* as antidiabetic in

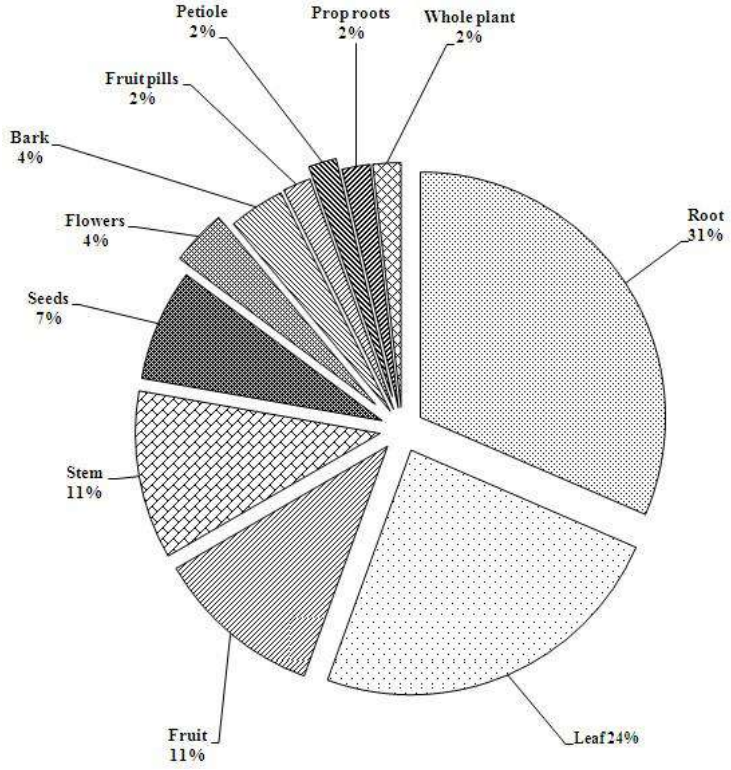

Fig. 2. Used plant parts in the preparation herbal remedies

Narail and Jessore Districts of Bangladesh supporting our result. However, Oyedemi *et al* (2009) accounted 15 plant species employed for the management of diabetes at entire Eastern Cape Province of South Africa. The study revealed that *Rauvolfia serpentina* and *Holarrhena antidysenterica* were exploited for the management of skin disorders whereas fruits of *Ficus glomerata* and roots of *Solanum xanthocarpum* were utilized to expel asthma (Table 1).

The study exhibited a high degree of ethnobotanical novelty and the use of plant resources for medicine by the aboriginal communities. In addition, the comparative studies on medicinal uses of plants among different regions showed similarities and dissimilarities in uses. In recent, the knowledge of medicinal plants or ethnobotany has gained worldwide attention due to its effectiveness, therapeutic and eco-friendly nature.

CONCLUSION

The present documentation is the first systematic study on local healthcare management of Chanchal block I and II as per authors' best knowledge. Despite the large scale environmental degradation and modernization, medicinal plants are still playing a significant role in the management of various human diseases in Malda District. The study was, therefore, intended to document the local knowledge of traditional healers treating different ailments in Chanchal block I and II. The study revealed that a total of 49 species had been utilized to treat 25 different diseases and disorders by local traditional healers. Root was the most frequently used plant part in the preparation of remedies followed by leaf. The common ailments treated in the area were bone fracture, diabetes, rheumatism, body pain, kidney stone, menstruation,

asthma, bronchitis, gastritis, skin disorders etc. A comprehensive phytochemical investigation of those studied plants would be a handy work for the invention of future eco-friendly drug. Besides, a serious awareness is needed be raised among the local people on sustainable utilization and management of the plant resources.

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REVIEW ARTICLE

Hepatotoxicity and the present herbal hepatoprotective scenario

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Most of the metabolic and physiological processes of our body as well as the detoxification of various drugs and xenobiotic chemicals occur in the liver. During this detoxification process, the reactive chemical intermediates damage the liver severely. There are several commercially available drugs, consumption of which results in idiosyncratic drug reaction mediated hepatotoxicity. Drug induced hepatotoxicity is a burning problem in this regard and several drugs are withdrawn from the market due to their hepatotoxic nature. Today, worldwide search of non-hepatotoxic drugs, especially potent hepatoprotective drugs have led towards the screening of numerous herbal products. Pharmaceutical companies and scientific communities have started to consider the therapeutic efficiency of the plant-based hepatoprotective remedies. Different herbs are mentioned in various ethnopharmacological practices possessing hepatoprotective capacities and around the globe, such herbs are still used by people to cure certain liver diseases. Therefore, we have documented the various aspects of hepatotoxicity and an overview on the current scenario of the hepatoprotective herbal remedies.

Key words: Ayurveda, drug induced hepatotoxicity, ethnopharmacology, hepatoprotective, hepatotoxicity, herbal, idiosyncratic drug reactions, liver

INTRODUCTION

Liver is the largest organ of the body, contributing about 2% of the total body weight in the average adult human. Liver is associated with most of the physiological processes, which include growth, immunity, nutrition, energy metabolism and reproduction. Synthesis and excretion of bile, albumin, prothrombin and production of the compliments, which is the major effector of the humoral branch of the immune system, occurs mainly in the liver. The detoxification of the harmful chemicals occurs in the liver, which in turn, results in various hepatic diseases. Other factors causing liver damage include chronic alcoholism, viral infections, hepatocarcinoma, etc., Drug induced hepatotoxicity (DIHT) resulting in liver damage has turned into a major medical concern in recent years. During DIHT, formation of pro-inflammatory cytokines and reactive free radicals from the hepatic neutrophils and Kupffer cells cause severe oxidative stress.[1]

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Use of herbal remedies as hepatoprotective therapy has been practised in various traditional medicinal systems. A wide variety of ethnomedicinal plants, which have been claimed to be hepatoprotective in the Ayurveda, Siddha, Unani and Amchi medicinal systems as well as in other traditional medicinal practices around the globe, are successfully tested *in vivo* for their therapeutic potential. Therefore, in this review work an effort has been made to highlight the phenomenon of DIHT and the present scenario of herbal drugs and supplements, which have shown promising signs in the amelioration of the hepatic system.

THE HEPATIC SYSTEM

The hepatic system performs hundreds of metabolic and physiological process either individually or in combination with other organs of the body. Lion's share of the protein, carbohydrate and lipid metabolism is performed by the liver. It synthesises various coagulation factors, insulin-like growth factor 1, anti-thrombin, thrombopoietin, angiotensinogen and albumin. Liver is also responsible for the catabolism of bilirubin and various hormones. One of the most important functions of liver is the detoxification of toxic substances. The detoxification process itself very often damages the hepatic system. Though liver possess tremendous regenerative capacity but toxic chemicals and their metabolic intermediates often cause subclinical liver injury.

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HEPATOTOXICITY

Liver being closely associated with the gastrointestinal system receives much of the blood from the portal veins, which drains the xenobiotic compounds to the liver. In the liver, the xenobiotic compounds get activated and forms reactive metabolic species (RMS). The RMS through the oxidative stress pathway damage cellular biomolecules, cause protein dysfunctions and damage to the nucleic acids.[2] Mitochondrial dysfunction results due to RMS mediated disruption of ionic gradients and intracellular Ca^{2+} storage, causing tissue injury. Hepatocellular inflammation is another outcome of DIHT. Activated natural killer T (NKT) cells and Kupffer cells secret inflammatory mediators such as tumour necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-1β also promotes tissue damage.[3]

HEPATOTOXICITY CLASSIFICATION

Hepatotoxicity in general can be coined in the following three cases[4]: (i) the level alanine amino transferase (ALT), that is glutamyl oxalacetic acid transaminase level in the serum increases three-fold, (ii) serum alkaline phosphatase (ALP) level increases two-fold and (iii) serum bilirubin (SBLN) level is also elevated two-fold (when serum ALT and ALP levels also increases).

Hepatotoxicity is of three major classes: (a) Hepatocellular injury: When serum ALT or ALP levels are elevated; (b) Cholestatic injury: When ALP and bilirubin levels in the serum increases; (c) Mixed injury: When both the ALT and ALP levels in the serum increases.[4]

ROLE OF AYURVEDA IN HEPATOPROTECTION

The Indian Ayurvedic medicinal system dates back to the mid-second millennium BCE describing the usage of various plants based products for the ailment of numerous diseases. About 77 herbal formulations are found in Ayurveda having hepatoprotective properties,[5] among which many of them have been tested for their hepatoprotective capacity [Table 1]. Many Indian ancient medicinal texts emphasise on the hepatoprotective capacity of certain plants such as Kalmegh, (*Andrographis paniculata*), Bhuia Amla (*Phyllanthus niruri*), Indian bearberry (*Berberis aristata*), Turmeric (*Curcuma longa*), Kutki (*Picrorhiza kurroa*), Mulethi (*Glycyrrhiza glabra*), Punarnava (*Boerhavia diffusa*), Tulsi (*Ocimum sanctum*), Chicory (*Cichorium intybus*), Bhringa Raja (*Eclipta alba*), Kanak champa (*Pterospermum acerifolium*), Guduchi (*Tinispora cordifolia*), Chirayata (*Swertia chirata*), etc.

Table 1: List of some important hepatoprotective medicinal plants mentioned in Ayurveda

Family	Name of plant	Parts used in Ayurveda
Acanthaceae	*Andrographic paniculata* (Burm.f.) Wall. ex Nees	Whole plant
	Asteracantha longifolia Nees.	Leaf, root and seed
	Hygrophila spinosa T. Ander.	Leaf, root, stem and seed
Asclepiadaceae	*Hemidesmus indicus* R.Br.	Root
	Gymnema sylvestre (Retz.) R.Br.ex Schult	Leaf
	Cosmpstigma racemosa Roxb.	Root and bark
Asteracceae	*Taraxacum officinale* F.H. Wigg	Root
	Pyrenthrum indicum DC.	Flower
	Cichorium intybus L.	Whole plant
	Achille millefolium L.	Whole plant
Berberidaceae	*Berberis lycium* Royle	Leaf
Cucurbitaceae	*Bryonia alba* Wild Hops	Root
	Luffa echinata Roxb.	Fruit and seed
Euphorbiaceae	*Euphorbia neriifolia* L.	Fruit
	Phyllanthus niruri L.	Whole plant
Fumariaceae	*Fumaria officinalis* L.	Whole plant
	Fumaria parviflora Lamarck.	Whole plant
Guttiferae	*Garcinia indica* (Linn.) Robs.	Fruit
Gentianaceae	*Swertia chirata* (Wall.) C. B. Clarke	Whole plant
	Gentiana kurroo Royle	Root
Labiatae	*Mentha longifolia* (L.) Huds.	Leaf
	Hyssopus officinalis L.	Whole plant
Leguminosae	*Trigonella foenumgraececum* Linn.	Seed
	Canavalia ensiformis (L.) DC.	Root
Moraceae	*Ficus carica* L.	Fruit
	Ficus heterophylla L. f.	Root
Meliaceae	*Aphanamixis polystachya* (Wall.) R.N. Parker	Bark
Myristicaeae	*Myristica fragrans* Houtt.	Seed
Menispermaceae	*Tinospora cordifolia* (Willd.) Hook. f.	Stem
Nymphaceae	*Nelumbo nucifera* Gaertn.	Flower
Pinaceae	*Pinus roxburghii* Sarg.	Oil
Polygonaceae	*Rumex crispus* L.	Root
Rosaceae	*Prunus armeniaca* L.	Fruit
Ranunculaceae	*Paeonia emodi* Wall. ex Royle	Tuber
	Delphinium zalil L.	Whole plant
Rubiaceae	*Hedyotis corymbosa* (L.) Lam.	Leaf
Solanceae	*Lycopersicon esculentum* L.	Fruit
Umbelliferae	*Apium graveolens* L.	Seed
Verbenaceae	*Vitex negundo* L.	Whole plant
Zingiberaceae	*Zingiber officinale* Roxb.	Rhizome

At the Rajiv Gandhi Post Graduate Government Ayurvedic Hospital, Paprola, India, a clinical trial was performed, with success, to evaluate the acclaimed hepatoprotective activity of Daruharidra (*Berberis aristata*), Kamachi

(*Solanum nigrum*), Ghrita kumara (*Aloe vera*) and Bhumyamalaki (*Phyllanthus fraternus*).[6] Presently there are around 89 Ayurvedic formulations used by 37 Indian pharmaceutical companies to prepare hepatoprotective medicines.[5] The Indian Medicinal Practitioner's Co-operative Pharmacy and Stores have approved some of the drug formulations form the Ayurvedic, Siddha and Unani medicinal system[7] and they have been listed in Table 2.

PLANTS AS HEPATOPROTECTIVE RESOURCE AND THEIR RECENT PERSPECTIVES

Use of herbal medicine for the treatment of various ailments dated back to thousands of years. Out of the 250,000 higher plant species, about 70,000 species have been used in different traditional medicinal formulations.[8] Plants have emerged as a great source of pharmaceutical products. In China, the plant-based pharmaceutical industry accounts for one-third of the entire pharmaceutical business and in Malaysia, the annual market of herbal medicine is around 1 billion Malaysian ringgit. In United States alone, the estimated plant-derived pharmaceutical market is of US$9 billion per year.[9] There has been increasing scientific and industrial interest in ethnobotanical medicine during the past few decades and thus, the global market of herbal medicine is expected to increase from $19.5 billion in 2008 to $32.9 billion in 2013 with an annual growth rate of 11%.[10] Very often the synthetic drugs and antibiotics are associated with adverse effects, which include hypersensitivity, immunosuppression and allergic reactions. With the emerging cases of hepatotoxicity, antibiotic resistance in bacteria and various side effects, there is a constant need for new and effective therapeutic agents. Thus, the plant-derived bioactive chemicals have drawn the main attention as a source of complementary and alternative medicine. The use of alternative medicines for the treatment of liver diseases has a long history and medicinal plants and their derivatives are extensively used around the globe for this purpose. Huge interest of the scientific and pharmaceutical community over the therapeutic use of plant-based materials used in various ethnobotanical practices have led to purification and characterisation of various bioactive compounds, which have proven to be hepatoprotective.

Table 2: A few herbal formulations approved by indian medicinal practitioner's co-operative pharmacy and stores

Ayurvedic	Siddha	Unani
Bhringarajasava	Arumuga chendooram	Jawarish-e-Amilasada
Chandraprabhavati	Annabedhi chendooram 1 and 2	Jawarish-e-Amila luluvi
Drakahadi rasayam	Ayakantha chendooram	Jawarish-e-Tabashir
Guduchi satwam	Mandooradi kudineer	Kurs-e-gul
Jambeeradi panakam	Ayabringaraja karpam	Rue-e-amila
Panchatiktakwatha churnam	Karisalai lehyam	Sherbeth-e-anarshreen
Dhathri loham	Kantha chendooram	Sherbeth-e-deenar
Tapyadi loham	Loha mandooram	Muffarah-e-Ahmedi
Pipilyadi loham	-	Gul-e-Nilofer
Saptamiruda loham	-	Bhoi-Amla

Herbal formulations have often been found to work better in a synergistic manner than working alone. One of such formulations is LIV 52, which is a mixture of extracts from *Capparis spinosa, Cichorium intybus, Solanum nigrum, Cassia occidentalis, Terminalia arjuna, Achillea millefolium, Tamarix gallica, Eclipta alba, Phyllanthus niruri, Berberis aristata, Taphanus sativus, Phyllanthus emblica, Plumbaga zeylanica, Boerhavia diffusa, Tinospora cordifolia, Embelia ribes, Terminalia chebula* and *Fumaria officianlis*.[11] One of the earliest mentions of the much acclaimed hepatoprotective plant Milk Thistle (*Silybum marianum*) is found in the Bible (Genesis 3:18) when God tells Adam and Eve, 'thorns also and thistles shall it bring forth to thee'. Later on, silymarin, a mixture of isomeric flavolignans-silybin, silydianin and silychristen was isolated from the plant, which has proven to be beneficial in liver related disorders. Similar plants [Table 3] acclaimed in various traditional medicinal practices are now being screened for their hepatoprotective efficiencies.

HEPATOPROTECTIVE PURE ISOLATED COMPOUNDS FROM PLANT SOURCE

Recent trends in the study of hepatoprotective herbal source have turned towards isolation and purification of pure compounds from plants and assessment of their hepatoprotective activity [Table 4]:

Silymarin from *Silybum marianum,* has been a standard hepatoprotective agent for numerous studies and accounts for 180 million US dollars business in Germany alone.[86] Various bioactive compounds from plant sources possessing antioxidant, anti-cancer, immunostimulatory effect are also being tested for their possible hepatoprotective potential. A wide variety of flavonoids such as quercetin (*Helichrysum arenarium*), myricitoside C (*Cercis siliquastrum*), stachyrin (*Stachys recta*), eupatolin (*Artemisia capillaris*); alkaloids such as atropine (*Datura metel*), pilocarpine (*Aristoclochia clementis*), berberine (*Beriberis vulgaris*); organic acids and lipids such as glycolic acid (*Cynara scolymus*), dihydrocholic acid (*Curcuma longa*) have shown potent anti-hepatotoxic activity.[87]

HERBAL HEPATOTOXICITY

Not all herbs are harmless. Many cases of herbal hepatotoxicity have been reported in recent years, which include confirmations of direct hepatic fibrosis, portal

Table 3: A concise list of hepatoprotective plants

Family	Name of plant	Part	Solvent	Important parameters studied	Reference
Acanthaceae	*Thunbergia laurifolia* Linn.	Leaf	Ethanol	*In vitro, in vivo* studies; ALT, AST, TGLY, ALP, SBLN, HS, MTT	12
	Hygrophila auriculata Schumach.	Seed	Petroleum ether and methanol	*In vivo* studies; SGOT, SGPT, ALP, SD, GD, SBLN, HS	13
Asclepiadaceae	*Decalepis hamiltonii* wight. and Arn.	Root	Water	LP, PC, SOD, CAT, GPX, GR, LG, HS	14
	Tylophora indica (Burm.f.) Merr.	Leaf	Water	*in vivo* studies; AST, ALT, ALP, TB, LDH, GR, SOD, CAT, GPX, GST, LP, HS	15
Asteraceae	*Chamomile capitula*	Capitula	Hydro-ethanolic	*In vivo* studies; LG, NKA, LG, TBARS, ALP, AST, ALT, TP, SBLN, GL	16
	Artemisia absinthium Linn.	Aerial part	Aqueous	*In vivo* studies; AST, ALT, TNF-α, IL-1, SOD, GPX, MDA, HS	17
	Epaltes divaricata (L.) Cass.	Whole plant	Aqueous	*In vivo* studies; ALT, AST, ALP, LG, HS	18
Amaranthaceae	*Amaranthus spinosus* L.	Whole plant	Petroleum ether	*In vivo* studies; AST, ALT, ALP, SBLN, MDA, GSH, SOD, CAT, HS	19
	Aerva lanata Juss. Ex Schult.	Whole plant	Hydroalcoholic	*in vivo* studies; AST, ALT, ALP, SBLN	20
Apiaceae	*Apium graveolens* L.	Seed	Petroleum ether, acetone and methanol	*in vivo* studies; SGOT, SGPT, ALP, TP, TA	21
Apocynaceae	*Nerium indicum* Mill.	Flower	Methanolic extract	*in vivo* studies; AST, ALT, ALP, SBLN, SOD, MDA, HS	22
Asparagaceae	*Asparagus racemosus* Willd.	Root	Ethanol	*in vivo* studies; AST, ALT, ALP, SBLN, SOD, CAT, HS	23
	Asparagus racemosus Willd.	Whole plant	Crude extract and aqueous fraction	*in vivo*; LP, PO, TBARS	24
Bignoniaceae	*Kigelia africana* (Lam.) Benth.	Leaves	Aqueous	*in vivo* studies; ALT, AST, TBARS, SOD, CAT, GPX, DAD	25
Bixaceae	*Bixa orellana* L.	Whole plant	Methanolic extract	*in vivo* studies; SGPT, SGOT, CLT, TG, HS	26
Coclospermaceae	*Cochlospermum planchoni* Hook.f.	Rhizomes	Aqueous	*in vivo* studies; SBLN, ALP, ALP	27
Cucurbitaceae	*Momordica dioica* Roxb. ex Willd.	Leaves	Ethanolic and aqueous	*in vivo* studies; AST, ALT, ALP, SBLN, HS	28
	Trichosanthes cucumerina L.	Whole plant	Methanol	*in vivo* studies; ALT, AST, ALP, SBLN, TP, TA, LG, MDA, HS	29
Capparidaceae	*Capparis spinosa* Linn.	Root	Ethanolic	*in vivo* studies; ALT, AST, HS	30
Casuarinaceae	*Casuarina equisetifolia* L.	Whole plant	Methanolic extract	*in vivo* studies; SGPT, SGOT, CLT, TG, HS	25
Dryopteridaceae	*Arachniodes exilis* (Hance) Ching	Rhizomes	Ethanol	*in vivo* studies; SGOT, SGPT, MDA, SOD, LP	31
Euphorbiaceae	*Baliospermum montanum* Blume	Root	Alcohol and chloroform extract	*in vivo* studies; SGPT, SGOT, ALP, HS	32
	Croton oblongifolius Roxb.	Aerial part	Petroleum ether, acetone and methanol	*in vivo* studies; SGOT, SGPT, ALP, TP, TA	33
	Mallotus japonicas (L.f.) Müll. Arg.	Cortex	Aqueous	*in vivo*; AST, ALT, SD, GST, GR, GGT, MDA	34
	Phyllanthus niruri L.	Leaves and fruits	Methanol and Aqueous	*in vivo*; LP, SGOT, SGPT	35
Fabaceae	*Cajanus cajan* (L.) Millsp.	Whole plant	Methanolic	*in vivo* studies; SGPT, SGOT, AST, ALT, CLT, TG, HS	25
	Tephrosia purpurea (Linn.) Pers.	Aerial part	Aqueous-ethanolic extract	*in vivo*; AST, ALT, SBLN, ALP, LG, GGT, MDA, HS	36
	Cassia fistula L.	Leaf	N-heptane	*in vivo*; SGOT, SGPT, SBLN, ALP	37
	Cassia fistula L.	Seeds	Methanol	*in vivo*; SGOT, SGPT, ALP, SBLN	38
	Acacia confusa Merr.	Bark	Hydroalcoholic	*in vivo*; AST, ALT, MDA, CP450, SOD, GPX, HS	39
Gentianaceae	*Gentiana olivieri* Griseb.	Ariel part	Ethanol	*in vivo*; MDA, LG, AST, ALT	40
	Halenia elliptica D. Don	Whole plant	Methanol	*in vivo*; ALT, AST, ALP, SBLN, HS	41
Juncaceae	*Juncus subulatus* Forssk.	Tuber	Hydromethanolic	*in vivo*; AST, ALT, ALP, TP, TA, TGLY, MDA	42

Contd...

Table 3: Contd...

Family	Name of plant	Part	Solvent	Important parameters studied	Reference
Lamiaceae	*Orthosiphon stamineus* Benth	Leaves	Methanol extract	*in vivo*; AST, ALT, ALP, GST	43
	Hoslundia opposite Vahl	Stem	Methanol and ethyl acetate	*in vivo*; AST, ALT, ALP, SBLN	44
	Ocimum sanctum Linn	Leaf	Hydroalcoholic	*in vivo*; AST, ALT, ALP, SBLN, HS	45
Liliaceae	*Aloe barbadensis* Mill.	Aerial parts	Petroleum ether, chloroform and methanol	*in vivo*; SGOT, SGPT, ALP, SBLN, TGLY, LP, LG, G6P, and MAH, AND, HS	46
Malvaceae	*Hibiscus esculentus* Linn.	Root	ethanol	*in vivo*; SGPT, SGOT, ALP, SBLN, LP	47
	Hibiscus sabdariffa Linn.	Calyx	Aqueous	*in vivo*; ALT, AST, TBARS, SOD, CAT, GPX, DAD	25
Meliaceae	*Azadirachta indica* A. Juss.	Leaf	Hydromethanolic	*in vivo*; GPX, GST, SOD, CAT	48
Myrsinaceae	*Embelia ribes* Burm.f.	Fruit	Aqueous	*in vivo*; SGPT, SGOT, ALP, SBLN, HS	49
Nyctaginaceae	*Boerhaavia diffusa* Linn.	Root	Aqueous	*in vivo*; GOT, GPT, ACP, ALP, GD, SBLN	50
Nelumbonaceae	*Nelumbo nucifera* Gaertn.	Leaf	Ethanolic	*in vivo*; ALT, AST, ALP, GGT, SBLN, SOD, CAT, TBARS, LG	51
Piperaceae	*Piper chaba* Hunter	Fruit	Hydroacetone	*in vivo*; AST, ALT, MTT, TNF-α	52
	Piper longum Linn.	Fruits and roots	Milk extract	*in vivo*; SGOT, SGPT, ALP, SBLN	53
Pittosporaceae	*Pittosporum neelgherrense* Wt. and Arn.	Stem bark	Methanolic	*in vivo*; AST, ALT, MDA, SOD, GSH, HS	54
Ranunculaceae	*Nigella sativa* Linn.	Seeds	Aqueous-ethanolic extract	*In vitro*; GSH, ALT, AST	55
Rubiaceae	*Rubia cordifolia* L.	Roots	Aqueous extract	*In vivo*; SGOT, SGPT, GGT, ALP, HS	56
Rutaceae	*Aegle marmelos* (L.) Corr.	fruit pulp/ seeds	Aqueous extract	*In vivo*; AST, ALT, ALP, SBLN, HS	57
	Glycosmis pentaphylla Retz.	Whole plant	Methanolic extract	*In vivo*; SGPT, SGOT, AST, ALT, CLT, TG, HS	24
	Zanthoxylum armatum DC.	Bark	Ethanolic	*In vivo*; SGOT, SGPT, ALP, SBLN, TP, SOD, CAT, LG	58
Sterculiaceae	*Ptrospermum acerifolium* Linn.	Leaves	Ethanol	*In vivo*; SBLN, TP, SGOT, SGPT, ALP	59
Scrophulariaceae	*Scoparia dulcis* L.	Whole plant	Methanol, diethyl ether and petroleum ether	*In vivo*; AST, ALT, ALP, TP, GLY, LP, SOD, GR, SBLN, HS	60
	Picrorrhiza kurroa Royle ex Benth	Aqueous	Underground stem	*In vivo*; AST, ALT, LL, CLT, TGLY	61
Saururaceae	*Saururus chinensis* (Lour.) Baill.	Whole plant	Ethanol	*In vivo*; ALT, AST, MDA, SOD, TC, TGLY, CLT, TA, CE, HS	62
Salvadoracaeae	*Azima tetracantha* Lam.	Leaves	Ethanol	*In vivo*; SGOT, SGPT, SBLN, TP, ALP, TP, TA, CLT, HS	63
Umbelliferae	*Bupleurum kaoi* Liu	Roots	Ethanol	*In vivo* and *in vitro*; AST, ALT, MDA, SOD, GPX	64
Verbenaceae	*Clerodendrum inerme* (L.) Gaertn.	Leaves	Ethanol	*In vivo*; ALT, AST, ALP, TGLY, CLT	65
	Vitex trifolia L.	Leaves	Ethanolic and aqueous	*In vivo*; SBLN, TP, ALT, AST, ALP, HS	66
Vitaceae	*Vitis vinifera* Linn.	Leaves	Ethanolic, chloroform, n-butanol, water	*In vivo*; MDA, AST LT, GSH, HS	67

SGOT – Serum glutamyl oxalacetic acid transaminase; SGPT – Serum glutamyl pyruvate transaminase; SBLN – Serum bilirubin; GST – Glutathione-S-transferase; HS – Histopathological studies; GPX – Glutathione peroxidase; ALP – Alkaline phosphatase; PC – Protein carbonylation; SD – Sorbitol dehydrogenase; LP – Lipid peroxidation; GD – Glutamate dehydrogenase; SOD – Superoxide Dismutase; CAT – Catalase; AST – Aspartate amino transferase; ALT – Alanine amino transferase; LG – Liver Glutathione; TP – Total Protein; TA – Total albumin; MDA – Malondialdehyde; DAD – d-aminolevulinate dehydratase; CLT – Cholesterol; TGLY – Triglycerides; GGT – γ-glutamyl transferase; TBARS – Thiobarbaturic reacting substrate; MTT assay – MTT; GR – Glutathione reductase; NKA – Na+ K+- ATPase activity; GL – Glycogen level; TG – Total glucose; PO – Protein oxidation; MAH – microsomal aniline hydroxylase; G6P – Glucose-6-phosphatase; AND – Amidopyrine N-demethylase; CP450 – Cytochrome P450; TNF -α – Tumor Necrosis factor alpha; LL – Lipoprotein level; CE – Collagen estimation

inflammation, cholestasis, chronic hepatitis, hepatic veno-occlusive and focal hepatic necrosis. Around 60 herbal formulations have been identified as hepatotoxic in 185 recent publications[88] and the United States Drug-Induced Liver Injury Network have recognised 10% of all DIHT to be associated with intake of herbal supplements. In countries like India and China, the main problem with herbal hepatotoxicity is that the vast traditional knowledge of both the countries leads people to use herbal supplements without any prior clinical trials, which sometime turns to

Table 4: A concise list of hepatoprotective active compounds

Name of compound	Source	Family	Chemical class	Reference
α-Amyrin and β-Amyrin	*Protium heptaphyllum*	Burseraceae	Triterpene	68
Anastatin A and Anastatin B	*Anastatica hierochuntica*	Brassicaceae	Flavonoid	69
Genistein, Orobol and 5,7,4'-trihydroxy-3'-methoxyisoflavone	*Erycibe expansa*	Leguminosae	Isoflavone	70
γ-Amyrone, γ-Amyrin, 18β-hydroperoxy-olean. 12-en-3-one and 3-epi-γ-amyrin	*Sedum sarmentosum*	Crassulaceae	Triterpene	71
Rutin	*Artemisia scoparia*	Asteraceae	Flavonoid	72
Rubiadin	*Rubia cordifolia*	Rubiaceae	Anthraquinone	56
Myristin	*Myristica fragrans*	Myristicaceae	Cetyl ester	73
Naringenin and wighteone	*Cudrania cochinchinensis*	Moraceae	Flavonoid	74
Kaempferol and salidroside	*Rhodiola sachalinensis*	Crassulaceae	Phenolic compound	75
Picroliv	*Picrorhiza kurroa*	Scrophulariaceae	Iridoid glycoside	76
Gentiopicroside and Sweroside	*Swertia japonica*	Gentianaceae	Iridoid	77
Tetrahydroswertianolin	*Swertia japonica*	Gentianaceae	Xanthione	77
Mangiferin	*Salacia reticulata*	Hippocrateacaea	Phenolic compound	78
Torilin and Torilolone	*Cnidium monnieri*	Apiaceae	Sesquiterpene	79
Acanthoic acid	*Acanthopanax koreanum*	Araliaceae	Diterpene	80
18β-glycyrrhetinic acid	*Glycyrrhiza uralensis*	Fabaceae	Glycyrrhetinic acid	81
Lithospermate B	*Salvia miltorhiza*	Lamiaceae	Caffeic acid	82
Corilagin	*Terminalia catappa*	Combretaceae	Tannin	83
Neoandrographolide	*Andrographis paniculata*	Acanthaceae	Diterpene	84
Scropolioside-A	*Scrophularia koelzii*	Scrophulariaceae	Iridoid glycoside	85

be deleterious for the liver. For instance, with an estimated $400 billion market of 7000 species of Chinese herbs, at least 10 species of them have been reported to be hepatotoxic. A case of severe hepatotoxicity was reported in a 64-year-old female caused by Indian ayurvedic herbal products, Bakuchi (*Psoralea corylifolia*), Khadin (*Acacia catechu*), Bramhi (*Eclipta alba*) and Usheer (*Vetiveria zizaniodis*).[88] A detail searchable database of numerous hepatotoxic herbal supplements is given by the National Institute of Health NIH[89] Some frequently reported herbs having hepatotoxic effects are germander (*Teucrium chamaedrys*), valerian (*Valeriana officinalis*), skullcap (*Scutellaria* sp.), comfrey (*Symphytum* sp.), chaparral (*Larrea tridentata*), mistletoe (*Viscum album*), kava kava (*Pipermet hysticum*), Chinese green tea (*Camellia sinensis*), greater celandine (*Chelidonium majus*), pennyroyal (*Menthapulegium*), margosa oil (*Azadirachza indica*), borage (*Borago officinalis*), gotu kola (*Centella asiatica*), broom corn (*Sorghum vulgare*), etc.[90] Toxic alkaloids from ackee fruit (*Blighia sapida*), *Callilepsis laureola*, *Erythroxylon coca*, *Cassia angustifolia*, *Borago officinalis* and *Sassafras albidum* also have been implicated in various hepatotoxic cases. Perhaps, the most inglorious of them all is Kava Kava, which is utilised as sedative throughout the south Pacific Polynesian cultures. A toxic alkaloid pipermethystine found in the stem and the leaves of Kava demonstrated positive signs of hepatotoxicity. Many commercial herbal products such Herbalife®, Enzyte® and Hydroxy cut® have also been reported to render toxic effects on the liver and therefore, the herbal formulations too must undergo proper clinical trials before commercialising.

CONCLUSION

Maintenance of a healthy liver is essential for the overall wellbeing of an individual. Detail clinical diagnostic criteria for assessment of hepatotoxicity has already been published.[88] The clinical research has confirmed hepatoprotective efficiency of plant-based traditional and alternative medicines and guided the pharmaceutical companies to formulate numerous hepatoprotective drugs. Today, the main problem with the herbal medicines is that many herbs are consumed as polyherbal formulations where multiple herbs work synergistically. The active component responsible for the disease treatment in most cases remains unknown. Therefore, very often some other components of the polyherbal formulations adversely affect the liver. The National Poison Information Service reports that, among 785 cases of adverse reactions by herbal drugs, hepatotoxicity was the most frequent phenomenon.[91] There are proper guidelines from World Health Organisation (WHO) for the assessment of hepatiotoxicity due to herbal products.[92] A regulated research policy to highlight the advantages of hepatoprotective herbal medicine with respect to their safety and efficacy could result in a better utilisation of these complementary systems of medicine. Thus, we may conclude with the words of Dr S.T. Han,[93] 'Herbal medicine holds great but still largely unexplored potential for the development of new drugs to combat major health problems' and thus, 'mechanisms for ensuring the safety and control of herbal medicine need to be introduced as part of its formal incorporation into the health service system'.[8]

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Review Article

AN OVERVIEW ON DRUG-INDUCED HEPATOTOXICITY

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ABSTRACT

Liver is a vital organ, contributing in most of the metabolic and physiological processes of our body. It plays principal role in detoxification of various drugs and xenobiotics. Though liver possess tremendous regenerative capacity, but metabolism of various chemicals severely damage the hepatic system. Drug induced hepatotoxicity (DIHT) is a major concern in this respect. The drugs we consume to treat various diseases, very often, their metabolic intermediates cause liver toxicity. The lion's share of the idiosyncratic drug reactions ultimately results either in liver transplantation or even death. DIHT is a major medical concern at present and several drugs have been withdrawn from the market due to their hepatotoxic phenotype. Therefore, considering the phenomenon of DIHT, we have documented various aspects of DIHT, also discussing about the mode of toxicity of the drugs.

Keywords: Drug-induced hepatotoxicity, Hepatoprotective, Hepatotoxicity, Idiosyncratic drug reactions, Liver,

INTRODUCTION

Liver performs central role in the transformation and metabolism of xenobiotic compounds, which in turn, results in various liver complications like ionic imbalance, formation of reactive metabolic species (RMS) causing oxidative stress, hindrance in signal transduction pathways, translational inhibition at multiple levels, Ca^{2+} shift and impairment of mitochondrial respiratory chain and β-oxidation. DIHT can lead to cholestasis, which in turn results in intrahepatic accumulation of toxic bile acids and excretion products, further promoting hepatic injury. Chronic hepatic diseases have emerged as major medical concern in recent days and hepatotoxicity due to drugs is among the major contributing factor in this regard [1]. In 2008, over 16,000 lives were lost in the UK due to liver diseases and 700 liver transplants are performed only in UK each year [2]. According to WHO report, 21,000 livers were transplanted in 2007 worldwide [3]. Therefore, safeguard of the hepatic system is of utmost importance from the physiological point of view.

Hepatotoxicity

In the context of DIHT, the words of Paracelsus, the father of toxicology that, "the dose makes the poison" seems to be most appropriate. There are more than 900 compounds marked as potent hepatotoxins [4]. Hepatotoxicity is one of the main reasons behind withdrawal of a drug from the market. 50% of all acute liver failures and 5% of all hospital admissions are associated with DIHT[4]. Liver transplantation is required and even death occurs as a results of idiosyncratic drug reactions in 75% of cases[5]. In the liver, bio-activation of these xenobiotics generates RMS, which reacts with cellular macromolecules leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress[6]. These RMS may further cause disruption of ionic gradients and intracellular Ca^{2+} storage resulting in mitochondrial dysfunction, which in turn releases harmful reactive oxygen species (ROS) causing tissue injury. Oxidative stress of hepatocytes may results in inflammation. ROS formed at the hepatocytes may stimulate activation of various immunoregulatory cells such as natural killer T (NKT) cells, Kupffer cells and natural killer (NK) cells. These cells further secret inflammatory cytokines such as tumour necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β which promotes tissue damage[7]. On the other hand, study on the knockout of immunoregulators such as prostaglandins, IL-10 and IL-6 has shown easy susceptibility to hepatotoxicity. Therefore, it may be the delicate balance between the detoxification machinery and the immune regulators which determines an individual's susceptibility and adaptation to hepatic injury. A model of the various events which occur during DIHT is shown in the figure1

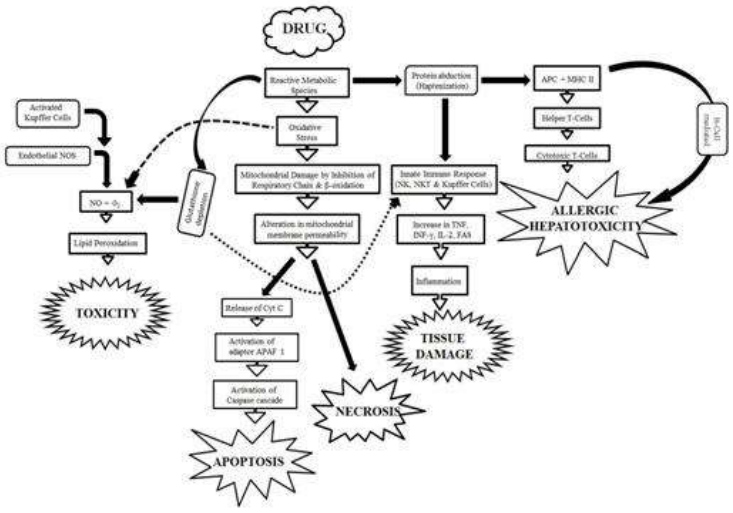

Figure 1: The figure displays the various events which occur during the DIHT. The reaction cascade starts with formation of reactive drug metabolic intermediate which can harm the hepatic system in various ways. By releasing the Cyt C, it may initiate the apoptotic pathway of it may induce hepatocellular necrosis by affecting the mitochondrial system. Activated Kupffer cells and endothelial nitric oxide synthase (NOS) may couple with the free oxygen radicals to subsequently form hepatic toxicity. Another arm of the downstream cascade leads to the activation of the immunoregulatory pathway, where it may cause direct inflammatory tissue damage by the innate immune response or it may results in T- cell mediated allergic hepatotoxicity.

Types of hepatotoxicity

Hepatotoxicity can be divided into three major categories and in general determined by three fold increases in alanine amino transferase (ALT) level i.e. glutamic oxaloacetic acid transaminase level, two fold increase in alkaline phosphatase (ALP) level and two fold increase in serum bilirubin (SBLN) level, if associated with increased ALT and ALP level[8]. Primarily elevation of ALT or ALP level is characterized as Hepatocellular injury; elevation of ALP and bilirubin level as Cholestatic and in mixed injury both ALT and ALP level increase[8]. **Table 1** show a list of various drugs associated with hepatotoxicity:

Table 1: Various drugs associated with various modes of hepatotoxicity

Hepatocellular Injury	Cholestatic Injury	Mixed Injury
Acetaminophen	Amoxicillin	Amitriptyline
Allopurinol	Anabolic steroids	Azathioprine
Amiodarone	Chlorpromazine	Captopril
Baclofen	Oral contraceptives	Clindamycin
Fluoxetine	Erythromycins	Cyproheptadine
Isoniazid	Estrogens	Enalapril
Ketoconazole	Mirtazapine	Nitrofurantoin
Lisinopril	Phenothiazines	Phenobarbital
Losartan	Terbinafine	Phenytoin
Methotrexate	Tricyclics	Sulfonamides
Omeprazole		Trazodone
Paroxetine		Verapamil
Pyrazinamide		
Rifampin		
Risperidone		
Tetracyclines		
Trovafloxacin		
Valproic acid		

Pathological Manifestations of DIHT

The mode of action of various drugs on the hepatic system is not the same. The following are the pathological manifestations of various hepatotoxic drugs on the liver[9]. Such as: Acute viral hepatitis (INH, halothane, diclofenac, troglitazone); Chronic hepatocellular injury (pemoline, methyldopa); Steatohepatitis (amiodarone, nifedipine, didanosine, synthetic estrogens); Massive necrosis (acetaminophen, halothane, diclofenac); Mononucleosis like condition (phenytoin, dapsone, sulphonamides); Seratosis (alcohol, methotrexate, TPN, nifedipine, corticosteroids, minocycline, tetracycline, valproic acid, piroxicam); Hepatocellular carcinoma (aflatoxin, arsenic, vinyl chloride, anabolic steroids); Cholestasis (amoxicillin-clavulanic acid, sulindac, erythromycin, tetracycline, chlorpromazine, sulfamethoxazole-trimethoprim, ibuprofen); Granulomatous hepatitis (tetracycline, chlorpromazine, sulfamethoxazole-trimethoprim, ibuprofen).

Acetaminophen and Hepatotoxicity

One of the highly studied cases of DIHT is of acetaminophen (also known as paracetamol), which accounts for worldwide highest number of drug-induced liver disease. Approximately 1000 cases of acute liver failure in United States are directly associated with DIHT, among which around 400 cases are due to acetaminophen toxicity and 130 cases due to idiosyncratic drug reactions[9]. Within the prescribed dose, though acetaminophen is well tolerated by the body, but overdose certainly results in acute liver damage. During the detoxification pathway, cytochrome P-450-2E1 reacts with acetaminophen and generates reactive metabolic intermediate N-acetyl-p-benzoquinone imine (NAPQI), which cause direct hepatotoxicity[10].

Standard hepatotoxic drugs and DIHT

Primarily due to hepatotoxicity, drugs such as nefazodone, trovafloxacin, troglitazone, ximelagatran, ebrotidine, pemoline were withdrawn from the market or given warnings [11]. DIHT has now emerged as the leading cause behind acute liver failure among the US patients. More than 10% of cases of acute liver failure are caused by idiosyncratic mediated hepatotoxicity[11].

Anti-tubercular drugs such as rifampicin, isoniazid and pyrazinamide are potentially harmful drugs causing hepatotoxicity and it has been estimated that anti-tuberculosis related hepatotoxicity is reported in 28% cases[12]. A vast array of non-steroidal anti-inflammatory drugs (NSAID) such as amphenac, benoxaprofen, bromofenac, cinchophen, fenclozic acid, fluproquazone, glafanine, isoxepac, pirprofenac, phenylbutazone, sudoxicam etc were withdrawn from the market as they caused hepatotoxicity[13] due to immunological idiosyncrasy[14]. Numerous aesthetic agents such as halothane, isoflurane, enflurane,

desflurane, chloroform, nitrous oxide have been found to cause direct hepatotoxicity (hepatocellular necrosis), immune mediated hypersensitivity, interference with bilirubin metabolism and cause cholestasis[15]. Anti-retroviral drugs (ARVD) very often elevate the liver transaminases level and are found to be associated with hepatotoxicity[16]. ARVD can be of several types depending on their mode of action[4]: nucleoside analogues reverse transcriptase inhibitors (abacavir, didanosine, lamivudine, tenofovir), non-nucleoside analogues reverse transcriptase inhibitors (efavirenz, emtricitabine, nevirapine) and protease inhibitors (indinavir, nelfinavir, saquinavir). Anti-hyperlipidemic drugs such as atorvastatin, simvastatin, pravastatin, lovastatin, gemfibrozil also displays hepatotoxic behaviour and their mode of action includes oxidative stress, immune-mediated inflammation and tissue damage, impairment of mitochondrial P450 system and bile acid transport[4]. Carbamazepine, felbamate, valproic acid and phenytoin belong to a huge family anti-epileptic drugs which are transformed to some reactive metabolic intermediates resulting in direct hepatic toxicity[17]. Similar cases of DIHT can be found for many other class of drugs such as anti-psychotic drugs, anti-Hypertensive Drugs, acetylcholine esterase inhibitors, anti-depressants, antibiotics etc.

Case study

Troglitazone (rezulin) was commercialised as antidiabetic agent in 1997, but 90 reports of hepatotoxicity over a period of merely 3 years made FDA place a ban on the drug[18]. In the same year, a nonsteroidal anti-inflamatory drug bromfenac (duract) was introduces to the orthopaedic patients as short-term analgesic drug, overdose of which resulted in over 50 cases of severe hepatic injury and subsequently withdrawal of the drug in the next year. Another potent hepatotoxic drug pemoline (cylert) was approved by the FDA in 1975for narcolepsy and attention deflect disorders. Later it was withdrawn by all the manufacturing companies in 2005 after 21 confirmed cases of liver failure induced by pemoline. Ximelagatran (exanta) was initially tested as anticoagulant but alter on during the clinical trials it was detected that ximelagatran uptake elevates the liver enzymatic levels and cause hepatotoxicity. Thus, in 2004, FDA rejected all the applications for the commercialization of ximelagatran. Ticrynafen (tienilic acid) was approved by FAD in 1979 as anti- hypertension drug, but after several reports of liver failure and association of ticrynafen with hepatitis resulted in its withdrawal in 1982. An anxiolytic drug alpidem (ananxyl) was withdrawn in 1995 due to the same reason[20]. Other drugs that have limited usage due to similar hepatotoxic fingerprints are trovafloxacin (trovan), an antibiotic; tolcapone (tasmar), used to treat Parkinson disease; zileuton (zyflo), for asthma; felbamate (felbatol), used for partial seizures; isoniazid, an anti-tubercular drug; dantrium (dantrolene), used for malignant hyperthermia; normodyne (labetalol), used in high blood-pressure etc. Interferon β-1a (also known as rebif, avonex and cinnovex) is used in the treatment of multiple sclerosis and has been reported to cause severe liver injury, which even results in total liver failure[21]. The risk of hepatic injury multiplies several folds when interferon β-1a is administered in combination with alcoholic products. Thirty two cases of severe liver injury was reported in 2009 by FDA, all of which were propylthiouracil mediated. For safety precautions various pharmaceutical companies are now including warnings stating that certain drug might cause hepatotoxicity. Such as, the package insert of an anti-depressant drug duloxetine mentions that, "cymbalta (duloxetine) should not be administered to patients with substantial alcohol use or any hepatic insufficiency"[9].

Risk factors Associated With DIHT

Statistical analysis of the cases of DIHT patients show that there are several underlying risk factors associated, with whom toxic effects of various drugs can be correlated. Alcoholism cause depletion of glutathione and cirrhotic changes in the liver, which alters the drug metabolism pathway. Age of a person may also play a profound role in susceptibility to hepatotoxicity, like it has been observed that elderly persons are at increased risk of DIHT because of their age related reduced hepatic blood flow and hepatic volume, variation in drug binding and decrease in clearance mechanism. Genetic factors may also play profound role in the susceptibility to DIHT such as in the case of an antiarrhythmic drug debrisoquine, which gets poorly metabolised due to abnormal expression in cytochrome P-450-II-D6 gene. The following are some risk factors associated with DIHT[9]: male (Amoxicillin-clavulanic acid); female (Halothane, Nitrofurantoin, Sulindac); young age (salicylates, valproic acid); old age (halothane, acetaminophen, amoxicillin-clavulanic acid); obesity (halothane); renal failure (tetracycline, allopurinol); Diabetes mellitus (methotrexate, niacin); hepatitis C (Ibuprofen, flutamide, ritonavir); Malnutrition (acetaminophen); AIDS (dapsone, trimethoprim-sulfamethoxazole).

CONCLUSION

Drugs are for the treatment of diseases, but it's utility turns in vain if it shows toxic phenotype. There are several commercial drugs possessing hepatotoxic character. Therefore, the pharmaceutical companies now have turned their attention towards the herbal formulations for their therapeutic and hepatoprotective capacity. Plants such as turmeric[22] have shown potent hepatoprotective capacity against various hepatotoxic models. In most cases, the phytochemical content[23,24] of these plants play the major role in their hepatoprotective activity. Numerous plants have been screened for their possible hepatoprotective capacity[25]. Plants possessing potent antioxidant capacities such as *Nerium indicum*[26,27] have been tested for their hepatoprotective status as antioxidant capacity is a major determinant of hepatoprotective behaviour. With the ever increasing cases of DIHT, we can hope for better and reliable complementary and alternative medicines in the future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Biology of useful plants and microbes
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Chapter 10

Ethnobotanically important plants of Malda and Uttar Dinajpur - a review

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Abstract

At the beginning of human civilization, knowledge of useful plants has been transmitted from generation to generation through visual memory and it has been an asset to every early civilization. In India such tradition can be traced back to 5000 B.C. The indigenous ethnic communities practice their own traditional treatment systems in their daily life to cure from different ailments. The ongoing growing recognition of local medicinal plants is due to less side effect and low cost. The medicinal properties of plants could be based on the antioxidant, antimicrobial and antipyretic effects of the phytochemicals present in plants. The Medical science has proved that the medicinal plants contain large amounts of antioxidants such as polyphenols, vitamin C, vitamin E, selenium, β-carotene etc., which is responsible for curing several diseases. So, medicinal plants would be the best source to obtain a variety of herbal drugs. Therefore, medicinal plants should be reviewed in every aspect for better understanding of their properties, safety and parallel efficacy. The present review deals with medicinal plants found in both two districts, based on the intensive survey as well as from various literatures. Such investigation will definitely contribute for better understanding of the plants and subsequently conservation and commercial strategy may be adopted as a whole.

Keywords

Ethnobotany, Malda, Uttar Dinajpur, Medicinal plants, Phytochemicals, Antioxidant

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Introduction

The history of medicine is as old as the history of the planet. The human body is not merely a biological entity; the possession of a soul gives rise to psychological, spiritual and psychosomatic values. Man being a sum total of biological and spiritual existence is liable to disease pertaining to both spheres of life. Needless to say, his desire for a quick recovery has sent him on the quest for Medicine.

Greece, Rome, China and India are the pioneering contributors of ancient medicines. The great physician Hippocrates, known as the "Father of Medicine", developed the '*Hippocratic Oath*' which is still using today in Unani system of medicine of Geek. Later, this system was further developed by the wisdom of many Greek scholars and spreaded in different parts of the world, such as, Egypt, Syria, Iraq, Persia, India, China and Middle East and Far Eastern countries[1].

The ancient Roman medicine included a number of specializations such as internal medicine, ophthalmology and urology. The Romans were great believers in a healthy mind equaling a healthy body and favored the prevention of diseases over the cures of them. Many Greek medical ideas were adopted by the Romans and Greek medicine had a huge influence on Roman medicine[2].

However, ancient China medical treatments were very different than those in the West, and there is much less reliance on more conventional methods such as prescription medications. Massage, acupuncture and manipulation of body part were more commonly used forms of treatment, and so natural is really the way to go in Chinese medicine. The Chinese believed that the patient's body should be viewed as an organic whole rather than as individual parts. This is why it is the preferred choice of a large number of people around the world[3].

The beginning of medical science in India started at the age of Vedic literature which is familiar with '*osadhi*' i.e. plants with medicinal properties. As time progressed, the domain of osadhi expanded at the expense of 'bhesaj' and osadhi itself transcended plants. The discipline of Ayurveda was born as an affiliate of the *Atharvaveda*[4]. Ayurveda can be defined as an ancient Indian health care system, comprising both practice and theory and devoted to a systematized quest for a long, healthy, vigorous and happy life comprising two distinct traditions: botanical and the metallurgical[5].

'*Charaka Samhita'* is the first treatise of Ayurveda in India, written by Charaka. About the same time Sushruta, an eminent surgeon of Vedic era, also wrote '*Susruta-samhita*' with more emphasis on surgery in the 4th and the 5th century[6].

Besides Ayurveda, the Unani and Siddha system of medicine was also popular in India. The

knowledge of Unani medicine came to India through Arabian and Persian and got enriched with the medicine of the sub continent and became a part of Indian systems of medicine. This system achieved great efficacy in curing diseases. Actually, the Unani and Ayurvedic medicines are contemporary systems of 400 BC, one took birth in Greek and the other one in India. Their philosophies are different but ideas can be correlated[7]. The Siddha system of medicine is one of the oldest traditional treatment system generated from Dravidian culture and flourished in the period of Indus Valley. Believed to be more than 10,000 years old, the Siddha system of medicine is considered to be one of the most antiquated traditional medical systems where use of metals, minerals and chemical products were predominant. The Siddha literature was also written in Tamil and is mainly practiced in Tamil speaking regions of the country and abroad. However, Ayurveda was prevalent in the north, Siddha was flourishing in the southern part of India[8].

Recent Perspective

The plant-based traditional medicine system, play a very essential role in human health care. Chandel *et.al,*[9] reported that nearly about 70% of Indian population depends on herbal medicines for various ailments. The information about medicinal properties of plants came down traditionally generation after generation since immemorial. The medicinal plants not only provide access and affordable medicine to poor people, but also a source of foreign exchange for developing countries. Most of herbs, shrubs and plant parts are used to prevent several human diseases. Almost all the developing countries have greater value of traditional healing treatments in these days[10,11]. According to Sahai[12], there are two levels of medicine system in India, the first one is classical well documented systems of Ayurveda, Siddha, Unani and the other one is folk medicine of system which is informal and exists in communities and passed orally from generation to generation and this folk system has survived for thousands of years because of their common use with cultural believes.

WHO[13] reported that more than 80% of the world's population relies on traditional herbal medicine for their primary health care. In almost every Asian country, there is a vast indigenous knowledge of the use of medicinal plants. However, the biodiversity of medicinal and aromatic plants is yet to be studied thoroughly in many countries. A few countries including China, India and Sri Lanka have formulated legislation to conserve their natural resources of medicinal and aromatic plants whereas some countries have ceased the practice of wild collection[14].

Area of interest

Having rich in biodiversity and with a handful of several ethnic communities (Santal,

Rajbansi, Polia, Oraon, Mundas, Malpaharias etc.), Malda and Uttar Dinajpur districts have great potential to be explored. Therefore, the present review deals with the medicinal properties of common medicinal plants which are commonly practiced in these two districts (Fig. 1) of West Bengal in India. The District of Uttar Dinajpur lies between latitude 25°11' N to 26°49' N and longitude 87°49' E to 90°00' E occupying an area of 3142 Sq. Km enclosed by Bangladesh on the East, Bihar on the west, Darjeeling & Jalpaiguri District on the north and Malda District on the south[15] whereas Malda District is located between the latitude and longitude figures of 24°40'20"N to 25°32'08"N and 88°28'10"E to 87°45'50"E respectively and surrounded by Bangladesh and South Dinajpur in the east, Santal Parganas

Figure 1: Map of Malda and Uttar dinajpur districts.

of Jharkhand state in the west, Uttar Dinajpur in the north and Murshidabad in the south. The total geographical area of this district is 3455.66 Sq Km[16].

***Culture with flora and fauna in the studied area*:**

Uttar Dinajpur is basically an agro and horticulture based city. Bengali is the main language but few portion of district consist of Urdu, Bihari and Hindi speaking people at Islampur Sub-Division showing mixture of culture. On the bank of Kulik River, the 'Raiganj Bird Sanctuary' is a major attraction of this district, spreading over an area of 35 acre and inhabited by a wide array of bird species including migratory birds such as night herons, open bill stroks, egrets, little cormorant etc. from South Asian countries and coastal regions.

Malda district shows a different type of colorful and enjoyable culture. Folk dances like, Gambhira, Jatra, Charak dance, Santhal dance, Lathi dance are most popular in this district performed by various types of ethnic communities. Besides, there are so many places of interest, such as Historical place like Gour and Jagjibanpur, Adina Deer Park, Ramkeli mela, Pandua Masjid etc. The soil of the district is particularly suited to the growth of mulberry and mango, for the production of both of which Malda has become famous, though the district is mainly agriculture based.

Literature on ethnobotany:

Previous literature on ethnobotany proved a number of immense works had been done in India. The importance of medicinal plants of India was described by Chopra *et al.*[17]. The ethnobotanical uses of plants by tribal of Madhya Pradesh were studied by Jain[18] whereas Rai *et al*.[19] described the ethnobotanical use of some areas Sikkim and Darjeeling Himalaya. S.G. Joshi[20] wrote a handbook on medicinal plants, comprising a huge data on the use of plants of India. H. Lalramnghinghlova[21] studied the ethnobotanical use of plants of Mizoram where as medicinal plants of Mysore were studied by Kshirsagar and Singh[22]. The folk usage of medicinal plants of Rajasthan was studied by Katewa *et al.*[23] and Sharma and Kumar[24] whereas Kuru[25] did the same kind of work for Tamil Nadu.

The eastern part of India particularly West Bengal has a wide array of natural vegetation which starts from the mountain of the Himalayan hills of Darjeeling in north to the unique vegetation of the coastal areas of Sundarbans in south. The Gangetic plain deals with the lush evergreen natural vegetation. During the last few decades, several works had been done with the ethnobotanical information among the different districts of West Bengal. An extensive ethnobotacinal survey of Bankura district, W.B., had been done by various workers[26-28]. De[29] reported the use of plants from Purulia District of W.B. whereas Chaudhuri *et al*.,[30-32] reported the same kind of work for 24- parganas, Midnapore and

Jalpaiguri Districts of W.B. Further survey was carried out by Molla and Roy[33] in Jalpaiguri district. Bandyopadhyay and Mukherjee[34,35] reported the floristic diversity and ethno-botanical survey of Koch Bihar District. A few workers[36-40] reported on the floristic composition and ethnomedicinal use of plants for Uttar and Dakshin Dinajpur districts.

A few floristic work had been done by Sur *et al*.[37,38] and Acharya and Das[41] for Malda district. Later, Chowdhury and Das[42] reported wet-land vegetation and some ethnomedicinal uses of plants of Malda district. Therefore, Malda and Uttar Dinajpur districts have a great potentiality to be explored.

Ethnomedicinal use

According to Schultes[43], ethnobotany is "the study of the relationship which exists between people of primitive societies and their plant environment". Ethnobotany is a new field of research and if this field is investigated thoroughly and systematically, it will yield results of great value to the ethnologists, archaeologists, anthropologists, plant-geographers and pharmacologists etc. It has been realized all over the world that much valuable knowledge about the uses of plants including medicinal uses is still endemic among many tribal or rural human societies. The traditional system of medicine not only provides cure for a large number of general and chronic diseases but it also strengthens the inner body strength. According to a survey of World Health Organization[13] and Siddiqui[44], the practitioners of traditional system of medicine treat about 80% of patients in India, 85% in Burma and 90% in Bangladesh.

According to the workers[45-47], the Indian medicinal plants have been successfully used in management of various ailments like bronchial asthma, chronic fever, cold and cough, malaria, dysentery, convulsions, diabetes, diarrhea, arthritis, emetic syndrome, skin diseases, insect bite etc. and in treatment of gastric, hepatic, cardiovascular and immunological disorders. Therefore, now-a-days there is a trend to switch over to the old traditional healing system of medicine which is highly effective and has no adverse side effect. The present review also deals with some common medicinal plants having different values in Malda and Uttar Dinajpur Districts.

Indigenous knowledge

Indigenous knowledge is derived from interaction between people and their environment and is a characteristic feature of all cultures. Rural India, inhabited by a number of ethnic groups with their diverse cultural practices, heavily depends on traditional system of medicine as a part of their lifestyle. It can be said that the same plant, used by different ethnic communities of different region, may treat same or different ailments. But, each

ethnic community has their own traditional system of methods and uses plant species either singly or with other remedies to treat diseases. De[48] described the close association of human society with the plant life, since time immemorial and also described that, from the ancient time plants have been a primary source of medicine and have influenced culture, thought and economic activity human beings through the ages[49].

Herbarium resources

Herbarium sheets and field notes have also proved to be a good source of ethnobotanical data. These sheets have been used for various purposes including plant species identification by various workers in their study. The most outstanding example of this type of research is findings of 5,178 useful notes of drugs and food value from about 2.5 million plant specimens in Harvard University Herbarium by Dr. Altschul[50].

Medicinal plants in curing diseases

No plants could be treated as weed as because of every plant has its own story. The literature showed the ethnomedicinal use of plants along with the mode of preparation of medicine and doses. The Indian folk medicinal knowledge and ethnobotanical uses of plants had been described by Jain[51,52]. Singh and Pandey[53,54] reported the medicinal uses of plants by ethnic communities of Rajasthan, India.

Sini, *et al*.[55] reported that *Cassia occidentalis* and *Clitoria ternatea*, which are commonly found beside the roadside or railway tracks or at garden, used for general infection and root juice in headache and swelling respectively, while *Datura metel* is used in asthma, depression, motion sickness, analgesic and hallucinations. They also worked on the uses of several other medicinal plants. Mitra and Mukherjee[56] reported more than 70 plants having ethnobotanical value of West Dinajpur district, West Bengal.

Joshi[57] reported that *Acorus calamus*, (known as 'bach' in the study areas) normally found in the wet-lands, used to treat dysentery and cough while *Cuscuta reflexa* commonly called 'swarnalata' used to treat jaundice, cough and stomach disorders. Ghimire and Bastakoti[58] reported on the ethnobotanical uses of several medicinal plants. They reported that *Achyranthes aspera* cures fever, swelling and rheumatism while *Artemisia vulgaris* is used for scabies, gastric, headache.

Sesbania grandiflora, (known as 'bakphul' in those two districts) used in dysentry, fevers, headaches, rheumatism, small pox and stomatitis, anaemia, bronchitis, tumors and biliousness[59]. Goyal *et al*.[60] reported that *Asparagus racemosus*, (known as 'satamul' in those two districts) is used to cure dyspepsia, diarrhoea, inflammations, hyperdipsia, infertility and leucorrhoea whereas *Azadirachta indica* have been reported to be a good

anthelmintic and also used for cough and cold, stomach worms, remittent fever, eczema, diabetes, leprosy and several skin infections[61].

The Ayurvedic Pharmacopoeia of India[62] reported that *Gymnema sylvestre* is used as antidiabetic, antiviral, diuretic, antiallergic, hypoglycemic and hypolipidemic.

Medicinal Properties

The study of various workers[17,20,63-69] proved that different aromatic and medicinal plants are used to cure various ailments. According to Chattopadhyay and Khan[70], a large number of traditional preparations are effective over modern allopathic drugs. For example, herpes virus infections, which cannot be completely cured by the available anti-herpes drugs like nucleoside analogs but curable only by traditional preparations as mentioned by Chattopadhyay and Khan[70]. Recently, interest has been raised in many countries on the commercial extraction of medicine from plants that contribute to cure for major diseases like cancer, AIDS etc.

The medicinal plants contain large amounts of antioxidants, such as polyphenols, vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein, and other carotenoids, anthocyanin, coumarin lignans, catechins and isocatechins, which play important roles in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides[71]. Therefore, there is an increasing interest in searching antioxidants from natural origin to scavenge free radicals to prevent human body from oxidative stress produced by ROS (reactive oxygen species) and RNS (reactive nitrogen species). The free radicals can lead to oxidative damage to biomolecules and may cause disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular diseases, neurodegenerative diseases and premature aging[72]. Plant secondary metabolites such as flavonoids and terpenoids also play an important role in defense against free radicals[73]. Data from various studies indicate that medicinal plants contain a wide variety of natural antioxidants, such as phenolics, flavonoids, and tannins, which possess more potent antioxidant activity than common dietary plants.

Middleton *et al*.[74] reported that the compounds responsible for antioxidant activity can be isolated and used for prevention and treatment of free radical-related disorders whereas Aqil *et al*.[75], reported that various herbs and spices exhibit antioxidant activity, including *Ocimum sanctum*, *Piper cubeba* Linn., *Allium sativum* Linn., *Terminalia bellerica*, *Camellia sinensis* Linn., *Zingiber officinale* Roscoe etc. and also reported this antioxidant activity is due to the flavones, isoflavones and flavonoids.

Gautam[76] reported that, several plant sources, like curcumin, resveratrol, baicalein,

boswellic acid, betulinic acid, ursolic acid, and oleanolic acid which are plant in origin, are the possible drugs for the future, against inflammatory diseases. Amara, *et al.*[77] showed various edible plant extracts have promising anti-tumorigenic activity. Various anti-cancerous agents like vinblastine, vincristine, nevelbine, etoposide, teniposide, taxol, taxotere, topotecan, and irinotecan, derived from plant sources, currently being used or undergoing clinical trials[78].

Therefore, the interest has refocused on traditional medicine because of the high cost of modern drugs, time and expenditure which is necessary to bring a drug to market after appropriate clinical trials. Besides these, there are various types of serious side-effects of modern drugs due to their carcinogenicity. As a result, scientists are now taking an active interest in traditional medicinal preparations of indigenous peoples, which are mostly plant based, having naturally occurring antioxidants and simultaneously trying to replace synthetic antioxidants.

This review summarizes the ethnomedicinal uses of some common medicinal plants which are found in Malda and Uttar Dinajpur districts and their antioxidant activities. This review is comprehensive enough to be useful to find as well as assemble the medicinal properties, main chemical constituents and antioxidant activity of those plants.

Study of the antioxidant activity

Antioxidants are the substances that when present in body at low concentration compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substrate. They have also been of interest to biochemist and health professionals because they may help the body protect itself against the oxidative damage caused by reactive oxygen species (ROS) and diseases like cancer, aging etc. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers[79].

The present review deals with a list of medicinal plants, found in Malda and Uttar Dinajpur districts, which are being used as a source of medicine since long, based on information collected from various literatures dealing with herbs having antioxidant properties.

Several methods have been used by the different workers to measure the antioxidant activity of medicinal plants. From the previous literature, the most widely used methods are the DPPH methods, super oxide radical scavenging method, scavenging of hydrogen peroxide, hydroxyl radical scavenging assay and ABTS etc. For example, Sini *et al*.[55] determined free radical scavenging assay by DPPH method and found 84.23±0.004 % and 82.87±0.246% inhibition (absorbance at 515 nm) in case of *Cassia occidentalis* and *Clitoria ternatea*

respectively in respect of Ascorbic acid standard solution 96.540 ± 0.652 %. They also determined free radical scavenging assay by DPPH method and presence of other chemical content in plant extracts.

Rachh *et al*.[80] showed maximum antioxidant activity of *Gymnema sylvestre* (Retz.) was 57.10% at 100 µg/ml conc. among different concentration and IC_{50} value is 85.28 µg/ml. (absorbance at 517 nm) while Fazal *et al*.[81] showed the alkaloids and ash value of *Gymnema* are 1.33% and 7.90 % respectively whereas the antioxidant activity of *Curcuma longa* was determined by Sathisha *et al.*[82] with the help of DPPH method at IC_{50} value of 0.32±0.12 mg/mL while *Trigonella foenum-graecum* shows 0.81±0.21 mg/mL at IC_{50} more antioxidant activity (absorbance at 517 nm).

Phytoconstituents in plants

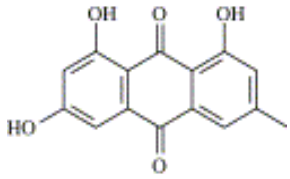
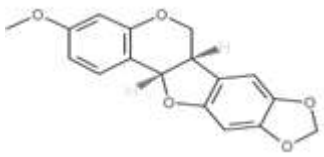
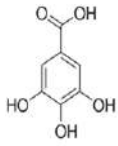
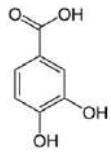
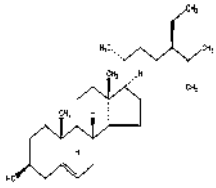
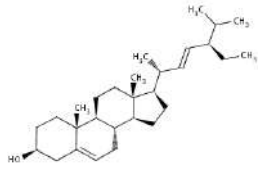
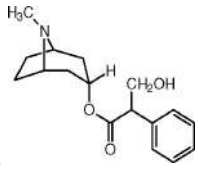
The active constituents in plants are the chemicals that have a medicinal effect on the body. Medicinal plants have been used for centuries as food, fooder, fertilizer and sources of chemicals for the pharmaceutical, food and chemical industries. These chemicals have been divided into several groups such as- alkaloids, flavonoids, phenols, saponins, coumarins, anthocyanins, essential oils etc. In this review, a few active constituents with structure of medicinal plants have been shown in Table 1.

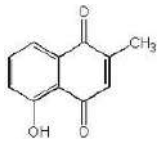
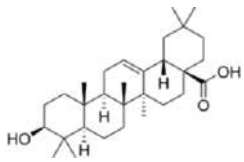
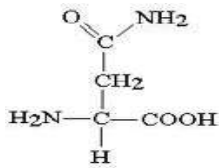
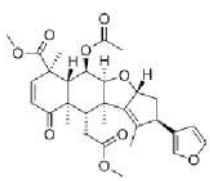
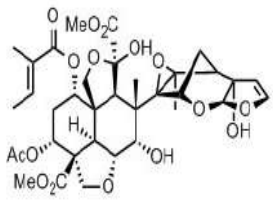
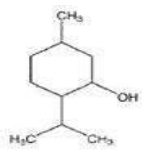
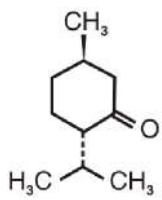
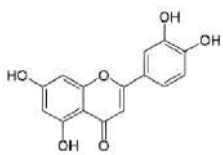
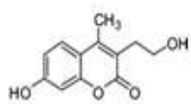
Conclusion

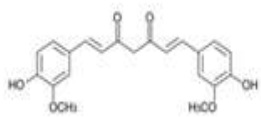
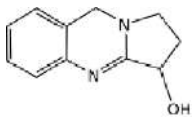
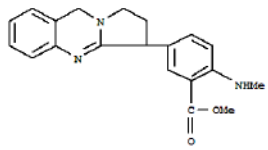
The aim of the present review was to develop a comprehensive and accurate list of some common plants, growing in Malda and Uttar Dinajpur districts that have identified as folk medical uses, and have antioxidant properties. The medicinal plants have occupied a distinct place in the human life right from the earlier era to till date and provide the information of the usage and management of various illnesses is due to their phytochemical constituents, which are naturally occurring in plants. There are so many evidences that indigenous antioxidants may be useful in preventing of oxidative stress in body and there is an increasing interest of natural antioxidants, found in medicinal plants, spices and in herbs. This knowledge of use of therapeutic plants has started to decline and become obsolete due to lack interest by younger generations as well as rapid socioeconomic, cultural changes, overgrazing, natural calamity and deforestation results lose of important medicinal plants from their habitats. Therefore, medicinal plants should be reviewed to better understand their properties, safety and efficacy.

Hence, the present review will play a significant role not only in documentation of this worthy knowledge of these districts but also for shorting out the plant species for conservation. So, proper documentation of this cultural heritage is urgently needed for the

Table- 1. Active constituents of medicinal plants

Plant Species	Active constituents (with structure)	References
Cassia occidentalis L.	 Emodin	83
Clitoria ternatea L.	 Pterocarpin	84
Acacia nilotica L.	 Gallic acid  Protocatechuic acid	85
Pergularia daemia (Forssk.) Chiov.	 ß- sitosterol  Stigmesterol	86
Datura metel L.	 Atropin	87

Plant Species	Active constituents (with structure)	References
Plumbago zeylanica L.	 Plumbagin	88
Achyranthes aspera L.	 Oleanic acid	89
Asparagus racemosus Willd.	 Aspargine	90
Azadirachta indica A. Juss	 Nimbidin  Azadirachtin	91
Mentha arvensis L.	 Menthol  Menthone	92
Ocimum sanctum L.	 Luteolin  Ocimarin	93

Plant Species	Active constituents (with structure)	References
Curcuma longa L.	 Curcumin	94
Justicia adhatoda L.	 Vasicine  Adhatodine	95

forthcoming future as well as it can generate new ideas in the field of modern drug development, which can be very useful in medical biology. So, further pharmacological and biochemical investigations are required to screen out some more effective user friendly potential medicine for human welfare. It is hoped that this review will serve as an encouragement to all the researchers, involved in this field.

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Survey of plants used for the treatment of diabetes in Dinajpur (Uttar & Dakshin) and Malda Districts of Paschimbanga

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Abstract

Diabetes is one of the most dreaded diseases and silent killer of the human body tissues. Several chemical drugs are available for its treatment; still peoples of low income group and tribal peoples are using their traditional knowledge for treating this disease. The objective of the present study is to find out the knowledge and plants which are being used by the tribals and lower caste peoples of three districts of Paschimbanga. The present paper, recorded 31 plant species belonging to 21 families those are frequently used for the treatment of diabetes by the different tribal and lower caste peoples of Malda, Uttar Dinajpur and Dakshin Dinajpur districts of Paschimbanga.

Key words: Diabetes, ethnobotany, Uttar Dinajpur, Dakshin Dinajpur, Malda.

INTRODUCTION

Diabetes mellitus is a clinical syndrome characterized by higher glucose level in blood caused by a relative or absolute deficiency of insulin or by a resistance to the action of insulin at the cellular level. It is estimated that about 25% of the world population is affected by this ailment (Maiti *et al* 2004).

According to International Diabetes Federation approximately by 2030, 438 million people (7.8 %) of the adult population, is expected to have diabetes. It is also estimated that the total number of people with diabetes in India, rising to 87.0 million by 2030. India has the world's largest share of diabetes followed by China (IDF 2009). According to the WHO's recent estimate, the prevalence of known diabetes was 5.6% and 2.7% among urban and rural areas, respectively (Mohan & Pradeepa 2009).

Recently, there has been a resurgent interest in the herbal treatments of diabetes. The growing public interest and awareness of natural medicines have led the pharmaceutical industry and academic researchers to pay more attention to medicinal plants (Day 1998). The apparent reversal of trend from western to herbal medicine is partly due to the fact that synthetic drugs have always shown adverse reactions and side effects. Whereas the common belief that natural products are safe because they are more harmonious with biological systems (Atal 1983; Erasto *et al* 2005). In addition, the cost of administering modern antidiabetic drugs is beyond the reach of people in the low income group peoples and those living in the rural areas. Therefore, plant extract or different folk plant preparations which are very very cheap as compared to modern drugs, are being prescribed by the rural traditional practitioners. Low income group peoples use these preparations with apparent hope of curing the disease.

The present study has undertaken to find out plants which are traditionally used to treat diabetes in Uttar & Dakshin Dinajpur and Malda districts. In this paper, we present the list of plants used for the treatment of diabetes as well as the parts used and also the various methods of preparation and administration especially in tribal and rural areas.

STUDY AREA

Uttar Dinajpur District is a narrow part of northern gangetic belt of Paschimbanga and lies between latitudes 25°11' N and 26°49' N and longitudes 87° 49' E and 90° 00' E occupying an area of 3142 sq km. To its east is Bangladesh, Bihar on the West, Darjeeling and Jalpaiguri Districts are on the North and Malda District is on the South. The Climate of this district is characterised by Hot-summer with high humidity, abundant Rainfall and Cold Winter (Sengupta 1964; Mitra 2002).

The Dakshin Dinajpur District principally made of old-gangetic alluvium lies to the south eastern part of North Dinajpur having area about 2162 sq km. The district lies between Latitude 26° 35' 15" N 25°10' 55" N Longitude 89°00' 30" E 87° 48' 37" E. The district has a tropical humid climate characterized by hot summer with high amount of humidity, abundant rainfall and a very cold winter (Sengupta 1964).

The principal communities of the above two districts are Hindus and Muslims, constituting the major portion of the population. Besides, Rajbanshis, Santal and Munda communities are also present in both Uttar & Dakshin Dinajpur districts (Sengupta 1964).

The total geographical area of Malda district is 3455.66 sq km. The District is situated between the Latitude and Longitude figures of 24°40'20" N to 25°32'08" N and 88°28'10" E to 87°45'50" E respectively and surrounded by Bangladesh and South Dinajpur in the east, Santal Parganas of Jharkhand state in the west, Uttar Dinajpur in the north and Murshidabad in the south. The climate of the district is rather extreme-very hot and sultry during summer season, with plenty of rains and moisture in the air throughout the year. The language of the majority of the people is Bengali. But a certain percentage of the population in the West and North is of Behar origin, and most of them speak adulterated Hindi. Among the scheduled castes population, there are 59 sub-communities of which Rajbanshi, Namasudra, Polia, Tiyer, Keet and Poundra are the main sub-castes. Among the tribal population there are 38 sub-communities of which Santala, Orao, Mundas and Malpaharia are main sub-communities (Sengupta 1969).

METHODOLOGY

Places for data collection were selected based on high concentration of tribal and local scheduled caste populations. The villages/ blocks were Chopra, Islampur, Kaliyaganj, Goalpara, Raiganj from Uttar Dinajpur; Mahipal, Kaldighi, Daulatpur, Balurghat and Hili etc. from Dakshin Dinajpur and Malatipur, Bandhapukur, Manikchak, Kaliyachak, Gazole from Malda District.

This study was conducted during November 2009 – October 2010. The information regarding the use of different medicinal plants for curing diabetes and several others ailments has been collected from the local traditional healers called 'Kaviraj', old women and aged knowledgeable persons by direct interview. Depending on person and his degree of practice, the interview took place more than 5-6 hours. We also enquired about method of preparation of anti-diabetic medicine. Some of the Kaviraj helped us to identify those medicinal plants and make us aware with the vernacular names of such plants. We also cross verified with some local aged and experienced practitioners regarding the use of those plants.

The method of collection of voucher specimen, preservation, herbaria and technique for the collection of ethnobotanical information were followed as described by Jain & Rao (1977). The voucher specimens were deposited in the Herbarium of the Botany Department, Raiganj College (University College), Uttar Dinajpur.

OBSERVATION AND RESULTS

We report in this communication that 31 plant species are being used for the treatment of diabetes and their parts used along with various methods of preparation (Table 1).

Table 1: Plants used by common low income group of people for the treatment of diabetes in the districts of Uttar Dinajpur, Dakshin Dinajpur and Malda

Scientific name [Family]; Voucher specimen	Vernacular name	Parts used	Ethnic practices	District
Ambroma augusta (L.) L.f. [Sterculiaceae]; UD – 211	*Ulatkambal*	Leaf	Dry leaf powder mixed with water is taken in empty stomach in morning	Uttar Dinajpr
Acacia nilotica L. [Fabaceae]; UD – 197; DD – 179	*Babla*	Bark	Bark decoction is taken twice daily	Uttar & Dakshin Dinajpur
Alternanthera sessilis (L.) DC. [Amaranthaceae]; UD – 141	*Sanchi*	Whole plant	Consumed as cooked vegetable regularly	U. Dinajpur
Andrographis paniculata (Burm. f.) Wall. *ex* Nees [Acanthaceae]; UD – 98; DD – 113; MLD – 107	*Kalmegh*	Leaf	Leaf decoction or boiled leaf taken orally	All the three districts
Azadirachta indica A. Juss. [Meliaceae]; UD – 69; DD – 79; MLD – 65	*Neem*	Leaf	Leaf juice given in the morning	All the three districts
Boerhavia diffusa L. [Nyctaginaceae]; UD – 135	*Punarnabha*	Whole plant	Leaves are boiled and the infusion is taken orally	Uttar Dinajpur
Catharanthus roseus (L.) G. Don [Apocynaceae]; UD – 153; DD – 177; MLD – 165	*Nayantara*	Leaf	Leaf juice is given daily morning	All the three Districts.
Coccinia grandis (L.) Voigt [Cucurbitaceae]; UD – 207; DD – 191; MLD – 173	*Telakucha*	Leaf	Leaf decoction is taken orally. Fruit used as vegetable	All the three Districts.
Desmodium gangeticum DC. [Fabaceae]; UD – 243	*Salpani*	Root	Root decoction is consumed regularly	Uttar Dinajpur
Ficus glomerata Roxb. [Moraceae]; UD – 88	*Dumur*	Leaf	Mixture of leaf juice of *Aegle marmelous*, *Syzygium cumini* and *Ficus glomerata* is taken in empty stomach	Uttar Dinajpur
Gmelina arborea L. [Verbenaceae]; UD – 319; DD – 341; MLD – 289	*Gamari*	Bark & fruit	Bark decoction and fruit infusion is taken orally	All the three district
Gymnema sylvestre (Retz.) R.Br. *ex* Schult. [Asclepiadaceae]; UD – 63; DD – 127	*Gurmar*	Leaf	Leaf decoction or dry leaf powder mixed with water is taken orally	Uttar & Dakshin Dinajpur
Helicteres isora L. [Sterculiaceae]; UD – 173	*Aatmora*	Root	Root bark decoction consumed daily	Uttar Dinajpur
Holarrhena antidysenterica (L.) Wall. [Apocynaceae]; UD – 118; DD – 307	*Kurchi*	Seed	Seed powder mixed with water and taken orally	Uttar & Dakshin Dinajpur
Hygrophila spinosa T. Anders. [Acanthaceae]; Ud – 159; DD – 258; MLD – 93	*Kulekhara*	Leaf	Leaf decoction or boiled leaf is taken orally	All the three district
Lagerstroemia reginae Roxb. [Lythraceae]; UD – 453; DD – 289	*Jarul*	Bark	Bark decoction is taken orally	Uttar & Dakshin Dinajpur
Luffa echinata Roxb. [Cucurbitaceae]; UD – 317	*Bon-kakrol*	Fruit	Fresh fruits are cooked and eaten as vegetable	Uttar Dinajpur
Melothria heterophylla (Lour.) Cogn. [Cucurbitaceae]; UD – 323; DD – 361	*Rakhal Sasa*	Fruit	Fresh fruits are eaten as vegetable	Uttar & Dakshin Dinajpur

Momordica charantia L. [Cucurbitaceae]; UD – 329; DD – 297; MLD – 312	*Karola*	Fruit	Fruit juice or seed powder is taken orally	All the three district
Morinda citrifolia L. [Rubiaceae]; DD – 421	*Noni*	Fruit	Fruit juice is taken orally	Dakshin Dinajpur
Murraya koenigii (L.) Sprengel [Rutaceae]; UD – 336; DD – 285; MLD – 285	*Curry pata*	Leaf	Leaf juice is taken in empty stomach	All the three district
Nyctanthes arbor-tristis L. [Oleaceae]; UD – 361; DD – 229; MLD – 188	*Shiuli/ Sephali*	Leaf	Leaf juice is taken orally	All the three districts
Phyllanthus emblica L. [Phyllanthaceae]; MLD – 297; UD – 155	*Amloki*	Fruit	Infusion of fruit is given in empty stomach	Malda & Uttar Dinajpur
Plumbago zeylanica L. [Plumbaginaceae]; MLD – 265	*Sadachita*	Root	Root decoction is consumed regularly	Malda
Premna corymbosa (Burm.f.) Rottl. & Willd. [Verbenaceae]; (UD – 407)	*Gonal*	Leaf	Dry leaf powder is mixed with water and taken orally	Uttar Dinajpr
Scoparia dulcis L. [Scrophulariaceae]; DD – 227	*Chinimichri/* mistipata	Leaf	Leaf decoction is consumed daily	Dakshin Dinajpur
Syzygium cumini (L.) Skeels [Myrtaceae]; UD – 249; DD – 331; MLD – 291	*Jam*	Seed	Seed powder is mixed with water and taken orally	All the three districts
Tinospora cordifolia (Willd.) Hook. f. [Menispermaceae]; UD – 137; DD – 185	*Gulancha*	Stem	Fresh Stem boiled and the infusion taken orally	Uttar & Dakshin Dinajpur
Trigonella foenum-graecum L. [Fabaceae]; UD – 254; DD – 333	*Methi*	Seed	Seed powder mixed with water and given in empty stomach	Uttar & Dakshin Dinajpur
Vernonia anthelminctica (L.) Willd. [Asteraceae]; UD – 281	*Somraji*	Seed	Seed powder mixed with water and given in empty stomach	Uttar Dinajpr
Vitex negundo L. [Verbenaceae]; MLD – 261	*Nishinda*	Leaf	Leaf juice is given daily in the morning	Malda

DISCUSSION AND CONCLUSION

All together 31 plants belonging to different families have been enlisted, those are used by tribal and lower caste peoples for the treatment of diabetes in the districts of Malda, Uttar & Dakshin Dinajpurs. The study also revealed these 31 plant species are frequently and commonly used for the treatment of diabetes. The highest number of plants belonged to Cucurbitaceae (Four species) followed by Fabaceae and Verbenaceae (Three species each). The Acanthaceae, Apocynaceae and Sterculiaceae were having 2 species each. Amaranthaceae, Asclepiadaceae, Asteraceae, Lythraceae, Meliaceae, Menispermaceae, Moraceae, Myrtaceae, Nyctaginaceae, Oleaceae, Phyllanthaceae, Plumbaginaceae, Rubiaceae, Rutaceae and Scrophulariaceae contributed 1 species each.

The plants were used either separately or in combination with some other plants. Most of these plants are rare available in natural sources in the study area. Most frequently used plants for all the three districts are *Andrographis paniculata*, *Azadirachta indica*, *Catharanthus roseus*, *Coccinia grandis*, *Gmelina arborea*, *Hygrophila spinosa*, *Momordica charantia*, *Murraya koenigii*, *Nyctanthes arbor-tristis*, *Syzygium cumini*, *Trigonella foenum-graecum*. Whereas *Acacia nilotica*, *Gymnema sylvestre*, *Holarrhena antidysenterica*, *Lagerstroemia reginae*, *Melothria heterophylla*, *Tinospora cordifolia* are commonly used in Uttar and Dakshin Dinajpur districts. In Uttar Dinajpur *Abroma augusta*, *Alternanthera sessilis*, *Boerhavia diffusa*,

Desmodium gangeticum, *Ficus glomerata*, *Helicteres isora*, *Luffa echinata*, *Premna corymbosa*, *Vernonia anthelminctica* are used. But only two plants viz. *Morinda citrifolia*, *Scoparia dulcis* used in Dakshin Dinajpur which are different from other two districts. On the other hand *Plumbago zeylanica*, *Vitex negundo* are used in Malda district and only one plant viz.- *Phyllanthus emblica*, commonly used in Uttar Dinajpur and Malda districts. The local Rajbanshis and tribal community of the study area have very good knowledge on medicinal plants. But such knowledge is restricted to a few people. So, knowledge of use of plants is in danger of being lost forever. These findings may lead to serious research towards developing new drugs for diabetes. Further investigations are being carried out for the active principles and chemical analysis of these medicinal plants.

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Allelopathic effects of three weed plants on mycorrhizal association of *Zea mays* root

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Plants with mycorrhizal association, are often more competitive and are capable of tolerating environmental stresses better than the non-mycorrhizal ones. The mycorrhizal fungus transfers many nutrients through their hyphal network and thus play a significant role in the soil ecology. Plants considered as weeds are involved in interaction with other plant species at different trophic levels through the production and release of allelochemicals in the soil. This work presents the data on the allelopathic effects of three common weeds on mycorrhizal association in *Zea mays* root system and spore population in soil. The alkaline phosphatase and succinate dehydrogenase activities of the active mycelium of AM fungi was also studied with respect to the most effective concentrations of the alleochemicals.

Key words: Allelopathy, *Croton bonplandianum, Glomus mosseae, Lantana camara, Parthenium hysterophorus*

INTRODUCTION

Certain plants exhibit allelopathy and influence other plants and microorganisms by releasing different chemicals such as phenol, glycosides, amino acid, alkaloids, terpenes and sugars. Since almost all higher plants in terrestrial ecosystem, are associated with mycorrhiza, there is bound to have some allelopathic effect on this group of microorganisms also. Report of mycorrhizal colonization being adversely affected in response to allelopathic effect of *Eucalyptus citriodora* is available (Rukhsana and Naz, 2005). Phenolic compound released by *Croton bonplandianum* has been reported to adversely affect microorganisms and other plants grown in the rhizosphere soil. The present study has therfore, been designed to investigate the allelopathic affect of the root and leaf extracts of the three common weeds - *Lantana camara, Parthenium hysterophorus,* and *Croton bonplandianum* on mycorrhizal association in *Zea mays* root. The effect on the activity of two important enzyme namely succinate dehydrogenase (SDH) and alkaline phosphatase (ALP) of the mycorrhizal fungi has, also been studied. SDH, a mitochondrial enzyme, is considered as an indicator of viability of mycorrhiza (Vierheilig and Ocampo 1989), while ALP activity, located within the phosphate accumulating vacuoles of AM hyphae, have been proposed as physiological marker for analyzing the efficiency of mycorrhiza (Gianinazzi *et. al.,* 1972; Tisserant *et al.,* 1993) Allelopathic effect of the selected plant on the mycorrhizal spore population in the soil has also been studied.

MATERIALS AND METHODS

Experiment was conducted in plastic pots (6"diam.), each containing 500 g of sterilized field soil, inoculated with *Glomus mosseae*- infected root pieces, external mycelium and spores (25 g soil / pot). The pot soil was rendered allelopathic by applying 10 % and 25% (w/v) aqueous extract of *Lantana camara, Parthenium hysterophorus,* and *Croton bonplandianum* leaves and roots separately @ 500 ml/pot, three and seven days after transplanting 20-day-old *Zea mays* seedlings. Two seedlings were transplanted / pot and three replications were maintained for each treatment. Plants were harvested 21 and 30 days after transplanting and the feeder roots were collected from each treatment. A control without allelochemical treatment was also maintained.

Roots were stained with trypan blue (TB) (Phillips and Hayman, 1970) and the degree of mycorrhizal infection of such roots were determined by the intersect method (Giovannetti and Mosse, 1980).

Succinate dehydrogenase (SDH) and Alkaline phosphatase (ALP) activity were determined following histochemicall techniques (Gianinaggi *et al.*, 1979; Tisserant *et al.*, 1993; Vierheilig and Ocampa 1989). Spore population of *G. mossae* in the differently treated soil was determined by conventional wet-sieving process.

Statistical analysis

Data obtained was subjected to one way analysis of variance (ANOVA) and treatment means were separated by a Student-Newman-Keul's test. Percentage data were arcsine-transformed before analysis.

RESULTS

The root system of young maize plants in the control showed 35.6% degree of active and viable mycorrhizal infestation, with ALP (35.6) and SDH (34.4) activity. Arbuscular percentage was 5.4 of which 75 % showed ALP and SDH activity. However, treatment with root and leaf extracts, irrespective of the concentration (10 and 25%) resulted in reducing mycorrhizal colonization and activity of ALP and SDH indicating negative allelopathic affect. The results (Table 1) indicated that The negative allelopathic effect of leaf extract of *P.hysterophorus* was higher in both concentrations than the leaf extract of *C. bonplandianum* and *L.camara*. Degree of mycorrhizal infestation was least in 25% (w/v) of *P. hysterophorus* leaf extract. negative allelopathic affect of *P.hysterophorus* and *C.. bonplandianum* leaf extracts at both concentrations was also reflected in the total absence of arbuscules in the treated roots.

The allelopathic affect of *C. bonplandianum* root extract was found to be more negative than its leaf extract at both concentrations on the total mycorrhizal infestation in the root system (Table2). The root extract of *L. camara* and *P. hysterophorus* was, however, found to be more positive than their respective leaf extracts in respect to the total mycorrhizal infestation, percentage of viable and active hyphae showing ALP and SDH activity. With increase in the age of the *Zea mays* plants (30 days of treatment), it was found there was an increase in the degree of the mycorrhizal infestation

Table 1 : Allelopathic effect of leaf extracts of three weed plants on AME enzyme activity

Treatment Leaf Extract	Concentration	M%			A%		
		TB	ALP	SDH	TB	ALP	SDH
Control	--	35.56*a	35.56a	34.44a	5.35a	4.05a	4.05a
Lantana camara	10%	25.52b	23.35b	22.4b	2.68c	0.5c	0.4c
	25%	14.48b	13.44b	12.34b	1.30c	0.02c	0.02c
Parthenium hysterophorus	10%	10.00b	6.83b	6.44c	0	0	0
	25%	7.30c	6c	5.88c	0	0	0
Croton bonplandianum	10%	10.24b	7.44c	6.92c	0	0	0
	25%	8.56c	7.14c	6.50c	0	0	0

*Each value is the mean of three replications. Data followed by the same letters within each column were not significantly different ($P<0.05$, student-Newman-Keuls' test). All percentage values were are sin transformed before statistical analysis.

(73%), which was also reflected in the increase percentage of ALP and SDH- active hyphae, total arbuscules in the root system and ALP and SDH-active arbuscules (Fig.2). Further it was observed that the negative allelopathic affect became gradually neutralized and increased mycorrhizal colonization, ALP and SDH-active and viable hyphae and arbuscule production in the root system

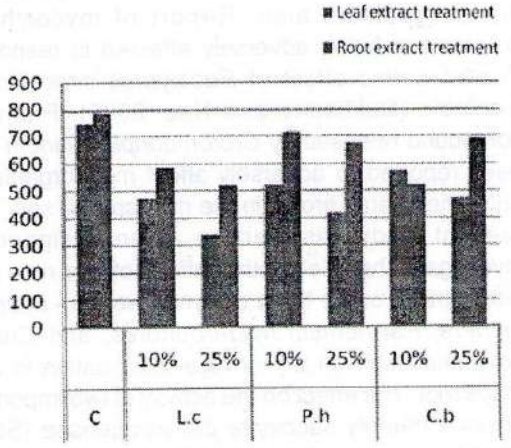

Fig. 1 : *G.mosseae* spore population/ 100 g soil after the treatment of allelochemical.

could be observed. However, no ALP and SDH activity was recorded in the arbuscules even a month after treatment.

Table 2 : Allelopathic effect of root extracts of three weed plants on AM fungi infestation.

Treatment Root Extract	Concen-tration	M%			A%		
		TB	ALP	SDH	TB	ALP	SDH
Lantana camara	10%	28.76*a	24.4a	23.56a	0.52c	0.41c	0.4c
	25%	19.40b	14.52b	13.08b	1.02c	1.02c	0.30c
Parthenium hysterophorus	10%	11.56b	7.8c	7.8c	0.10c	0.03c	0.02c
	25%	8.44c	7.03c	6.8c	0	0	0
Croton bonplandianum	10%	9.72c	7.28c	5.68c	0	0	0
	25%	5.6c	4.96c	3.8c	0	0	0

*Each value is the mean of three replications. Data followed by the same letters within each column were not significantly different ($P<0.05$, Student-Newman-keuls' test). All percentage values were arc sin transformed before statistical analysis.

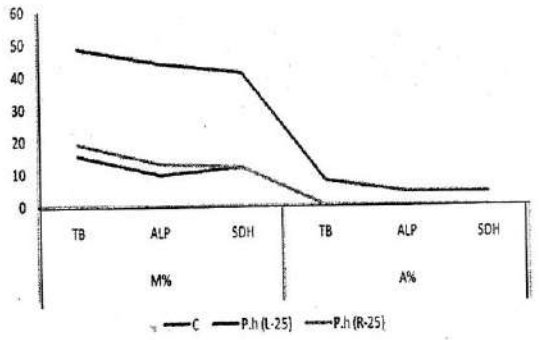

Fig. 2 : Mycorrhizal infestation percentage & SDH-ALP activity after 30th day of treatment

Negative allelopathic affect of both leaf and root extract of the three weeds plant could also be observed on the total spore count of *G. mosseae* / 100g of soil. The maximum negative affect was seen in 25% (w/v) of leaf extract of *L. camara* (Fig.1).

DISCUSSION AND CONCLUSION

Negative effect of allelochemicals on mycorrhizal fungus has been reported by several worker observed that allelopathic grasses decreased the mycorrhizal colonization of other plants including *Zea mays* L. It has also been recorded the exotic weed can interfere with nearby species by releasing allelochemicals that either directly inhibit growth and cause disturbance of associated species or affect then indirectly by disrupting their interaction with soil organisms such as arbuscular mycorrhizal fungi (Karasawa *et al.*, 2002). In the present study a similar trend has been observed and it is found that allelochemicals present in the root and leaf extract of the three plants selected for the study could not only reduce the AMF inoculum present in the soil, but considerably reduce the degree of mycorrhizal infestation in maize roots (Elmer, 2002) Further, the allochemicals present in the leaf and root extracts are found to reduce the ALP abd SDH associated active and viable hyphae in the roots to varying degrees. The negative affect is, however, found to be transient and appeared to wear off after a certain priod of time, when mycorrhizal infestation of root system gradually increased again. This study is specially relevant since there has been an increasing trend of use of botanicals for plant disease control. such botanicals while affecting the plant pathogens, may also, for a short period of time, reduce the positive roleof mycorrhiza in uptake of nutrients and enhancing plant growth.

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