ANTILEISHMANIAL AND ANTIFUNGAL ACTIVITIES OF ETHNO - MEDICINALLY IMPORTANT PLANT EXTRACTS USED BY TRIBAL POPULATIONS OF NORTH BENGAL

Thesis submitted to the University of North Bengal
for the award of doctor of philosophy in
Biotechnology

Submitted by
Anindita Chakraborty

Supervisor
Prof. (Dr.) Dipanwita Saha

DEPARTMENT OF BIOTECHNOLOGY
UNIVERSITY OF NORTH BENGAL

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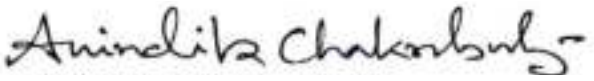
Declaration

I, Anindita Chakraborty hereby declare that the work embodied in my thesis entitled "Antileishmanial and Antifungal activities of Ethno-Medicinally important plant extracts used by Tribal populations of North Bengal" has been carried out by me under the supervision of Dr. Dipanwita Saha, Professor, Department of Biotechnology, University of North Bengal for the award of the degree of Doctor of Philosophy in Biotechnology. I also declare that, this thesis or any part thereof has not been submitted for any other degree/ diploma either to this or other University.



(**Anindita Chakraborty**)
Date: 05/05/2022
Place: Department of Biotechnology
University of North Bengal
Siliguri-734013

Department of Biotechnology
UNIVERSITY OF NORTH BENGAL
Accredited by NAAC with Grade 'A'

Dr. Dipanwita Saha
M.Sc., Ph.D.
Professor

P.O. NBU West Bengal, India (734013)
Contact: +91-9434429800
E-mail: dsahanbu@yahoo.com

TO WHOM IT MAY CONCERN

This is to certify that Ms. Anindita Chakraborty has worked under my supervision at the Department of Biotechnology, University of North Bengal. Her thesis entitled "Antileishmanial and Antifungal activities of Ethno-Medicinally important plant extracts used by Tribal populations of North Bengal" *is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Biotechnology in accordance with the rules and regulation of the University of North Bengal. She has fulfilled all requirements according to the rules of the University of North Bengal regarding the works embodied in her thesis. This thesis or any part thereof has not been submitted for any other degree/Diploma either to this or other university.*

Dipanwita Saha
.....
(Dipanwita Saha)
Supervisor
Dr. Dipanwita Saha
Professor
Department of Biotechnology
University of North Bengal

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I am the luckiest mother in the world to be blessed with an incredible son, Deeptarko, who is the source of inspiration and strength behind all my successes.

Anindita Chakraborty

Date: 05/05/2022 *(Anindita Chakraborty)*

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Dipanita Saha

Anindita Chakraborty

Abstract

The green part of the universe is the backbone for the existence of all life. Utilization of plants for survival is practiced since ages. Evidences of use of plants for therapeutic purposes and well-being of mankind are there in ancient history and literature. India, being a country with mega diversity, has rich indigenous knowledge of natural world and their uses. The present study aims to explore these age-old knowledge and natural resources to fight against diseases like leishmaniasis and related challenges of fungal infections of modern era.

At the onset of this study, a survey was conducted among the rural and tribal communities belonging to Jalpaiguri district and parts of Alipurduar district of West Bengal. The plants used by them for therapeutic purposes against leishmaniasis (Kala-azar) and fungal infections were enlisted, from which four plants, *Rauvolfia serpentina*, *Moringa oleifera*, *Nyctanthes arbor-tristis* and *Clausena excavata* were chosen for further study. A thorough literature survey was conducted on the shortlisted plants to know their reported bioactive compounds.

Phytochemical analysis of leaf extracts of plants showed presence of secondary metabolites like alkaloid, flavonoid, terpenoid, tannin and phenolics. Quantitative estimation of phenolic compounds showed that all the four tested plants contained good amount of phenolics with *N. arbor-tristis* and *C. excavata* recording the highest and lowest content respectively. Flavonoid content was found to be highest in *M. oleifera* extract. Plant leaf extracts were further studied for antioxidant activity by DPPH free radical scavenging assay. While all the tested plants showed positive results, *C. excavata* extract was found to be most potential antioxidant.

Antifungal activities of the crude leaf extracts were tested by agar cup assay against *Candida albicans*. In addition, the antifungal compound excavarin-A was also tested by similar method. This compound was purified from the dichloromethane extract of *C. excavata* leaves by bioassay guided fractionation following silica gel column chromatography. The identity of the compound was confirmed through UV–VIS, IR and NMR (1H- and 13C-) spectroscopic analysis. All the plants showed positive results and the MIC value was lowest for excavarin-A (0.078mg/ml) followed by *R. serpentina* (0.156mg/ml).

Anti-leishmanial activity of the selected botanicals was studied against the protozoa *Leishmania donovani*. For *in vitro* screening, the pathogen, cultured in 96-well plate, were treated with graded doses of test substances (crude leaf extracts of *R. serpentina, C. excavate* and the molecule excavarin-A). The percentage of growth inhibition of parasitic promastigotes were calculated to estimate anti-leishmanial activity. Results showed that all the test substances had antileishmanial activities *in vitro*. The IC_{50} values for excavarin-A and the crude leaf extracts of *R. serpentina* and *C. excavata* was recorded as 1.24 mg/ml, 4.04 mg/ml and 32.1 mg/ml respectively. The purified compound was found to be much more effective than the crude extracts. The selected botanicals were also tested on amastigotes cultured in hamster macrophage in laboratory condition. Excavarin-A was found to be most efficacious followed by crude extracts of *R. serpentina* and *C. excavata.* However, both *C. excavata* leaf extract and excavarin-A purified from its leaves showed cytotoxicity in test for viability of hamster macrophages in the range of effective therapeutic doses. On the other hand, *R. serpentina* leaf extract was not found cytotoxic in its effective range of concentrations.

Botanicals showing good antipathogenic potentiality *in vitro* were also tested *in vivo,* excluding those that were found to be cytotoxic

in the therapeutic range of doses. In a preliminary *in vivo* experiment, infections were created on the skin of experimental animals (male albino rats) using cell suspension of *C. albicans.* The experimental candidiasis models were treated with crude extracts of *R. serpentina* and *M. oleifera.* Both the tested leaf extracts significantly decreased and cured the infection upon superficial application. *R. serpentina* surfaced as a potentially better antifungal agent. The elevation in total count of WBC which was found by haematological analysis of blood samples of treated animals, suggested that the leaf extracts of both the plants have protective roles in improving host defence to counter fungal attack.

Crude leaf extract of *R. serpentina* was also tested for its antileishmanial activity *in vivo*. The test animals, Syrian golden hamsters, were first infected with *L. donovani* parasite, and after development of infection, the graded doses of leaf extract were administered intramuscularly. Parasitic burden of spleen and liver of infected hamsters were reduced after treatment in a dose-dependent manner. *R. serpentina* extract was also found to be hepatoprotective as evident from the plasma levels of SGOT and SGPT enzymes.

Studies on mechanisms involved in antileishmanial activity showed that the action of the *R. serpentina* extract is mediated through inhibition of leishmanial superoxide dismutase (SOD) evident by measuring the inhibition of pyrogallol autoxidation rate. Further an enhanced release of toxic superoxide radical which was measured spectrophotometrically through the formation of blue formazan was also observed. Polyacrylamide gel electrophoresis of *Leishmania* promastigote lysate confirmed these results. On gel activity staining through non-denaturing PAGE as well as SDS-PAGE showed degeneration of SOD bands with increasing concentration of *R. serpentina* extract. The results obtained from these experiments suggest that inhibition of SOD and simultaneous release of

superoxide radicals impose toxic effects to destroy intracellular parasites during experimental visceral leishmaniasis. The findings of this study may be significant in the field of development of new therapeutic agents from natural resources against fungal infections and leishmaniasis.

Preface

India is a mega diversity country having numerous species of flora and fauna. Due to their proximity to rich natural resources, Indians have enriched their knowledge about the natural world, and this knowledge is also reflected in their daily life including treatment of illness. As such, we have a long heritage of using plants or plant products for medicinal and aromatic purposes. This study is a step to explore that age-old knowledge for the betterment of human civilization.

The essence of true India comes alive when we come to Dooars because of its rich biodiversity and vibrant rural life. The name Dooars is derived from 'doors' as the region is the gateway to the entire North-East India and Bhutan. The Dooars region which cover a vast area of 8800 sq. km. is famous for its rich wildlife and forests; the most notable of which are Gorumara National Park, Jaldapara Wild Life Sanctuary, and Buxa Tiger Reserve. The region is characterized by a sub-tropical and humid type of climate. The average maximum temperature is 37°C and the average minimum temperature is 6°C. The average annual rainfall of this area is 3300mm. The strong rain is of hydro-meteorological significance, causing deluges and flood in the area. The average relative humidity of this region is about 82%.

Almost all the forest villages and remote areas of this region are inhabited by various tribal populations. The local people use various plant species to cure common diseases, which became evident during a preliminary survey that involved direct interaction with tribal medicine men and healers practicing herbal medicines in their original primitive forms. Still, there are a large number of tribal areas having no healers or medicine men. Indigenous or traditional knowledge of human health and medicine can form a strong base of primary health care. There has been renewed interest

in plant medicine for the treatment of different diseases, as herbal drugs are generally devoid of toxic side-effects (Sharma et al., 2013). Studies show that different primary and secondary metabolites of systematic pathways of plants, mainly alkaloids, flavonoids, tannins, and phenolic compounds produce definite therapeutic actions on the human body. Knowledge of chemical constituents of phytoextracts is desirable for understanding their mode of action as therapeutic agents, and in the field of new drug discovery. At present time, phytochemical research based on ethnopharmacological information is the most effective and emerging approach to the world of medication.

Not only as curative agents, traditionally natural products, plants, vegetables, and fruits are also being used as tools for the prevention of diseases, infections and also for improvement of nutritional status. In today's world, natural antioxidants are a matter of great interest, and consumption of green tea, green vegetables, and organic foodstuffs have become a trend to overcome oxidative stress and delay aging. Research says there is an inverse relationship between intake of natural antioxidant-rich food and incidents of diseases in humans. Plants, having good antioxidant properties, are reported to have phenolic content of up to 30% of their dry weight. Many such antioxidant compounds have been isolated in recent studies (Lin et al., 1998; Rio et al., 2013).

Researchers of the modern age are concentrating their efforts to develop a new line of treatment and are trying to isolate novel molecules from natural resources to fight against the diseases which become a real threat to mankind. Chemical medicines and artificial drugs are proven to be less effective, with lots of side effects, which are used for diseases like cancer, microbial infection, systematic disorder, etc. Co-infection of two or more diseases, the evolution of new strain of microbes with different pathogenic characteristics, environmental changes and many other factors are

making the situation adverse day by day. Here lies the importance of cost-effective natural remedies with fewer or no side effects. Leishmaniasis is an example of a protozoan disease affecting millions of people in tropical countries with no proper line of treatment and effective vaccine. Moreover, in the last few decades, reports of co-infection of AIDS and Visceral leishmaniasis are making the situation worse. Visceral leishmaniasis (VL) accelerates the onset of AIDS in HIV positive people, and the chances of VL rise by 100-1000 times in immune-compromised patients (Fuzibet et al., 1988; Lindoso et al., 2009). This is a potentially fatal tropical disease, whose study is currently being neglected (de Paula et al., 2019), is considered by the World Health Organization (WHO) as the second most important protozoan disease in regard to public health (annual report 2009. Geneva, WHO).

Manifestation of leishmaniasis ranges from mild cutaneous lesions to fatal visceral form if left untreated. The first line of treatment, based on antimonial drugs, becomes useless when antimonial resistant *Leishmania* strains emerge and frequent relapses occur after treatment. Amphotericin β based second line of drugs also has severe limitations due to their toxic effects and high costs. Despite continuous efforts by scientists to develop a vaccine, no one has yet been protected against *L. donovani*. Visceral leishmaniasis attacks the immune system of the host, characterized by defective cell-mediated immunity, increase in cell membrane fluidity, hampering of antigen-presenting ability and also impairment of specific T-cell response (Chakraborty et al., 2005). In such immuno-compromised condition, other opportunistic microbes like fungi (*Candida sp., Cryptococcus sp., Aspergillus sp.* etc.) can easily attack humans, making the situation complicated. Immuno-modulator drugs can improve the situation by activating the microbicidal mechanism of macrophages and by stimulating the host's overall immune system. Studies say many plant extracts or natural products have immuno-

modulatory activities. In this scenario, it is necessary to develop a novel line of treatment having direct and selective microbicidal effects – which can eradicate age-old diseases like leishmaniasis and related complications. In the humid and wet weather of sub-Himalayan West Bengal, fungal infections are most common cause of skin diseases. Hepatomegaly and splenomegaly are very common among these people. During the preliminary survey through local people, it was found that they use particular herbs to cure skin diseases, liver disorders, and diseases like leishmaniasis (Mitra and Mukherjee, 2009; Mitra and Mukherjee, 2010). This potential of the herbs can be explored to discover bioactive molecules relating to new drug development against leishmaniasis and fungal infections.

LIST OF TABLES

LIST OF FIGURES

Figure: 7.3 Effect of *R. serpentina* on serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels (units/litre) in *Leishmania* infected hamsters.

Figure: 7.4. Leishmanial SOD activity when enzyme (promastigote lysate) and *R. serpentina* leaf extracts were added to the reaction mixture either simultaneously ( )or after preincubation for 30 minutes ( ).

Figure: 7.5 Rate of superoxide radical release before ( ) and after ( ) treatment of Leishmania pathogen by *R. serpentina*. Formation of blue formazan derived from reduced nitroblue tetrazolium in the presence of superoxide radical was measured spectrophotometrically.

Figure: 7.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of *Leishmania* cell free extract in different doses (50 µg protein was loaded in each lane). A. 40 mg/ml *R. serpentina*; B. 30 mg/ml *R. serpentina*; C. 20 mg/ml R. serpentina; D. 10 mg/ml *R. serpentina*; E. 5 mg/ml *R. serpentina*; F. Without *R. serpentina*; G. Pure Fe^{3+}-SOD.

Figure: 7.7 Activity staining of *R. serpentina* leaf extract treated leishmanial SOD after separating by native polyacrylamide gel electrophoresis in a 10% gel. In each lane 60 µg of protein was loaded. Gel was incubated in nitro blue tetrazolium and riboflavin solutions and finally illuminated. SOD activity which inhibits the formation of blue formazan, was visualized as white bands on blue background. (A) Without extract; (B) treated with 10 mg/ml leaf extract; (C) treated with 20 mg/ml leaf extract; (D) treated with 30 mg/ml of leaf extract.

ABBREVIATION

ABCD	Amphotericin beta lipid complex
APC	Antigen Presenting cell
BSA	Bovine serum albumin
BSI	Blood stream infection
CL	Cutaneous leishmaniasis
Cm	Centimetre
CPG	Cytosine-phosphate guanosine
DCL	Diffuse cutaneous leishmaniasis
Dia	Diameter
DMSO	Dimethyl sulfoside
DPPH	2, 2'-diphenyl-1-picryl Hydrazyl Radical
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
ER	Endoplasmic reticulum
EtoAc	Ethyl acetate
FBS	Fetal bovine serum
FCS	Fetal calf serum
$FeCl_3$	Ferric chloride
FML	Fructose mannose ligand
gp63	Glycoprotein 63 kd
H_2O_2	Hydrogen peroxide
HASPBI	Hydrophilic acylated surface protein beta1
HCl	Hydrochloric acid
HIV	Human Immuno-deficiency Virus
HOCL	Hypochlorous acid
Hr	Hour
IFAT	Indirect fluorescent antibody test
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL	Interlukein
IVDU	Intervenous drug user
kDa	Kilo Dalton
kDNA	Kinetoplastid DNA
Kg	Kilogram
Km	Kilometre
LAMB	Liposomal amphotericin beta

LD	*Leishmania donovani*
LPCB	Lactophenol cotton blue
LPG	Lipophosphoglycan
M	Molar
MCL	Mucocutaneous leishmaniasis
MeOH	Methanol
Mg	Milligram
MHC	Major Histo-compatibility Complex
Min	Minute
mL/ ml	Milliliter
mm	Millimetre
µg	Microgram
µl	Microlitre
µm	Micrometre
mM	Millimolar
Mmol	Millimole
MTCC	Microbial type culture collection
MTT	3(4,5dimethylthiazole-2-yl)-2,5 diphenyl
N	Normal
NAC	Non albican species
13C NMR	Carbon nuclear magnetic resonance
1H NMR	Hydrogen nuclear magnetic resonance
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PEC	Peritoneal exudates cells
Pet ether	Petroleum ether
PKDL	Post Kala-azar Dermal Leishmaniasis
ROS	Reactive oxygen species
Rpm	Rotation per minute
S	Seconds
SAG	Sodium antimony gluconate
SD	Standard deviation
SE	Standard error
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
SOD	Superoxide dismutase

sp.	Species
SSG	Sodium stibo gluconate
TC	Total count
TGFβ	Transforming growth factor beta
Th	T helper
Th-1	T helper-1 cells
Th-2	T helper-2 cells
TLC	Thin layer chromatography
TNFα	Tumor necrosis factor alpha
UV	Ultraviolet
v/v	Volume by volume
VL	Visceral leishmaniasis
Vol.	Volume
VV	Vaccinia virus
WBC	White blood cell
w/v	Weight by volume
WHO	World health organization
ZOI	Zone of Inhibition

Contents

1.1. Medicinal plants in prevention and treatment of diseases

The life of an individual is concerned with 'health'. The idea of ill-health disturbs the rhythm of life and the performance capabilities of the individual being. After the basic need for survival is met i.e., food, cloth, and shelter, the human community feels the need for medicine to prevent diseases and illness and lead a healthy life. All human groups, no matter how small or technologically primitive, have always been devising ways and means for taking care of health because health is a property and illness is a state. Health and illness have close linkages with therapeutic and preventive practices, which have shown variations throughout the ages. Human beings over the ages have used the plants in their surroundings to treat and prevent different diseases they suffer from. They have acquired the experience to use herbal medicine against different diseases through their continuous usage and observations regarding the diseases, their symptoms, and the particular herbal drugs to cure them. At present, a major part of the world's population depends upon the traditional system of medicine. So, it is important to protect and preserve the traditional knowledge for health care of people and this can be achieved only by recording ethnobotanical knowledge scientifically (Dutta and Dutta, 2005).

In developing countries, there is an increasing attempt to incorporate traditional medicines, especially herbal preparations, into the local health care systems. Many modern researchers are involved to explore the huge potential of ethnobotanical knowledge for treating various diseases (Dutta and Dutta, 2005; Jain et al., 2010; Jeyaprakash et al., 2011). However, the ethnomedicinal plants are under threat due to deforestation, overgrazing, and their reckless utilization. This indicates the urgent need for their conservation. Conservation of biological resources as well as their sustainable use is important in the preservation of traditional knowledge (Payyappallimana and Fadeeva, 2013).

1.2. Heritage of natural remedies in India

India is known for its rich heritage of knowledge of natural products, particularly herbal medicine. Indian people have been using medicinal plants from prehistoric periods (Singh and Lahiri, 2010). Tribals, living mostly in remote forest areas, still depend on the indigenous system of medication to a great extent. Indigenous healing practices have been culturally accepted during all phases of human civilization and environmental evolution. About 85% of traditional medicines are plant- derived (Fransworth, 1988). Medicinal plants have a long-standing history in many indigenous communities and are an integral part of treating various diseases, particularly to cure daily ailments; and this practice of traditional medicine is based on hundreds of years of belief and observations. With enormously diversified ethnic groups and rich biological resources, India represents one of the great emporia of ethnobotanical wealth (Kala, 2005).

1.3. Phytopharmacological studies in today's world

Today, medicinal plants have considerably high importance in international trade; and their therapeutic, pharmaceutical and economic values are increasing day by day. It is evident, that natural resources will continue to play an important role as a health aid. Research on medicinal plants is based on their phytopharmacological studies because validation of medicinal plants and evolution of the active constituents in them are revealed by phytochemical analysis. So, for pharmacological research, drug development, or industrialized investigation on complementary or alternative medicinal invention, phytochemical studies are the basic steps (Ali et al., 2008). Phytochemical analysis shows that mainly the secondary metabolites of medicinal plants confer therapeutic properties in them, and so intake of vegetables and plants as food or therapy may offer protection from different health hazards. For example, oxidative stress or imbalanced production of superoxides or free radicals causes tissue damage, biodegradation of membrane lipid, protein, or DNA, giving rise to early aging, diseases, or health hazards. But intake of

natural antioxidants, present in fruits, green leafy vegetables, and green tea may delay or reduce the decay. Studies show that the antioxidant property of plant and phenolic content has established a co-relation (Govindarajan et al., 2005). Moreover, evidence suggested that medicinal plants have microbicidal effects too. Virus, bacteria, parasites, or fungus-infected diseases are long being treated by plant extracts by indigenous people of countries of Asia, Europe, or other continents. Ancient documentation has revealed information about plants with active effects on microbes. More advanced studies may explore some new lines of treatment against such dangerous microbes which are emerging as threats to mankind, such as the parasite-infected disease leishmaniasis (Govindarajan et al., 2005).

1.4. Prospect of natural remedies in treatment of leishmaniasis and opportunistic fungal infections

Leishmaniasis is responsible for the second-highest number of deaths due to parasitic infection globally and is overwhelmingly associated with poverty. It has an estimated prevalence of 12 million humans infected and causes a burden estimated at 2,357,000 disability-adjusted life years (WHO, 2009). Visceral leishmaniasis (VL) is almost always fatal if not treated, and morbidity caused by cutaneous leishmaniasis (CL) is also important. Treatments for all forms of leishmaniasis are few, toxic, and/or expensive; and furthermore, drug resistance is on the rise (Croft et al., 2006). There is no vaccine available for the disease, and the medications of the first choice – the pentavalent antimonials – are toxic and administered exclusively by the parenteral route (Rocha et al., 2005; Kumar et al., 2009; Joshi et al., 2006). Moreover, resistance to these medications has been reported – which is increasing for all forms of leishmaniasis, especially in areas endemic to such threats to human health (Brendle et al., 2002). Leishmaniasis emerged as a threat to mankind when studies revealed that AIDS and VL work in a vicious cycle of mutual reinforcement (Fuzibet et al., 1988; Lindoso et al., 2009). Though the parasite is sensitive to humoral defence mechanisms, its intracellular habitat offers almost complete protection. Only if the macrophages are activated, the

parasite may be killed and degraded by the host cell (Olliaro et al., 1993; Ram et al., 1992; Braconier and Miomer, 1993; Badirzadeh et al., 2020). Moreover, opportunistic fungal infections, mainly caused by the species of *Candida*, *Cryptococcus*, and *Aspergillus*, are life-threatening in immuno-compromised patients (with AIDS, cancer, visceral leishmaniasis, or organ transplant) (Badiee et al., 2014). Macrophage activation and destruction of the intracellular parasite may be a potent mode of therapy against microbial infections like leishmaniasis and related co-infections. Laboratory experiments showing immunomodulatory activities in many plant extracts (Khoshzaban et al., 2011) attract the attention of researchers to test the natural resources for their microbicidal effects which are nontoxic, less costly, and more effective.

1.5. Objective

The present work aims to explore the indigenous knowledge of our rich culture and community and use the natural resources to fight against infectious microbial agents, stress and hazards caused by adverse environmental conditions of present time. The focus is also on search of active novel molecules and their mode of action to offer a new dimension to the modern science of pharmacology, drug designing and therapeutic approach. So, the basic objectives of the present study are -

1. Ethno-botanical studies on plant species used as antileishmanial and antifungal agents among tribal population of North Bengal.

2. *In vitro* screening of potential plant preparations for antileishmanial and antifungal activities.

3. Purification and characterization of bioactive molecules from plant extracts.

4. Evaluation of plant extract under *in vivo* condition in suitable animal model.

5. Study the immunomodulatory role and possible side effects, if any, of the purified substance.

2.1. Literature review

Greek words 'Ethnos' and 'Botane' (meaning 'people' and 'herb' respectively) make the word ethnobotany. The term ethnobotany was coined in the year 1895 by John. W. Harshberger, an American taxonomist, who defined it as 'the study of the utilitarian relationship between human being and vegetation in their environment' (Choudhary et al., 2008). Now it is defined more precisely as 'the study of the interaction between plants and people with a particular emphasis on traditional tribal culture' (Mesfinet al., 2013). Ethnobotanical studies are conducted to explore the traditional knowledge and plants or natural resources used for human benefits and for therapeutic purposes.

2.1.1. The Indian heritage of using medicinal plants

In India the medicinal use of plants is as old as civilization itself, and is a part of our cultural tradition. Ancient religious literature has mentioned the great heritage of treating human and domestic animals with herbs. Several ethnic indigenous groups in India possess vast knowledge and experience of using medicinal plants and in their own way (Jain, 1994; Kala, 2005). People of olden times were wise enough to have the knowledge of usage and preservation of natural resources and plant kingdom in sustainable manner, and prevention of their extinction by offering such natural resources divinity, connecting them with customs and rituals, and growing them nearby (Kala et al., 2006). The knowledge of medicinal plants is based on interactions, observations and experiences; those are confined in the indigenous population verbally, generated, preserved and carried out generation after generation as a secret and community specific functional knowledge. Very few literary evidence has depicted proper identification of characteristics and utility of medicinal plants which are used year after year by the people.

2.1.2. Conservation of plant resources

In recent times, the pharmaceutical world has understood the importance of use of natural resources to avoid and treat different life threating diseases. Efforts of cultivation and conservation of naturally growing medicinal plants are made worldwide to ensure uninterrupted supply of good quality raw drugs which are pre-requisite to invent new biomolecules and open new lines of

treatment to the modern world. In developing countries, there is an increasing attempt to incorporate traditional medicines, especially herbal preparations, in the local health care systems. Many modern researchers are involved today to explore the huge potential of ethnobotanical knowledge for treatment of various diseases (Dutta and Dutta, 2005; Jain et al., 2010; Jeyaprakash et al., 2011). However, the ethnomedicinal plants are under threat due to deforestation, overgrazing and reckless utilization, indicating the urgent need of their conservation. Conservation of biological resources as well as their sustainable use is important in preservation of traditional knowledge (Payyappallimana and Fadeeva, 2013).

2.1.3. Documentation of traditional knowledge

Study on plants is part of history in India. Relevant evidence has been traced in Vedic literature, Charak, Shusruta and Ayurveda. Except all the written evidence, huge information about plants and their therapeutic uses are traditionally carried on verbally generation after generation among indigenous people in different tribal communities. In modern era, scientists have shown efforts to assimilate this traditional knowledge and explore the vast botanical resources in India to reveal a new direction to the science of medication. Scientific researches are also being initiated to explore, preserve and conserve the indigenous line of medication from the end of Government of India and different non-Government or educational bodies throughout the country. Traditional Knowledge Digital Library (TKDL) in one of the major initiatives taken by Government of India to preserve all traditional knowledge on Ayurveda, Oname, Siddha and Yoga, to prevent the misuse of this knowledge and to protect it from exploitative activities such as bio piracy or unethical patents (https://www.csir.res.in/documents/tkdl). In recent times, several such investigations have been made on traditional medicine and medicinal plants in different places throughout India. This includes Meghalaya (Rao, 1981); Rajasthan (Katewa et al., 2003); Rajasthan (Choudhary et al., 2008); Himachal Pradesh (Prakash and Aggarwal, 2010); Arunachal Pradesh (Das and Tag, 2006); Arunachal Pradesh (Khongsai et al., 2011) and Haryana (Yadav and Bhandoria, 2013).

2.1.4. Ethnobotanical studies from North East India

North-East India, comprising of seven states *viz*. Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tripura, is the hot spot for wild variety of medicinal plants. More than 250 tribes of different ethnic groups with distinct cultural entities inhabit this region, who have immense knowledge on forest, plants and there uses (Mao and Roy, 2016).

There are studies on the use of different plant parts by different tribal communities in Assam (Shankar et al., 2012). Traditional herbal treatment of jaundice (Borthakur et al., 2004; Bora et al., 2012), malaria (Paul et al., 2013), or anaemia (Nath and Choudhury, 2009) are reported in different ethnomedicinal studies of present time. Even some studies reveal use of different plant species by tribal population in Assam, for pest control during harvesting and post-harvest storage (Majumdar et al., 2013). Different ethnobotanical studies in Manipur on tribes like Mao, Kabui, Meitei, Tang Khul-Naga, Paite, Thadou and Zou (Mao and Roy, 2016) show extensive use of plants in curing diseases, agriculture, pest control, or in dye preparation. There are reports on potential anticancer medicinal plant (Mao, 2002) and herbal vapour therapy (Ningombam et al., 2012). Singh et al., 2012 reported about using *Stemona tuberosa* Lour., a wild medicinal plant, for treating malaria by the Garo tribe in Meghalaya. Typical usage of plants as agricultural season indicators is a very popular practice in tribes of North-Eastern states (Jeeva et al., 2006; Mao and Hynniewta, 2011). Lalramnghianglova (2011) reported on knowledge of tribal population in Mizoram on use of plants on cattle for the treatment of snake or insect bite.

Nagaland is the land of very primitive tribes like Ao, Angane, Lotha, Konyak, Rengma, Phom, Sangtan, Zeliang, Yaumchungar and Sum, but very few studies are there on these population. Pioneeringethno-medicinal study by Rao and Jamir (1982), and a more recent study by Kichua et al. (2015) revealed immense knowledge of these indigenous population on medicinal and regular use of different wild plant species. Darlong, Halaim and Reang are some of the tribal communities residing in the state of Tripura. Report on use of traditional

antifertility active plants (Das et al., 2014) shows the diversified utilization of natural resources in these indigenous population.

2.1.5. Ethnobotanical studies in Jalpaiguri, West Bengal

In West-Bengal, very few ethnobotanical studies have been conducted till date. 'Study on medicinal plant used by Rajbangshi community in Cooch Behar' by Sushmita Roy published in 2015 is one of such few studies. Other few studies done by Sinhababuand Banerjee (2013) and Hussain and Hore (2007) at Darjeeling are also reported.

Jalpaiguri is a district in North-Bengal habitat of many tribal populations, but any ethnobotanical study has rarely been done in this area. Covered with dense forests and riverine grasslands, Jalpaiguri is the harbour of one of the richest bounties of Wild life. In Jalpaiguri district, we can see heavy concentration of all the major Chhotanagpur tribes, the hill tribes and many other tribes like the Mech, Toto, Rabha, Garo, Magh, Hajang etc. The later mentioned tribes are the ones which are usually found in the states of North-east India (Roy, 2005). All these various tribes brought along with them their culture and beliefs. Although Jalpaiguri is known for its rich floristic diversity, no serious scientific research work was done on medicinal plants in this area before 90's. Even those earlier studies either documented all the plants with medicinal value in the wild (Nandi, 2005) or tabulated the medicinal plants used by a few tribes in a particular zone of the district (Chaudhuri et al., 1982; Das et al., 1983). So, we designed this study to survey the use of medicinal plants among tribal people of entire Jalpaiguri district. At mid phase of this study (2014) the district has been divided in to two parts, Jalpaiguri and Alipurduar. The aim of present study is to first conduct ethnobotanical investigations in the vast areas of this district inhibited by tribal population. Based on initial findings, almost 50 plant species have been studied at the preliminary level; subsequently four medicinal plants have been chosen for further studies for antioxidant, antileishmanial and anti-fungal screening and also for phytochemical analysis. They are also analysed and purified for isolation of novel molecules.

2.2. Materials and methods

2.2.1. Study Area

The district of Jalpaiguri in West Bengal is located in the foothills of the Eastern Himalayas. At the time of initiation of the study, it was the largest district of North Bengal covering an area of 6, 245 km^2. It is situated between 26°16' and 27°0' North latitudes and 88°4' and 89°53' East longitudes. On 2014, Jalpaiguri district was divided in to two parts, Jalpaiguri and Alipurduar. The entire topography of the area is crisscrossed with several rivulets and rivers, and is lying between the Sikkim – Darjeeling Himalayas and the Gangetic West Bengal. Main forest cover comprises of semi-moist deciduous vegetation. Sal forest is predominant with pockets of various other types like evergreen forests, savannahs, riverain forest and swamps (Fig. 2.1).

The climate of the district is characterized as sub-tropical and humid type. The average annual humidity in the district is of 82%. The average maximum temperature is 37°C and the average minimum temperature is 6°C. The average annual rainfall of this district is about 3300 mm, with an average of more than 100 rainy days.

2.2.2. Data Collection

A simple but very basic work plan was adopted for this survey work. At first various government departments like Forest Department, Department of Backward Classes, Panchayat Offices etc. of the erstwhile Jalpaiguri District were approached for getting information about checklist of forest villages with relevant demographic information, and to get detailed information about tribal population and tribal villages of the district. Relevant information was also collected from various websites. On the basis of that information a plan of work was chalked out for our survey. Then extensive survey was conducted during the period of July, 2012 to December, 2012 and some of the places were revisited again during July to December of 2013.

During field survey, detailed information on types, traditional method of preparation, mode of consumption, shelf life and ethnic value of the medicinal plants were collected from elderly persons and traditional healers of tribal

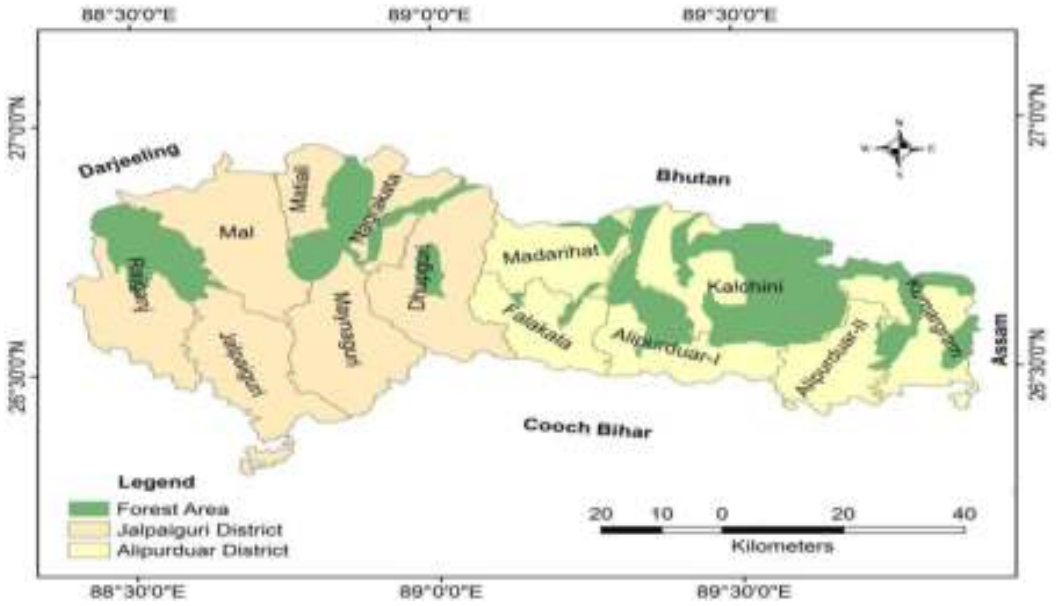

Figure: 2.1. Geographical map of Jalpaiguri and Alipurduar districts (area of study)

(Prepared using ***ArcGIS 10.3.1*** *software*)

communities. Information was collected through well-structured pre-tested questionnaires and discussions among the informants in their local language.

The plant specimens were collected as directed by the resource persons in flowering and fruiting conditions. Digital photographs of the plants were also taken. Collected specimens were dried, chemically treated, and herbarium sheets were prepared for possible identification. Identifications were made using available literature (Prain, 1903; Bhattacharyya, 1997). The herbarium sheets were deposited in the Herbarium of Ananda Chandra College and in West Bengal State Council of Science and Technology Department.

2.3. Results

In this study 50 plant species of 30 families (Table 2.1) were found to be used for medicinal purposes by various tribes of Jalpaiguri and Alipurduar districts. Most of this knowledge was transmitted from one generation to the next. The traditional medicine-men are integral part of the community and take care of the common ailments of the folks in their home settings. The reported plants were arranged according to their scientific name, family, local status on availability, parts used, therapeutic uses and method of usage of herbal preparations. However, we were not able to collect information about method of usage of herbal preparations in all cases; because many of the traditional healers believe that upon disclosure of the knowledge (particularly to urban people) the effect of medicine will diminish.

The tribal populations use these 50 species of medicinal plants to treat 56 various types of physical ailments. Most of the plants reported in this study were collected from natural vegetation (76%) and few of them from home gardens (24%). Of the 30 families, Lamiaceae is represented by the highest number of species (6 species), followed by Apocynaceae which has five species and Asteraceae which has four species. Acanthaceae, Euphorbiaceae, and Malvaceaeis represented by 3 species in each family. Amaranthaceae, Fabaceae and Rutaceaehave two species each. Ancardiaceae, Apiaceae, Annonaceae, Cucurbitaceae, Cleomaceae, Cyperaceae, Caryophyllaceae, Heliotropiaceae, Meliaceae, Myrtaceae, Moringaceae, Menispermaceae,

Onagraceae, Oleaceae, Pedaliaceae, Plantaginaceae, Plumbaginaceae, Phyllanthaceae, Rutaceae, Solanaceae and Zingiberaceae are represented by only one species each.

Table: 2.1. Medicinal plants used by tribal population of districts of North Bengal (Jalpaiguri and Alipurduar)

Name of Plants (Common name/Local name)	Family	Local Distribution	Use
Ageratum conyzoides L. (Uchunti)	Asteraceae	Common wild	Leaf used to treat cut
Alstonia scholaris (L.) R. Br. (Saptparni)	Apocynaceae	Common wild	Bark extract used to treat intestinal worm; bark juice used to treat fever
Amaranthus spinosus L. (Kantanotya)	Amaranthaceae	Common wild	Leaf used to treat anaemia; root paste applied on stomach to treat urinary disorder
Andrographis paniculata (Burn.f.) Wall. ex Nees (Green Chireta)	Acanthaceae	Commonly cultivated	Leaf extract to treat jaundice; dried leaf extract to treat body pain
Azadirachta indica A. Juss. (Neem)	Meliaceae	Common wild	Extract used to treat jaundice; dried leaf extract to treat body pain
Calotropis gigantea (L.) W.T. Aiton	Apocynaceae	Common wild	Leaf used to treat Rheumatism
Calotropis procera (Aiton) W.T. Aiton (Rubber-bush)	Apocynaceae	Common wild	Leaf used to treat rheumatism and cuts; latex used in dog bite
Centella asiatica (L.) Urb. (Thankuni)	Apiaceae	Common wild	Leaf used to treat diarrhea and dysentery; leaf extract to treat eczema
Chenopodium album L. (Bathua)	Amaranthaceae	Common wild and cultivated	Leaf used to treat intestinal worm
Clausena excavata Burm. f. (Agnijol)	Rutaceae	Common wild	Used in abdominal pain, detoxifying agent and also in snake bite
Cleome rutidosperma DC. (Fringed spider flower)	Cleomaceae	Common wild	Seeds used in menstrual problems
Coccinia grandis(=*indica*) (L.) Voigt. (Tindora)	Cucurbitaceae	Common wild	Leaf used to treat hypertension
Croton bonplandianum Baill. (Ban tulsi)	Euphorbiaceae	Common wild	Leaf extract used to treat cut and wounds
Curcuma longa L. (Turmeric)	Zingiberaceae	Commonly cultivated	Rhizome paste applied in cuts and wounds
Cyperus rotundus L. (Nutgrass)	Cyperaceae	Common wild	Root extract used to treat cuts

Name of Plants (Common name/Local name)	Family	Local Distribution	Use
Dalbergia sissoo Roxb. ex DC. (Indian rosewood)	Fabaceae	Cultivated for timber	Leaf juice used to treat stomach disorder
Drymaria diandra Blume (West Indian Chickweed)	Caryophyllaceae	Common wild	Dried leaf smoked to treat cough
Eclipta prostrata (L.) L. (Bhringaraj)	Asteraceae	Common wild	Leaf extract used to disinfect cuts and wounds
Emblica officinalis Gaertn. (Amla)	Phyllanthaceae	Common	Fruits used to enhance digestion, health and intellect, strengthen the heart, purify bloody eyes. Also used against constipation, fever and cough and promote longevity.
Eupatorium odoratum L. (Christmas bush)	Asteraceae	Common wild	Fresh leaf juice externally applied to cuts and wounds to stop bleeding
Euphorbia hirta L. (Asthma weed)	Euphorbiaceae	Common wild	Leaf used to treat menstrual problems and extract used to stop irregular periods
Glycosmis arborea (Roxb.) DC. (Orange Berry / Ash-sheora)	Rutaceae	Uncommon wild	Root powder used in fever, hepatopathy, eczema, skin diseases, wounds and liver problems
Gmelina arborea Roxb. (Gamhar)	Lamiaceae	Commonly cultivated for timber	Root extract used in stomach disorder
Heliotropium indicum L. (Hatisura)	Heliotropiaceae	Common wild	Juice of plant used to treat eye infection
Hibiscus rosa-sinensis L. (Hibiscus Joba)	Malvaceae	Commonly cultivated	Leaf used to treat burning sensation, fatigue and skin diseases
Hygrophila schulli (Buch.-Ham.) M.R. Almeida & S.M. Almeida (Gokulakanta)	Acanthaceae	Restricted wild	Leaf extract used to treat anaemia
Justicia adhatoda L. (Vasaka)	Acanthacea	Common in wild and also cultivated	Leaf juice taken as expectorant to treat chronic bronchitis, cough and cold
Leonurus sibiricus L. *(Guma)*	Lamiaceae	Common	Anti-inflammatory, anti-diarrhoea. Leaf extract used in haemorrhage, weakness.
Leucas plukenetii (Roth) Spreng. syn *L. aspera* (Willd.) Link (Dondokolosh)	Lamiaceae	Common wild	Leaf extract used in jaundice
Ludwigia perennis L. (Paddy Clove)	Onagraceae	Common wild	Boiled plant extract used externally to reduce fever
Malvaviscus arboreus Cav. (Lanka Joba)	Malvaceae	Common cultivated	Flower buds are used to stop bleeding

Name of Plants (Common name/Local name)	Family	Local Distribution	Use
Mangifera indica L. (Mango)	Ancardiaceae	Common wild and cultivate	Bark used for the treatment of loose motion
Moringa oleifera Lam. (Drumstick)	Moringaceae	Common in wild and cultivated	To get relief from stress and pain, nutrient supplement
Nyctanthes arbor-tristis L.(Seuli)	Oleaceae	Common wild	Juice of leaf is used as antidote to reptile venom, laxative digestive element and diuretics
Ocimum basilicum L. (Ram tulsi)	Lamiaceae	Cultivated in marshy places	Seed paste applied against stings of wasps, bees and other venomous insects
Ocimum gratissimum L. (Tulsi)	Lamiaceae	Uncommon wild	Leaf extract applied on cut to stop bleeding
Plumbago zeylanica L. (Leadwort)	Plumbaginaceae	Uncommon cultivated	Root used to treat high fever; leaf used to treat cut
Polyalthia longifolia Var.*pendula* (Devdaru)	Annonaceae	Common	Powder of stem bark is given orally to cure diarrhea and in the treatment of gout.
Psidium guajava L. (Common Guava)	Myrtaceae	Common in wild and cultivated	Bark used as contraceptive; young leaf used to treat stomach pain
Rauvolfia serpentina (L) Benth. exKurz (Sarpagandha)	Apocynaceae	Rare wild	Root extracts used in stomach pain and to treat intestinal worm
Rauvolfia tetraphylla L. (Devil-pepper)	Apocynaceae	Uncommon cultivated	Root extract used in stomach pain and to treat intestinal worm
Ricinus communis L. (Castor oil plant)	Euphorbiaceae	Common wild	Seed oil is used as painkiller
Scoparia dulcis L. (Sweet-broom)	Plantaginaceae	Common wild	Leaf juice used against stomach disorder
Sesamum indicum L. (Sesame)	Pedaliaceae	Cultivated	Fried fruit taken in case of fever
Sesbania grandiflora (L.) Pers. (Humming bird tree)	Fabaceae	Cultivated	Extract of leaf used in jaundice
Sida acuta Burm. f. (Clock Plant)	Malvaceae	Common wild	Root extract used against blood urea, boils and nephritis
Solanum indicum L. (Brihati)	Solanaceae	Common wild	Seed applied on teeth and gum to treat infection
Stephania glandulifera Miers. (Jaluko)	Menispermaceae	Common wild	Root used in headache
Vitex negundo L. (Nishinda)	Lamiaceae	Common wild	Extract of leaf used against whitening of hair and memory loss
Xanthium strumarium L. (Cocklebur)	Asteraceae	Common wild	Used as anti rheumatic, appetizer and laxative.

Among different plant parts used for the preparation of medicine, leaf (33.9%) was found to be the most frequently used plant part followed by root (18.2%), whole plant (14.7%), seed (10.4%), rhizome (9.5%), fruit (9.5%), latex (3.4%), flower (3.4%), and in rare occasion, fleshy scale, flower bud, root bark and only stem. Different plant parts of a single medicinal plant may have multiple medicinal use and importance. An important example of such plant is *A. spinosus* (Kantanotya).Additionally, several plants were used for the treatment of multiple diseases. *G. arboroea* is used for the treatment of six diseases; *H. rosa-sinensis* for the treatment of four diseases; *S. acuta* and *V. negundo* are used for the treatment of three diseases each; and 14 other plants are used to treat two diseases.

Most of the ethno botanical studies confirmed that leaves are the major portion of the plant used in the treatment of diseases. The methods of preparation of the botanicals fall into four categories, viz. plant parts applied as a paste, juice extracted from the fresh parts of the plant, and plants used to prepare decoction in combination with water and powder made from fresh or dried material.

2.4. Discussion

Almost in every corner of the Jalpaiguri and Alipurduar districts, plants were found to be used as medicines. The study reveals unquestionable dependency of tribals of Jalpaiguri and Alipurduar districts on medicine-men called as gunin. They are integral part of the medication process and provide treatment to the members of the tribe in their home setting depending primarily on medicinal plants. There is a conscious effort to preserve this traditional knowledge exclusively within their own community. The herbal preparations made from the traditional medicinal plants were mostly used to treat common ailments prevalent in this region like cuts and wounds, cough and cold, skin diseases, pain and inflammation, bone fracture and stomach ache and abdominal disorder, for treatment of jaundice and liver problems, and fever. Some frequently used plant species used by local tribal population of the studied area were found to be in common with those used by the tribal population of Tripura (Debbarma et al., 2017), Nagaland (Bhuyan et al., 2014),

Assam (Bora et al., 2012), Rajasthan (Choudhary et al., 2008) and Bangladesh (Khatun and Rahman, 2019). In the present study, 50 plant species of 30 families were listed, which the tribal population use in their regular health care in different forms such as raw extract of the whole plant, part of plant or in dried form. It was found that leaves are the most frequently used plant parts, followed by roots. This finding is in agreement with previous studies in different parts of India and Bangladesh (Debbarma et al., 2017; Bhuyan et al., 2014; Bora et al., 2012; Khatun and Rahman, 2019).

Use of medicinal plants among the tribals of Jalpaiguri and Alipurduar districts in treatment of various diseases has definitely been outnumbered today by the allopathic treatment. But still their dependence on plants of their surroundings to get relieved from day-to-day ailments is unquestionable. However, all persons who are using plants as medicine, complain about the gradual fading out of many of the medicinal plants from their surroundings. It is presumable that availability of such plants in the vicinity may increase the use of plants as medicine. So, possibilities of propagation, preservation and cultivation of these plants in this area should be explored to achieve the goal of sustainable development. Further research on the medicinal plants mentioned in this study might provide some potential leads to fulfil the needs of search for bioactive compounds and the discovery of new drugs to fight diseases. Four very common and well-known medicinal plants, *R. serpentina* (sarpagandha), *M. oleifera* (drumstick), *N. arbor-tristis* (seuli or night jasmine) and *C. excavata* (agnijol), whose utilizations were found to be vast and versatile among the above said population, were selected for further study both in laboratory set up and on animal models.

3.1. Literature review

The plant kingdom is a treasure house of potential therapeutic compounds. The use of herbal medicines for the treatment of diseases and infections is as old as mankind. In the last few decades, there has been an exponential growth in the field of herbal medicines. They are getting popularized in developing and developed countries owing to its natural origin, lesser side effects and cost-effective qualities (Khan and Ahmad, 2019). The World Health Organization supports the use of traditional medicines provided they are proven to be efficacious and safe (WHO, 2020a). Medicinal plants have been used as a source of remedy against many pathological conditions since ancient times in India. The present study aims to screen the plants short listed during ethnobotanical studies and also well known as medicinal plants, for their phytochemical constituents and antioxidant properties. The present study also focuses on establishing a relationship between phytochemical content of selected plants with their antioxidant potentiality. The review presents the report of a literature search on importance of different phytochemicals. Additionally, an overview of four medicinal plants, *C. excavata*, *M. oleifera, N. arbor-tristis* and *R. serpentina* selected for experimental studies are described here.

3.1.1. Plants as source of therapeutic compounds

Traditional medicine systems consist of large number of plants with medicinal and pharmacological importance and hence represent an invaluable reservoir of new bioactive molecules (Sharma et al., 2013). In the recent years, awareness about the importance of these compounds is increasing as they are the richest bio-resource not only of traditional therapeutic systems and folk medicines, but also of modern medicines, food supplements, nutraceuticals, pharmaceutical intermediates and chemical entities for chemical drugs. Moreover, compounds from the plants are easily available, less expensive, efficient and proven to have no side effects (Zaffer et al., 2015). In different previous studies, some plants have been selected for examination and have proven therapeutically effective for development of new drugs such as anticancer drugs (Dewick, 1996; Ali et al., 2000), antimicrobial drugs

(Phillipson and Wright, 1996; Sunthitikawinsakul et al., 2003), antihepatotoxic (Hong et al., 2015) compounds. Some organic compounds isolated from medicinal plants, which are primary or secondary metabolites, provide definite physiological action on the human body. So, to explore the therapeutic value of a plant and to test its prospect as a candidate for further research, aiming drug development, analysis of its phytochemical constituents, primary and secondary metabolites content in different parts like leaf, stem or root is the foremost requirement.

3.1.2. Phytochemicals or secondary metabolites

In addition to basic nutrients like carbohydrate, protein, fat, vitamins and minerals, analysis of plant materials shows presence of some phenolic compounds, tannin, saponin, alkaloids, flavonoids etc. These materials are known as secondary metabolites, which are synthesized by plants as intermediate or by-products of their metabolism (Zaffer et al., 2015). Studies show that secondary metabolites confer the therapeutic values to the plants. So, phytochemical research based on ethnopharmacological information may prove to be an effective approach to discover medicinal, therapeutic and anti-infective agents from plants (Chhetri et al., 2008).

Primary and secondary metabolites of plant metabolism, collectively called phytochemicals, are synthesized in or during different metabolic pathways (Fig. 3.1). Primary metabolites are basic needs of life, synthesized for energy, food or defence. Secondary metabolites occur in low quantities, have no obvious role in plant growth and development, but have significant ecological function as they serve to reduce the impact on insect and animal predation or provide protection against microbial attack. They have significant economic and medical value and have been found to be used in antiquity as folk remedies, soaps and essences. They include drugs, dyes and feed stocks for chemical industries (gum, resins and rubber) and variety of substances used to flavour food and drink. These bioactive substances include tannins, alkaloids, carbohydrates, protein, glycosides, terpenoids, steroids and flavonoids (Mann et al., 1978; Edoga et al., 2005). They are widely used in the human therapy,

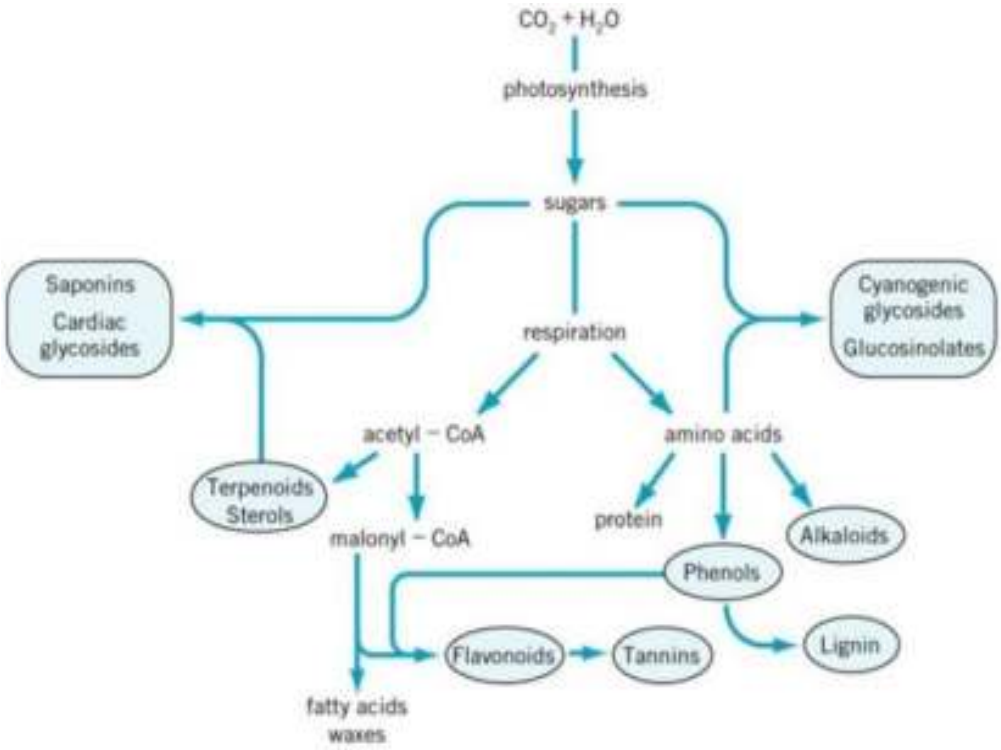

Figure: 3.1. Secondary metabolites are derived from primary metabolites

as well as in veterinary, agricultural, scientific research and many other different areas of human interest (Vasu et al., 2009). Moreover, the content of different secondary metabolites and their biological activity are shown to differ significantly with extraction procedure, solvent, time of sample collection, environment, place etc. (Shan et al., 2005).

3.1.3. Phenolic compounds

Among the secondary plant metabolites, phenolic compounds such as flavonoid, phenolic acids, tocopherols etc. are among the most ubiquitous groups (Singh et al., 2007). The phenolic compounds are of considerable interest and mostly studied in recent time. These are found to be antioxidant, antimicrobial, antiviral, anti-inflammatory, and anti-cancerous (Ignat et al., 2011). It is found that, phenolic compounds are therapeutically active on humans in various ways. More than 8000 poly phenolic compounds including 4000 flavonoids have been identified (Harborne et al., 1999).

In response to microbial infection, plants synthesize hydroxylated phenolic substances.Studies revealed that plants rich in phenolic compounds possess biological properties such as anti-apoptotic, anti-ageing, antioxidant, anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, cardiovascular protective and endothelial function improvement properties, and inhibit angiogenesis and cell proliferation activities (Han et al., 2007; Brown and Rice-Evans, 1998; Krings and Berger, 2001; Ali et al., 2008).

3.1.4. Flavonoids

Flavonoids have antioxidant, metal chelating potentials (Tsao and Yang, 2003). Studies show that if consumed regularly, flavonoids cause reduction in the incidence of prostate cancer (Jaganathan et al., 2014) and breast cancer (Yiannakopoulou, 2014). Isoflavones have structural similarities with ß-esterdiols, so it is referred to as 'phytoestrogen'. They are effective in prevention of atherosclerosis (Klejdus et al., 2007). Tannins interfere with protein synthesis by binding with proline rich proteins. Studies show that tannins have diverse biological forms and activities too, they work as metal ion chelator, protein precipitant and antioxidants (Hagerman, 2002).

3.1.5. Lignins, saponins and others

Lignins, produced by oxidative dimerization of phenylpropene in plant in response to pathogenic attack (Bavaresco, 2003), have potential application in cancer chemotherapy and pharmacological purposes (Saleem et al., 2005). The plant extracts containing saponins have anti-inflammatory (Just et al., 1998) and hemolytic properties (Okwu, 2004).Studies show that lycosides lower the blood pressure (Nyarko and Addy, 1990). Steroids are very important compounds, having relationship with sex hormones (Okwu, 2001). They also have antibacterial activities (Epand, 2007; Taleb-Contini et al., 2003). Alkaloids have been used for centuries for their medicinal value. They have cytotoxic (Nobori et al., 1994), analgesic, antispasmodic and antibacterial (Stray et al., 1998; Okwu and Okwu, 2004) properties.

3.1.6. Antioxidants from plants

Reactive oxygen species (ROS), super oxides like hydrogen peroxide (H_2O_2) and free radicals (hydroxyl group, $^{\cdot}OH$, O_2^{-}) are produced during normal cellular metabolism, but rapid production of such molecules can cause damage to body tissue and bio molecules, leading to cancer, asthma, diabetes, inflammatory disorder, neurodegenerative problem or premature aging (Young and Woodside, 2001). These free radicals and super oxides may biodegrade membrane lipids, cellular proteins and DNA, leading to several diseases or even death (Pourmorad et al., 2006). The lipid or fatty acid destruction causes rancidity and rotting of food items or ageing of biological tissues. Though biological systems have their own mechanisms to neutralize these naturally produced free radicals and maintain equilibrium, some external factors like environmental pollutant, radiation, chemical and physical stress may disrupt the natural procedure. So, in oxidative stress, there is an imbalance between generation and elimination of ROS and RNS (Reactive Nitrogen Species) which may cause critical diseases and aging (Serafini, 2006).

Antioxidants are the substances which delay or inhibit oxidation of oxidizable substrate by neutralizing free radical (Antolovich et al., 2002). Antioxidants work in two ways, 1. Primary antioxidants donate electron to free radical, 2. Secondary antioxidants remove ROS molecules by quenching the chain

initiating catalysts. In biological system they may exert their effects by electron donation, chelating metal ion or by regulating gene expression (Krinsky, 2002). Antioxidants may be enzymatic, like super oxide dismutase, catalase, glutathione synthase, or non-enzymatic, like, ascorbic acid, glutathione, melatonin, tocopherol, uric acid etc. Number of phenolic antioxidants like butylate hydroxytoluene (BHT), butylated hydroxyanisole (BHA) are being used for years in food industries) to stop food rotting (food containing oil or fat).

Table: 3.1. Phytochemicals or secondary metabolites from plants used for therapeutic purposes and their mechanisms of action

Phytochemicals	Activity	Mechanism of action
Quinones	Antimicrobial	Binds to adhesins, complex with cell wall, inactivates enzymes
Flavonoids	Antimicrobial Antidiarrheal	Complexes with cell wall, binds to adhesins, inhibits release of autocoids and prostaglandins, inhibits contractions caused by spasmogens, stimulates normalization of the deranged water transport across the mucosal cells, inhibits GI release of acetylcholine
Polyphenols and Tannins	Antimicrobial Antidiarrheal Anthelmintic	Binds to adhesins, enzyme inhibition, substrate deprivation; complexes with cell wall, membrane disruption, metal ion complexation; Makes intestinal mucosa more resistant and reduces secretion; astringent action Increases supply of digestible proteins by animals by forming protein complexes in rumen; interferes with energy generation by uncoupling oxidative phosphorylation
Coumarins	Antiviral	Interaction with eucaryotic DNA
Terpenoids and essential oils	Antimicrobial Antidiarrheal	Membrane disruption Inhibits release of autocoids and prostaglandins
Lectins and Polypeptides	Antiviral	Blocks viral fusion or adsorption, forms disulphide bridges
Glycosides	Antidiarrheal	Inhibits release of autocoids and prostaglandins
Alkaloids	Antimicrobial Antidiarrheal Anthelmintic	Intercalates into cell wall and DNA of parasites inhibits release of autocoids and prostaglandins, possess anti-oxidating effects, thus reduces nitrate generation which is useful for protein synthesis, suppresses transfer of sucrose from stomach to small intestine, diminishing the support of glucose to the helminthes, acts on CNS causing paralysis
Saponins	Antidiarrheal Anticancer Anthelmintic	Inhibits histamine release *in vitro,* possesses membrane permeabilizing properties, to vacuolization and disintegration of teguments
Steroids	Antidiarrheal	Enhance intestinal absorption of Na+ and water.

But carcinogenic nature of some synthetic antioxidants along with high volatility and/or instability shifted the attention of both manufacturers and researchers towards the search of antioxidants of natural origin (Lourenco et al., 2019).

Plant secondary metabolites like phenolic compounds and flavonoids are found to be working as antioxidants in various studies. It has been reported that there is a reciprocal relationship between dietary intake of antioxidant rich food or plants and incidence of disease in human. Strong antioxidant properties are found in berries, cherries, citrus fruits, olives, green tea etc. Green tea contains up to 30% of its dry weight as phenolic compounds (Rio et al., 2013) and is a good antioxidant. Attempts have been made to explore antioxidant properties in regular food and vegetables like potato, spinach, legumes, tomatoes and seasonal fruits (Furuta, 2010; Wang, 2008) with positive results.

3.1.7. Overview of selected medicinal plants

3.1.7.1.*Clausena excavata*

Common Names: Agnijol, Pink lime berry, Cama, Cemama, CerekHitam, KemantaHitam, Seemere etc. *C. excavata* (Fig. 3.2) is a wild woody shrub (6 m-1m) belonging to Rutaceae family (Burkill, 1935). It is a terrestrial, tropical plant, with bushy appearance. The native distribution of the plant covers an area stretching along the Himalayas (Manosroi et al., 2005). India, Myanmar, South China, Taiwan, Philippines, Indonesia are the countries where at least fourteen species of genus *Clausena* are found growing wildly (Shier, 1983). This evergreen, flowering seed plant (angiosperm) can withstand heavy pruning and are free from pests and diseases, so they grow easily (Swarbrick, 1997).

3.1.7.1.1. Traditional use and established therapeutic properties of *C. excavata*

C. excavata is well-known from a long time for its medicinal value. Traditionally it is used in treatment of abdominal pain, as a detoxifying agent, and also in snake bites (Ridley, 1922). Tamil people use it as potherb (Arbab et

al., 2012). The root extract is used to treat ulceration of nose; decoction of root is consumed for bowel complaints for years. Decoction of leaves is also given after child birth (Wiart et al., 2004; Arbab et al., 2012). It is found from previous studies that the leaves of *C. excavata* are also used for cold and even in malaria, and root in powdered form is applied for decayed teeth whereas stem is consumed by indigenous people in colic with or without diarrhoea (Arbab et al., 2012). Reports of such a diversified use of *C. excavata* has been motivating traditional scientists and researchers to screen extracts of leaf, root and different parts of the plant for their biological activity. Table 3.2 shows some of such biological studies. In addition, table 3.3 shows in detail the therapeutic activities of various phytochemicals, secondary metabolites and essential oil, isolated from *C. excavata.*

Table: 3.2. Biological activity of the crude extracts of different parts of *C. excavata*

Plant Part	Extract	Biological Activity	Reference
Leaf	70% ethanol	Anti-hyperglycaemic, Anti-rhinitic	Sakong et al., 2011
Stem	Methanol	Oral toxicity	Puongtip et al., 2011
Leaf	Methanol	Antioxidant	Guntupalli et al., 2012
Root	Acetone	Cytotoxicity	Sharif et al., 2011
Wood	Aqueous	Immunomodulating	Manosroi et al., 2005

Table: 3.3. Phytochemicals isolated from *C. excavata* and their established therapeutic activities

Phytochemical	Classof phytochemical	Plant part	Biological activity	Reference
Clausena D and B	4-Prenyl carbazole alkaloids	Stem bark	Anticoagulant	Wu and Huang,1992
Clausendin nordentatin Clausarin, dentatin-cumarin	Pyronocoumarin	Root bark	Anticancer antibacterial	Albaayit et al., 2021; Wu and Furukawa, 1982)
Clausines B, E, H, I and K	Carbazole	Stem	Inhibit platelet aggregation in rat	Wu et al.,1996
Clausenamine-A	Biscarbazoles	Stem and root bark	Cytotoxic activity	Zhang and Lin, 2000
Mukonal	Limonoid	Stem bark	Antifungal	Takemura et al., 2000
Xanthoxyletin Murrayanine	Carbazole derivatives	Leaf	Antibacterial	Sunthitikawinsakul et al., 2003
Clausenolide-1 ethyl ether	Limonoid	Rhizome; root	HIV-1 inhibitort activity	Sunthitikawinsakul et al., 2003
Sansoakamine	Carbazole alkaloid	Stem	Anti-malarial	Lastra-Gonzalez, 2005
Clausine-TY Clausine-H Clausine	Carbazole Alkaloid	Stem; Bark	Cytotoxicity	Taufiq-Yap et al., 2007
Clausine-E and 2, 7 Dihydroxy-3 formyl-1 carbazole	Carbazole alkaloid	Leaf; Stem	Anti topisomerase II	Xin et al., 2008
Clausine	Carbazole alkaloid	Stem; Bark	Anti-proliferative	Zain et al., 2009
Clausendin	Pyrano-coumarins	Root	Anti-HIV	Kongkathip et al., 2010
Xanthyletine and Clausenarin	Coumarins	Root; Bark	Cytotoxicity	Sharif et al.,2011
Excavarin	Coumarins	Leaf	Antifungal	Saha et al., 2012

3.1.7.2. *Moringa oleifera*

Common Names: Subhanjana, Sainjana, Sojna, Suragavo, Shevga, Mulaga, Raktaka, Drumstick tree, Horseradish tree, Banzoil tree etc.

M. oleifera (Fig. 3.3) is the most cultivated species of the monogeneric family Moringaceae, which is native to India, the Himalayan tract, Pakistan, Bangladesh, and Afghanistan. *M. oleifera* is a perennial, softwood fast-growing, deciduous tree; its height may be up to 10-12m and diameter of its stem may be up to 45cm. The flowers are fragrant and asexual, surrounded by five unequal, yellow/white petals. Flowering can be seen once in a year, between April and June. Fruits are brown, shaped like a capsule, hanging and are 20-45 cm in size.

M. oleifera is an extremely popular tree for its medicinal property. In Philippines, moringa leaves are cooked and fed to babies, so it is also called "mother's best friend" or malunggay.

3.1.7.2.1. Traditional use of *M. oleifera*

M. oleifera is widely used for its therapeutic and nutritional value. All parts of *M. oleifera* tree are edible and consumed by indigenous people. History says ancient kings used to consume *M. oleifera* leaves and fruit to maintain mental alertness and for good skin. In India, Maurian soldiers were reported to take its leaves to get extra energy and to get relief from stress and pain (Jahn, 1996). With extensive use as food and medication, *M. oleifera* gets the title "the miracle tree" from the common people. Even dried leaves in powdered form are used as nutrient supplement (Makkar and Becker, 1997).

3.1.7.2.2. Biological activity of *M. oleifera*

The Iron and protein content and bio–availability of moringa leaves are found to be very high. Studies show 100 gm dry leaves contain 29.6 gm of protein (twice than that of milk), 28.9 mg of iron, 1924.28 mg of calcium and 15620.6 IU of vitamin A (Wangcharoen and Gomolmanee, 2013). It contains 7 times more vitamin C than oranges, and 3 times more potassium than a banana (Gopalan et al., 1989). It has anti proliferative, antiepileptic, anti-inflammatory, anti-hypertensive, anti-oxidant, anti-diabetic, anti-bacterial and antifungal activities (Table 3.4). It can also control blood cholesterol. *Moringa* seed oil, called Ben oil, resists rancidity (Tsaknis et al., 1999). Several

Figure: 3.2. Plant *C. excavata*(Agnijol)

Figure: 3.3. Plant *M. oleifera* (Drumstick tree)

phytochemical or biomolecules have been isolated and identified in previous studies from different parts of *M. oleifera* plant (Table 3.5).

Table: 3.4. Therapeutic activities of crude extracts from different parts of the plant *M. oleifera*

Plant Part	Biological Activity and used for	Reference
Leaf	Inflammation urinary tract infection, herpes, simplex virus anti-hypertensive	Chuang et al., 2007; Fahey, 2005; Faizi and Siddiqui, 1992
Leaf and root	Pulmonary disease	Omino and Kokwaro, 1993
Root	Kidney pain, flatulence	Fuglie, 2001
Bark	Stomach disorder	Navie and Csurhes, 2010
Pods	Joint pain	Fuglie, 2001
Root bark with gum	Dental caries	Fahey, 2005
Flower	Common cold	Fuglie, 2001
Seeds	Inflammation	Chuang et al., 2007

Table: 3.5. Compounds isolated and identified from different parts of *M. oleifera*

Compound	Plant part	Reference
Kaempferol 3,7-diglycopyranosyl (ß- D-glucopyranosyl-(1-2) - (αrhamnopyranosyl-(1-6)-ß-d-glucopyranoside)	Leaf	Faizi et al., 1995; Bushra and Anwar, 2008
Amino methoxysulfinylpentasilfide	Pod	Faizi et al., 1998
4-hydroxybenzaldehyde O-(4-O-acetyl-α-rhanmopyranoside)	Pod	Faizi et al., 1998
3,3´,4,4´,5,5´,7 heptahydro3-0-(ß-D galactopyranose,D-glucopyranose)	Bark, leaf	Asem and Laitonjam, 2008
Niazidin	Leaf	Francis et al., 2004
Rhamnose; α-l-pyranose-form, glycoside	Leaf	Francis et al., 2004
(4-hydroxybenzyl) carbamic acid	Pod, leaf	Faizi at al., 1998; Tiwari et al., 2011
(4-hydroxybenzyl) thiocarbamic acid	Fruit	Francis et al., 2004
p-Salcylic acid	Leaf	Strohl and Seikel,1965
4-Hydroxybenzyl glucosinolate	Leaf	Fahey et al., 2001

3.1.7.3. *Nyctanthes arbor-tristis*

Common Names: Seuli, Sephalika, Parijat, Harsinghar, Manjatpu Pavelam, Night Jasmine, Jayaparvati etc.

N. arbor-tristis (Fig: 3.4) is a large shrub of height up to 10m with rough leaves and flaky grey bark. Flowers are fragmented white with orange coloured corolla producing cluster of 2-8 together, which opens at dusk and closes at dawn (Das et al., 2008). Flowering usually occurs from July to October. It is a very well-known plant in India and is native to southern Asia, from northern Pakistan to Nepal, Northern India to Southeast Thailand. It is a terrestrial woody perennial tree with a lifespan of 5-20 years.

3.1.7.3.1. Traditional uses and biological activities of *N. arbor-tristis*

The plant is traditionally used for medication of diversified diseases or problems from ancient time in India. It is reported to be used to provoke menstruation, treat skin disease and scabies (Jain and Pandey, 2016).

Table: 3.6. Therapeutic activities of different parts of *N. arbor-tristis*

Plant part	Extract	Activity	Reference
leaves and fruits	Ethanolic	Anti-arthritis leaves and fruits extracts reduced TNF α, IL-1, IL-6	Rathore et al., 2007
Whole plant	Ethanolic	Antidiabetic, Subdued TBARS; antioxidant	Das et al., 2008
Leaves	Methanolic	Antibacterial, against *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhi, Salmonella paratyphi*.	Mahida and Mohan, 2007
Leaves	Methanolic	Antileishmanial isolated compound, calceolarioside A works against visceral leishmaniasis	Poddar et al., 2008
Leaves	Methanolic	Hepatoprotective decrease the elevated levels of biochemical parameters of liver Hepatoregenerative in hepatic damage	Vishwanathan and Juvekar, 2010
Seed	Aqueous	Hepatoprotective against CCl4 induced hepatotoxicity	Lucas and Sekhar, 2000
Whole plant	Ethanolic	Immunostimulant, Enhanced total WBC count and DTH reaction	Tiwari et al., 2011

Table: 3.7. Different compounds derived from *N. arbor-tristis* with biological value

Chemicals isolated	Pharmacological activity	Parts of the plant	Reference
Arbortristoside-A Arbortristoside-B Arbortristoside-C	Antileishmania, Antihistaminic	Corolla Tubule	Sasmal et al., 2007
Nyctoside A	Not known	Seed	Sasmal et al., 2007
Carotenoid aglycone Ag-NY	Orange tubular calyx	Good membrane stabilizing agent	Siddique et al., 2006
4-hydroxy hexahydrobenzofuran-7-one Rengyolone	Flower	Antibacterial, larvicidal, Antimalarial	Tuntiwachwuttiku et al., 2003

Tribal people of India, especially in Orissa and Bihar use different parts of the plant for medication. Ayurveda, Unani, Sidho has referred *N. arbor-tristis* as having therapeutic values. The juice of its leaves is used as antidote against reptile venom, laxative digestive element and diuretics (Nadkarni, 1954). Crude extracts of different parts of the plant like leaf, bark, seed and root have been reported to have therapeutic properties (Table 3.6). In various previous studies, different bioactive compounds have been derived from *N. arbor-tristis* (Table 3.7).

3.1.7.4. *Rauvolfia serpentina*

Common Names: Sarpagandha, Chandrabhaga, Snakeroot plant, Chotachand, Chandrika, Harkaka etc.

The genus name of the plant was given in honour of a 16th century German botanist, physician, and explorer, Dr. Leonhard Rauwolf. The plant is established to have medicinal value for years. The root of *R. serpentina* has been used in India from centuries, for hypertension, as sedative and for its hypnotic properties (Gawade and Fegade 2012). *R. serpentina* is a glabrous herb or shrub and about 1-2ft long (Fig: 3.5). Leaves are arranged in whorls of 3-4, rarely opposite, ecliptic-lanceolate or acuminate or obovate acute. Soft

Figure: 3.4. Plant *N. arbor-tristis* (Night jasmine/Seuli)

Figure: 3.5. Plant *R. serpentina* (Sarpagandha)

plants are present with leaves that are light or dark green in colour. Flowers are white or finged with red; there are many-flowered cymes, corolla is salver-shaped. Fruits are pre-sized drupes, purple-black colour in ripe condition. Seeds are ovoid shape (Vaidyaratnam, 2010); root is branched and 0.5-2cm in thickness, long (8-15cm); on breaking it is circular and with centripetal lines. It is found in India, Pakistan, Sri Lanka, Burma, and Thailand. In India, it is widely distributed in sub-Himalayan area, from Punjab to Nepal, Sikkim and Bhutan.

3.1.7.4.1. Traditional uses and biological activities of *R. serpentina*

R. serpentina is being used since pre-Vedic era for the treatment of snake bite, thus the name Sarpagandha. It is also used for insect sting, hypertension, insomnia, psychological problem, epilepsy, gastro intestinal disorder, fever, wounds and also in schizophrenia (Ayurvedic Pharmacopoeia of India, Govt. of India, 2006). In ayurveda, it is well described as important therapeutic plant. It is used for its medical values in Siddha Unani system from long time. IUCN (International Union for Conservation of Nature) has marked it as endangered plant and its conservation is recommended. Different parts of this plant have been reported to have several biological activities (Table 3.8) and several bioactive compounds have been isolated from the plant parts (Table 3.9).

Table: 3.8. Biological activities of different parts of *R. serpentina*

Plant Part	Extract	Activities	References
Leaf	Methanolic	Antioxidant	Nair et al., 2012
Leaf	Aqueous	Antihypertensive	Ranjini et al., 2015
Root	Ethanolic	Antibacterial(*K. pneumoniae*,*K. aeruginosa*)	Kumari et al., 2013
Root and leaf	Methanolic	Antibacterial	Murthy and Narayanappa, 2015
Rhizome	Aqueous ethanolic or Methanolic	Hepato-protective	Gupta et al., 2010
Root	Methanolic	Antidiabetic	Azmi and Qureshi, 2013
Leaf	Methanolic	Antidiarrheal	Ezeigbo et al., 2012

Table: 3.9. Compounds purified from different parts of *R. serpentina* with their biological activities

Compound	Plant part	Reference
Reserpine	Root	Rosen and Shoolery, 1961
Yohambinine	Root	Lohse, 2002
Reserpinine	Root	Shamma and Richey, 1963;
Rescinnamidine	Root	Rosen and Shoolery, 1961
Rauwolfine; Rescinnaminol; Reserpenediol	Root	Bose, 1952, 1954;
Raumacline; Isoraumacline; 6 α-hydroxyraumacline	Whole plant	Endreß et al., 2007
Papaverine	Bark of the root	Han et al., 2010
7-Epiloganin	Dried root	Itoh et al., 2005
Indobine; Indobinine	Root	Okabe and Adachi, 1998
Ajmalicine; Ajmalicinial; N^+-Methoxycarbonyl	Stem, bark	Nasser and Court, 1984

3.2 Materials and methods

3.2.1. Plant sample collection and extraction

Leaves of all the four plants, *R. serpentina M. oleifera, N. arbor-tristis, C. excavata* were collected in adult stages, from large bushes in sub-Himalayan region of West Bengal, India in August – September 2013 when they grow abundantly at road side and adjoining forest areas. A voucher specimen of each of the plants was deposited in the herbarium of Department of Botany, Ananda Chandra College Jalpaiguri for identification.

Fresh leaves of each of the plants were thoroughly washed with distilled water thrice and dried at room temperature for 5–10 days in shade. The dried leaves of each of the plant (500 g) were ground to moderately fine powder using REMI Mixer Grinder (REMI group, India) and soxhlet extracted with methanol (1500 ml) for 15 h at 32 °C. The extract was concentrated to dryness under vacuum

in a rotary evaporator (Eyela, Japan). A sticky brown to black residue was obtained in every case in different amounts. These solid residues were considered as crude extracts of plants.

3.2.2 Phytochemical analysis

Tests were carried out on the extract and on the powdered specimens following standard procedures as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973) to identify the constituent phytochemicals.

3.2.2.1 Qualitative analysis

3.2.2.1.1 Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish green or a blue-black coloration.

3.2.2.1.2. Test for phlobatannins

Deposition of a red precipitate when the crude extract from each plant sample was boiled with 1% aqueous HCl was taken as evidence for the presence of phlobatannins.

3.2.2.1.3. Test for saponins

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. Then 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The froth was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

3.2.2.1.4. Test for flavonoids

Shinoda test

Crude extract of the leaf of each plant was mixed with a few fragments of magnesium ribbon and then concentrated hydrochloric acid was added to it drop by drop. After few minutes the appearance of pink-scarlet coloration was observed which indicated the presence of flavonoids.

Alkaline reagent test

Crude extract of plant leaf was mixed with 2ml of 2% solution of sodium hydroxide. An intense yellow colour was observed which disappeared on addition of few drops of diluted acid, indicating the presence of flavonoids in the extract.

3.2.2.1.5. Test for steroids and terpenoids

Libermann Burchards test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added to the solution. Formation of brown ring at the junction and green upper layer indicated the presence of phytosterols and formation of deep red color indicated the presence of triterpenoids.

3.2.2.1.6. Test for cardiac glycosides

Keller-Killani test

Five ml of each extract (dissolved in water) was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicates a deoxysugar characteristic of cardenolides. Another ring may appear below the brown ring; while in the acetic acid layer, a greenish ring may form gradually throughout the thin layer.

3.2.2.1.7. Detection of alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered.

- **Mayer's Test:** Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow-colored precipitate indicated the presence of alkaloids.
- **Wagner's Test:** Filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation of brown/reddish precipitate indicated the presence of alkaloids.
- **Hager's Test:** Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids was confirmed by the formation of yellow colored precipitate.

3.2.2.1.8. Detection of carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

- **Molisch's test:** Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicated the presence of carbohydrates.
- **Benedict's test:** Filtrates were treated with Benedict's reagent (qualitative) and heated gently. Orange-red precipitate indicated the presence of reducing sugars.
- **Fehling's test:** Filtrates were hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with Fehling's A and B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3.2.2.1.9. Detection of organic acids

- **Oxalic acid test:** To the test solution few drops of 1% potassium permanganate and dilute sulphuric acid were added; disappearance of the colour showed the presence of organic acid.
- **Malic acid test:** To the test solution, 2-3 drops of 40% ferric chloride was added, appearance of yellowish color proved the presence of organic acid.

3.2.2.2. Quantitative analysis

3.2.2.2.1. Test for total phenol

Determination of total phenolic content was performed using the Folin–Ciocalteu assay, following the method of Kim et al., 2007 with some modifications, where the reducing capacity of sample was measured. The reagent containdhetero-polyphospho tungstate-molybdate. One mililitre of each extract (in different concentrations) was added to a test tube containing 5 ml of the Folin–Ciocalteu (F-C) reagent and 5 ml of sodium carbonate (Na_2CO_3) (20% in water, v/v) was added and vortexed. A reagent blank was prepared using 5 ml of F-C reagent, 1 ml of methanol and 5 ml of sodium carbonate (Na_2CO_3). The final mixture was vortexed and then incubated for 20 mins in the dark at 25°C temperature. The absorbance was measured at 760

nm using a UV- spectrophotometer (V 530; Jasco, Tokyo, Japan). A standard curve was prepared using serially double diluted solution of gallic acid (1000-15.625 µg/ml) in methanol: water (50:50, v/v). Total phenolic values are expressed in terms of gallic acid equivalents (GAE) in milligrams per gram dried leaf extract. All determinations were performed in triplicate. The standard error represents the mean of three replicas.

3.2.2.2.2. Test for flavonoids

Total flavonoid content of test plant extract was determined by aluminium chloride ($AlCl_3$) colorimetric method (Moreno et al., 2000), where $AlCl_3$ forms complex with hydroxyl group of flavonoid compounds. It had maximum absorbance at 420 nm. The value of flavonoid content is expressed as quercetin equivalent (QE) per gram of dried leaf extract. For that a standard curve was prepared using quercetin in different concentrations (10µg/ml, 50µg/ml, 100µg/ml, 200µg/ml). Then 3 ml of methanol was added to 1ml of plant extract or standard solution, taken in a test tube. Subsequently 200 µl of 10% $AlCl_3$ and 200 µl of 1M potassium acetate solution were added to it. Next 5.6 ml of distilled water was added and the mixture was kept for 30 mins at room temperature. Finally absorbance was measured at 420 nm in UV-spectrophotometer. Significant difference with P value <0.05 was measured by one-way analysis of variance (ANOVA). The standard error represents the mean of three replications.

3.2.3. Antioxidant activity

Antioxidant potential of plant leaf extract was estimated using 2, 2'-diphenyl-1-picryl Hydrazyl Radical (DPPH) free radical scavenging assay (Patel and Patel, 2011). For the experiment, 1ml of methanolic solution of test compound in different graded concentrations (stock 5mg of extract in 20ml of methanol) were taken in test tubes. To each sample, 0.5 ml of 0.2 µM DPPH-methanolic solution (1.6 mg DPPH in 20 ml of methanol) was added and the volume was made to 2ml with methanol. The control solution contained only DPPH. The mixture components of each test tube were mixed vigorously and allowed to stand for 30 minutes in dark at room temperature. The absorbance was

measured at 570 nm by spectrophotometer. The percentage inhibition of free radical activity was measured using following formula:

% inhibition = [(absorbance of control – absorbance of test sample)/ absorbance of control]×100

A scattered line graph of percentage inhibition of free radical activity was plotted against concentration of crude extracts and for each plant, concentration of 50% inhibition (IC_{50}) was obtained from the graph. The standard error represents the mean of three replicas.

3.3. Results

3.3.1. Phytochemical analysis (Qualitative)

The leaf extracts and the dried powder of leaf of *R. serpentina,M. oleifera, N. arbor-tristis* and *C. excavata* showed the presence of tannin, flavonoids, alkaloids, carbohydrate, protein and organic acids (Table 3.10). The leaf extracts of *R. serpentina* and *M. oleifera* tested negative for triterpenoids. The leaf extracts of *N. arbor-tristis* and *C. excavata* tested negative for saponin.

Table: 3.10. Phytochemical constituents (qualitative) of leaf extracts of *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata*

Phytoconstituents	*R. serpentina*	*M. oleifera*	*N. arbor-tristis*	*C. excavata*
Alkaloids	++	+	++	+
Flavonoids	+	+	+	++
Tannin	+	+	++	+
Phlobatannin	++	++	-	+
Saponin	+	++	-	-
Steroid	+	++	+	-
Triterpinoid	-	-	+	+
Cardiac Glycoside	-	++	+	+
Organic acid	+	+	+	+
Carbohydrate	+	+	+	+
Protein	+	+	+	+

++ present in good amount, + present, - absent

3.3.2. Phytochemical constituents quantitative of leaf extracts

3.3.2.1. Phenolic content

Total phenolic content (TPC) of leaf extract was determined in terms of gallic acid equivalents (GAE) in milligrams per gram of dried leaf extract and the results are shown in Table 3.11. In a comparative graph, the total phenolic contents of four plants are shown by bar diagrams depicting the highest value for *N. arbor-tristis,* and lowest value for *C. excavata* (Fig 3.6).

Table: 3.11. Phenolic content of leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE)

Plant	Sample conc. mg/ml (M/V)	Absorbance At 760nm	GAE[a] conc. µg/ml	GAE Conc. mg/ml (C)	TPC[b] as mg GAE/gm DLE# (C x V/M)	mg GAE/gm of DLE[c] ± SEM
RS	0.025	2.902	956.133	0.956	38.25	38.24±0.00
		2.902	956.066	0.956	38.24	
		2.902	956	0.956	38.24	
MO	0.013	1.223	396.266	0.396	31.70	31.70±0.01
		1.222	396	0.396	31.68	
		1.224	396.533	0.397	31.72	
NA	0.02	2.592	852.66	0.853	42.63	42.65±0.01
		2.590	852	0.852	42.66	
		2.594	853.33	0.853	42.67	
CE	0.01	0.700	222.1	0.222	22.21	22.22±0.01
		0.701	222.166	0.222	22.22	
		0.701	222.3	0.222	22.23	

[a]GAE=Gallic acid equivalent, [b]TPC=Total phenolic content, [c]DLE=Dried leaf extract

3.3.2.2. Flavonoid content

The value of total flavonoid content (TFC) was expressed as mg of quercetin equivalent (QE) per gm of dried leaf extract. The result is shown in Table 3.12.The highest flavonoid content was observed in *M. oleifera* and lowest content in *C. excavata* (Fig. 3.7).

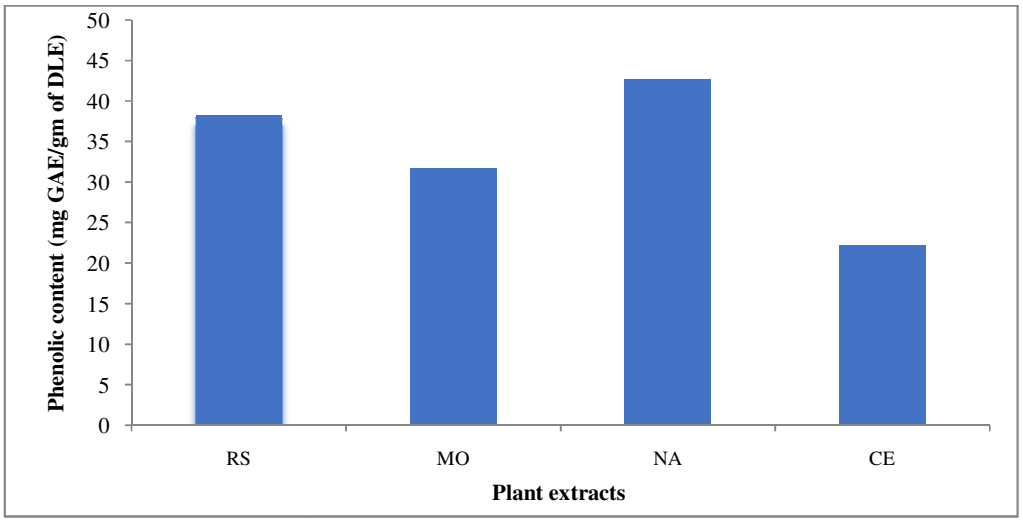

Figure: 3.6. Phenolic content of crude methanolic leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE) (GAE=Gallic acid equivalent, DLE=Dried leaf extract)

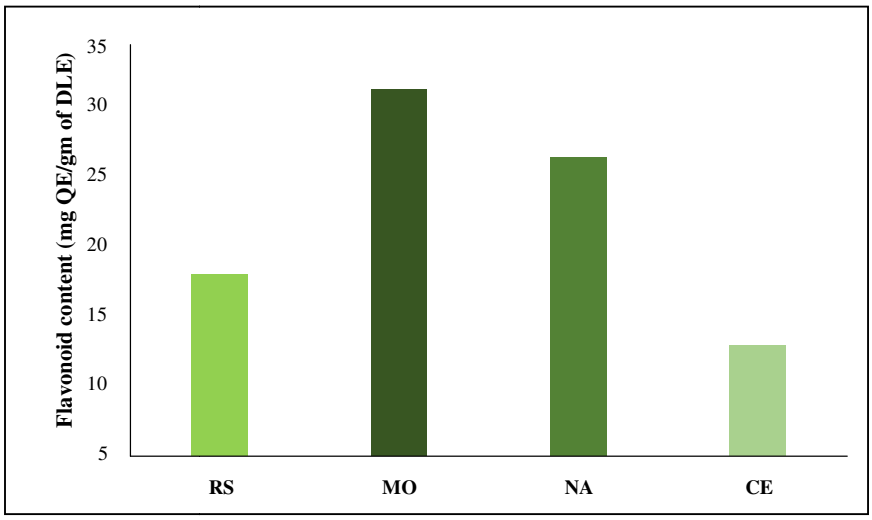

Figure: 3.7. Flavonoid content of crude methanolic leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE) (DLE = Dried leaf extract, QE = Quercetin equivalent)

Table: 3.12. Flavonoid content of leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE)

Plant	Sample conc. Mg/ml (M/V)	Absorbance at 420nm	QE[a] conc. µg/ml	QE[a] Conc. mg/ml (C)	TFC[b] as mg QE/gm of DLE[#] (C x V/M)	mg QE/gm of DLE[C] ± SEM
RS	0.01	1.53	156.3	0.156	15.63	15.43±0.12
		1.51	154.33	0.154	15.43	
		1.49	152.33	0.152	15.23	
MO	0.01	3.128	316.1	0.316	31.61	31.15±0.41
		3.001	303.43	0.303	30.34	
		3.117	315.3	0.315	31.5	
NA	0.01	2.58	261.3	0.261	26.13	25.4±0.68
		2.37	240.33	0.240	24.03	
		2.62	265.33	0.265	26.03	
CA	0.01	0.9	93.21	0.093	9.32	9.39±0.05
		0.915	94.823	0.095	9.48	
		0.903	93.633	0.094	9.36	

[a]QE=Quercetin equivalent, [b]TFC=Total flavonoid content, [c]DLE=Dried leaf extract,

3.3.3. Antioxidant activity

Antioxidant activities of leaf extracts of the four plants weredetermined by free radical scavenging capacity in DPPH assay. Table 3.13 shows the percentage inhibition of DPPH activity of there spective plants and IC_{50} values were also determined. All the four test samples were in concentration range of 20-100µg/ml. At the concentration of 40 µg/ml, *C. excavata* showed highest free radical scavenging activity (58%) and *R. serpentina* showed lowest activity (39%**).** Fig. 3.8 graphically shows the percentage inhibition of DPPH by all four plant leaf extracts in a given set of concentrations, representing their capacity of scavenging free radicals.

Comparison of phenolic and flavonoid content of leaf extracts of four test plants with their corresponding IC_{50} value of antioxidant (DPPH assay) activity reveals that *R. serpentina, M. oleifera* and *N. arbor-tristis* have high phenolic

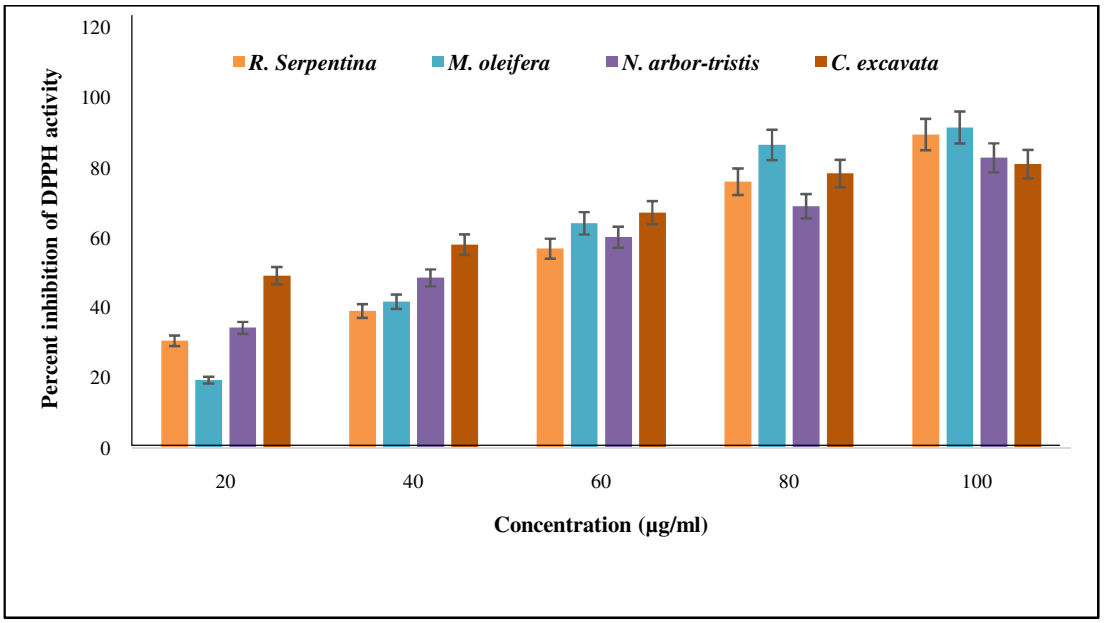

Figure: 3.8. Percent inhibition of DPPH activity of crude leaf extract of *R. serpentina*, *M. oleifera*, *N. arbor-tristis* and *C. excavata in* different concentrations.

and flavonoid content and low IC_{50} values. Only *C. excavata* with low phenolic and flavonoid content still showed lowest IC_{50}value in DPPH assay (Table 3.14).

Table: 3.13. Percent inhibition of DPPH activity by crude leaf extract of *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* in different concentrations and IC_{50} values

Sample concentration µg/ml	% Inhibition			
	R. serpentina	*M. oleifera*	*N. arbor-tristis*	*C. excavata*
20	30.6	19.35	34.27	49.21
40	39.1	41.74	48.55	58.05
60	56.9	64.14	60.15	67.14
80	76	86.54	69.03	78.36
100	89.5	91.5	82.85	81.07
IC_{50}	53.6	48.5	44	20.65

Table: 3.14. Phenolic and flavonoid content of leaf extracts of four test plants with their corresponding IC_{50} values of DPPH free radical scavenging assay*

Test plant	Phenolic content (mg of GAE/gm of DLE)	Flavonoid content (mg of QE/gm of dried extract)	IC_{50} (% inhibition) of antioxidant activity(µg/ml)
R. serpentina	38.24±0.00	15.43±0.12	53.6
M. oleifera	31.70±0.01	31.15±0.41	48.5
N. arbor-tristis	42.65±0.01	25.40±0.69	44
C. excavata	22.22±0.01	9.39±0.05	20.65

*DLE=Dried leaf extract, QE=Quercetin equivalent, GAE=Gallic acid equivalent

3.4. Discussion

In this study, four well known medicinal plants, *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* were analysed for their phytochemical contents. It was observed that leaf extracts of each of the four plants gave positive result in qualitative test for alkaloid, flavonoid, terpenoid and tannin. Steroid was found to be present in three of them except *C. excavata;* saponin was found to be present in *R. serpentina, M. oleifera*; cardiac glycoside was found to be present in *M. oleifera, N. arbor-tristis* and *C. excavata;* macronutrients like protein and carbohydrate were found to be present in every plant leaf extract. In the quantitative assay, it was seen that phenolic compound was present in each of the four plant leaf extracts, with values ranging from 22.22±0.01 to 42.65±0.01 mg GAE/gm dried leaf extract. *N. arbor-tristis* contained the highest amount and *C. excavata* contained the lowest amount. Amount of flavonoid ranged from 9.39±0.05 to 31.15±0.41 mg QE/gm dried leaf extract where *M. oleifera* contained the highest amount and again *C. excavata* contained the lowest amount. It is reported that medicinal plants have high contents of organic compounds and bioactive substances including phenolic acids, tannins, alkaloids, terpenoids, steroids and flavonoid which provide definite physiological and therapeutic action on human body (Yadav and Agarwala, 2011; Criagg and David, 2011; Edoga et al., 2005). The present study confirms the previous findings. Furthermore, flavonoids are active against a wide array of microorganisms *in vitro*, which may be due to their ability to complex with extracellular and soluble proteins, and bacterial cell wall (Marjorie, 1996). Phenolic compounds have electron donating and electron withdrawing substituent in their ring structure, and thus act as antioxidant by donating hydrogen ions to highly reactive radicals (Lapornik et al., 2005).

Several studies have described that, plants rich in phenolic compounds also have antioxidant properties (Brown and Rice-Evans, 1998; Krings and Berger, 2001; Ali et al., 2008). In our study, all of the four plants showed positive results in free radical scavenging activities in DPPH assay. *C. excavata* showed

the lowest IC_{50} implying good antioxidant property, and *R. serpentina* showed the highest IC_{50} value. In a comparison graph, it is depicted clearly that there is a relationship between phenolic and flavonoid content and antioxidant activities of respective plants. *N. arbor-tristis* had higher phenolic content and low IC_{50} value compared to *M. oleifera,* which had lower phenolic content and comparatively more IC_{50} value in percent inhibition of DPPH.A clear correlation between secondary metabolite content, mainly phenolic, and antioxidant activity of plant extract has been reported by several previous studies (Brown and Rice-Evans, 1998; Krings and Berger, 2001; Ali et al., 2008). In this study, *R. serpentina, M. oleifera* and *N. arbor-tristis* showed a good supportive result in establishing the previous findings. In contrast, *C. excavata*, which showed lowest phenolic and flavonoid content but exhibited good antioxidant property (lowest IC_{50}), indicating that active compound of different polarity might be present in the plant. Studies also reveal that the content of different secondary metabolites and their biological activity differ significantly with extraction procedure, solvent, time of sample collection, environment, place etc. (Shan et al., 2005).

In conclusion, it can be said that the leaf of test plants, *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* have shown the presence of a variety of secondary metabolites and a good source of phenolic compounds and flavonoids. The plants have also exhibited different degrees of antioxidant activities. So, they can be considered as promising source of phytochemicals and natural antioxidants for therapeutic uses. However, detailed studies are required to establish the correlation between the presence of phenolic or flavonoid contents and antioxidant activity and the role of different phytochemicals involved in antioxidant activity before using them therapeutically.

4.1. Literature review

Fungi are extremely diversified group of eukaryotes and may be single celled or multinucleated organism. They are set for survival in virtually all kinds of environments, from soil to water to even air. Fungi maintain their submissive presence, and manifesting existence mainly by opportunistic infection in immuno-compromised hosts. Fungal infections are common in different parts of human body, which include superficial skin, hair, nail, vagina, or internal organs. Most common fungus causing infection are mainly ubiquitous colonizers like *Candida* sp., *Cryptococcus* sp. and *Aspergillus* sp. (Perfect and Casadevall, 2002; Denning and Hope, 2010). Global Action Fund for Fungal Infections (GAFFI, 2018) says over 300 million people of all ages suffer from a serious fungal infection every year globally, and over 1.5 million of them are estimated to die from fungal disease. More than 1 million eyes go blind yearly due to keratitis; fungal spores contributed reactive airway diseases is seen in over 10 million individuals, and almost one billion people get attacked by skin mycoses every year. More than 400,000 people develop pneumocystis pneumonia annually and most of them die without treatment. Every year around 220,000 new cases of cryptococcal meningitis are reported worldwide, causing 181,000 deaths concentrated only in sub-Saharan Africa (GAFFI, 2018).

In human, fungal infections are the 4^{th} common cause of life-threatening infection, as a co-factor in AIDS epidemic, cancer chemotherapy and organ or bone marrow transplantation (Pfaller and Diekema, 2007). AIDS patients, who are already immuno-compromised, are reported to be attacked by *Pneumocystis carinii* (pneumonia) and oesophageal candidiasis in 60% and 20% cases respectively (Moore and Chaisson, 1996). During the current COVID-19 pandemic, cases of fungal infection in COVID-19 infected patients are reported worldwide, which are most commonly caused by *Aspergillus* sp., *Candida* sp., *Cryptococcus neoformans* and *Mucoralis* order (Hoenigl et al., 2022, Chiurlo et al.,2021). The distribution of fungal infection, particularly mucormycosis (commonly known as 'black fungus disease') was found to affect India during second wave (Aranjani et al., 2021). Treatment with corticosteroids and

mechanical ventilation predispose COVID-19 patients to get infected by these opportunistic fungi (Kumar et al., 2021). The triad of uncontrolled diabetes, use of corticosteroids and SARS-CoV-2 was evident for the increase of maxillofacial mucormycoses (Al-Tawfiq et al., 2021).

Climatic diversity and the hot and humid weather of India is suitable for fungal infection. Candidiasis, aspergillosis, cryptococcosis andzygomycosis are common opportunistic fungal infections in India. Although there have been major advancements in drug development against fungal infection, the morbidity and mortality still remain high due to therapeutic limitations, ranging from diagnostic challenges, toxicity of available drugs, limited routes of drug administration, drug solubility, stability to drug resistance (Denning and Hope, 2010). Current treatment for fungal infection is based on antifungal drugs such as Ketoconazole, Fluconazole, Amphotericin-B, Clotrimazole etc., which are proved to be inefficient in some cases or often cause nephrotoxicity or hepatotoxicity (Zapata-Garrido et al., 2003). Moreover, some fungal species are reported to be resistant to commonly used drugs (Rodrigues and Nosanchuk, 2020). All these facts emphasize the need for novel antifungal agents with a broad spectrum of activities and fewer side effects.

4.1.1. Phylogeny and classification

Fungi are filamentous microscopic spore bearing organisms. They have cell wall made with chitin and cellulose and they lack chlorophyll. Heterotrophic nutrition using organic source of nutrients is a common feature of all types of fungi. Traditionally they are a member of plant kingdom but in recent time scientists constitute a separate fungi kingdom. Fungal life cycle comprises of sexual or teleomorph state and asexual or anamorph state. Some filamentous fungi predominantly or exclusively produce conidia, external asexual spore and they are termed as deuteromycetes. Some fungi, called allergen, produce air borne spores and are called ascomycetes (Simon-Nobbe et al., 2008).

4.1.2. Infection caused by fungus

An impressive feature for survival of fungi is that, it is widespread. They survive everywhere, from air to water and soil, even as lichens inside Antarctic rocks. They can withstand a wide range of temperature. Another prominent factor for fungal growth is moisture. They can flourish well in hot and humid environment. The infection ranges from superficial skin to fatal systemic mycosis (Table 4.1). Fungal diseases have alarming impact on human health but remain neglected over the years. As it affects the poor population, it develops slowly and become worse progressively (if left untreated) and have long term consequences (Rodrigues and Nosanchuk, 2020).

Depending on degree of infected tissue the fungal infection may be classified into: a. Superficial or localized infection affecting skin, hair and nails, b. Subcutaneous tissue infection confined to dermis, c. Systemic infection involving deep internal organ, d. Opportunistic infection, where host is already immuno-compromised or immuno-deficient (Horner et al., 1995; Friedman et al., 1982; Salvaggio and Aukrust, 1981). Systemic fungal infection may be caused by either primary pathogenic fungi or by opportunistic fungi with marginal pathogenicity (Rodrigues and Nosanchuk, 2020). Sometimes central nervous system also gets infected by microorganisms as primary infection or uncommon ectopic site of infection. For example, *Aspergillus* sp. may create infection in brain abscess and gets in by inhalation. Some strains like *Candida* sp., *Mucor* sp., *Rhizopus* sp. also may attack CNS after entering through inhalation (Gilligan et al., 2014).

Table: 4.1. Common fungi infecting human and their clinical manifestations

Disease	Fungal Species	Common clinical manifestations of infection	Clinical status during infection	References
Aspergillosis	*Aspergillus* genera: *A. fumigatus, A. flavus, A. terreus, A.niger,*	Nodules, ulcers, skin necrosis, vesicles, indurated erythema, fever, chest pain, cough, hemoptysis, dyspnea, fatigue, shortness of breath, runny nose, headache, congestion, loss of smell, wheezing	People with weakened immune systems, lung diseases, COVID-19	Schmiedel and Zimmerli., 2016; Kniemeyer et al., 2011; Samson, 2007; Amin et al., 2021
Mucormycosis	*Mucorales* order: *Rhizopus* sp., *Mucor* sp., *Rhizomucor* sp, *Absidia* sp, *Lichtheimia* sp, *Apophysomyces* sp, *Cunninghamella* sp, *Saksenaea* sp,	Ecthyma gangrenosum-like lesions, muco-cutaneous ulceration and eschar, necrotic papulonodules, cellulitic plaques, necrotizing fasciitis, Rhino-cerebral: unilateral facial swelling, headache, congestion, fever, cough, nasal congestion, chest pain, shortness of breath, nausea, vomiting, gastrointestinal bleeding	Most common in diabetics, especially those with ketoacidosis, poorly controlled diabetes mellitus, hematopoietic stem cell transplant (HSCT) recipients and burn patients, COVID-19	Gnat et al, 2021, Gilligan et al., 2014; Amin et al., 2021
Candidiasis	*Candida albicans, C. glabrata, C.parapsilosis*, *C.tropicalis, C.auris*	Thrush or oropharyngeal candidiasis, vaginal infection, bloodstream infection, fever, chills, loss of taste, sore throat, Odynophagia	Use of catheters, intravascular or intracranial devices, solid organ transplantation, haematopoietic stem cell transplantation, COVID-19	Schmiedel and Zimmerli., 2016; Amin et al., 2021
Cryptococcosis	*Cryptococcus gattii*, *C. neoformans*	Cellulitis, ulcers, Pulmonary: fever, cough, fatigue, dyspnea, cryptococcal meningitis in HIV patients, Shortness of breath, headache, nausea, vomiting,confusion, chest pain, light sensitivity CNS: meningeal signs, focal neurological deficits	Tuberculosis, HIV/AIDS, Immunocompromised condition, COVID-19	Kohler et al., 2017; Miceli et al., 2011; Amin et al., 2021
Coccidioidomycosis (Valley Fever)	*Coccidioides immitis, C. posadasii*	Self-limiting mild flu-like in healthy people, severe disseminated infection	immunocompromised condition	Gnat et al., 2021

Disease	Fungal Species	Common clinical manifestations of infection	Clinical status during infection	References
Histoplasmosis	*Histoplasma capsulatum*	Self-limiting mild flu-like in healthy people,Pneumonia, immunocompromised condition	Healthy and immunocompromised condition	Gnat et al., 2021
Blastomycosis	*Blastomyces dermatitidis B. gilchristii*	Self-limiting mild flu-like in healthy people Pneumonia, immunocompromised condition	Healthy and immunocompromised condition	Kohler et al., 2017
Pneumocystis pneumonia	*Pneumocystis jirovecii*	Pneumonia	Immunocompromised individuals, especially with AIDS and Auto immune disease	Gnatet al., 2021; Chakrabarti, 2005
Necrotizing encephalitis, Rhinocerebralzygo mycosis	*Mucor sp., Rhizopus sp.*	Aseptate hyphae in tissue, neural complications	Diabetics, neutropenic individuals	Gilligan et al., 2014; Firacative,2020
Dermatophyte infection	*Epidermophyton floccosum Microsporum canis, M. gypseum, M. audouinii, Trichophyton* sp.	Skin lesions, infection on keratinized tissue	Healthyor immunocompromised subject	Gilligan et al., 2014; Firacative,2020

4.1.3. Epidemiology

Systemic fungal infection has rapidly evolved in the last two decades. Medical advancement though improved general medication also led individuals at risk; such as people with indwelling catheter, patient in ICU, individual receiving immunosuppressive therapy or facing organ or stem cell transplantation, are prone to fungal infections. The most common fungal infection following organ transplantation is candidiasis (53%) followed by invasive aspergillosis (19%), cryptococcosis (8%), and zygomycosis (2%) (Pappas et al., 2010). Increase in size of population in general also causes increase in the size of immuno-compromised population, due to diseases such as blood cancer, bone marrow cancer and AIDS. Fungal infection is prevalent in patients treated with immunosuppressive drugs (Skalski et al., 2016; Wang et al., 2017). Massive population growth, rapid urbanisation and climatic change are increasing the risk of fungal infection day by day. Frequent natural calamities also contribute

to the changing epidemiology of fungal infection (Casadevall et al., 2019). Many fungi have developed the ability to grow and multiply within the host macrophage itself. In a healthy individual, at the site of infection, adaptive immunity cells form granulomas and later degenerate to scars and often calcify which may be seen after years on X-ray imaging. But patients with impaired immunity cannot control the infection and almost any organ can be affected by invading fungus (Wilson et al., 2002; Dasbach et al., 2000; Ben Ami et al., 2009; Rodrigues and Nosanchuk, 2020). There are some common fungi infecting HIV patients, imposing clear symptoms (Table 4.2). According to the Center for Disease Control and Prevention (CDC), fungi are among the leading causes of opportunistic infections. In fact, fungi cause most of the coinfections affecting patients with HIV/AIDS (CDC, 2017). Reports say, meningitis caused by the genus *Cryptococcus* is the second leading cause of death after tuberculosis in people living with HIV. Only in Latin America, approximately 30% of patients living with HIV/AIDS, die from histoplasmosis caused by *Histoplasma capsulatum*.

Table: 4.2. Common fungal co-infections in HIV patients and their areas of occurrence

Fungal disease	Causative agent	Main epicenters	References
Cryptococcal meningitis	*Cryptococcus neoformans,* *Cryptococcus gatii*	Sub-Saharan Africa, Southeast Asia	Armstrong-James et al., 2014
Pneumocystis pneumonia	*Pneumocystis jirovecii*	Asia, Latin America, sub-Saharan Africa	Skalski and Limper, 2016
Disseminated histoplasmosis	*Histoplasma capsulatum*	North America, sub-Saharan Africa, Asia (parts of China, India, Thailand, and South Korea)	Wheat et al., 2016
Disseminated penicilliosis	*Penicillium sp.* (Mainly *Penicillium marneffei*)	Southeast Asia, mainly North Thailand	Armstrong-James et al., 2014
Aspergillosis	*Aspergillus sp.* (Prevalently *Aspergillus fumigatus*)	Worldwide	Patterson et al., 2016
Coccidioidomycosis	*Coccidioides sp. (C. immitis* and *C. posadasii)*	Semiarid regions of North and South America	Wang et al., 2017
Paracoccidioido Mycosis	*Paracoccidioides brasiliensis*	South and Central America (most common in parts of Brazil, Colombia, and Venezuela)	Wang et al., 2017
Disseminated Emmonsiosis, adiaspiromycosis	*Emmonsia pasteuriana* (for disseminated emmonsiosis). *Emmonsia parva* and *Emmonsi acrescens* (for adiaspiromycosis)	Italy, Spain, China, and India	Schwartz et. al., 2015; Wang et al., 2017

4.1.3.1. *Candida* sp.

Candida sp. is the most common fungus affecting human subjects, and candidiasis ranges from superficial invasion of mucosal surface to systemic organ infection (Miceli et al., 2011). All *Candida* species are of round or oval shaped budding yeast found single, in chain or forming cluster. After entering the host tissue, they form both pseudohypha and hyphae using chain of blastoconidia, which helps to invade the host defence system. They grow rapidly and form colonies. Healthy human tissues normally targeted by *Candida* sp. are, oral site, gastro intestinal tract and women genital organ like vagina (Soliman et al., 2017). In situation of impaired host defence or change in ecological niche, they become pathogenic (Miceli et al., 2011). The virulent attributes of *Candida* sp. are wide, like adherence to the host surface and enzyme production (phospolipase, proteinase). They form biofilms to penetrate the epithelium with ease and to adapt in stressful condition (Samaranayake and Samaranayake, 1994). Different strains of *Candida* sp. target different system or organ of host to infect. For example,*C. albicans* mainly causes invasive fungal infection on the mucosal membranes (Miceli et al., 2011). *C. parapsilosis* causes candidal endocarditis and onychomycoses (Ge et al., 2019). *C. krusei* (*Issatchenki aorientalis*) mainly attacks patient after stem cell or bone marrow transplant. Non-albican species (NAC) isolated between 1952 and 1992 were *C. glabrata, C. lusitaniae, C. lipolytica, C. guilliermondii, C. dubliniensis* etc. Population based study in USA shows that *C. albicans* is the most common species followed by *C. parapsilosis, C. tropicalis* and *C. glabrata*. Blood stream infection (BSI) is increasingly caused by NAC species mainly by *C. glabrata* in western world (Pfaller and Diekema, 2002). In India among the NAC species, *C. tropicalis* is predominant in all age groups (Ahmed et al., 2020).

4.1.4. Treatment available

There is no effective antifungal vaccine till date, the diagnostic tools are very limited and the therapeutic options are restricted to a few conventional drugs which are found to be either toxic or otherwise expensive. Most recently

approved antifungal agent is echinocandin, developed on 2002 (GAFFI, 2018). It shows that the drug development field is progressing at a very slow pace. Classical antifungal drug amphotericin B, discovered in 1955, still remain the first line medication for some fungal infection like cryptococcal meningitis. United State Food and Drug Administration (FDA) approved five categories of drugs to be used clinically for treating fungal infection from 1955 to 2010. They are polyenes, pyrimidine analogues, azoles, allylamine and echinocandins. Among these five categories of drugs, azoles are intensively studied and modified to increase the efficacy (Vandeputte et al., 2012) which act on fungal cell membranes and disrupt normal functioning. Echinocandins destruct fungal cell wall structure impairing the synthesis of β-1, 3-glucan. Fungal nucleic acid synthesis and mitotic division are inhibited by intracellularly activating antifungal drugs of pyrimidine analogue group (Lewis,2011). Deoxymulundocandin, a drug of echinocandin group, showed positive effect during*in vitro* and *in vivo* study on *Candida* and *Aspergillus* sp. (Mishra and Tiwari, 2011). Ambruticin is found effective in aspergillosis and coccidioidomycosis (Chiang et al., 2006).

The conventional drugs are found to be effective, but showed side effects in multiple occasions. Most effective and least toxic formulations like liposomal AmB are very expensive, more over it is available only in six out of 116 developing countries worldwide (GAFFI, 2018). Sometimes, it is found that if some low-cost antimetabolites (for e.g., 5-fluorocytosine) is combined with other approved antifungal drugs, it is beneficial for treating systematic mucosis. But the fact is, most of the drugs are not available in many countries, especially where the disease is most lethal. High price antifungal drugs face market failure in developing countries (Rodrigues and Nosanchuk, 2020). In the present days, the field of research too is lacking in developing new effective and low-cost antifungal agents. Sometimes proper diagnosis can improve the situation; for example, the symptoms of infection caused by *H. capsulatum* are similar to tuberculosis and in most of the cases, without proper diagnosis, the patients are treated for tuberculosis and as a result they usually die within 1-2

weeks out of drug toxicity (WHO, 2019). Considering these intrinsic and extrinsic difficulties, it is more realistic and impactful to focus on the available diagnosis and antifungal tests and use natural resources derived agents for treatment to minimise the cost and number of deaths. There are ongoing initiatives for developing antifungal vaccines, antifungal agents and diagnostic tools; but emergence of multi-resistant and largely unknown pathogens such as *C. auris* (Casadevall et al., 2019) is making the situation complex.

4.1.5. Antifungal drug resistance

Persistence of progressive infection caused by any pathogen despite of application of drugs is called drug resistance. Many synthetic antifungal therapeutic drugs are found to be resisted by some fungal pathogen. These fungal species are considered to inherit the resistance to those drugs (Sanglard, 2003). For example, *A. terreus* and *Fusarium* sp. are resistant to amphotericin ß; whereas *C. neoformans* and *Zygomycetes* sp. are resistant to caspofungin. Fluconazole resistance is seen in *Candida krusei*and *Aspergillus* sp. (Rogers, 2006). The multi-resistant pathogen *Candida auris* has emerged as a serious global threat to human health in the recent years, which, in immunocompromised patients, cause infections resistant to all major classes of antifungal drugs. Climate change and the widespread use of antifungal drugs are some of the determining factors for the emergence of the drug resistant strains of *Candida* (Clancy and Nguyen, 2017; Casadevall et al., 2019).

4.1.6. Prospect of medicinal plants as antifungal agents

Medicinal plants are proved to be a rich source of antimicrobial agents. From ancient times they are used to fight against different types of microbial pathogens by the indigenous people of countries like India. In the present time considering the toxic effect and resistant variety of pathogens, scientists concentrate on developing novel molecules from natural resources against these pathogens or to fight against diseases. A wide range of medicinal plants, plant parts, leaves, roots etc. are screened for antifungal activity with satisfactory result (Table 4.3). Scientists have isolated some compounds from

plant sources with antifungal activity (Cowan, 1999; Ahmad et al., 2006; Dzoyem et al., 2007; Endo et al., 2010; Hu et al., 2014; Sukieum et al., 2017).

Table: 4.3. Medicinal plants and identified antifungal compounds

Scientific name	Common name	Class of Compound	Compound	References
Allium cepa	Onion	Sulfoxide	Allicin	Cowan,1999
Allium sativum	Garlic	Sulfoxide, terpenoids	Allicin, ajoene	Ahmad et al., 2006
Arnica montana	Mountain tobacco	Lactones	Helanins	Cowan,1999
Camellia sinensis	Green tea	Flavonoid	Catechin	Ahmad et al., 2006
Clausena excavata	Agnijol	Coumarin	Excavarin-A	Kumar et al., 2012
Croton cajucara	Chandra Sacaca	Essential oil	Linalool	Ahmad et al., 2006
Diospyros crassiflora	African ebony	Naphthoquinone	Plumbagin	Dzoyem et al., 2007
Gloriosa superba	Glory lily	Alkaloid	Colchicine	Cowan,1999
Humulus lupulus	Hops	Phenolic acids	Lupulone,humulone	Cowan,1999
Malus sylvestris	Apple	Flavonoid Derivative	Phloretin	Ahmad et al., 2006
Mentha piperita	Peppermint	Terpenoid	Menthol	Ahmad et al., 2006
Moringa oleifera	Drum stick	Protein	Mo-CBP2	Neto et al., 2017
Papaver somniferum	Poppy	Alkaloids	Opium	Cowan,1999
Piper betel	Betel pepper	Essential oils	Catechols, eugenol	Ahmad et al., 2006
Piper nigrum	Black pepper	Alkaloid	Piperine	Ahmad et al., 2006
Podocarpus nagi	Tree bard	Lactone	Negilactone	Ahmad et al., 2006
Punica granatum	Pomegranate peel	Polyphenols	Punicalagin	Endo et al., 2010;
Ranunculus bulbosus	Buttercup	Lactone	Protoanemonin	Cowan,1999
Rauvolfia serpentina	Rauvolfia	Alkaloid	Reserpine	Cowan,1999
Satureja Montana	Winter savory	Terpenoid	Carvacrol	Ahmad et al., 2006
Syzygium aromaticum	Clove	Terpenoid	Eugenol	Cowan,1999
Toddalia asiatica	Orange climber	Coumarin Alkaloid	8S-10-O demethylbocconoline	Sukieum et al., 2017
Vinca minor	Periwinkle	Alkaloid	Reserpine	Ahmad et al., 2006
Withania somnifera	Ashwagandha	Lactone	Withafarin A	Cowan,1999

To fulfil the demand of antifungal drugs which improve patient's recovery rate and shortens treatment time, there is no better way than exploration of vast natural resources. Our ancient therapeutic history and current research have given evidence that plant extracts exhibit antimicrobial and antifungal activities. The present study screens four such well known medicinal plants for antifungal activity.

4.2. Materials and methods

4.2.1. Plant materials

Leaves of all the four plants, *R. serpentina, M. oleifera, N. arbor-tristis, C. excavata* were collected from road side and forest areas of Jalpaiguri district of sub-Himalayan West Bengal.

4.2.2. Extraction and purification of excavarin-A from *C. excavata* leaves

Crude extracts from fresh leaves of each of the four plants were prepared as described in previous chapter (section 3.2.1). Purification of the antifungal compound excavarin-A was done from *C. excavata* leaves following the method of Kumar et al. (2012). The dry powdered leaves (200 mg) were extracted in dichloromethane in Soxhlet apparatus at 35^0C for 24 h and then concentrated under vacuum to obtain a brown sticky solid (14g). This crude extract was further subjected to silica gel column chromatography. Elution with n-hexane, n-hexane: dichloromethane (3:1, 1:1, 1:3), dichloromethane, ethyl acetate: dichloromethane (3:1, 1:1, 1:3), ethyl acetate, methanol: ethyl acetate (3:1, 1:1, 1:3) and methanol gave thirteen fractions (F1 to F13). Based on activity tested through bioautography on TLC plates, fraction F7 (2 g) was rechromatographed on a silica gel column, eluting with petroleum ether (200 ml) and ethyl acetate: hexane (2%, 5%, 10%, 15%, 20%, 25%, 30% and 35%) by which a total of 8 fractions (f1 to f8) were collected. The bioactive fraction f7 and f8 were fractionated into 40 sub-fractions (Sf1 to Sf40). Six sub-fractions (Sf30–Sf35) were pooled and crystallized with hexane–ethyl acetate to give the purified compound (100mg). Colourless needle shaped crystals of excavarin-A, a γ-lactone coumarin, was obtained whose identity was confirmed after being

analysed by UV–Vis, IR and NMR (1H- and 13C-) spectroscopy. The excavarin -A was also tested for antifungal activity by agar cup bioassay and MIC was also determined.

4.2.3. Test organism

The antifungal activity of the plant leaf extract was screened against the fungal strain *C.albicans* (MTCC183) obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

4.2.4. Agar cup bioassay

The agar cup bio assay was performed to evaluate the antifungal activity of the selected crudeplant extracts and the purified compound excavarin-A following the method of Saha et al.(2005b). First potato dextrose agar (PDA) medium was autoclaved at 121°C for 15 minutes, then cooled to 45°C. Subsequently 1ml of pure fungal suspension ($1x10^5$cells/ml) of the test pathogen (*C. albicans*) was mixed with 19 ml molten medium. Then the mixture was poured into sterile Petri plates of 9 cm diameter. After solidification of the medium, agar cups were prepared with sterile cork-borers (4 mm diameter) in PDA plate, which were seeded with cell suspension of the test fungi. For screening the plant leaf extracts for their antifungal activities, different concentration of the methanolic extracts were placed (50 µl) into each well and the plates were incubated at 28^0C for 48-72 hours. Antifungal activity was determined by measuring the zones of inhibition of fungal growth around the wells. Measurement was done by using centimetre scale. The experiments were done in triplicate in complete aseptic condition and the mean value was noted.

4.2.5. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of the four medicinal plants and the isolated compound were tested against *C. albicans* by using the 96-well micro-titer plate assay following the method of Kumar et al. (2012). The crude leaf extracts and active constituent of *C. excavata*, excavarin-A, were serially

double diluted in methanol (5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 mg/ml) and 50 µl of each of the different concentrations were poured into the wells of the micro-titer plate. The antifungal antibiotic nystatin was used for comparison. When the solvent was evaporated, a mixture (100 µl) of sterile PDB and fungal inoculums were loaded in each well. For positive control, only inoculum and PDB was taken. A negative control set was taken which contained the test compound and PDB only. The covered plates were incubated in a growth chamber at 28°C. Fungal growth was monitored after 48 h by measuring absorbance at 600 nm using a microtiter-plate reader (Mios Junior, Merck). A zero-hour reading was taken as blank. MIC was considered as the lowest concentration that did not record any growth.

4.3. Results

Methanolic leaf extract of *R. serpentina* of different doses were applied against pure cell suspension of the test pathogen (*C. albicans*) and after the period of incubation zone of inhibition (ZOI) was measured. Table 4.4 shows the ZOI (in cm) for three different concentrations of leaf extracts of four plants and also for excavarin-A. The ZOI was high in the case of *R. serpentina* leafextract. It was 4.87 cm at the concentration of 100 mg/ml. At the same concentration ZOI for *M. oleifera, N. arbor-tristis, C. excavata* were 3.2cm, 1.6cm and 2.4cm respectively. Excavarin-A emerged as most effective antifungal agent with a ZOI value of 4.6 cm at a concentration of only 10mg/ml.

The least MIC value was recorded for excavarin-A (0.078 mg/ml) followed by *R. serpentina* (0.156 mg/ml) (Table 4.5). Results indicated that both *R. serpentina* leaf extract and the molecule excavarin-A have antifungal activities close to known antifungal antibiotic nystatin (0.039 mg/ml).

Table: 4.4. Antifungal activities of crude methanolic leaf extracts of tested plants and the purified compound excavarin-A against *C. albicans*

Tested plant species	Zone of inhibition (cm) at different concentrations			
	100 mg/ml	10 mg/ml	1 mg/ml	0.1 mg/ml
R. serpentina	4.87	3.17	1.27	Not tested
M. oleifera	3.2	1.9	0.73	Not tested
N. arbor-tristis	1.6	0.8	0	Not tested
C. excavata	2.4	1.12	0.41	Not tested
Excavarin-A	Not tested	4.6	2.9	1.13

Table: 4.5. Minimum inhibitory concentration (MIC) of crude methanolic leaf extracts of tested plants and the purified compound excavarin-A against *C. albicans*

Studied extracts sample	MIC (mg/ml)
R. serpentina	0.156
M. oleifera	0.313
N. arbor-tristis	2.5
C. excavata	0.625
Excavarin-A	0.078
Nystatin	0.039

4.4. Discussion

The present study screened crude leaf extracts of medicinal plants for antifungal activities *in vitro*. *C. albicans* was used as test pathogen as it is the most common opportunistic fungal strain that may cause epidermal as well as potentially life threatening invasive-systematic infection, especially in immuno-compromised subjects or in immuno-suppressed patients. Commonly used antifungal drugs have toxic side-effects, moreover cases of drug resistance are increasing sharply, especially with long-term usage of the drug (Whaley et al., 2017). This creates an urgent need for development of new line

of treatment to fight against this noxious human pathogen, for which herbal remedy may be a good option.

Several medicinal plants have shown promising activities against *C. albicans in vitro* (Duarte et al., 2005; Soliman et al., 2017). In a recent study a protein molecule isolated from seeds of *M. oleifera*, named Mo-CBP2 was shown to possess antifungal activity against *C. albicans* (Neto et al., 2017). In the same year, extract from aerial parts of seven known medicinal plants were reported to have anticandidal activities (Soliman et al., 2017). Santos and Pereira (2018) enlisted several Brazilian traditional medicinal plants which were found to be effective against different strains of *Candida* sp. collected from mouth of infected population. Reports say that phytocompounds are potentially effective antifungal agents or they may work as synergistic agents with antifungal drugs (e.g., Fluconazole) particularly against *Candida* sp. (Lu et al., 2017). Plants having high flavonoid content showed good antimicrobial activity, probably due to their ability to complex with extracellular or soluble proteins of microbial cell walls (Marjorie, 1996; Tsao and Yang, 2003).

In the present work, leaf extracts of all four plants had shown good flavonoid content in phytochemical analysis, so they were further screened for antifungal activity. Excavarin-A emerged as most effective antifungal agent, which is in agreement with the previous finding of Kumar et al. (2012) who had isolated the novel molecule excavarin-A from leaf extract of *C. excavata* and screened it for antifungal activities. In our study, the crude methanolic leaf extract of *C. excavata* was also found to be antifungal when tested against *C. albicans*, *in vitro*. Leaf extract of *C. excavata* has been reported as potent therapeutic agent having wound healing and antioxidant activities *in vivo* (Albaayit et al., 2015). Antibacterial activity of ethanolic extract from the leaves of four Rutaceae species, including *C. excavata*, was reported in a recent study (Van et al., 2020).

The MIC value was lowest for excavarin-A followed by *R. serpentina*. There are previous reports on screening of extracts of different plant parts of

R. serpentina for antimicrobial activities (Negi et al., 2014). Elizabeth (2017) found significant antifungal activity of crude extract of *R. serpentina* when tested against human pathogen *C. albicans*. Our study also showed that the antifungal activities of *R. serpentina* leaf extract and excavarin-A were close to the activity of the antifungal antibiotic nystatin. Therefore, these may be potent candidates for effective antifungal agents of natural origin.

M. oleifera is a well-known traditional medicinal plant in Asia, it has also been a part of food habit in India. We found methanolic leaf extract of the plant had good antifungal activity against *C. albicans*. Similar trend was observed in some previous reports which screened different parts of *M. oleifera* for antimicrobial activities (Kalpana et al., 2013; Zaffer et al., 2015; Neto et al., 2017). Reports say extracts of different parts of *N. arbor-tristis* were screened for *in vitro* antifungal activity and showed significantly positive results previously. Different extract of bark of *N. arbor-tristis* was found active inhibitor of *C. albicans* and *Aspergillus fumigates* when compared with ketoconazole as standard antifungal drug (Sharma et al., 2013). Shrivastava et al.(2018) reported antifungal activities of different parts of *N. arbor-tristis,* which is in accordance with our findings. Further work is needed for isolation of biomolecules responsible for the antifungal property of these tested leaf extracts.

5.1. Literature review

Leishmaniasis is a zoonotic disease, caused by 20 different species of an obligate protozoan parasite of the genus *Leishmania*. The disease is vector-borne, and all species of *Leishmania* are transmitted by at least 70 different types of phlebotomine sand flies. Mammalian species, mainly humans, play the role of host. Leishmaniasis is responsible for the second-highest number of deaths due to parasitic infection globally and is overwhelmingly associated with poverty. It has an estimated prevalence of 12 million humans infected and causes a burden estimated at 2,357,000 disability-adjusted life in a year (Annual Report 2009, WHO, Geneva). It is a neglected infectious disease affecting nearly 98 countries worldwide (de Paula et al., 2019) with about 1.7 billion people at risk of contracting the infection (Sosa et al., 2019). The manifestation of the disease shows a vast range froms an asymptomatic localized skin lesion, to life-threatening progressive visceral form (https://www.who.int/news-room/fact-sheets/detail/leishmaniasis).

5.1.1. History

Long before identification, leishmaniasis was reported as early as the first century AD, in pre-Incan pottery from Ecuador to Peru as skin lesions and facial deformities. From the 15th to 16th century in Spain, it was known as 'White Leprosy', resembling a leprosy lesion. It was marked as 'Andean Sickness', 'Valley Sickness', and many other names. In Africa and India, in the mid-18th century, it was known as Kala-azar or Black fever. In 1776, Alexander Russel made an important advance in this field and called visceral leishmaniasis 'Allepo Boil'. The disease got its name after William Leishman, who worked as a doctor with the British army in India, and identified and named the strain in the splenic smear of a patient, who died of 'Dum-Dum' fever in 1901. In 1903, Charles Donovan for first time characterized the causative agent as protozoan and morphologically related to Trypanosomes (Cox, 2002; Akhoundi et al., 2016). Later, in 1930, Sir Ronald Ross named the parasite *L. donovani*, and eventually, thus, established the link between the very old disease and the causative organism. Species belonging to the genus *Leishmania* are the

etiological agent of a variety of manifestations of the disease collectively known as leishmaniasis. The common species of disease causing *Leishmania* are *L. donovani, L. infantum* and *L. archibedi* in the old world, and *L. chagasi* in the new world. Sandfly was identified as a vector by Swaminath et al. (1942) by using human volunteers and humans, wild animals, and domestic animals were known to act as reservoir hosts. Later it was established that *Lutzomyia longipalpis* is the only sand fly vector species that transmit visceral leishmaniasis and reservoir host is a wild or domestic dog (WHO expert committee report 1991, https://apps.who.int/iris/handle/10665/37432;de Paula et al., 2019).

5.1.2. Clinical manifestations and classification of leishmaniasis

The range of clinical manifestations of leishmaniasis is wide and is specified by the type of invading parasite and also the genetic determinants present in the human host which influences the overall immune response. These clinical features collectively called leishmaniasis are classified as (i) visceral leishmaniasis (VL) or 'kala-azar' (ii) cutaneous leishmaniasis (CL), diffused and localised; (iii) post kala-azar dermal leishmaniasis (PKDL), (iv) mucocutaneous leishmaniasis (MCL) and (v) leishmaniasisrecidiva. (Torres-Guerrero et al., 2017; Valpedo et al., 2021).

Visceral leishmaniasis

This is one of the most deadliest kind of the leishmaniasis commonly known as kala-azar and caused by *L. donovani* in India and Africa, *L. donovani chagasi* in South America and *L. donovani infantum* in Mediterranean areas. *L. tropica* is also marked as causative agent of VL in recent studies. If remain untreated, VL can emerges in fatal form with 100% morality. VL is characterised by long term low-grade fever, hepatosplenomegaly, that is enlarged spleen and liver with biological signs of anaemia and weight loss (Woodruff, 1972; Rajagopala et al., 2008; Singh and Sundar, 2015), affecting the organs and tissues like liver, spleen, lymph nodes, bone marrow, lung, intestine, skin and rarely oral mucosa, placenta and thymus. In the year 2020, 90% cases of VL have been reported in 10 countries, those are Brazil, China, Ethiopia, Eritrea, India,

Kenya, Somalia, South Sudan, Sudan and Yemen (WHO, 2020, https://www.who.int).

Post Kala-azar dermal leishmaniasis (PKDL)

PKDL is characterised by non-ulcerative cutaneous lesion developed in post kala-azar subject mainly in India and Sudan following drug treatment (WHO, 2020). It is proven to be due to accumulation of heavily infected macrophage under the skin (Zijlstsa et al., 2003) and manifesting nodules, papules, hypopigmented macules mainly on face first then a spread on other parts of body are seen.

Localised cutaneous leishmaniasis

This is the most common form of leishmaniasis worldwide, with approximately one million new cases that are diagnosed every year, and about 90% of the cases are found in developing countries (Badirzadeh et al., 2020). It is caused by *L. tropica, L. aethiopic*and *L. major* in old world and by *L. mexicana, L. guyanesis, L. braziliensis* and *L. amazonensis* in new world. CL is characterised by development of self-healing benign lesions on skin without any mucosal involvement. In *L. mexicana* infections, lesions (Chiclero's Ulcer) develop on pinna of ear (Saito and Murray, 2020). Sometimes, the disease exists in host body for a long time (*L. tropica*), leading to disfigured body parts.

Diffuse cutaneous leishmaniasis (DCL)

DCL is the rare form of the disease caused by *L. mexicana*, *L. aethiopica* and *L. amazonesis* reported from Venezuela, Brazil, Ethiopia and Dominic republic. The disease is featured by non-ulcerating nodules like lepromatous leprosy over the entire body without pain which can be healed only by treatment (Volpedo et al., 2021).

Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis is caused by *L. braziliensis braziliensis*. The disease occurs mainly (90%) in South America. MCL is characterised by two phases, primary cutaneous lesion followed by mucosal involvement (metastatic

infected macrophages). The primary lesions are spontaneously cured still infection may remain inside the host and without effective early treatment it may cause permanent disfigurement (Volpedo et al., 2021).

Leishmaniasis recidiva

Chronic recurrence of *L. tropica* causes a hypersensitive dermal response with nodular lesion and satellite lesions at margins of primary lesion of DCL which have already been healed. It is mainly found in people of Middle East (Volpedo et al., 2021).

5.1.3. Mode of transmission of disease

Leishmaniasis may be transmitted from vector to host, host to non-infected subject in many ways among which the most common mode of transmission worldwide is vector borne transmission. Other modes are parenteral, congenital, sexual and occupational from host to non-infected person (Fig. 5.1).

5.1.4. Epidemiology

As the world's most neglected disease, leishmaniasis is still affecting the poor people of developing and underdeveloped countries; prevalent throughout the tropical and sub-tropical regions of Asia, Africa, Southern Europe, South, and Central America. Moreover, many cases go misdiagnosed and unreported, and therefore the exact scale of impact on public health cannot be predicted. According to WHO (2022), most cases occur in Brazil, East Africa and India (Fig. 5.2). An estimated 50,000 to 90,000 new cases occur worldwide annually, with only 25% to 45% reported to WHO. In 2019, more than 90% of new cases reported to WHO occurred in 10 countries viz. Brazil, Eritrea, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan. Visceral leishmaniasis (VL) brings India on focus in the world and in India, mainly the state of Bihar, eastern Uttar Pradesh (Marwaha et al., 1991) and West Bengal (Chandra et al., 1995) are heavily affected. According to a recent report of WHO (2022), the four states of India where kala-azar is still a threat to public health are: Bihar (33 districts, 458 blocks), Jharkhand (4 districts, 33 blocks), West Bengal

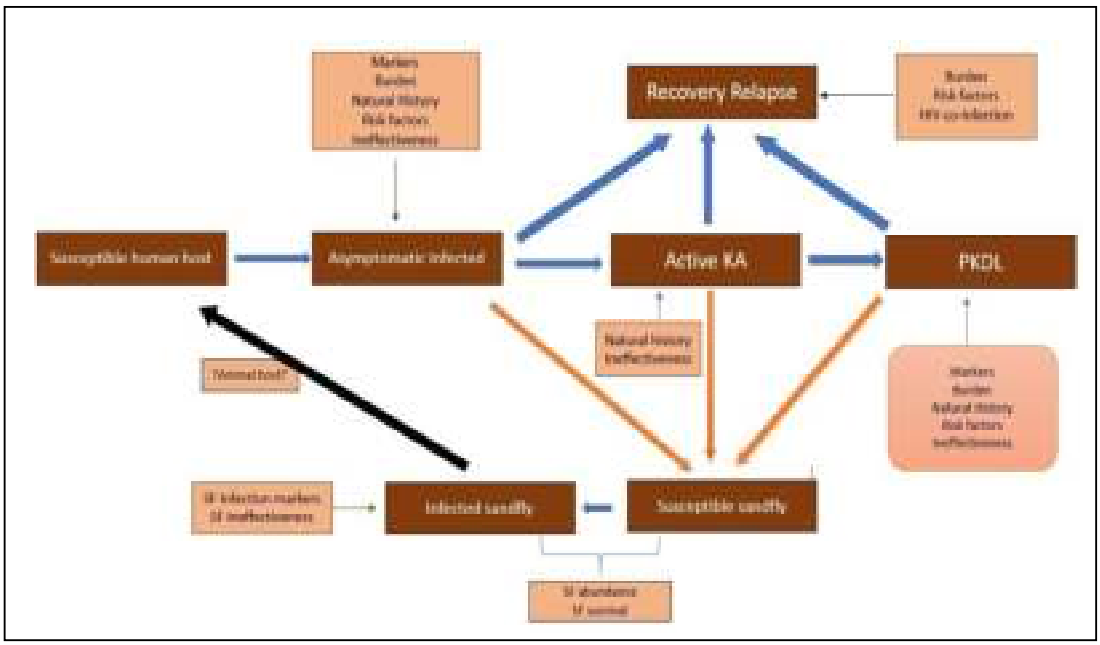

Figure:5.1. Schematic framework showing transmission of visceral leishmaniasis

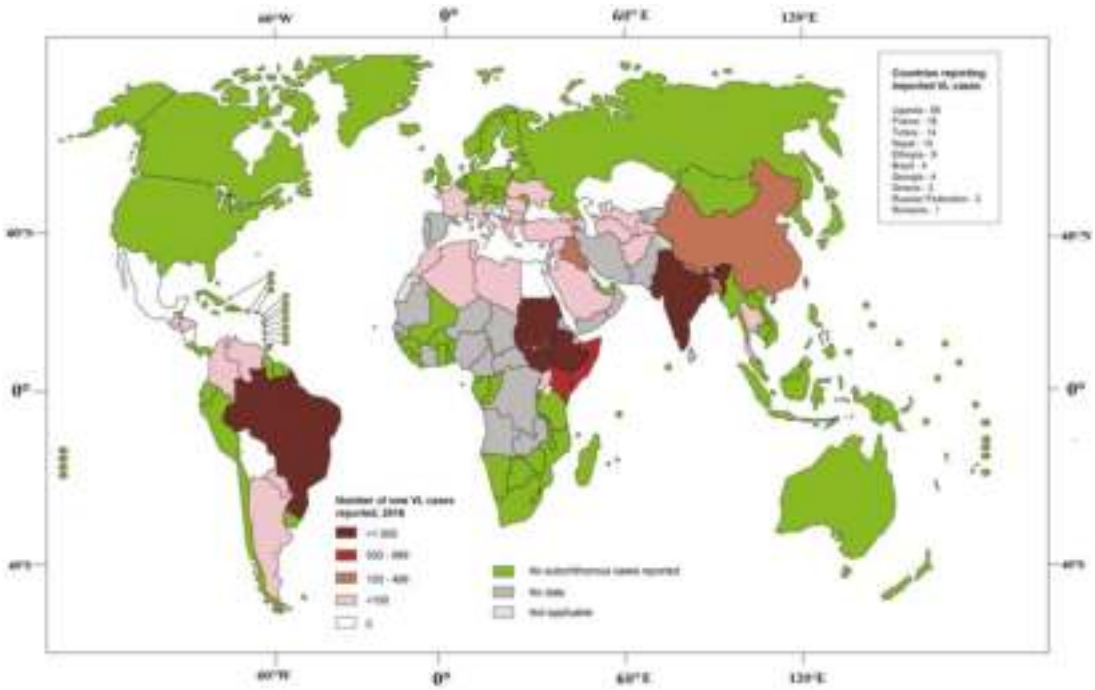

Figure: 5.2. Status of endemicity of visceral leishmaniasis Worldwide 2016 (WHO) (Prepared using ***ArcGIS 10.3.1*** *software*)

(11 districts, 120 blocks) and Uttar Pradesh (6 districts, 22 blocks). Recent news of leishmaniasis in some blocks of Malda districts, West Bengal, increases the concern (Uttar Banga Sambad, 18th Feb 2019) (Fig. 5.3). National Center for Vector Borne Diseases control, Ministry of Health and Family Welfare & Government of India keeps the record of cases of VL, post Kala-azar dermal leishmaniasis (PKDL) and AIDS -VL coinfection in India. They reported some cases from states other than above four, like Assam, Sikkim and Uttarakhand from the year 2014 to 2017**(**https://nvbdcp.gov.in**).**

5.1.5. Leishmaniasis and AIDS-a vicious cycle

Leishmaniasis is a well-recognized opportunistic infection in HIV infected people (Kooli et al., 2021). The first case of leishmaniasis associated with HIV infection was reported in 1985, and thereafter persistent rise in the number is seen worldwide. Studies said, in areas of endemicity, HIV infection increases the risk of developing VL by 100 to 2,320 times, reduces the likelihood of a therapeutic response, and increases the probability of relapse. At the same time, VL helps the clinical progression of HIV disease and the development of AIDS (Alvar et al., 2008). In the 1.5-9% of AIDS patients are reported to develop visceral leishmaniasis, and on the other side, 25-70% of people affected with leishmaniasis are related to HIV infection. For the last few decades, AIDS and VL are observed to work in a vicious cycle of mutual reinforcement (Fuzibet et al., 1988; Lindoso et al., 2009). According to WHO (2022), *Leishmania*-HIV coinfections have been reported from 45 countries. High *Leishmania*-HIV coinfection rates are reported from Brazil, Ethiopia and the state of Bihar in India. In a recent study, incidence of hemophagocytic lymphohistiocytosis (HLH) in HIV-positive person with visceral leishmaniasis has been reported (Kooli et al., 2021).

5.1.6. Morphology of parasite

Leishmania parasite, a protozoan, exists in two morphological states, and thus, is known as a dimorphic parasite. As a promastigote, it exists in sand fly and as an amastigote, it exists in the vertebrate host.

Figure: 5.3. Some articles published in leading newspapers depicting news of kala-azar in India and West Bengal in recent time

5.1.6.1. The promastigote forms

The promastigote form is the extracellular form of the parasite, seen in the insect vectors and also in laboratory culture(Herwaldt,1992). It is flagellated, having a spindle-like shape, 15-20 nm in length, and 1.5-3.5 nm in breadth (Fig: 5.4 A and B). When sandfly ingests the blood of an infected person or vertebrate host, the amastigotes are transformed into promastigotes within hours in the midgut of the sandfly and are transformed into different stages (Ashford, 1997). In metacyclic promastigotes, the nucleus is in the centre and the Kinetoplast in the anterior end. The flagellum of promastigote is 15-28 nm in length with a tubular and latticed structure.

5.1.6.2. The amastigote forms

The amastigote forms are seen within the body of vertebrate hosts; it resides in the reticuloendothelial cells of humans, dogs, or hamsters. It is a round or oval-shaped flagellated form with a delicate filament extending from the basal body. It is 2-4 nm long, with little or no motility (Fig: 5.4 A and C).

5.1.7. Life cycle of parasite and the role of host

Leishmania parasite has dimorphic life cycle between primary reservoir (human) and secondary host (sand fly) (Fig: 5.5). When a female sand fly attacks *Leishmania* infected human host and feeds on blood, it ingests a number of amastigotes and these non-flagellated amastigotes are transformed into flagellated promastigotes in posterior midgut of female sand fly within hours. The promastigotes divide by binary fission and get converted into procyclic promastigotes, which are transformed into nectomonad form. Within 3 days of blood feeding, peritrophic membrane of gut of sand fly containing these parasitesbegins to break down and promastigotes are set free and move towards anterior midgut of the host (Ashford, 1997). In the anterior midgut, the parasite changes its form into nectomonad and then to haptomonads within the next five days and are attached to the stomodeal valve (Vickerman and Tetley, 1990). Thereafter, the highly motile metacyclic parasite localized

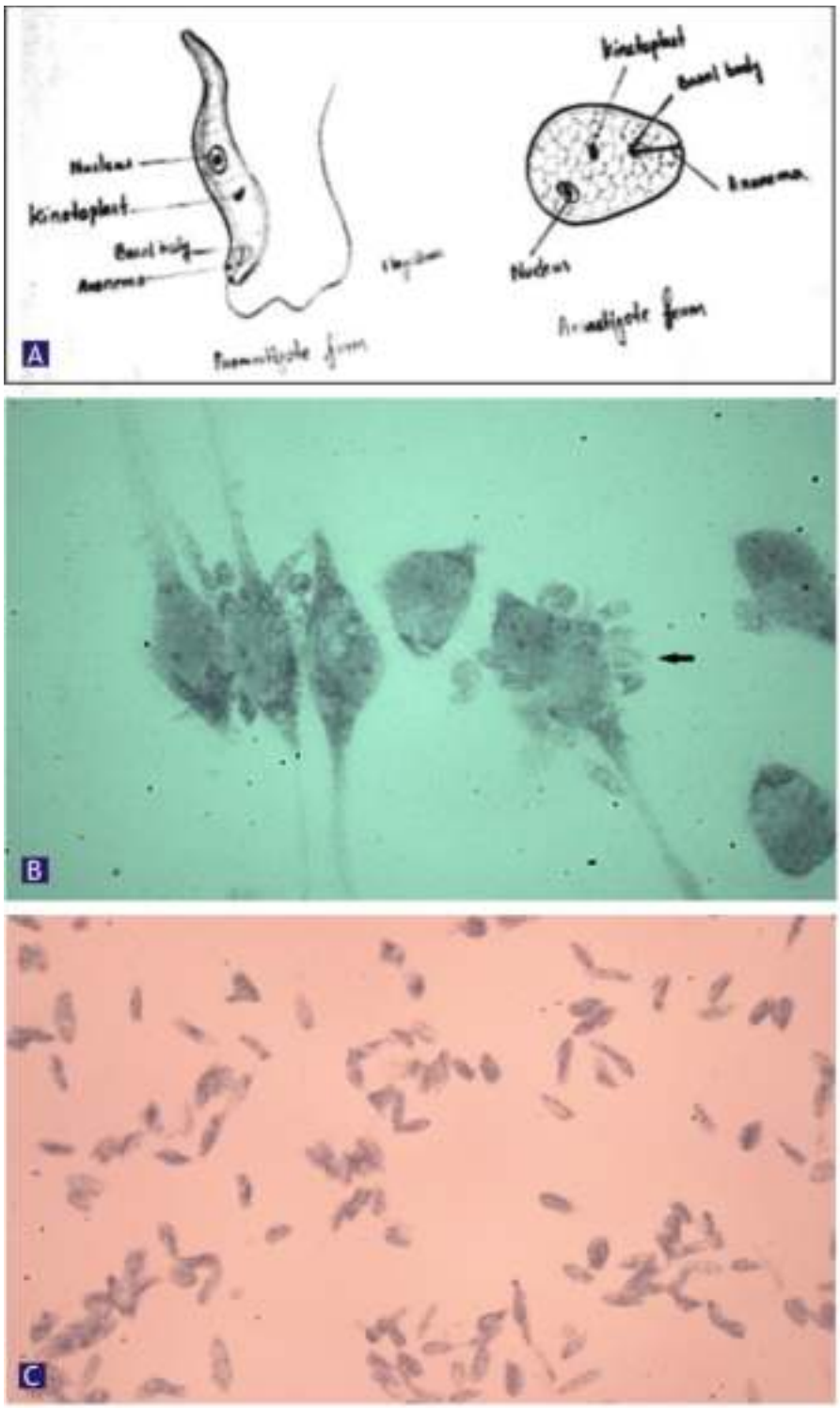

Figure: 5.4. A: Diagram of promastigote and amastigote form of *L. donovani*; B: Image of promastigotes in culture as seen under microscope; C: Image of amastigotes attacking macrophage in culture as seen under microscope

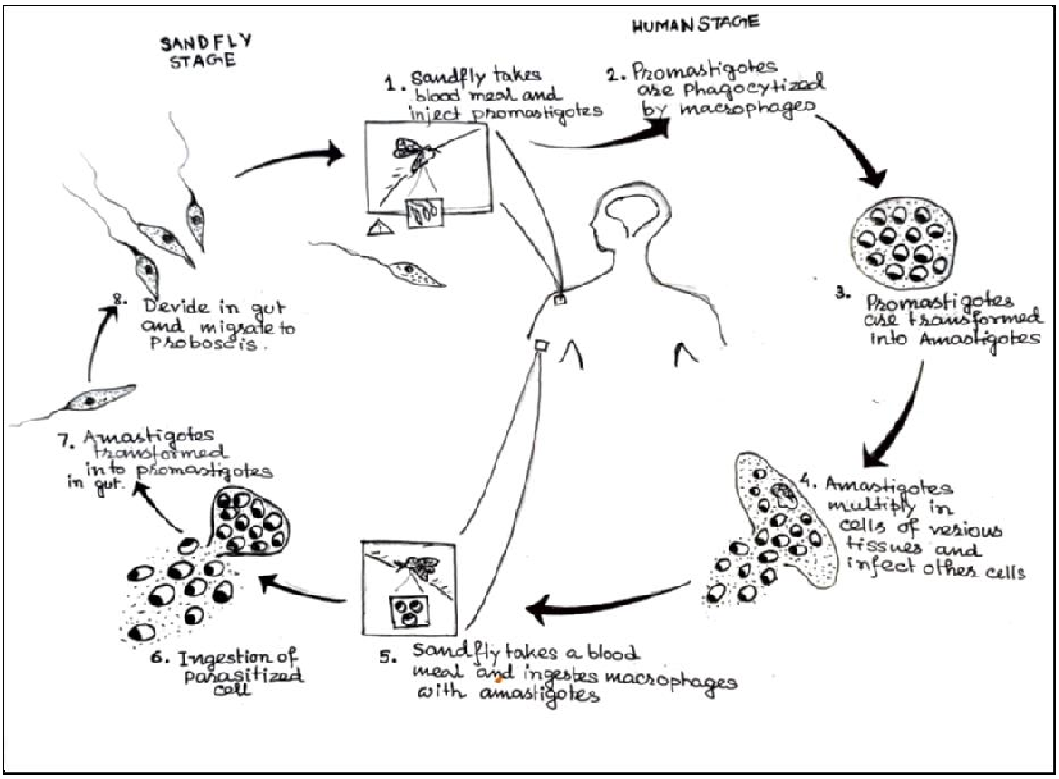

Figure: 5.5. Life cycle of *Leishmania* parasite

in the lumen of anterior midgut and foregut is ready for spreading into primary host during the next blood meal of the sand fly. The metacyclic promastigotes enter into vertebrate host, are taken up by macrophages, and transformed into amastigotes with in parasitophorus vacuoles (Kima, 2007). The manifestation of disease in various forms of leishmaniasis depends on the different tissues where the infected macrophages are present (Torres-Guerrero et al., 2017).

Change in temperature in different host initiates the changes in form of the parasite. Amastigotes, when face a drastic change in temperature from 37° Celsius of mammalian body to 22-28°C in insect body, the onset of change in form starts. Not only temperature, the environment, motility of two types of hosts implies significant physiological difference in two forms of the parasite. All these findings give the possibility of *in vitro* transformation of promastigote in culture in laboratory. Transformation from amastigote to promastigote is associated with increase in respiratory rate, synthesis of cytochrome C, polyamines (Morrow et al., 1980), increase in cell volume and acquisition of antigen and expression of heat shock gene, enhancement in glycolytic enzyme activities and reduction in enzyme activities of fatty acid (Hart and Coombs, 1982).On the other hand, in amastigotes, glutathione peroxide, catalase and superoxide dismutase are active, which helps the parasite to survive in mammalian host's immuno-attack. There are changes in antigenic properties and gene expression.

The phlebotomine sand flies recognized as vectors of leishmaniasis causing parasites are of the order *Diptera,* Family *Psychodidae,* subfamily Phlebotominae. Among the 800 known species or subspecies of Phlebotomine only 40 species are responsible for transmission of leishmaniasis (Killick-Kendrick, 2002). The two main genera, *Lutzomyia* in new world and *Phlebotomus* in old world (Mann et al., 2021) are medically important as vectors. In India, *Phlebotomus argentipes*is prevalent. The parasite develops and multiplies in wide range of temperature (16°celsius to 34°celsius) in sand fly; and bites of sand fly are very common in Kala-azar endemic areas throughout the year. According to the observation, the number of flies reaches

a peak between February and July in Bihar, and July to October in West Bengal (Torres-Guerrero et al., 2017).

5.1.8. Pathogenesis of leishmaniasis and role of immune system in host body

Blood meal by a female sand fly caring *Leishmania* promastigote initiates the infection with *Leishmania* in vertebrate host or human. The saliva of sand fly is infused with virulent metacyclic promastigotes which are injected into skin and then into circulation during insect bite. The saliva also plays role in proper injection of parasite as it carries vasodilating molecule, substances having anticoagulant and immunomodulatory role (Sacks and Kamhawi, 2001). From the circulation in the host body, the promastigotes are ingested by phagocytes by complement aided opsonization and lectinlipophosphoglycan (LPG) – mediated phagocytosis (Wozencraft and Blackwell, 1987). Phagosome releases superoxides and hydroxyl radicals to encounter invading pathogen. But *Leishmania* parasite has several mechanisms to resist host immune attack and survive.

First the parasite produces acid phosphatase on their cell surface which blocks the production of superoxide (Glew et al., 1988). LPG of parasite also inhibits protein Kinase C which is essential for generating oxidative metabolites in host macrophage (McNeely et al., 1989). Growth factor withdrawal induced apoptosis of macrophage in host is also inhibited by invading parasite because viability of infected macrophages is required to be up taken by vector (sand fly) as much in numbers as possible (Delgado-Altamirano et al., 2017) (Fig: 5.6). Various studies reported the role of different components of acquired immunity of host body, such as interleukin (IL-10, IL-12), interferon (IFNγ) andtumour necrosis factors (TNFα) in disease development (Gazzinelli et al., 1996; Rittig and Bogdan, 2000; Maynard and Weaver, 2008; Volpedo et al., 2021) (Fig.5.6). Besides, the enzyme super oxide dismutase (SOD) which catalyses the dismutation of superoxides (O_2^-) into H_2O_2 and O_2, plays a vital role in the survival of parasites of leishmaniasis in host body. Its activity is also

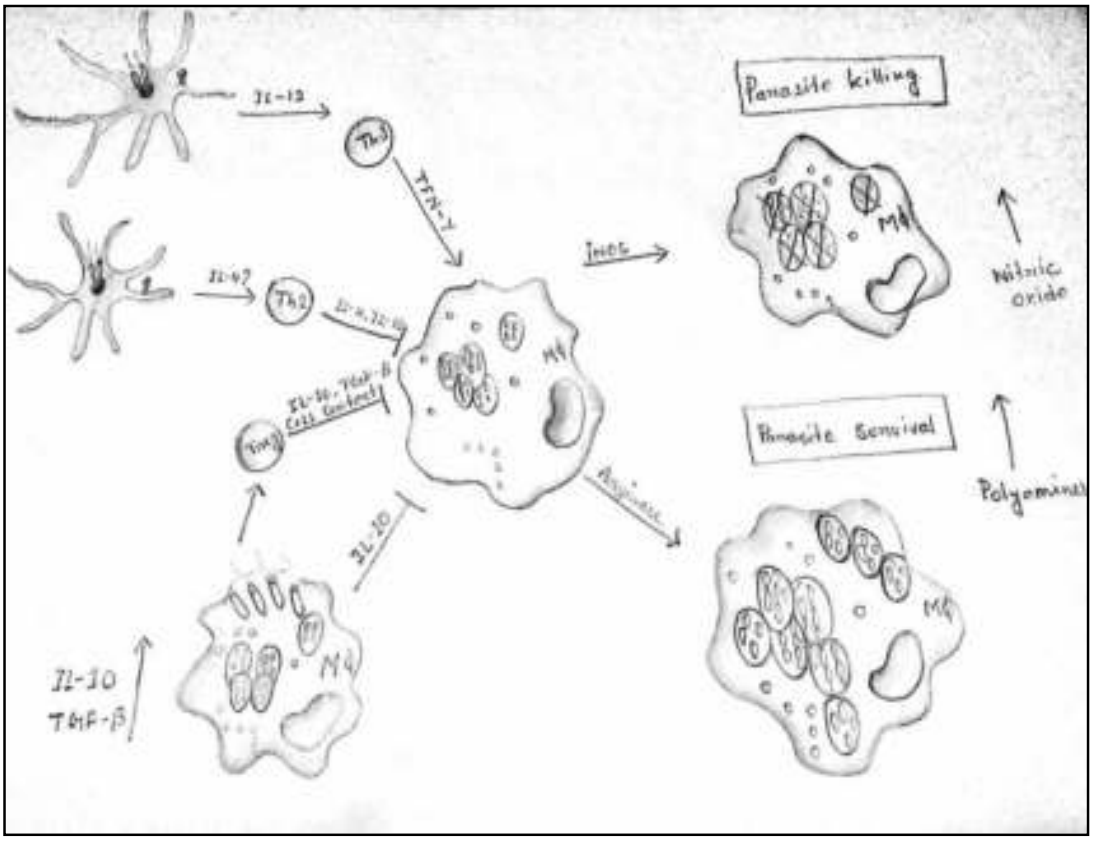

Figure: 5.6. Immunological response of host body towards invading pathogen of leishmaniasis

found to be elevated when the parasite is within the host (Dey et al., 1995; Ghosh et al., 2003).

5.1.9. Available line of treatment

Since 1940, antimonial drugs are first line of treatment of all forms of leishmaniasis worldwide. Meglumine antimoniate, (marketed as glucantine and prostib), sodium stiboglucanate or SSG (commercially available as pentastam, solustibosan and stibanate) are the widely used compositions. But with these compositions some complications are emerging in different studies, like requirement of high doses and drug resistance (Rees et al., 1980; Thakur et al., 2004). After the rise in SSG resistance, amphotericin ß is extensively used even as first line of drug in Bihar (India) with 98-100% positive results (Sundar et al., 2002). Newer drugs used include paromomycin, miltefosine and sitamaquine but these therapeutic agents are facing either the problem of drug resistance, inefficiency or adverse side effects (Sundar et al., 2002).

The concept of vaccination against leishmaniasis was materialised a long time ago when intuitive knowledge of mother of Lebanese children motivated them to expose their children's arms to the bite of sand flies, which developed a self-healing single first lesion which would protect them from severe attack of the same disease in future (Vanloubbeeck and Jones, 2004). This practice inspired Professor Adler from Hebrew University of Jerusalem, Israel to develop first vaccine against leishmaniasis. At first scientifically an axenic culture of parasite was prepared, administration of which was known as leishmaniazation and it became usual in Israel and Russia, which was the first step in preparation of vaccine. Then killed parasites were used instead but the leishmaniazation process was discontinued due to uncontrolled long-lasting skin lesion. At present its use is limited worldwide (Murray, 2001). In past different attempts were made to discover an effective vaccine, such as live non-attenuated vaccines, live-attenuated vaccines, killed vaccines and DNA vaccines (Handman, 2001; Giunchetti et al., 2008) but none were found to be totally efficient or safe. Therefore, till today, there is no definite product which offers all-round protection against the disease (Badirzadeh et al., 2020).

In this context, natural remedies and novel molecules from natural sources provide an alternative to fight against leishmaniasis.

5.1.10. Natural remedies

From ages indigenous people of different countries are using vast resources of plant kingdom for medication without knowing the science behind it. Local people of leishmaniasis affected countries are using different plants or extracts of plant part like root, leaf, fruit etc to get rid of this endemic disease. Local literature and ayurveda have mentioned about these natural products. From ancient knowledge, scientists and researchers get inspirations to screen different referral plants or new ones against leishmania pathogen *in vitro* or in animal models (Table: 5.1).

In the present study, we have screened methanolic leaf extract of *R. serpentina* and *C. excavata* and the molecule excavarin-A for antileishmanial activity in search of a new therapeutic option.

Table: 5.1. Some studies on the efficacy and activity of herbal medicines used against leishmaniasis *in vitro* and *in vivo*.

Plant	Plant part/ extract used	Species of pathogen	Mode of action	Reference
Alkanna tictoria	Stem and root	*L. major*	IC_{50} 200µg/ml	Yousefi et al., 2009
Allium hirtifolium	Bulbs	*L. infantum*	Parasite growth inhibited in one day at 0.2 mg/cc	Amnzadeh et al., 2006
Artemisia annua	Aerial parts	*L. major*	IC_{50}400 µg/ml for ethanolic extract	Emami et al., 2012
Berberis vulgaris	Root	*L. tropica*	IC_{50}16.1 µg/ml for methanolic extract and 26.6 µg/ml for aqueous extract	Mahmoudvand et al., 2014
Berberis vulgaris	Whole plant	*L. major*	With 20% preparation, the lesions had decreased with complete healing in 5 mice(27.7%).	Kazemi et al., 2007
Calotropis gigantea	Aerial parts	*L. major*	IC_{50}0.18 mg/ml for methanolic extract and 0.17 mg/ml for hexane extract	Oskuee et al., 2012
Capsicum frutescens	Capsaicin from seeds	*L. infantum*	IC_{50} 5.01 µg/ml against promastigotes and 24.18 µg/ml against amastigotes	Vieira-Araújo et al., 2018
Ixora brachiate	Root	*L. major*	IC_{50} 0.078 mg/ml for ethanolic extract	Sadeghi-Nejad et al., 2014
Matricaria chamomilla	Flower	*L. major*	Treatment with chamomilla tea (for 5mins/day) for 2 weeks wound healing (skin ulcer) was 58.3%(in diameter)	Dashtpeima et al., 2015
Myrtus communis	Leaves	*L. tropica*	IC_{50} 28.9 µg/ml	Mahmoudvand et al., 2015
Peganum harmala	Seed	*L. major*	IC_{50}40 µg/ml	Yousefi et al., 2009
Phytolaca americana	Fruits	*L. major*	IC_{50}171.1 µg/ml against promastigotes	Jafroodi et al., 2015
Piper aduncum	Essential oil	*L. braziliensis*	IC_{50} 77.9 µg/ml against promastigotes	Ceole et al.,2017
Pistacia atlantica	Gum obtained from trunk and branches	*L. major*	Eight weeks treatment with gum (60 mg/kg body weight) decrease skin lesion size (mice) from 68.4 to 35.3 mm	Taran et al., 2010

Plant	Plant part/ extract used	Species of pathogen	Mode of action	Reference
Pistacia khinjuk	Stock	*L. tropica*	IC_{50} 58.6 µg/ml against promastigotes, IC_{50} 37.3 µg/ml against amastigotes	Ezatpour et al., 2015
Sature jakhuzestanica	Leaf	*L. major*	IC_{50}0.3 mg/ml for ethanolic extract and 0.6 mg/ ml for methanolic extract	Sadeghi-Nejad et al., 2011
Scrophulara striata	Whole plant	*L. major*	The parasites were killed at 20% concentration within two days.	Dalimi et al., 2013
Urtica dioica	Leaves	*L. major*	IC_{50} 4500 µg/ml against promastigotes	Badirzadeh et al.,2020

5.2. Materials and Methods

5.2.1. Preparation of leaf extract

Leaf extract of plants were prepared following the procedure described in previous chapter (section 3.2.1). The molecule Excavarin-A was isolated from the leaf extract of *C. excavata* following the procedure described in section 4.2.2.

5.2.2. Propagation of parasites

L. donovani, strain MHOM/IN/83/AG was originally derived from an Indian kala-azar patient (Ghosh et al., 1985). The strain was maintained by intracardial passage every 8 weeks in a laboratory animal model, Syrian golden hamsters. The amastigotes were isolated from the spleen of infected animals in Medium-199 (GIBCO BRL, Gaithersburg, MD, USA), pH 7.4, supplemented with 10% heat inactivated foetal calf serum. Promastigotes were obtained by transforming amastigotes isolated from infected spleen (Jaffe et al., 1984) *in vitro* and sub-cultured in the same medium at 22°C, at an average density of $2x10^6$ cells/ml (Kar, 1990).

5.2.3. Maintenance of promastigotes

Solid Media: The promastigote form of *L. donovani* was maintained in modified Ray's solid medium at 22-25°C (Ray, 1932).

Liquid Media: Parasites were also grown in sterile Medium-199, as and when required. The medium was sterilized by passing through 0.22 µm sterile Millipore filters (Millipore Corporation, USA). Maintenance of *L. donovani* strain was done as described above. Promastigotes were obtained by transforming amastigotes ($1X10^7$cell concentration) isolated from infected spleen (Raychaudhury et al., 2005) and maintained in Medium-199 supplemented with 10% foetal calf serum (FCS) *in vitro*.

5.2.4. Model animal

Syrian golden hamsters were originally obtained from Haffkine Research Institute, Mumbai, India and bred in animal house of IICB, Jadavpur, Kolkata.

5.2.5. Isolation of amastigotes

Spleens of hamsters infected with *L. donovani* were suspended in liquid Medium-199, homogenized using a glass homogenizer and centrifuged at 1,000 rpm for 10 minutes. The supernatant was centrifuged at 2,000 rpm for 10 minutes and the pelleted parasites were washed in the same medium by centrifugation again at 2,000 rpm for 10 minutes and resuspended again in the same medium (Channon et al., 1984). These amastigotes were injected intracardially into hamsters for the maintenance of virulent strain and also used for further study. The animal ethics committee of the institute approved all animal experiments.

5.2.6. Screening for Leishmanicidal activity

The assessment of the *in vitro* leishmanicidal activities of leaf extracts of *R. serpentina* (RS), and *C. excavata* (CE)and a molecule excavarin-A were carried out as described below following the method of Gupta et al. (2010).

5.2.6.1. *In vitro* inhibitory assay of plant extracts against promastigote proliferation

All three samples were aseptically dissolved in 1% DMSO (dimethyl sulfoxide) and diluted appropriately with the growth medium. Promastigotes from the logarithmic phase were seeded into the wells of a 96-well plate (5×10^5 cells/ml) and kept for 24, 36 and 48 hrs. This was followed by the addition of the test compounds at different dilutions (in triplicate). The standard drug SAG

was used for comparison, whereas the medium and 1% DMSO was included as negative control. After treatment with extracts, the plates were incubated for an additional 48 hrs at 26°C. Following incubation, 10 µl MTT (4 mg/ml) was added and the cells were incubated for another 4 h at 37°C. Thereafter, the plates were centrifuged at 2,000 rpm for 5 min and the supernatant was removed. The insoluble formazan produced by the parasites was dissolved in DMSO (100 µl) and the absorbance was noted at 570 nm. The absorbance is a measure of living cell amount. Leishmanicidal effect of extracts was expressed as IC_{50} values which is the concentration of the extracts which caused death of 50% *Leishmania* pathogen cells. The result was computed as an average of three replications.

5.2.6.2. Test of efficacy of crude plant leaf extracts and the purified molecule excavarin-A on intracellular survival of *Leishmania*-infected hamster macrophages

5.2.6.2.1. Isolation of macrophages

The host body macrophages are the cells where parasites reside. To test the effect of plant extracts on parasites dwelling in the cells, the host macrophages were collected from host body, cultured over coverslips, infected with parasites and studied further. For collection of macrophages from host peritoneal cavity, RPMI-1640 [GIBCO Laboratories, Grand Island, New York, USA], supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS) was used. Syrian golden hamsters were injected intraperitoneally with 2ml of sterile 4% thioglycollate. After 72 hrs, ice cold RPMI sterile medium was again injected in a volume of 10 ml (with a 20-gauge needled syringe). After gentle massage at abdominal region, the fluid from peritoneal cavity was withdrawn. This fluid was then centrifuged at 400x g for 10 minutes at 4°C. After washing the pellet twice with same media, it was resuspended in fresh RPMI 1640 supplemented with 10% FCS with a cell concentration of $5x10^5$/ml.

5.2.6.2.2. Antileishmanial activity of plant extracts on amastigotes

The isolated macrophages were cultured on coverslips (20x25 cm^2) placed in sterile disposable petri dish. For this, the macrophages were counted in a haemocytometer and the concentration was adjusted with the medium. Cell suspensions containing $2x10^5$ cells in 0.2 ml were distributed to sterile coverslips and incubated for 2 hr at 37°C. Non-adherent cells were removed by wash using same media. Now again, the adhered macrophages were overlaid by same media and incubated overnight at 37°C in 5% CO_2 incubator. On the next day, these macrophages were infected with *L. donovani* promastigote (in stationary phase) suspended in media RPMI 1640 (with FCS) at the macrophage parasite ratio of 1:10 and incubated for 5h at 37°C in 5% CO_2. After incubation, cover slips were washed with RPMI 1640 medium containing 10% FCS to remove non infected promastigotes. Plant extracts and the standard drug were then added at different concentrations and incubated for 48h.Following incubation, the cover slips were washed with media fixed with cold methanol and stained with GIEMSA to examine intracellular parasite load under microscope. The experiment was performed in triplicate and number of viable amastigotes was determined in average.

5.2.6.2.3. GIEMSA staining

Initially the stock solution of GIEMSA stain was prepared and subsequently the working solution was prepared by diluting stock solution in 1:5 ratio with doubled distilled water. The cover slips with attached macrophages were fixed with ice-cold methanol for 5 minutes and allowed to dry. Then the working GIEMSA solution was poured over the film of macrophages and kept for next 5 minutes at room temperature. After that, with a gentle flow of tap water, the stain was flushed well and slides were examined under microscope, with oil immersion lens.

5.2.7. *In vitro* cytotoxicity assay

In a separate experiment, cellular viability of hamster macrophages after treatment with test substances, were determined by using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to detect

living cells which have the ability to reduce yellow MTT to a blue formazan product (Mosmann, 1983). The experiment was conducted following the method of Gupta et al. (2010). Macrophage cells were maintained in 96-well plates using RMPI 1640 containing 10% FCS AT 37°C and 5% CO_2 for 24 h. After appropriate treatment for 30 minutes at 37°C with varying concentration of experimental plant leaf extracts,10 µlMTT (0.5 mg/ml) was added and plates were incubated for 4 h. *R. serpentina* and *C. excavata* were taken in the concentrations of 10 mg/ml, 20 mg/ml, 40 mg/ml, 80 mg/ml and 100 mg/ml. Excavarin-A was taken in the concentrations of 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 2.5 mg/ml 3 mg/ml and 3 mg/ml. Next 1N hydrogen chloride–isopropanol (1:24, v/v) was added, left for 15 minutes at room temperature and optical density was then read at 570 nm on an ELISA reader. The whole experiment was performed in triplicate and result was calculated as average of three separate experiments. IC_{50} was calculated by fitting the values to non-linear curve analysis.

5.3. Results

5.3.1. *In vitro* inhibitory assay of plant extracts against promastigote proliferation

The parasites in promastigote form were treated with leaf extracts of test plants and a molecule excavarin-A in different concentrations and percentage of viable promastigotes were determined as:

(Number of live cells after treatment/initial number of live cells) X 100

The percentage of growth inhibition of promastigotes were calculated for respective concentrations of test samples.

Excavarin-A was found to be most effective in inhibiting the growth of promastigotes. With application of graded doses of excavarin-A, the percent inhibition of promastigotes increased progressively and almost linearly. Its IC_{50} value was recorded as 1.24 mg/ml (Fig.5.9), which was found to be even less than the standard drug SAG which produced an IC_{50} value of 1.42 mg/ml (Fig.5.10).*R. serpentina* was found to be the most effective among crude

extracts and next to excavarin-A. It showed a different trend, maximum progression in the percent of growth inhibition of promastigotes was observed at lower concentrations.Thereafter, the increase in the % inhibition showed a declining trend. The IC_{50} was recorded as 4.04 mg/ml (Fig: 5.7). Much more concentration of leaf extract of *C. excavata* was required to inhibit the growth of promastigotes, and the IC_{50} value was found as 32.1 mg/ml (Fig: 5.8) (Table 5.2).

5.3.2. Antileishmanial activity of plant extracts on amastigotes

Study was done to test the effect of plant extracts on parasites, dwelling in the host macrophages in the amastigote form. Effectiveness of the test substances was determined by calculating percentage of viable amastigotes within macrophages after treatment. Excavarin-A emerged as most effective botanical in inhibiting the growth of internalized amastigotes. At 4 mg /ml concentration of excavarin-A, 52% viability of amastigotes was observed (Fig: 5.13). After treatment with leaf extracts of *R. serpentina*, a gradual decline in number of viable amastigotes with increasing concentration of leaf extract was observed (Fig: 5.11). At a concentration of 40mg/ml, there was 50% growth inhibition of amastigotes (Fig: 5.12). In case of *C. excavata* leaf extract, percentage of growth inhibition of amastigotes progressed with increasing concentration but the range of concentrations required were much higher than *R. serpentina*.

5.3.3 Comparative analysis between effective doses of tested extracts against promastigotes and amastigotes

All three botanicals showed growth inhibition of promastigotes *in vitro*. IC_{50} values of *R. serpentina* and *C. excavata* leaf extracts, and excavarin-A for growth inhibition of promastigotes were 4.04 mg/ml, 32.1 mg/ml and 1.24 mg/ml respectively (Table 5.2). Percentage of viable amastigotes within macrophages was also determined after treatment with graded doses of test samples and IC_{50} value was calculated by fitting the values to nonlinear curve analysis. IC_{50} values of *R. serpentina* and *C. excavata* leaf extracts, and

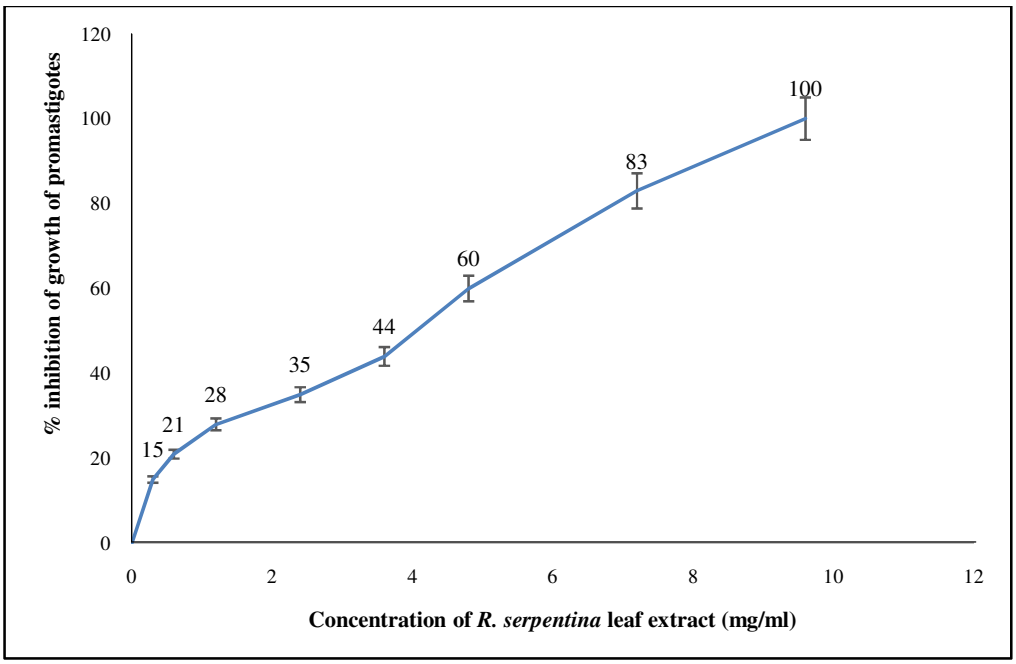

Figure: 5.7. Effect of *R. serpentina* leaf extract on growth of *Leishmania* promastigotes

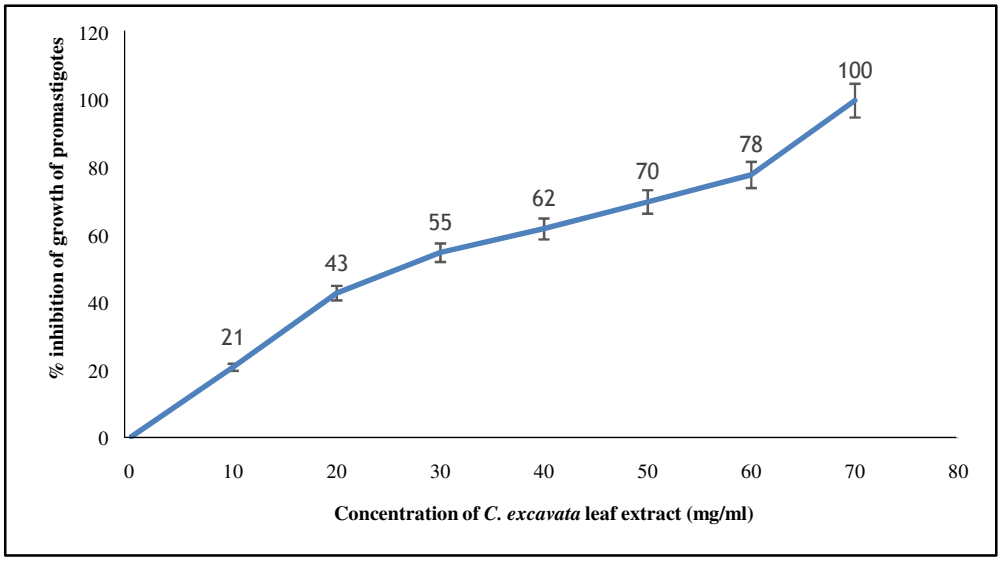

Figure: 5.8. Effect of *C. excavata* leaf extract on growth of *Leishmania* promastigotes

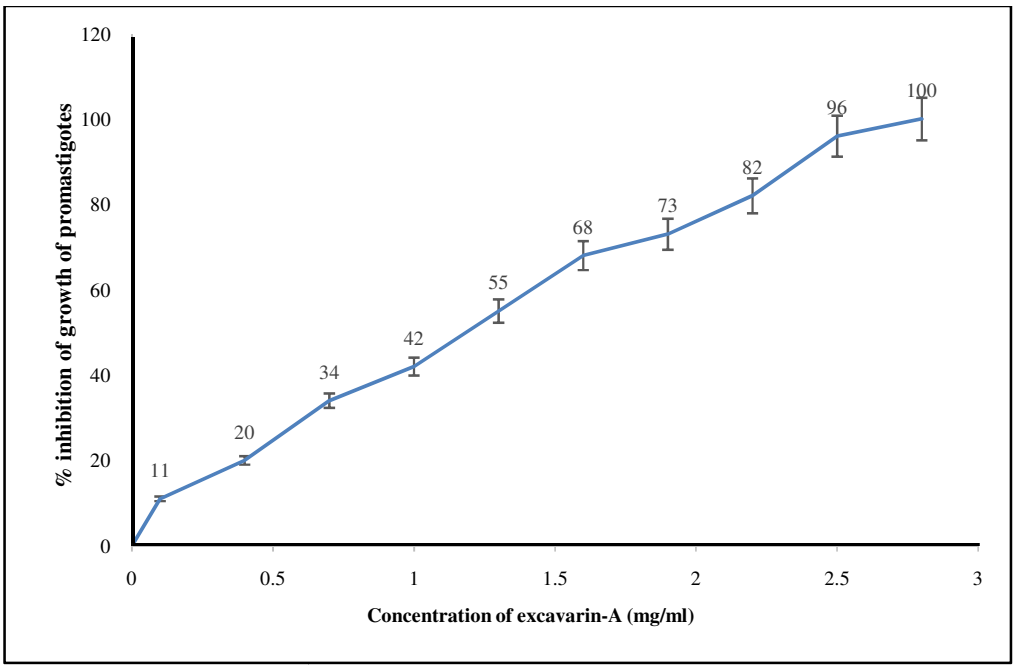

Figure: 5.9. Effect of excavarin-A purified from *C. excavata* on growth of *Leishmania* promastigotes

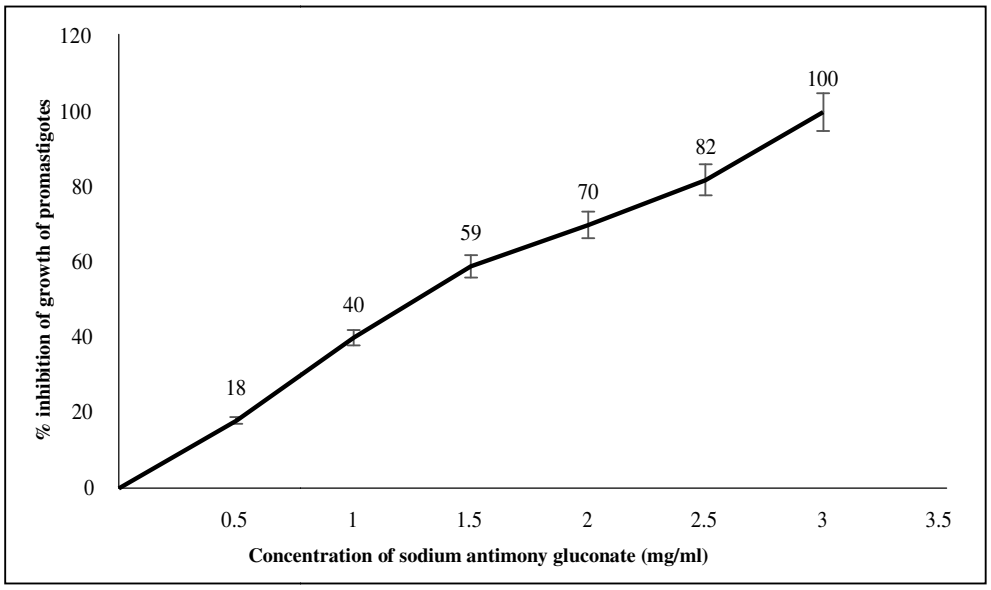

Figure: 5.10. Effect of sodium antimony gluconate (SAG) on the growth of *Leishmania* promastigotes

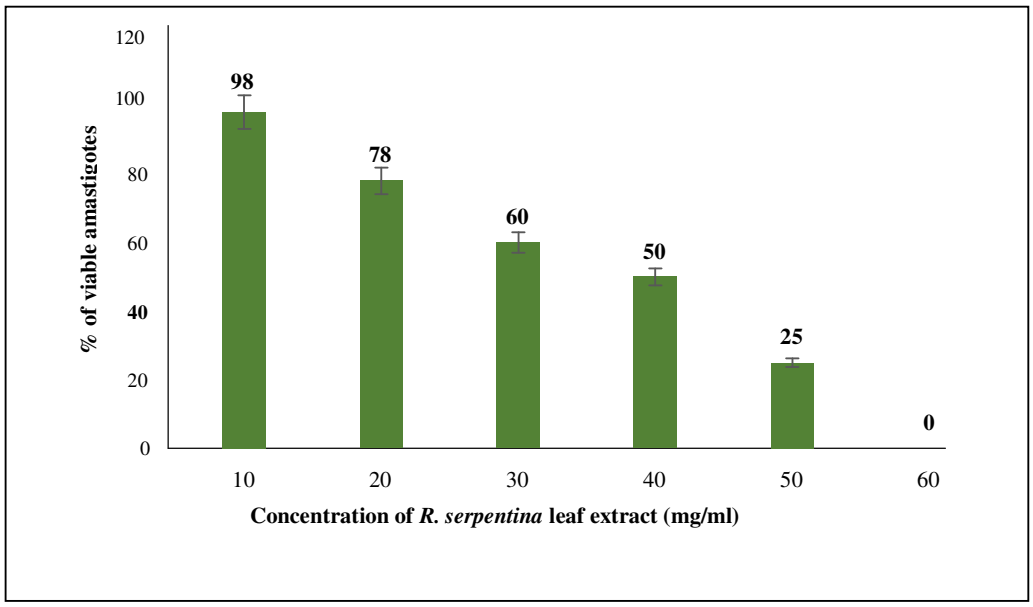

Figure: 5.11. Effect of *R. serpentina* leaf extract on growth of amastigotes within macrophages

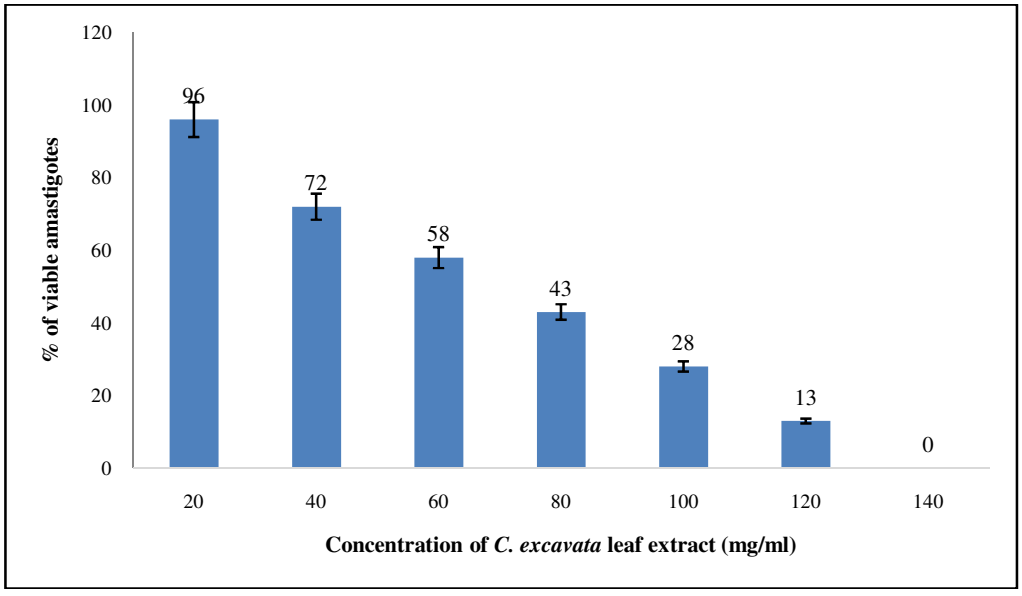

Figure: 5.12. Effect of *C. excavata* leaf extract on the growth of amastigotes within macrophages

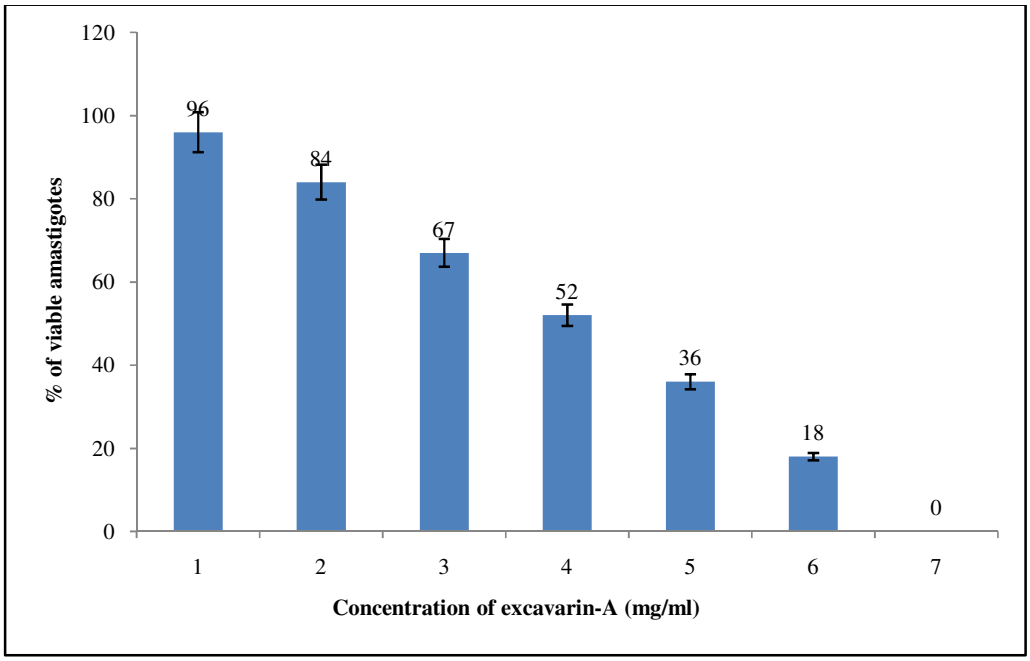

Figure: 5.13. Effect of excavarin-A on the growth of amastigotes within macrophages

excavarin-A for % of viable amastigotes were 40.0 mg/ml, 72.7 mg/ml and 4.8mg/ml respectively. It was found that concentration of plant extracts required for growth inhibition of internalized amastigotes were higher than that of promastigotes growth inhibition in case of all three test samples.

Table 5.2. Comparative analysis between effective doses of tested extracts against promastigotes and amastigotes

Plant extracts/Drug	**IC_{50} value for growth inhibition of promastigotes (mg/ml)**	**IC_{50} value for growth inhibition of amastigotes in macrophages (mg/ml)**
Excavarin-A	1.24	4.8
R. serpentina leaf extract	4.04	40
C. excavata leaf extract	32.1	72.7

5.3.4. *In vitro* cytotoxicity assay

R. serpentina and *C. excavata* were taken in a concentration range of 10 to 100 mg/ml as they were found to be antileishmanial in these range of concentrations. The coumarin molecule excavarin-A showed antileishmanial activity against promastigotes and amastigotes in the range of 0.5 mg/ml to 3mg/ml, therefore, it was tested for its cytotoxicity effect in that range of concentrations. Percentages of viable macrophages after treatment with test substances in different concentrations is shown in Fig: 5.14, 5.15 and 5.16. *R. serpentina* was found to be nontoxic at the IC_{50} value of 4.04 mg/ml (for promastigotes), and continued to show low toxicity even at much higher doses and the maximum cell viability was 52% at the highest dose of 100 mg/ml. On the other hand, *C. excavata* and excavarin-A were found to be cytotoxic to hamster macrophages in the range of effective doses.

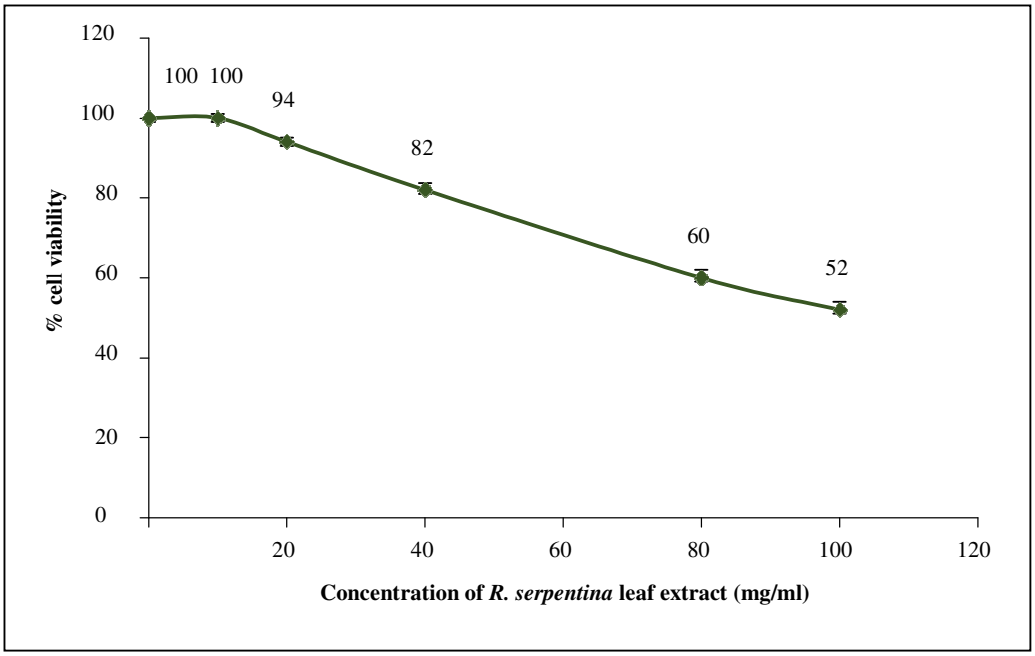

Figure: 5.14. Viability of hamster macrophages after treatment with different concentrations of *R. serpentina* leaf extract

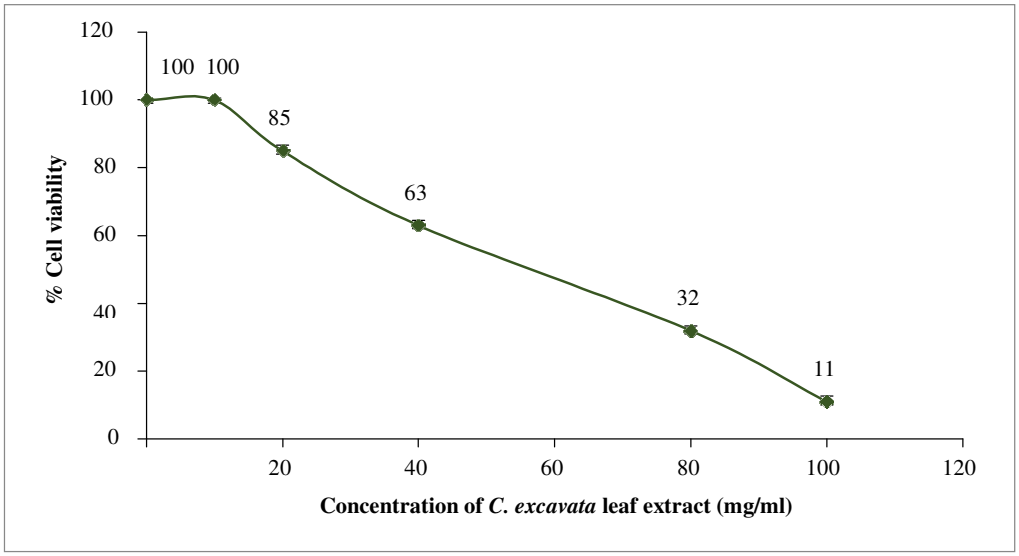

Figure: 5.15. Viability of hamster macrophages after treatment with different concentrations of *C. excavata* leaf extract

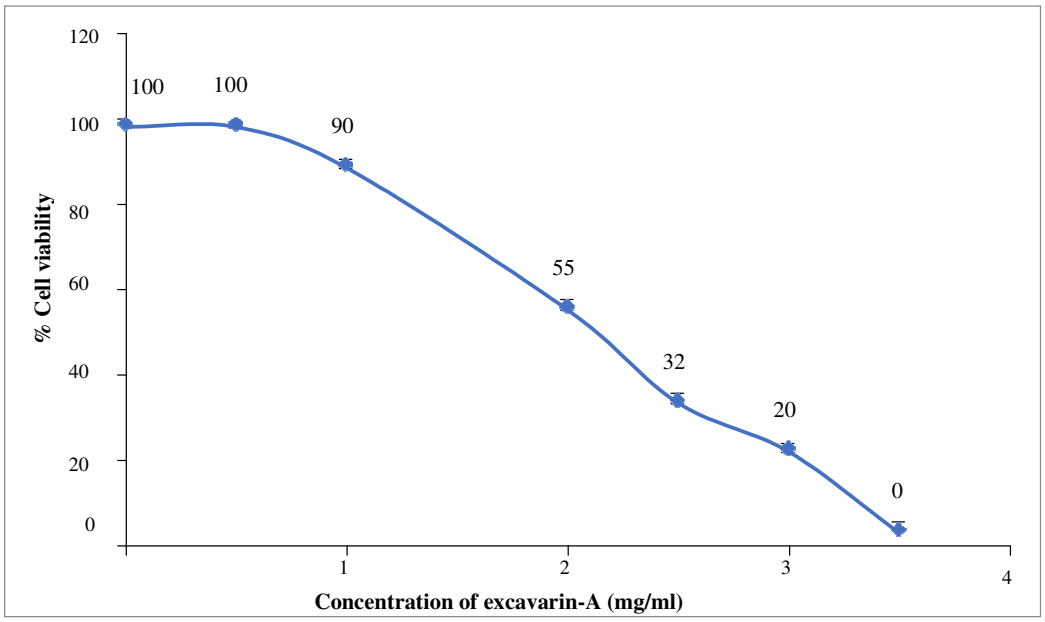

Figure: 5.16. Viability of hamster macrophages after treatment with different concentrations of excavarin-A

5.4. Discussion

The pathogen *Leishmania* sp.is capable of undergoing profound biochemical and morphological adaptations in order to complete its life cycle, to multiply and survive in different hosts. *Leishmania* amastigotes resist phagosomal enzymes and are able to thrive and multiply within the acidic hydrolase rich parasitophorous vacuoles. Though the parasite is sensitive to humoral defence mechanisms, its intracellular habitat offers almost complete protection. This makes the treatment for leishmaniasis complicated. The medication of first choice, the pentavalent antimonials, are toxic and administered exclusively by the parenteral route (Rocha et al., 2005; Joshi et al., 2006; Kumar et al., 2009). Second-line drugs include amphotericin B, pentamidine, miltefosine and paromomycin but limitations in terms of safety, resistance, stability and cost, low tolerability, long duration, and difficult administration, hinder its treatment. Hepatotoxicity, nephrotoxicity and cardiotoxicity, results in poor adherence to treatment, and compromises the effectiveness of therapy (Macêdo et al., 2021). Given this scenario, studies are needed that aim to identify an ideal antileishmanial agent which will have both direct and selective leishmanicidal effect.

To date many *in vitro* studies have been carried out to screen different referral plants or new ones, against *Leishmania* pathogen showing promising results (Yousefi et al.,2009; Maspi et al., 2010; Emami et al.,2012; Mahmoudvand et al.,2014; Kheirabadi et al.,2015; Bernuci et al.,2016; Vieira-Araújo et al., 2018; Badirzadeh et al., 2020). Different previous works showed antihypertensive (Ranjini et al., 2015), antidiabetic (Azmi and Qureshi, 2013), antioxidant (Nair et al., 2012), antibacterial (Rathi et al., 2013), antidiarrhoeal (Ezeigbo et al., 2012) and hepato protective (Gupta et al., 2010) activities of extracts of different parts of *R. serpentina*. Extracts of root, leaf and stem of *C. excavata* also showed different therapeutic activities previously (Sakong et al., 2011; Puongtip et al., 2011; Guntupalli et al., 2012). High content of secondary metabolites may offer good therapeutic value to both these plants

as we found in our work. In this study we screened the methanolic leaf extract of *R. serpentina* and *C. excavata* and excavarin-A for their antileishmanial activities.To the best of our knowledge, this is the first report of antileishmanial activity of *R. serpentina*.

We applied graded concentrations of methanolic leaf extract of *R. serpentina* and *C. excavata* along with a coumarin compound excavarin-A purified from *C. excavata* and a standard drug SAG against the pathogen *L. donovani*. Result showed that excavarin-A was most effective in inhibiting promastigotes and amastigotes of *L. donovani* and its activity was comparable to the standard drug included in this study. Coumarins belong to an active class of heterocyclic compound present in plant extracts, which are reported to have wide spectrum of therapeutic and biological activities like anticoagulant, antifungal, antioxidant, antitumor, antibacterial and anti-inflammatory (Rao et al.,2020). Excavarin-A was reported as a new coumarin compound by Kumar et al. (2012) with strong antifungal activity. We have also found, excavarin-A was to be a good antifungal, antileishmanial and antioxidant agent.

Many earlier reports evaluated leishmanicidal activity of plants or biomolecules on extracellular promastigotes as well as internalized amastigotes in animal macrophages, which showed that the active compounds acted differently against promastigotes and amastigotes. Compounds have direct contact with promastigotes, in contrast substances have to cross the cell membrane to reach the internalised amastigotes; moreover, amastigotes are adapted to survive in hostile intracellular environment. Thus, the promastigotes are more sensitive to active compounds (Ribeiro et al., 2014; Queiroz et al., 2014). The findings of Vieira-Araújo et al. (2018) also confirmed the fact. They isolated one molecule piperine from fruits of the plant *Piper nigrum* L. and tested for growth inhibition activities against both promastigotes and internalised amastigotes of *L. infantum*. They found lower IC_{50} values for promastigotes growth inhibition than that of amastigotes. Few more previous works concluded with similar findings (Monzote et al., 2010;

Bernuci et al., 2016) and ourresults are also in agreement with those observations. In each case, the concentration of substances needed for inhibition of amastigotes is higher than that of promastigotes.

Before recommending any natural product for therapeutic purposes, it is necessary to test whether the substance is cytotoxic for the animal cell in the limit of its effective concentration. Very frequently it is seen that several plant extracts give outstanding inhibitory effect against pathogens or has potentiality to cure diseased conditions, but it is cytotoxic to the living tissues which may cause immediate or long-term side effects. So, the samples were tested for viability of hamster macrophages in different doses. In a number of old and recent studies, cytotoxic activities of extracts of different plant or plant parts were tested on different cell lines and the inhibition of cell growth or cell proliferative activities were tested by MTT assay (Horiuchi et al., 1988; Hashemi et al., 2011; Rahman et al., 2014). In the present study, the crude extracts of *R. serpentina*, *C. excavata* and excavarin-A were checked for cytotoxicity by MTT assay. The macrophages were isolated from hamster peritoneal cavity, and tested with samples of plant leaf extracts in different concentrations. In a similar study, cytotoxicity test was performed on BLBC mice macrophage cell using MTT and the leaf extract of the plant *U. dioica* was found to be toxic at higher concentration (Badirzadeh et al., 2020). *C. excavata* leaf extract and excavarin-A showed good antileishmanial activity, however they were found to be cytotoxic within effective therapeutic ranges. But *R. serpentina* leaf extract was not found to be cytotoxic in cell viability test in its effective range of doses. At a higher concentration of 100mg/ml, which is much far from its therapeutic range, *R. serpentina* showed 52% viable cells when tested on host macrophages.

From the present work, it was evident that, crude leaf extract of *R. serpentina* had significant antileishmanial activity *in vitro*, and showed no cytotoxic effect in its therapeutic ranges of concentrations. Further study on animal model has been done with *R. serpentina* extract to explore its antileishmanial activity in

systematic environment. The *C. excavata* extract and excavarin-A were not considered for *in vivo* tests due to their cytotoxic activity observed in this study.

6.1. Literature review

In the humid and wet weather conditions of North Bengal, fungal infection is very common among local people. In general, fungal infections are the most common cause of many skin diseases in most of the developing countries. Opportunistic fungal infections, mainly resulting from the species of *Candida*, *Cryptococcus* and *Aspergillus* are life-threatening in immuno-compromised patients especially when affected with AIDS, cancer, or organ transplant. Resistance of many pathogens towards antifungal drugs is also a major factor that limits appropriate treatment. Therefore, it is necessary to search for more effective and less toxic novel antifungal agents from natural sources.

C. albicans is the most common causative agent of human infection among all *Candida* species, where infection ranges from mucosal surface to systemic organ (Miceli et al., 2011). Previous studies have shown that some plant extracts possess significant antifungal activities against different strains of *Candida* sp. but all are mainly screened *in vitro* (Soliman et al., 2017; Neto et al., 2017). The preliminary stage of clinical investigation for identification of biomolecules having antifungal activity is *in vitro* testing of natural materials which are mainly crude botanical extracts. However, this is not enough to invent new line of drugs because *in vitro* studies do not consider the individuals body system, organic metabolism or idiosyncrasies. Very few studies show antifungal activities of medicinal plant extracts on animal models. The latest advancements have focused primarily on mechanisms of action of natural compounds against opportunistic fungal pathogens at the genomic, molecular or proteomic levels (Martins et al., 2015).

6.1.1. *In vivo* evaluation of antifungal activity against candidiasis

In recent past, several studies on *in vivo* antifungal activities of medicinal plants evaluated on plant pathogens have been reported (Seepe et al., 2020; Dhar Purkayastha et al., 2018); but *in vivo* study on fungal pathogen of clinical importance still remains scarce. In this aspect, most of the *in-vivo* studies are concerned with candidiasis as this is the most frequently encountered fungal disease (Garber 2001). In a search for new antifungal treatments, the

mammalian animal models play a very important role for observing *in-vivo* efficacies. While mice are the most widely used models (Scorzoni et al. 2016), the ultimate choice of animal species along with the route of inoculation of the organism depends on the desired goal. In this review, the findings of few such studies are deliberated.

Zhang et al. (2005) studied the antifungal activity of eight steroid saponins isolated from n-butanol extract of *Tribulus terrestris* against six fluconazole resistant yeasts. *In vivo* testing of the purified saponin TTS-12, which showed maximum efficacy in preliminary tests was performed by the authors in a vaginal infection model, oestrogen-dependent rat vaginitis, developed with fluconazole-resistant *C. albicans*. The results showed remarkable therapeutic effect on candidal vaginitis upon vaginal administration of TTS-12. In another *in vivo* study, methanolic extract of *Syngonanthus nitens* scapes was tested in animal model (rat) infected by different *Candida* strains collected from patients with valvovaginal candidiasis. The treatment with cream containing the extract at different doses showed rapid clearance and eradication of vaginal fungal burden in experimental animals after 8 days of treatment (Araújo et al., 2013). Fabri et al. (2021) tested the antifungal activity of spilanthol, a bioactive alkylamide from the native Amazon plant species, *Acmella oleracea*. *In vivo* evaluation in an experimental vulvovaginal candidiasis model showed that infected rats treated with spilanthol recorded steady reduction of fungal burden when compared to the untreated animals.

The antifungal activity of aqueous extract of *Nigella sativa* seeds was studied on candidiasis in mice (Khan et al. 2003). Treatment of mice that were injected intravenously with *C. albicans* with the plant extract resulted in considerable inhibitory effect on the growth of the organism at the level of 5-fold decrease in cfu in kidneys, 8-fold in liver and 11-fold in spleen. In another study,antifungal activities of acetone extracts of leaves from *Combretum* species were tested against *C. albicans* and other pathogenic fungi associated with wound infections on immunocompromised Wistar rats (Masoko et al., 2010). The authors made wounds on the back of the rats and treatments were

administered topically using the extracts at 20% concentrations in aqueous cream. Their results showed that in the treated group, wound healing was evident as a rigid, dark, and thick crust formation after three days, but the lesion without treatment took longer time to heal. In a similar study, Sahgal et al. (2011) used crude methanolic seed extract of *Swietenia mahogany* against *C. albicans*. Here, experimental mice were infected with *C. albicans* suspension via injection at lateral tail vein and subsequently treated with 2.5 g/kg of seed extract by intraperitoneal injection after 24 hours. Their results showed significant reduction of the pathogen counts in both blood and kidney when compared to control group. In histopathological observations, the authors found that the treated kidney failed to exhibit the presence of either *C. albicans* or pseudomycelia. Similar study was performed by Jothy et al. (2012) with methanolic seed extract of the plant *Cassia fistula*. Mice were injected with *C. albicans* via intravenous route and treatment was performed with seed extract at 2.5 g/kg body weight administered intraperitoneally once daily for 3 days. Results revealed a six fold decrease in *C. albicans* burden in kidney and blood sample in the curative group mice when compared with those of the control group.

Dzoyem et al. (2014) studied the antifungal activity of methanol-dichloromethane extracts of twenty- one spices used in Cameroon. *In vivo* activity of *Olax subscorpioidea* extract which exhibited maximum activity during *in vitro* evaluation was tested in rat model of disseminated candidiasis caused by *Candida albicans*. The results revealed that oral administration to artificially infected rats at the dose of 2 g/kg of body weight recorded a significant reduction of cfu of *C. albicans* cells in the blood while a moderate decrease was observed in the kidney.

6.1.2. *In vivo* evaluation of antifungal activity against other superficial infections

Superficial fungal infections are found in the outermost layers of the skin, nails, hair and mucous membranes. There has been an increase in the incidence of these infections, mainly because of the increasing number of immunocompromised patients (Garber, 2001). The most common types of skin

diseases are caused by dermatophytes such as *Trichophyton* sp., *Microsporum* sp. and *Epidermophyton* spp. although non-dermatophyte yeasts such as *C. albicans* and other non-albicans *Candida* sp. along with the moulds, *Aspergillus* sp., *Fusarium* spp., *Acremonium* sp., *Scopulariopsis* sp. and *Scytalidium* sp. are also reported as causative agents in a growing number of cases (Tosti et al., 2000).

One of the past studies screened 50% ethanolic leaf extract of *Leptadenia reticulata* for antifungal activity against *Aspergillus flavus*. Here, wounds were made surgically in male albino rats on the posterior mid dorsal side of the animal and fresh spore suspension of *A. flavus* was introduced in the wounds by injection as a single dose. Treatment was done by application of cotton swab soaked in the plant extract on the wound at regular intervals of twice a day for 8 days which showed visible healing sign and the swab made from the healing wound region showed absence of the fungal hyphae (Sureshkumar, 2008).

Li et al. (2015) studied the antifungal activity of ellagic acid purified from the plant *Euphorbia humifusa*. Dermatophytosis was induced in guinea-pig infection model using suspension of *Trichophyton rubrum* as inoculum. Subsequently, the test compound was administered topically mixed with an ointment base once a day for 14 days. The treatment significantly enhanced the cure rate at all tested doses and microscopic examinations for the pathogen in the treated animals yielded negative result.

Garrido et al. (2015) tested efficacy of five Mexican traditional medicinal plants against human superficial mycoses causing fungus *in vivo*. Dermatophytosis was induced with *Trichophyton rubrum* in the sole of feet in mice to develop the tinea pedis model and subsequently from day 14 post-infection, the mice were treated intragastrically daily for seven days with 2.5 and 5 mg/kg of acetone extract of *Berberis hartwegiior* and *Zanthoxylum caribaeum* respectively. After 7 days of treatment with *B. hartwegii,* an improvement of100 with no signs of disease was observed. *Z. caribaeum treated* mice also showed significant improvement of disease condition.

In the present study, methanolic leaf extracts of *R. serpentina* and *M. oleifera* was screened for antifungal activities *in vivo* using rats as model animal after getting significant results in the *in vitro* experiments with the same leaf extracts against *C. albicans*.

6.2. Materials and methods

6.2.1. Preparation of plant extracts

Leaf extract of plants were prepared following the procedure described in previous section (Section 3.2.1).

6.2.2. Effect of plant extracts *in vivo*

For *in vivo* experiment, male albino rats of similar weight (80-100 gm) and age groups (10-12 months) were taken. Altogether 15 animals divided into 3 groups were infected with *C. albicans*. Of these five infected rats were untreated and marked as control group. Rest of the 10 animals were treated with plant extracts.

6.2.2.1. Infection with *C. albicans*

A wound of skin depth (round, 1 cm diameter) was made with sterilized surgical blade at mid posterior dorsal side of each animal. The test fungal strain *C. albicans* was cultured in PDA medium and a cell suspension of$1x10^5$ cells/ml was used as inoculum. The prepared solution was applied superficially on wounds on the skin of test animals with cotton swab at a dose of 1ml in each case. It took 2 days to develop skin infection at the site of application of fungal spore suspension (Sureshkumar, 2008). The animal ethics committee of the institute (IICB, Jadavpur, Kolkata) approved all animal experiments.

6.2.2.2. Treatment with plant extract

Ten infected animals were treated with diluted plant extracts superficially on the wounds. One milligram of crude extract was dissolved in minimum volume of physiological saline (0.9% NaCl) and applied over the wound of each infected animal. Five infected rats were treated with *R. serpentina* leaf extract and remaining five were treated by *M. oleifera* leaf extract. This process was repeated in regular intervals of twice a day for next 8 days. The whole

experiment was performed thrice. All the animals were kept in uniform diet and environment throughout the period of experiment.

6.2.3. Recovery of pathogen from test animals

After 8 days of treatment with plant extracts, fungal loads on the wounds were estimated by standard Lacto-Phenol Cotton Blue (LPCB) and Gram staining methods. For this, sample was recovered from the dry wound using a sterile swab from both treated and control groups and smeared on the microscopic slides. The slides were then stained and finally the stained samples were observed under the microscope (Olympus).

For LPCB staining, one or two drops of the stain was added to cover the smear. One edge of the drop of stain was touched with a cover slip edge, and lowered gently, avoiding air bubbles to cover the liquid. The preparation was thus ready for examination (Procop, 2017).

Although Gram stain is useful in staining bacteria, certain fungi such as *Candida* and *Cryptococcus* are observed to be Gram positive yeasts. For Gram staining fungal strain, the smear collected from the wound was spread on glass slide and fixed and dried by rapidly passing the slide through the flame of spirit lamp (keeping the smear uppermost). The fixed smears were then covered with crystal violet stain for 30-60 seconds. The stain was rapidly washed off with clean water, and the smear was covered with Lugol's iodine for 30-60 seconds to form crystal violet-iodine complex. Next, iodine was washed off with clean water and decolorized rapidly (within few seconds) with acetone-alcohol, and again washed immediately with clean water. At last, the smear was counterstained with Safranin solution for 30 seconds. The stain was then washed off with clean water and examined under microscope (Procop, 2017).

6.2.4. Analysis of blood sample of test animals

After the period of treatment, on 9^{th} day of experiment, blood samples were collected from both control and treated rats. The blood samples were collected on glass vial containing EDTA (anticoagulant) and the total count (TC) of the leukocytes was determined. For finding the total leukocyte count,

a sample of blood was diluted with Turk's fluid (1.5 ml glacial acetic acid, 1.5 ml of 1% gentian violet solution and 100 ml distilled water) which destroyed the RBC and stained the nuclei of the leukocytes to make them visible. Initially 1 ml of Turk's fluid was taken in a watch glass, the counting chamber was placed on a microscope stage containing 16 WBC squares. A WBC pipette was filled with the blood sample up to the mark 0.5 and Turk's fluid was sucked to the mark of 11, and the blood and fluid were mixed thoroughly. That gave the dilution of ratio 1:20. In another pipette, blood was drawn up to mark of 1 followed by Turk's fluid up to mark of 11. After discarding first two drops of fluid from the pipette, the chambers on its both sides were charged. The cells were allowed to settle for the next 3 to 4 minutes and the chambers were transferred under the microscope, first under low magnification and then switched to high magnification. The leukocytes were seen as round shaped cells with clear unstained cytoplasm and deep blue-violet nuclei. WBCs were counted in six squares marked in the counting chamber, where cells were in four groups of sixteen squares, i.e., in a total of 64 squares. In a counting chamber, the volume of a square is 1/160 mm^3, therefore the volume of 64 squares is 4/10 mm^3. Thus, the total volume of diluted blood, in which WBCs were counted, was 4/10 mm^3. By multiplying the number of WBC count by 10/4, the WBC count in 1 mm^3 diluted blood was found. The blood was diluted 20 times, so 1 mm^3 of undiluted blood from the first pipette contained A x 10/4 x 20=A x 50 WBCs (A=WBC count). In case of second pipette, it was A x 10/4 x 10=A x 25. Two counts were taken for comparison and confirming the accuracy (Procop, 2017).

6.2.5. Data analysis

Total count of WBC (mean $\pm$ SD) were analysed by student "t" test.

6.3. Results

The wounds caused by the infection of *C. albicans* were treated with leaf extracts of *R. serpentina* and *M. oleifera*; this caused healing of infection which was reflected by the reduction of fungal load in the samples collected from treated rats. The observation was confirmed by LPCB and Gram staining

methods. On the other hand, in infected but untreated rats, a significant presence of *C. albicans* was observed in samples collected from the wound after 8 days (Table 6.1).

The process of recovery from infection is faster in *R. serpentina* treated animals in comparison to *M. oleifera* treated group. On the fourth day of treatment with *R. serpentina* leaf extract, the scar on skin of the experimental animal totally disappeared; but in case of *M. oleifera,* few signs of infection were still present (Fig: 6.1). On the eighth day of treatment, the body fur was regained at the site of the wound in *R. serpentina* treated group, but very little skin hair was observed in case of *M. oleifera* treated animals.

In both *R. serpentina* and *M. oleifera* treated rats, there was a significant increase in mean WBC count in comparison to untreated group indicating that both the plant leaf extracts have a protective role in reducing fungal burden in the animals. (Table: 6.2).

Table: 6.1. Treatment of skin lesion induced by *C. albicans* using plant extracts.

Treatment	Occurrence of *C. albicans* at different days during treatment			Nature of wound at different days during treatment		
	0 d	**4 d**	**8 d**	**0 d**	**4 d**	**8 d**
untreated control	+++	+++	+++	Severe	Severe	Severe
Infected and treated with *R.serpentina* leaf extract	+++	+	-	Severe	Superficial	Absent
Infected and treated with *M. oleifera* leaf extract	+++	++	+	Severe	Moderate	Superficial

+++ High occurrence, ++Moderate occurrence, +Low occurrence, -Absence

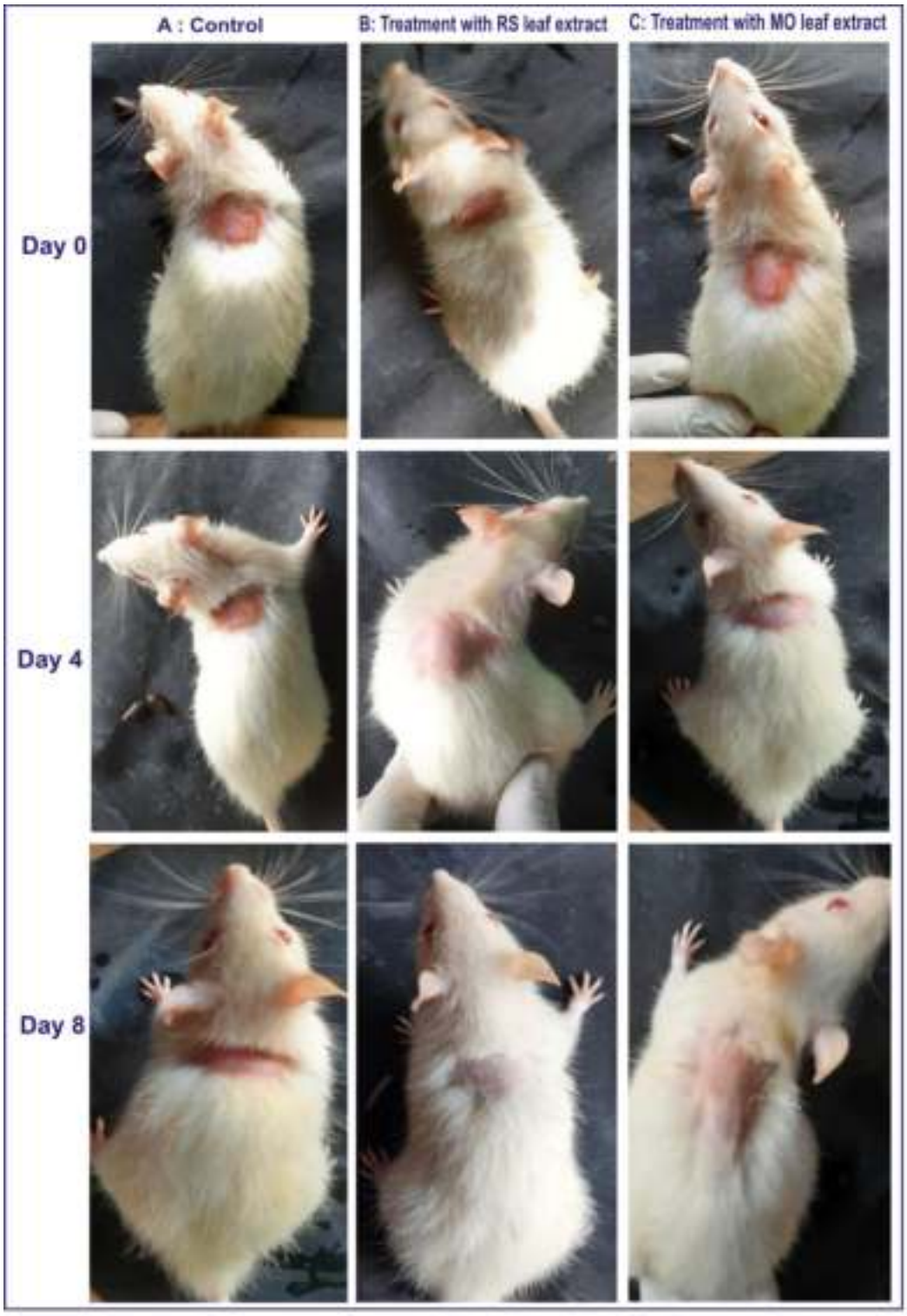

Figure: 6.1. Effect of plant extracts on superficial infections by *C.albicans* on experimental animals: A. Control group experimentally infected with *C.albicans*. B. Infected animals treated with leaf extracts of *R. serpentina* (RS) and C. Infected animals treated with leaf extracts of *M. oleifera* (MO)

Table: 6.2. Total leukocyte count in blood samples of experimental rats infected with *C. albicans* and treated with plant extracts.

Sample	White blood cell count[#] Cells/mm^3
Infected but untreated rat	7634±141
Infected rats treated with *R. serpentina* leaf extract	8735±130
Infected rats treated with *M. oleifera* leaf extract	8642±122

[#] (average no. in 5 rats± SD)

6.4. Discussion

As eukaryotic pathogens, human fungal diseases differ from other infections fundamentally. Fungi share many similarities with their host cells, this impairs the activity of antifungal compounds. A single fungal pathogen can infect a wide range of cell types and multiple tissues in the same patient especially if the host is immune compromised in any way (Rodrigues and Nosanchuk, 2020).The Global Action Fund for Fungal Infections has highlighted the devastating impact of focal fungal diseases in patients in advanced stage of AIDS(GAFFI,2018). In addition, patients on anticancer therapies and other immunosuppressive medications are affected, which have contributed to the excess number of deaths due to fungal diseases worldwide. The Center for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have emphasized on this fact in their reports (CDC, 2017; WHO, 2020b). Despite this fact, fungal diseases have been continually neglected over the years and persistence of its neglected status is caused by failures in science, market, and public health. The financial support for fungal disease research is incredibly low, drug development field is progressing at a very slow pace. Classical antifungal drugs Amphotericin ß discovered in 1955, still remain the 1st line medication for fungal infection. The affordable conventional formulations include significant side effects. On the other hand, most effective and least toxic formulation like liposomal Amß, is available at a very high cost.

As the deadliest fungal infections affect mainly the neglected and poorest populations of the world, it faces market failure in the form of lack of interest from the pharmaceutical sector in the development of medicines, vaccines, and diagnostic tests for human fungal diseases or for drug commercialization (Rodrigues and Nosanchuk, 2020). In this scenario, it is the need of the hour to unturn the rubble from natural resources in search of new therapeutic formulation which is effective, low cost and devoid of any side effect.

In the last two decades, phytotherapeutic agents derived from natural resources with antimicrobial and antifungal potentiality, has grabbed the attention of scientific researchers (Martins et al., 2014). Various *in vitro* studies have been conducted to screen the antifungal activities of different medicinal plants. But plant-derived bioactive molecules show synergic, antagonistic and polyvalent relationship when they enter into human body. Their mode of action, structural and functional conformations are also modulated due to organic metabolism or in complex environment of the host body (Martins et al., 2015). Thus, *in vitro* screening in laboratory set up is not enough to establish the activity of any plant extract or any biomolecule derived from medicinal plants; and so, *in vivo* screening and clinical trials are needed (Sealbert et al., 2000; Alviano et al., 2009). Some studies are reported in past showing antifungal activities of medicinal plants *in vivo* (Masoko et al., 2010; Araújo et al., 2013; Sahgal et al., 2011; Jothy et al., 2012; Garrido et al., 2015). The present study intended to further extend the positive findings of *in vitro* screening in animal model.

A very simple *in vivo* experiment was designed where skin infection was developed in experimental animals with *C. albicans,* and then effectiveness of the plant extracts was observed by superficial application. Although the study was very basic in nature, it showed visible results. Superficial infections on experimental animals (male albino rats) were treated with diluted methanolic leaf extract of *R. serpentina* and *M. oleifera*, twice a day for the next 8 days, and the control group remained untreated. Standard LPCB and Gram staining showed significant recovery which were also visible prominently with the

naked eye. *R. serpentina* showed better healing in comparison to *M. oleifera,* but both the plant extracts reduced skin infections when compared to the untreated group. On the fourth day of treatment, the scar completely faded out in *R. serpentina* treated animals, and on 8th day the skin hairs reappeared. In case of *M. oleifera* treated group of animals, on the 8th day of treatment, the scar disappeared totally but very little amount of skin hair was observed at the site of wound. Haematological analysis of blood samples from animals of both treated and untreated groups showed significant results.

Total count of WBC had increased in both cases of treated animals. This finding was in agreement with previous work described by Suresh Kumar (2008). As a part of host's innate immune system, neutrophil, leukocytes or polymorphonuclear leukocytes (PMNLs) are known to function as major phagocytic cells, which cause elimination of the fungi during fungal infection (Demirezen et al., 2015). The elevation in total count of WBC which was found by haematological analysis of blood samples of treated animals, suggests that the leaf extracts of both the plants have protective roles in improving host defence to counter fungal attack. From the findings of the present work, it is evident that methanolic leaf extracts of *R. serpentina* and *M. oleifera* possess antifungal activities against *C. albicans*. The active principles responsible for the antifungal activities, and their mode of action may be explored through further research.

7.1. Literature review

Leishmaniasis is one of the main endemic parasitic infections worldwide, which is found in around 98 countries, especially in developing countries with about 1.7 billion people at risk of contracting the infection (Sosa et al., 2019). Leishmaniasis is considered as one of the six major neglected tropical diseases by WHO, due to its remarkable effect on global public health. The disease has great importance in HIV infected individuals as it is found as an opportunistic infection in those areas where both infections are endemic (Martínez et al., 2018).

A literature review reveals that several antileishmanial chemical compositions have already been reported (Murray, 2001; Marty and Rosenthal, 2002) but none of these proved to be the ultimate choice of drug due to varying degrees of efficacy and toxicity. Pentavalent antimonials, that are the first-line drugs in the leishmaniasis treatment, could result in severe toxic side effects including cardiotoxicity, pancreatitis, hepatotoxicity and nephrotoxicity. Discovery of antimony salt resistant pathogenic strains has made the situation worse to treat the patients against these parasites (Sundar, 2001). On the other hand, second-line drugs like pentamidine and miltefosine may cause diabetes when applied in high doses. Furthermore, the recently developed resistance of the pathogen against certain antileishmanial drugs has resulted in treatment failures. Therefore, new treatment approaches are immediately required (Badirzadeh et al., 2020). There is a need to identify new chemotherapeutic agents for effective therapy against all forms of leishmaniasis.

7.1.1. Experimental evidence of antileishmanial activity using plant extracts

In search for effective treatment for leishmaniasis, natural products may offer unlimited source of chemical diversity for identification of new drug templates (Fournet and Munoz, 2002). In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects (Zaffer et al., 2015). In rural areas, traditional medicine for

leishmaniasis largely remains the only source of treatment being handled by the local people without proper scientific information. Government of India has also started to focus more on the potential of herbal preparations (The Times of India, 2003). The interest in the plant products, especially medicinal plants or their extracts, surfaced all over the world due to the belief that many herbal medicines are known to be free from side effects. In a study, activity of *Haplophyllum myrtifolium* against *L. tropica* was screened, both *in vitro* and *in vivo*, where the efficacy of the medicinal plant was tested by measuring the lesion size (developed in cutaneous leishmaniasis) in infected animal but the study was devoid of any experiment to determine the probable mode of action or cytotoxic effect, if any (Ostan et al., 2007).

In another study, leishmanicidal activity of the extracts of five well known Turkish medicinal plants were evaluated against *L. tropica* infected mice, taking glucantime as reference drug. Promastigote solution was injected subcutaneously and after development of lesion, extracts of five plants (both in water and chloroform) were administrated. Effectivity of test substances were determined by measuring the lesion size on foot pad, showing good results but two of the plants among five had cytotoxic activities in therapeutic dose limits (Ozbilgin et al., 2014). The work was confined only to screen the antileishmanial activity *in vitro* and *in vivo* condition but no experiment was done to establish probable mode of action. Some studies were done on human subjects too. In a study, leishmanicidal activity of a medicinal plant having anti-malarial effect (*Artemisia annua* L.) was tested on human subjects with uncomplicated cutaneous leishmaniasis who were not taking any other medication and were cured 45 days after initiation of treatment (Mesa et al., 2017). In another work, the ethanolic root bark extract of *Berberis vulgaris* was examined for treatment of *L. major* infected mice, with cutaneous leishmaniasis. About 90% recovery was found by treatment with 20% root bark extract. No tests for cytotoxicity or other experiments were performed which might establish any immunological or enzymatic modifications in test animal causing recovery (Salehabadi et al., 2014).

7.1.2. Mode of action of leishmanicidal plant extracts

Several research works were performed to evaluate mode of actions behind leishmanicidal activities of different medicinal plants but most of the studies were done on specific enzyme activity or any other internal environmental situation created in laboratory set up (Zhai et al.,1999). Antileishmanial activity of extracts from *Allium sativum* along with a molecule purified from the plant (allicin) was reported by Foroutan-Rad et al. (2017). The author observed that the molecule interfered in the function of thiol-redox proteins like glutathione and trypanothione/trypanothione reductase which led to the damage of the *Leishmania* parasites. It also caused microtubule disruption. In another investigations, aqueous and ethanolic extracts of garlic as well as allicin inhibited the growth of *L. mexicana* and *L. chagasiin vitro* and it was found that, allicin caused disruption of the activity of cysteine proteases enzyme which might be the reason of its antileishmanial activity (Eslami et al., 2013). Essential oil from the plant *Tetradenia riparia* was evaluated for the antileishmanial activity against *L. amazonensis*. It was found to inhibit some of the most critical cytokines for parasite growth and the establishment of infection, including granulocyte-macrophage colony-stimulating factor, interleukin-4 (IL-4), IL-10, and tumour necrosis factor (Demarchi et al., 2015). It has been demonstrated that the iridoid glucosides isolated from *N. arbor-tristis* seeds promoted the increase of reactive oxygen species (ROS) in parasites, causing redox imbalance induced oxidative stress, cell membrane damage and apoptosis-like death in both promastigote and amastigote of *L. donovani* (Arraché Gonçalves et al., 2021).

7.1.3. Role of SOD in antileishmanial activity

Super oxide dismutase (SOD, EC 1.15.1.1) is an enzyme which acts as a component of first line defense systems in the cell. It is a major detoxification enzyme and the most powerful antioxidant in the cell (Ighodaro and Akinloye, 2018). It protects the living tissues from oxidative damages caused by reactive oxygen species (ROS) such as super oxide anions, hydroxyl radicals, or free radicals. These super oxides and free radicals are generated by the effect of toxins, ultraviolet ray and nuclear exposure or during body defence activation

(Wang et al., 2018). SOD is a metalloenzyme which requires a metal cofactor for its activity. Various forms of SOD are distributed in the living systems depending on the type of metal ion required as a cofactor. These forms include (i) Fe-SOD which is commonly found in prokaryotes and chloroplasts of some plants (ii) Mn-SOD which is present in prokaryotes and mitochondria of eukaryotes and (iii) Cu/Zn-SOD which is found mainly in eukaryotes (Ighodaro and Akinloye, 2018).

During microbial attack the human body releases super oxides to destroy the microbes and microbes use their own SOD to encounter and neutralize host's super oxides. SOD is an enzyme, which exists both in host and parasites and functions differentially and this attract the interest of scientists and pharmacologist from years (Nishikimi et al., 1972; Ghosh et al., 2003; Raychaudhury et al., 2005). Variation in genomic structures, activities and sensitivities have made this enzyme a potential diagnostic target. One recent study was conducted on different types of SODs (characterised by presence of different metallic co factor, $Mn^{2+,}$ Fe^{3+} Cu^{2+}/Zn^{2+}) which differ in sensitivity to cyanide, azide, hydrogen peroxide etc (Folgueira et al., 2019). In another study, it was found that a molecule momordicatin, isolated from fruits of *Momordica charantia*, acted as antileishmanial agent both *in vitro* and *in vivo* (Gupta et al., 2010). It was also observed that Fe^{3+} containing parasitic SOD was totally inhibited by both the fruit extract and molecule within therapeutic dose but Cu^{2+}-Zn^{2+} containing SOD, present in host cell remain unaffected and that emerged as a probable mechanism of leishmanicidal activity of the studied plant or molecule.

7.1.4. Antileishmanial medicinal plants change liver function in host

Previous studies showed parasitic infection caused toxicity of liver in animals which increased the levels of two important serum enzymes, viz. serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate Transaminase (SGOT) (Kaur et al., 2016). In a previous work, liver function test was performed to assess the toxicity effect of a molecule, momordicatin in *Leishmania* infected experimental animals. Serum alkaline phosphatase, SGPT

and SGOT were measured in normal as well as DMSO and momordicatin treated infected hamsters and it was found that all the enzyme levels were changed in *Leishmania* infected but untreated animals whereas the enzymes level reached to nearly normal level after the momordicatin treatment (Gupta et al., 2010).

7.2. Materials and methods

7.2.1. Preparation of leaf extract of *R. serpentina*

Methanolic leaf extract of *R. serpentina* was prepared following the procedure described in previous chapter (section 3.2.1).

7.2.2. Parasite isolation

L. donovani strain MHOM / IN /AG / 83 was obtained from Indian kala-azar patient (Ghosh et al., 1985) and maintained by intracardial passage every 8 weeks in Syrian golden hamsters. Promastigotes were obtained by transforming amastigotes isolated from infected spleen (Jaffe et al., 1984) and maintained in Medium – 199 supplemented with 10% fetal calf serum (FCS) *in vitro* (section 5.2.2).

7.2.3. *In vivo* antileishmanial activity determination

Estimation of the *in vivo* antileishmanial activity was done following the method of Raychaudhury et al. (2005). Golden hamster was obtained from Haffkine Research Institute, Mumbai, India and bred in animal house of CSIR-IICB, Jadavpur, India. Eight-week-old hamsters weighing between 100-120 gm were first infected individually with freshly purified *L. donovani* promastigotes (2×10^5), administered through cardiac route. Therapy with target plant extract and reference drug were started after 30 days following infection. The plant leaf extract was administrated in graded doses (50 mg/kg body weight (bw), 100 mg/ kg bw and 200 mg/kg bw) and the reference drug was sodium antimony gluconate (SAG) (40 mg/ kg bw). The animals were divided into 5 groups, with 5 animals in each group. Intramuscular injections of intended drug or extract (200 µl) were given in every alternative day for one month. All the compounds were dissolved in 0.1 % (v/v) DMSO, which was found nontoxic to animal cells. Animal of control group received 200 µl of 0.1% (v/v) DMSO in transcellular way. After one month of administration of last dose of therapy,

animals of all groups were sacrificed and parasitic burden of liver and spleen were determined from impression smears after GIEMSA staining. Before sacrificing, blood was withdrawn for SGPT and SGOT assay.

7.2.4. Determination of parasitic burden in liver and spleen

To observe the anti-leishmanial effect of *R. serpentina* leaf extract, the parasitic load in spleens and livers of infected hamsters were determined before and after treatment from impression smears after GIEMSA staining (Gupta et al., 2010). For preparing spleen/liver impression stamp smear on glass slide, first the glass slides were made grease free. The spleen and liver were collected from experimental animals. Weight of the organs were recorded. The organs were cut with scissors and forceps and putting the cut part of the organ downwards (by the help of forceps), a stamp was made over the slide (about 10-12 stamp on one slide) with just touching the organ over the slide. The slide was allowed to dry and then fixed by methanol wash (for 10 minutes). Then the slide was stained with GIEMSA (section 5.2.6.2.3) and observed under the microscope for calculation of parasitic burden. During measurement, at least 10 nucleated cells were examined for each set. The total parasitic burden was calculated using the formula as follows –

Parasitic burden = Organ weight (mg) x number of amastigotes per cell nucleus x 2 x 10^5

7.2.5. Animal serum enzyme assay

To test the toxic effect of the plant extract in animal, the blood sera of untreated normal hamsters, infected hamsters (with *L. donovani*), and infected hamsters receiving treatment were subjected to assay for liver enzymes, SGPT and SGOT (Gupta et al., 2010) using kit from Dr. Reddy's Laboratory, Hyderabad. Protocol was followed as per the manufacturer's instruction. SGPT and SGOT values were expressed as units/L (Mayne, 1994).

7.2.6. Superoxide dismutase (SOD) assay

The activity of SOD was assayed by measuring the inhibition of pyrogallol autooxidation rate following the protocol of Marklund and Marklund, (1974) with some modifications. Promastigotes of *L. donovani* strain maintained in

Ray's solid medium at 22°C were collected (12g cell) and taken in ice cold potassium phosphate buffer (50mM) containing 10 mM TES, 1 mM EDTA, 0.5 mM phenyl methyl sulphonyl fluoride and 0.25 leupeptin (pH 7.6). The mixture was then sonicated in a sonicator [Soniprep, Mes (UK) Ltd.] (5-6 strokes of 20 sec) at 4°C. The mixture was then centrifuged for 100000 g for 60 minutes and the supernatant was collected. The protein content of supernatant was measured following Lowry's method (Lowry et al., 1951) using BSA as standard.

Superoxide dismutase activity was measured by pyrogallol autooxidation assay. The assay mixture contained 0.2mM pyrogallol in air equilibrated 50mM tris-cacodylic acid buffer (pH 8.2) and 1mM diethylene triamine pentaacetic acid. The promastigote lysate and *R. serpentina* leaf extracts at different concentrations (10-40 mg/ml)were added to the reaction mixture either simultaneously or after 30 min preincubation of lysate with extract. The rate of autooxidation was obtained by monitoring the increasing absorbance at 420nm (spectrophotometer, (Hitachi, No U 2000). The enzymatic activity of SOD was determined by its ability to inhibit autooxidation. One unit of SOD was defined as the amount of enzyme which inhibited the pyrogallol autooxidation rate by 50% (Raychaudhury et al., 2005).

7.2.7. Determination of superoxide radical release

Superoxide radical release was determined following previously described method (Yasuka, 1978) with necessary modification. Promastigotes lysate was incubated for 30 min with different concentrations of crude leaf extract of *R. serpentina* (Raychaudhury et al., 2005). Release of superoxide radical was measured spectrophotometrically by measuring the formation of blue formazan (derived from reduction of NBT).

7.2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecryl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) was performed to study the effect of crude leaf extract of *R. serpentina*on the Fe-SOD enzyme of *L. donovani*. Cell free extract of *L. donovani* promastigotes (50 µg protein) (section 7.2.6) was mixed with *R. serpentina* leaf extracts in

different concentrations (5-40 mg/ml) and incubated for 30 min. Next, the samples were loaded in each lane of polyacrylamide gel and electrophoresed at 4°C. Gel was made according to the method of Laemmli (1970). A 10% resolving gel was used for separating the denatured proteins (Appendix 2, No. 28). Electrophoresis was accomplished in electrode buffer at 30 mA for 4 h using a Bio-Rad Mini Protean Gel electrophoresis system. Following electrophoresis, the gel was stained with 0.1% Coomassie blue (in 50% methanol). Pure Fe^{3+}-SOD was taken as reference. Intensity of SOD protein band in presence of different concentrations plant extracts indicated the amount of active protein.

7.2.9. SOD on-gel activity staining

Leishmanial SOD (in cell free lysate of *L. donovani* promastigotes) was treated with different concentration of *R. serpentina* leaf extracts (10 mg/ml, 20 mg/ml, 30 mg/ml) and then separated in 10% non-denaturing polyacrylamide gels. For preparing the gels, the method described by Davis (1964) was followed with some modifications (Appendix 2). In each lane 60 µg of protein was loaded. For activity staining the gels were incubated first in nitroblue tetrazolium solution (Solution A: 20 mg nitroblue tetrazolium and 10 ml of distilled water, soaked for 20 mins) and next in solution B containing riboflavin, potassium phosphate and TEMED (tetramethylethylenediamine) (4 mg riboflavin, 0.4 g potassium phosphate and 600 µl TEMED in 50 ml glass distilled water soaked for 20 min). After staining, the gels were illuminated and white band appears on blue background.

7.2.10. Statistical analysis

Experiments were performed in replication and standard error was determined. Statistical analysis was conducted through Student's t-test as described (Mishra and Mishra, 1983).

7.3. Results

7.3.1. *In vivo* effect of *R. serpentina* extract on intracellular amastigotes

Responses of *R. serpentina* treatment on intracellular parasite burden within macrophages (per 10 macrophages) in *Leishmania* infected animals was

studied**.** Parasite burden in liver and spleen of *Leishmania*-infected hamsters were significantly decreased after treatment with *R. serpentina* leaf extract in a dose dependent manner (Table 7.1). Giemsa-stained micrographs showing the effect of the plant extract are given in Fig: 7.1 (liver) and Fig: 7.2 (spleen). At a concentration of 50mg/kg bw, intracellular amastigote counts in the infected hamster liver (Fig: 7.1B) and spleen (Fig: 7.2B) was reduced upto 70%, whereas with dose of 200 mg/kg bw, the parasite burden was diminished upto 94% in infected spleen (Fig: 7.1C) and liver (Fig: 7.2C). Control group did not show any detectable change (Fig: 7.1A and 7.2A).

Table: 7.1. Dose–dependent responses upon treatment with *R. serpentina* extract on intracellular parasite burden within macrophages during *Leishmania* infection.

Dose of *R. serpentina* (mg/kgbw*)	**Parasitic burden (Count) per 10 macrophages**		**% Reduction in parasitic burden**	
	Liver	**Spleen**	**Liver**	**Spleen**
0 (untreated)	52±4	65±5	-	-
50	15±2	23±2	71%	65%
100	9±1	12±1	83%	82%
200	3±1	5±1	94%	92%
SAG[#]	1±1	2±1	98%	97%

*bw=body weight;[#]SAG at a dose of 40 mg/kg bw.

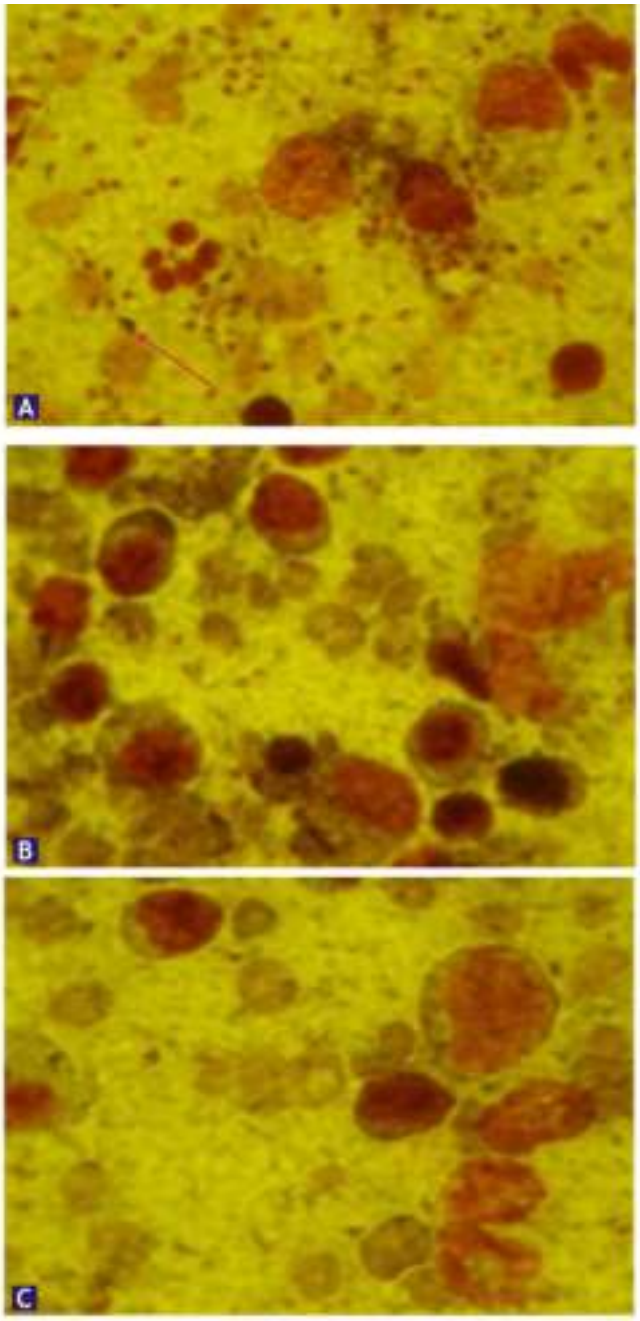

Figure: 7.1. GIEMSA-stained micrographs showing effect of *R. serpentina* leaf extracts on intracellular amastigotes in liver. Black spots within the macrophage indicate amastigotes (arrow). [A] Untreated *Leishmania* infected macrophage. [B] *Leishmania* infected macrophages treated with 50 mg/kg body wt. leaf extract. [C] *Leishmania* infected macrophages treated with 200 mg/kg body wt. leaf extract.

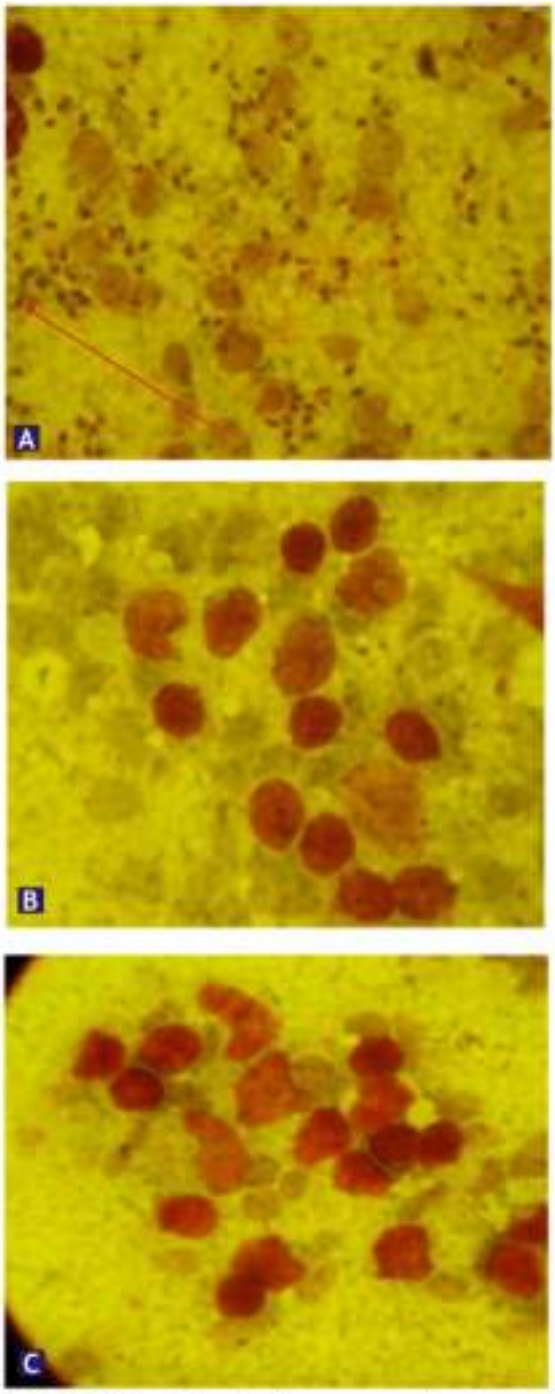

Figure: 7.2. GIEMSA-stained micrographs showing effect of *R. serpentina* leaf extracts on intracellular amastigotes in spleen. Black spots within the macrophage indicate amastigotes (arrow). [A] Untreated *Leishmania* infected macrophage. [B] *Leishmania* infected macrophages treated with 50 mg/kg body wt. leaf extract. [C] *Leishmania* infected macrophages treated with 200 mg/kg body wt. leaf extract.

7.3.2. Animal serum enzyme assay

To check the liver function, the level of the specific serum enzymes and SGOT of hamster undergoing experimental visceral leishmaniasis were studied (section 7.2.3). The results are shown in table 7.2, and figure 7.3. The levels of SGOT and SGPT were markedly higher in *L. donovani* infected hamster compared to uninfected control. The level of both these enzymes decreased in infected hamster upon treatment with *R. serpentina* (at a dose of 200mg /kgbw). These results suggest that *R. serpentina* is non-toxic to liver within the limit of therapeutic doses.

Table: 7.2. Effect of *R. serpentina* extracts on serum enzyme levels

Serum	SGOT(U/L)	SGPT(U/L)
Uninfected and untreated	75 ± 3.3	46 ± 3.3
Infected but untreated	107 ± 4.5	72 ± 3.1
Infected and treated	78 ± 3	49 ± 2.8

7.3.3. Superoxide dismutase (SOD) assay

When promastigote lysate and *R. serpentina* extracts (10-40 mg/ml) were added simultaneously in the pyrogallol assay mixture to determine SOD activity, 50.5% inhibition was observed at the concentration of 20 mg/ml. However, at the same concentration, 85% inhibition was found when promastigote lysate was pre-incubated with *R. serpentina* prior to SOD assay (**Fig: 7.4**).

7.3.4. Impact of *R. serpentina* on SOD activity and superoxide radical release

When cell free promastigote lysate was tested for superoxide free radical release assay, it was observed that the rate of superoxide radical release before and after treatment of *Leishmania* pathogen with *R. serpentina* leaf extract, varies. Fig: 7.5 shows the rate of superoxide free radical release from different amount of cell free lysate after treatment with *R. serpentina* leaf extract at a concentration of 20 mg/ml. Preincubation with same amount of plant extract caused increase in free radical release.

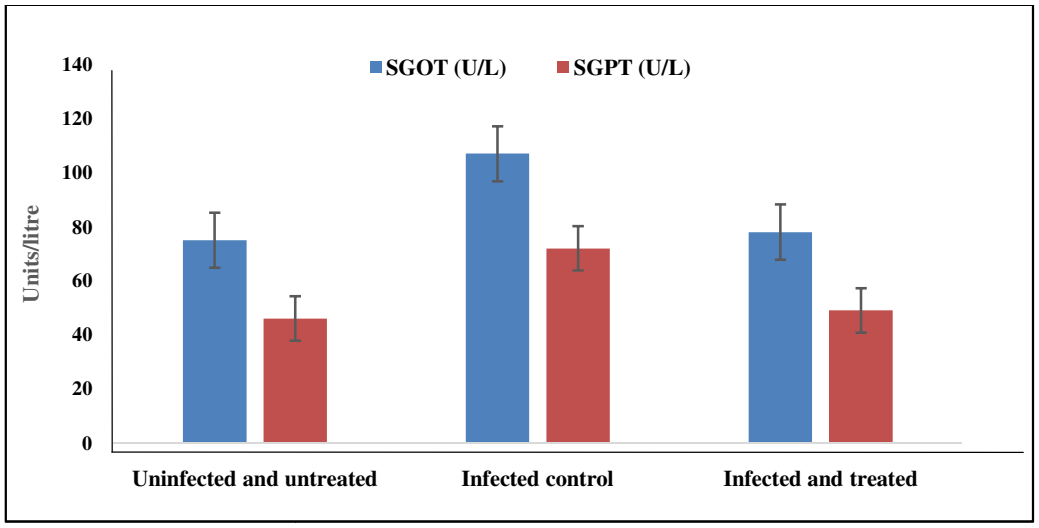

Figure: 7.3. Effect of *R. serpentina* on serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels (units/litre) in *Leishmania* infected hamsters.

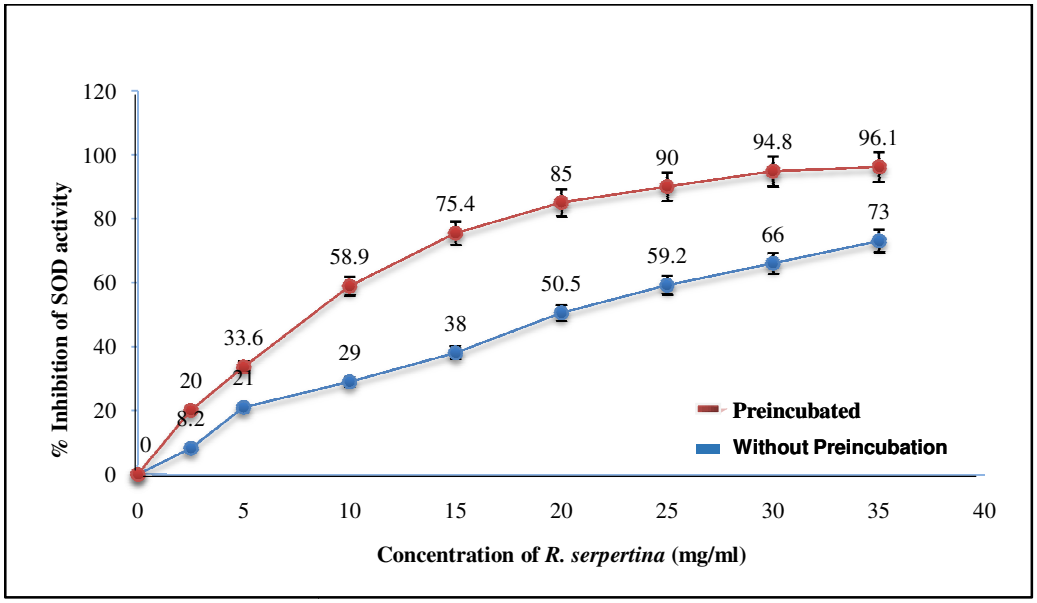

Figure: 7.4. Leishmanial SOD activity when enzyme (promastigote lysate) and *R. serpentina* leaf extracts were added to the reaction mixture either simultaneously (▬) or after preincubation for 30 minutes (▬)

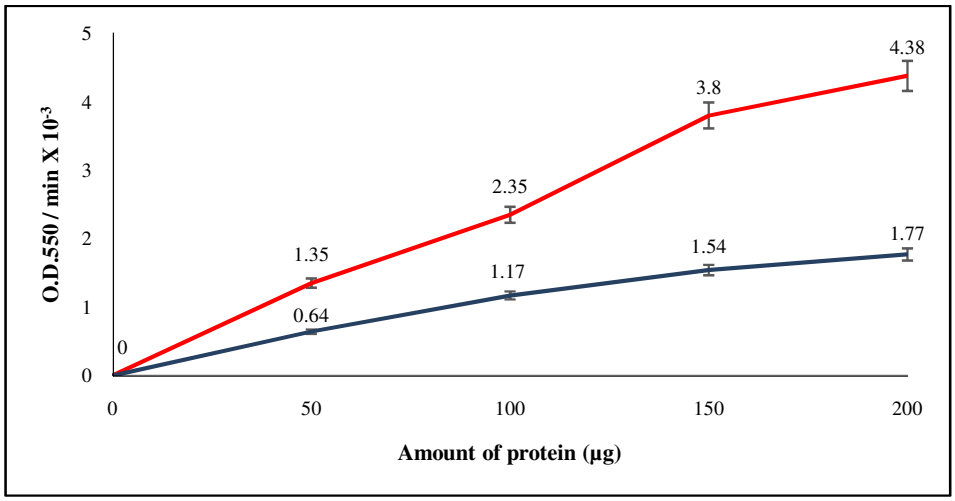

Figure: 7.5. Rate of superoxide radical release before (▬) and after (▬) treatment of *Leishmania* pathogen by *R. serpentina*. Formation of blue formazan derived from reduced nitroblue tetrazolium in the presence of superoxide radical was measured spectrophotometrically.

7.3.5. SDS-PAGE

When the cell free promastigote lysate was treated with different concentrations of leaf extracts of *R. serpentina,* and visualized on polyacrylamide gel,the intensity of the Fe^{3+}-SOD protein band gradually decreased. At 40mg/ml and 30 mg/ml concentration of *R. serpentina*, the SOD activity disappeared completely from the gel (Fig: 7.6).

7.3.6. SOD activity assay

In activity staining, leishmanial SOD activity was found to be inhibited with increasing concentration of *R. serpentina* leaf extract. SOD activity inhibited the formation of blue formazan which was visualised as white band on blue background (Fig: 7.7). In lane A, clear white band was seen where the sample without plant leaf extract was used. Then in lane B and C, the band size gradually decreased where leishmanial SOD was treated with increasing concentrations of leaf extracts (10 mg/ ml and 20 mg/ ml respectively). At the dose of 30 mg/ ml of leaf extract no activity band was observed.

7.4. Discussion

R. serpentina is known as an effective antihypertensive agent from long time. It is traditionally used in psychotic disorders like schizophrenia, anxiety, epilepsy, insomnia, insanity, and is also used as a sedative and a hypnotic drug (Bunkar, 2017).

The plant is mentioned in classic Indian medicinal text. Sarpagandha is included in AparajitGana indicating the use in mental disorder (susrutauttartantra 60/47), in EkasarGana (susrutakalpa 5/84) indicating its use against ‘visha’ and ‘musakavisha’ (susrutakalpa 7/29) (Bunkar, 2017). Its immense traditional use has inspired modern scientists to screen its extract for therapeutic value. Different studies showed its antihypertensive (Ranjini et al., 2015), antidiabetic (Azmi and Qureshi, 2013), antioxidant (Nair et al., 2012), antibacterial (Rathi et al., 2013), antidiarrhoeal (Ezeigbo et al., 2012) and hepatoprotective (Gupta et al., 2010) effects. Few works have been done on antimicrobial activity (Elizabeth, 2017) of *R. serpentina* but, no work has yet been done on its antileishmanial activity to the best of our knowledge.

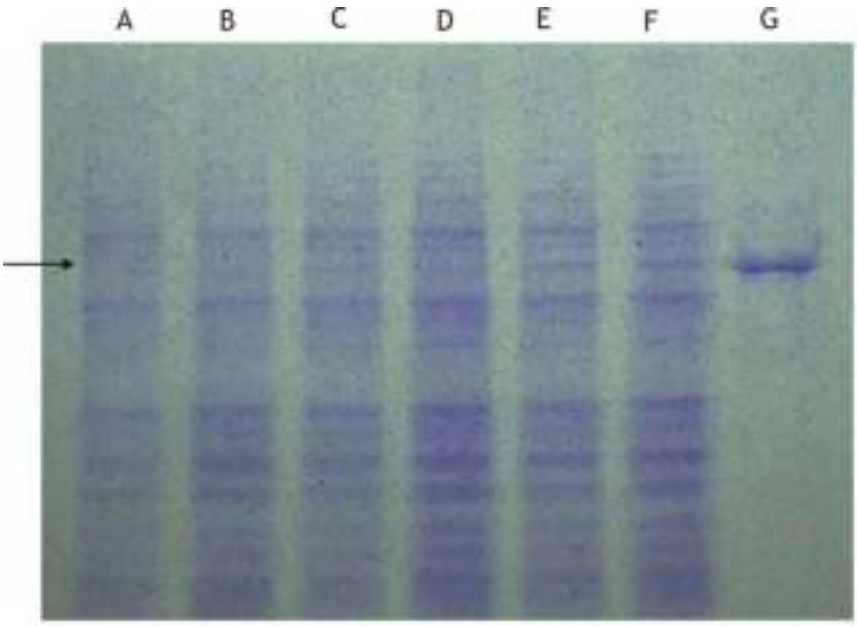

Figure: 7.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of *Leishmania* cell free extract after treatment with *R. serpentina* leaf extract in different doses (50 µg protein was loaded in each lane). A. 40 mg/ml *R. serpentina*; B. 30 mg/ml *R. serpentina* C. 20 mg/ml *R. serpentina*; D. 10 mg/ml *R. serpentina*; E. 5 mg/ml *R. serpentina*; F. Without *R. serpentina*; G. Pure Fe^{3+}- SOD.

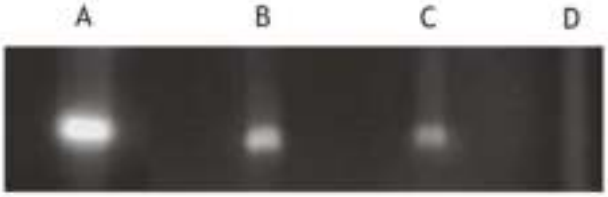

Figure: 7.7. Activity staining of *R. serpentina* leaf extract treated leishmanial SOD after separating by native polyacrylamide gel electrophoresis in a 10% gel. In each lane 60 µgm of protein was loaded. Gel was incubated in nitro blue tetrazolium and riboflavin solutions and finally illuminated. SOD activity which inhibits the formation of blue formazan, was visualized as white bands on a blue background. (A) Without extract B) treated with 10 mg/ml leaf extract (C) treated with 20 mg/ml leaf extract (D) treated with 30 mg/ml leaf extract

The present study screened the leaf extracts of *R. serpentina* for antioxidant, antifungal and antileishmanial activities both *in vitro* and *in vivo* models, and *R. serpentina* had emerged as the promising agent with no cytotoxic effect within therapeutic range.

In vivo studies for antileishmanial activity of *R. serpentina* on *L. donovani* infected golden hamsters showed positive results. *R. serpentina* reduced the parasite burden of liver and spleen of infected hamsters in a dose-dependent manner when administered intramuscularly. Our previous experiment showed that in the administered doses, the extract had no cytotoxic effect on hamster macrophages. Thus, the data suggested that *R. serpentina* can serve as an effective drug in reducing *L. donovani* infection in hamsters. *Leishmania* amastigotes resist phagosomal enzymes and are able to thrive and multiply within the acidic hydrolase rich parasitophorous vacuoles. Though the parasite is sensitive to humoral defense mechanisms, its intracellular habitat offers almost complete protection. Only if the macrophages are activated, the parasite may be killed and degraded by the host cell (Ram et al., 1992; Braconier and Miomer, 1993; Olliaro and Bryceson, 1993; Badirzadeh et al., 2020). Macrophage activation occurs both in natural and adaptive immune reactions in an immuno-competent host. It is induced by INF-γ, a cytokine released mainly by appropriately stimulated natural killer (NK) or T cells (Dasgupta et al., 2003; Volpedo et al., 2021). Activation of microbicidal mechanisms with macrophages may also be done by their exposures to immunomodulating agents. in humans, the protozoan *Leishmania* sp. is an obligated intracellular parasite of macrophages, (Rocha et al., 2005) this makes the treatment for leishmaniasis complicated.

Superoxide dismutase (SOD), which is one of the key enzymes of oxygen defence system, is known to be an essential factor in mediating normal cellular functions (Fattman et al., 2002). Presently the enzyme has been targeted for the treatment of several diseases (Briedbach et al., 2002, Hortelano et al., 2017). SOD also plays a vital role during host–parasite interaction. Its activity is elevated when *Leishmania* parasite infects host cells (Dey et al., 1995). The

parasites use their own SOD to encounter host's superoxides and their own super oxides to get relief from oxygen stress. It is known that the main form of leishmanial SOD contains Fe^{3+} as a co-factor. In the present study the enzyme activity is lost following *R. serpentina* treatmentas shown by activity staining on non-denaturing gel. Leishmanial Fe-SOD was also found to diminished progressively with increasing concentrations of *R. serpentina* leaf extracts in SDS-PAGE. SOD activity completely disappeared at greater concentration of test extract.

It presumed that inhibition of enzymatic activity was caused by protein denaturing effect of the leaf extract (Raychaudhury et al., 2005). Due to deficiency in SOD activity, which is responsible to detoxify released superoxide radicals by the host immune system, toxic free radicals cannot be scavenged up to the maximum limit. The result of present work showing the rate of superoxide radical release in parasitic promastigote lysate before and after treatment with *R. serpentina* leaf extract established the fact clearly. This finding was also in agreement with the previous work of Martin-Montes et al. (2017). Super oxide dismutase has been demonstrated to be a key enzyme to play a vital role in the survival of intracellular parasites (Hortelano et al., 2017), where importance of this enzyme in the host–parasite interaction was established by generating SOD-deficient *L. donovani* (Ghosh et al., 2003). The results obtained from different experiments suggest that inhibition of SOD and simultaneous increase in release of superoxide radicals impose toxic effect to destroy intracellular parasites during experimental visceral leishmaniasis (Gupta et al., 2010). Parasitic infection causes toxicity of liver in animals which is manifested by increased levels of specific serum enzymes such as SGPT and SGOT (Kaur et al., 2016). The present study showed the levels of SGOT and SGPT were markedly higher in *L. donovani* infected hamster compared to uninfected control. The levels of both these enzymes decreased to normal level upon treatment with *R. serpentina*. The collected data suggests that *R. serpentina* may be a good choice to act as an antileishmanial agent with better efficacy. Results also reveal that *R. serpentina* seems to be non-toxic up to therapeutic dose.

The present study was undertaken with an aim to explore the indigenous knowledge on medicinal plants and ayurveda in order to evolve new compositions for fighting against leishmaniasis and fungal infections. The ethnobotanical studies were conducted in the remote areas of two districts of North Bengal, Jalpaiguri and Alipurduar. An intensive survey was done to gather information about medicinal plants used by the indigenous population and tribal communities inhabiting in the jungles of tarai – dooars and tea garden areas. Fifty plant species of 33 families were found to be used for medicinal purposes by various tribes of Jalpaiguri and Alipurduar districts. These plants are mostly (76%) collected from natural vegetation to treat cuts and wounds, cough and cold, fever, skin diseases, pain and inflammation, bone fracture, stomach ache and abdominal disorder, jaundice and liver problems. Leaf was found to be the most frequently used (33.9%) plant part followed by root (18.2%). Four very common and well-known medicinal plants, *R. serpentina* (sarpagandha), *M. oleifera* (drumstick), *N. arbor-tristis* (seuli or night jasmine) and *C. excavata* (agnijol) were selected for screening, as the utilizations of these plants were found to be vast and versatile among the studied population. Crude leaf extracts of *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* were analysed for their phytochemical contents and were also screened for antioxidant, antifungal and antileishmanial activities.

In phytochemical analysis, leaf extracts of each of the four plants showed positive result in qualitative test for alkaloid, flavonoid, terpenoid and tannin. In the quantitative assay, it was seen that the amount of phenolic compound in all four plant leaf extracts ranged from 22.22 to 42.65 mg GAE/gm extract. Flavonoid presence ranged from 9.39 to 31.15 mg QE/gm extract.

All the four plants were found to have free radical scavenging activities in DPPH assay. The percent inhibition of free radical neutralizing activities was determined. *C. excavata* showed the lowest IC_{50}, implying good antioxidant property, and *R. serpentina* showed the highest IC_{50} value. The relationship between phenolic and flavonoid content and antioxidant activities of respective plants were also quite evident. *N. arbor-tristis* had higher phenolic

content and lower IC_{50} value, whereas *M. oleifera* had low phenolic content and comparatively more IC_{50} value in percent inhibition of DPPH. In contrast, *C. excavata*, which showed lowest phenolic and flavonoid content but good antioxidant property (IC_{50} lowest), indicated the probability of presence of active compound of different polarity in its leaf extract.

The methanolic extracts of leaves of all the four plants showed good antifungal activities against human fungal pathogen *C. albicans in vitro*. *R. serpentina* leaf extract showed highest range of zone of inhibition (4.87cm) at the concentration of 100mg/ml. At the same concentration the size of ZOI for *M. oleifera, N. arbor-tristis*and *C. excavata* were 3.2cm, 1.6cm and 2.4cm respectively. But coumarin molecule, excavarin-A (isolated from *C. excavata* leaf extract) showed highest antifungal activity with a ZOI of 4.6cm at a concentration of 10mg/ml. The MIC value was lowest for excavarin-A (0.078 mg/ml) followed by *R. serpentina* (0.156 mg/ml), which was nearer to the known antifungal antibiotic nystatin having MIC of 0.039 mg/ml.

The methanolic leaf extracts of *R. serpentina* and *C. excavata,* and excavarin-A were screened for their antileishmanial activities *in vitro* on the promastigotes of *L. donovani* strain in graded doses. Result showed all the test substances had antileishmanial activities *in vitro*. The IC_{50} values for excavarin-A and the crude leaf extracts of *R. serpentina* and *C. excavata* was recorded as 1.24 mg/ml, 4.04 mg/ml and 32.1 mg/ml respectively. The purified compound was found to be much more effective than the crude extracts. The selected botanicals were also tested on amastigotes cultured in hamster macrophage in laboratory condition. Excavarin-A was found to be most efficacious followed by crude extracts of *R. serpentina* and *C. excavata.*

Superficial infections were created on experimental animals (male albino rats) with cell suspension of fungal strain of *C. albicans* ($1x10^5$cells/ml). Infection developed at the site of application after two days of pathogen application and the animals were treated superficially with diluted plant extracts on the wounds. Significant recovery after 8 days of treatment was evident in the treated animals in comparison to untreated group. The therapeutic action of

R. serpentina was found better than *M. oleifera*. Total count of WBC was increased in both cases of treated animals. This model of *in vivo* experiment using animal sample with open wounds and application of crude extracts superficially provides a preliminary but direct visible evidence of the efficacy of the plant extracts. Study with more pathophysiological parameters is needed to further evaluate the effectiveness and mode of actions of these botanicals.

The crude leaf extracts of *R. serpentina* and *C. excavata*, and the purified compound excavarin-A were checked for cytotoxicity by MTT assay, and tested for viability on macrophages isolated from hamster peritoneal cavity. *C. excavata* leaf extract and excavarin-A were found to be cytotoxic within effective therapeutic ranges. But *R. serpentina* leaf extracts were not found to be cytotoxic in cell viability test in its effective range of doses. So, on the basis of this observation, the crude methanolic leaf extract of this plant was studied further *in vivo*.

The parasitic burden of spleen and liver of golden hamsters experimentally infected with *L. donovani* decreased significantly following treatment with graded dose of leaf extracts of *R. serpentina*. Further an increase in serum SGOT and SGPT levels in infected animals, and the lowering of these levels by application of *R. serpentina* leaf extract to almost normal level indicated the therapeutic effect of the extract. Pyrogallol auto oxidation assay showed inhibition of activity of parasitic superoxide dismutase. The release of toxic superoxide radicals was also found to increase by the application of *R. serpentina* leaf extracts which indicated the probable mode of action of the active substances present in the applied sample. Polyacrylamide gel electrophoresis of *Leishmania* promastigote lysate confirmed these results. On gel activity staining through non-denaturing PAGE as well as SDS-PAGE showed degeneration of SOD bands with increasing concentration of *R. serpentina* extract.

The present study reported significant results of some preliminary screening tests of a few very common and widely used medicinal plants having anti-

oxidant, antifungal and antileishmanial properties. The study also proposed immunological modifications induced by application of test samples which may be helpful in further research to find the mode of action of therapeutic agents gained from natural resources. Some easy and effective protocols of experiments were also tried, which has very limited references so far. *R. serpentina* emerged as most effective plant with significant antimicrobial values and without cytotoxic properties. This plant is strongly recommended for further study and isolation of active substances to develop a new line of treatment. The present work is definitely a step forward in the utilization of natural resources of unexplored areas of North Bengal to elucidate the local knowledge scientifically to discover bioactive molecules related to new drug development against parasitic diseases, whose prevalence and impact is universal.

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APPENDIX - A

Chemicals used

Chemicals	Company
Acetic acid (glacial)	SRL Pvt. Ltd., Mumbai, India
Acetic anhydride	Sigma Aldrich Chemicals Pvt. Ltd., India
Acetone	SRL Pvt. Ltd., Mumbai, India
Agar powder, Certified	HiMedia Laboratories Ltd, Mumbai, India
Agarose	SRL Pvt. Ltd., Mumbai, India
Aluminium chloride	Sigma Aldrich Chemicals Pvt. Ltd., India
Aluminium oxide (Neutral) for column chromatography	SRL Pvt. Ltd., Mumbai, India
Ammonia	SRL Pvt. Ltd., Mumbai, India
Ammonium oxalate	Sigma Chemical Company, St. Louis, Missouri, USA
Ammonium sulphate	HiMedia Laboratories Ltd., Mumbai, India
Ascorbic acid	SRL Pvt. Ltd., Mumbai, India
Barbituric acid	HiMedia Laboratories Ltd, Mumbai, India
Benzene	SRL Pvt. Ltd., Mumbai, India
Coomassie blue	Sigma Chemical Company, St. Louis, Missouri, USA
Crystal violet	Sigma Chemical Company, St. Louis, Missouri, USA
Dextrose	HiMedia Laboratories Ltd., Mumbai, India
Diethyl ether	SRL Pvt. Ltd., Mumbai, India
Dimethyl sulfoside (DMSO)	Sigma Chemical Company, St. Louis, Missouri, USA
1,1-diphenyl-2-picryl-hydrazil (DPPH)	HiMedia Laboratories Ltd., Mumbai, India
EDTA	Sigma Chemical Company, St. Louis, Missouri, USA

Ethidium bromide	Bangalore Genei (India) Pvt. Ltd.
Ethyl acetate	SRL Pvt. Ltd., Mumbai, India
Ferric chloride	HiMedia Laboratories Ltd., Mumbai, India
Foetal bovine sera	Sigma Chemical Company, St. Louis, Missouri, USA
Formazan	Sigma Chemical Company, St. Louis, Missouri, USA
Gallic acid	Sigma Aldrich Chemicals Pvt. Ltd., India
GIEMSA	Sigma Chemical Company, St. Louis, Missouri, USA
Glutamine	Sigma Chemical Company, St. Louis, Missouri, USA
HEPES	Sigma Chemical Company, St. Louis, Missouri, USA
Hexane	SRL Pvt. Ltd., Mumbai, India
Hydrochloric acid	SRL Pvt. Ltd., Mumbai, India
Iodine	HiMedia Laboratories Ltd, Mumbai, India
Lactophenol- cotton blue	HiMedia Laboratories Ltd, Mumbai, India
Leishman's stain	Sigma Chemical Company, St. Louis, Missouri, USA
Lipophilic Sephadex	Sigma Aldrich Chemicals Pvt. Ltd., India
Magnesium chloride	HiMedia Laboratories Ltd., Mumbai, India
Magnesium ribbon	ISOCHEM, India
Methanol A.R.	SRL Pvt. Ltd., Mumbai, India
Medium-199	GIBCOBRL, Gaithersburg, Maryland, USA
Molecular Biology Grade Chloroform	SRL Pvt. Ltd., Mumbai, India
Molybdate	Sigma Aldrich Chemicals Pvt. Ltd., India
MTT	Sigma Chemical Company, St. Louis, Missouri, USA

Nitro-blue tetrazolium	Sigma Chemical Company, St. Louis, Missouri, USA
Olive Oil	BIO VEDA ACTION RESEARCH CO., HARYANA, INDIA
Percoll	Pharmacia Fine Chemicals, Sweden
Petroleum ether	SRL Pvt. Ltd., Mumbai, India
Picric acid	Sigma Aldrich Chemicals Pvt. Ltd., India
Potassium mercuric iodide	Sigma Aldrich Chemicals Pvt. Ltd., India
Quercetin	HiMedia Laboratories Ltd., Mumbai, India
RPMI-1640 medium	GIBCO Lab, Grand Island, New York, USA
Riboflavin	Sigma Chemical Company, St. Louis, Missouri, USA
Safranin	Sigma Chemical Company, St. Louis, Missouri, USA
SGOT-SGPT kit	Dr. Reddy's Laboratory, Hyderabad, India
Silica Gel GF-254 (60-120 mesh)	SRL Pvt. Ltd., Mumbai, India
Silica gel (100-200 mesh) for column chromatography	SRL Pvt. Ltd., Mumbai, India
Silica gel (60-120 mesh) for column chromatography	SRL Pvt. Ltd., Mumbai, India
Sodium antimony gluconate (SAG)	Sigma Chemical Company, St. Louis, Missouri, USA
Sodium carbonate	HiMedia Laboratories Ltd., Mumbai, India
Sodium chloride	Merck Specialities Pvt. Ltd., Mumbai, India
Sodium stibogluconate	Gluconate Health Ltd., Sweden
Sodium sulphate	HiMedia Laboratories Ltd., Mumbai, India
Sodium thioglycolate	GIBCO Lab, Grand Island, New York, USA
Sucrose	SRL Pvt. Ltd., Mumbai, India
Sulphuric acid	SRL Pvt. Ltd., Mumbai, India

Superoxide dismutase (SOD)	Sigma Chemical Company, St. Louis, Missouri, USA
TEMED	Sigma Chemical Company, St. Louis, Missouri, USA
Trichloroacetic acid	Merck, Germany
Vanillin	SRL Pvt. Ltd., Mumbai, India
0.22 µm pore size millipore filter	Millipore Corporation, USA
Gel loading buffer (6X)	Bangalore Genei (India) Pvt. Ltd.
TLC Silica gel 60 F254 aluminium sheets	Merck, Germany
100 µg/ml streptomycin sulphate	Sigma Chemical Company, St. Louis, Missouri, USA
100U/ml peniciline G-Sodium	Sigma Chemical Company, St. Louis, Missouri, USA

APPENDIX-B

1. **Mayer's reagent (potassium mercuric iodide)**

   Mixture A: 1.358 g of mercury (II) chloride dissolved 60 mL of distilled water

   Mixture B: 5 g of potassium iodide dissolved in 10 mL of distilled water. Mixture A was poured into Mixture B, and the final volume was made up to 100 mL with distilled water.

2. **Wagner's reagent (iodine in potassium iodide)**

   Two grams of iodine and 6 g of KI dissolved in 100 mL of distilled water.

3. **Hager's reagent (saturated picric acid solution)**

   Picric acid(1g) dissolved in 100 mL of distilled water.

4. **Molisch's reagent**

   15 g of 1-naphthol dissolved 100 mL of 95% (vol/vol) ethanol or chloroform.

5. **Benedict's reagent (qualitative)**

   Solution A:173 g sodium citrate and 100 g of sodium carbonate dissolved in 800 mL of distilled water, filtered, and diluted to 850 mL with distilled water.

   Solution B: 17.3 g of copper (II) sulphate pentahydrate in 100 mL of distilled water.

   Solution B was poured, with constant stirring, into Solution A and diluted to 1 L with distilled water.

6. **Fehling's A and B solutions**

   Fehling's Reagent A: 70g of copper sulphate and concentrated sulphuric acid 1ml dissolved in distilled water (200 ml) and made up to volume 1 litre with distilled water.

Fehling's Reagent B: 352 g of sodium potassium tartrate and 154 g sodium hydroxide dissolved in 200 ml distilled water and made up to volume 1 litre with distilled water.

7. **Folin-Ciocalteu reagent**

   Hetero-polyphospho tungstate-molybdate diluted with distilled water in 1:1 ratio

8. **Phosphate buffered saline (PBS) (0.15M)**

   Solution A: Na_2HPO_4 4.2588 g, Distilled water 200ml.

   Solution B: Na_2HPO_4, $2H_2O$ 9.3606 g, Distilled water 400ml.

   140 ml A was mixed with 360 ml of Solution B, and 4 g of NaCl and 100 mg of KCl was added.

9. **Potassium phosphate buffer (50mM, pH7.2)**

   Solution A: 0.1 M solution of Na_2HPO_4 1.42 g, Distilled water 100ml

   Solution B: 0.1M solution of Na_2HPO_4, $2H_2O$ 1.56 g, Distilled water 100ml

   Solution A and Solution B were separately autoclaved and stored at 40°C. 81 ml of Solution A were mixed with 19 ml of Solution B, diluted to 200 ml with distilled water to make final phosphate buffer.

10. **EDTA (0.1 mM, pH 8.0)**

    Disodium EDTA - dihydrate 18.6g

    Distilled water 100ml

    Measured amount of disodium EDTA-dihydrate was dissolved in distilled water and mixed vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH. Solution was sterilized by autoclaving at 15 lbs psi pressure for 15 min at 121°C.

11. **Turk's fluid**

    Glacial acetic acid 1.5 ml, gentian violet 1% solution 1.5 ml, and distilled water 100 ml.

12. **Pyrogallol**

    0.2 mM Pyrogallol in air equilibrated, 50mM Tris-cacodylic acid buffer, and 1 mM diethylene triamine penta acetic acid, (pH 8.2)

13. **Crystal violet stain**

    Solution A- Crystal violet 2g, 95% Ethanol 20ml

    Solution B- Ammonium oxalate 0.8 g, Distilled water 80 ml

    Solution A and B was mixed for 30-60 seconds.

14. **Lugol's iodine**

    Iodine 1.0 g

    Potassium iodide 2.0 g

    Distilled water 300 ml

15. **Lactophenol Cotton-Blue (LPCB)**

    Phenol 200.0 gm
    Cotton Blue 0.5 gm
    Glycerol 400 ml
    Lactic Acid 200 ml
    Deionized Water 200 ml

16. **Safranin solution**

    Stock solution – Safranin 0 2.5 gm, 95% Ethanol 100 ml

    Working solution – Stock solution 10 ml, distilled water 90 ml

17. **1,1-diphenyl-2-picryl-hydrazil (DPPH) solution (0.2 mM)**

    3.94 mg of DPPH was dissolved in 50 ml methanol to make a solution, fresh each time.

18. **Richard's medium**

KH_2PO_4	5 g
KNO_3	10 g
$FeCl_3$	0.02 g
$MgSO_4.7H_2O$	2.5 g
Sucrose	50 g
Distilled water	1000 ml

All the constituents were taken and mixed with required distilled water by stirring and sterilized at 15 lb psi for 15 minutes.

19. **Potato Dextrose Broth (PDB)**

    Peeled Potato 40 g

    Dextrose 2g

    Distilled water 100ml

    The potato was peeled and boiled in double volume distilled water of required amount. The potato broth was filtered through cheese cloth and then required amount of dextrose was added. Finally, the medium was sterilized at 15 lb psi for 15 minutes in autoclave.

20. **Potato dextrose agar (PDA)**

    Peeled potato 40 g

    Dextrose 2 g

    Distilled water 100 ml

    Peeled potato was boiled and filtered through cheese cloth. The required amount of dextrose and 2% agar powder were added in filtrate. The agar was completely melted by heating the media before sterilization. Finally, the medium was sterilized as 15 pb psi for 15 min in autoclave.

21. **RPMI-1640**

    About 0.584 g L-glutamine, 5.95 HEPES buffer, 100 mg/ ml streptomycin and 100 IU/ml penicillin, supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) was used.

22. **Medium-199 (pH 7.4)**

    Supplemented with 10% heat inactivated foetal bovine serum, 2mM glutamine, 25mM N-2-ethansulfonic acid (HEPES), 100U/ml peniciline G-sodium, 100 µg/ml streptomycin sulphate.

23. **Ray's solid medium**

    Brain heart infusion 3.7%, Sodium chloride (w/v) 0.1%, Agar 1.5%; pH was maintained at 7.4. The ingredients were dissolved in glass-distilled water

and autoclaved. To this, 1% glucose (w/v) and 1% blood (v/v), drawn from normal rabbit heart, was added.

24. **Complex-forming reagent (For protein estimation following Lowry's method) (Lowry, 1951)**

This reagent was preparedby mixingthree stock solutions in the proportion of 100:1:1 (vol / vol).

Solution A: 2% (w/v) Na_2CO_3 in distilled water.

Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water.

Solution C: 2% (w/v) sodium potassium tartrate in distilled water.

25. Polyacrylamide gel electrophoresis (PAGE):

Stock solutions

Solution A: Acrylamide stock solution (resolving gel)

For preparing acrylamide stock solution, 29.2 g of acrylamide and 0.8 gm of N N' methylene bisacrylamide were dissolved in 100 ml of warm distilled water. The stock solution was filtered with Whatmann No.1 filter paper in dark and stored in dark bottle at 4^0C.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for the stacking gel, 10 gm acrylamide and 2.5 gm of bisacrylamide was dissolved in 100 ml warm distilled water. The stock solution was then filtered with Whatmann No.1 filter paper and stored at 4^0C in dark bottle.

Solution C: Tris-HCl (resolving gel)

Tris HCL buffer was prepared by dissolving 36.6 gm of Tris base in distilled water and 0.25 ml of TEMED was added. The pH of the solution was adjusted to 8.9 with conc. HCl. The final volume of the solution was made upto 100 ml with distilled water. The solution was then stored at 4^0C for further use.

Solution D: Tris HCl (stacking gel)

5.98 gm of Tris base was mixed with distilled water and 0.46 ml of TEMED was added to it. Finally the pH of the solution was adjusted to 6.7 with conc. HCl. The final volume of the solution was made upto 100 ml with distilled water and stored at 4^0C for further use.

Solution E: Ammonium per sulphate

Ammonium per sulphate was prepared by dissolving 0.15 g of APS in distilled water. The final volume was made up to 10 ml. This was prepared fresh each time.

Solution F: Riboflavin solution

Fresh riboflavin solution was prepared by dissolving 2 mg of riboflavin in 2 M sucrose (100 ml). The solution was kept in dark bottle to protect it from light.

Solution G: Electrode buffer (pH 8.4)

Fresh electrode buffer was prepared by dissolving 6 g of Tris base and 28.8 g of glycine in 1000 ml distilled water.

Preparation of gel

Resolving gel (10%)
30% Acrylamide 2.55 ml
1.5 M Tris (pH 8.8) 1.95 ml
10% SDS 0.075 ml
10% APS 0.075 ml
TEMED 0.003 ml
Water 2.85 ml

Staking gel (5%)
30% Acrylamide 0.5 ml
1.0 M Tris (pH 6.8) 0.38 ml
10% SDS 0.030 ml
10% Ammonium persulphate 0.030 ml
TEMED 0.030 ml
Water 2.1 ml

Protocol:

Polyacrylamide gel electrophoresis (PAGE) was done following the method as described by Davis (1964) with some modifications. A mini slab gel (8 X 5 cm) was prepared for which, two glass plates ware thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with 1% agar solution. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. A 10% resolving gel was prepared by mixing the acrylamide stock solution (for resolving gel), Tris-HCl (pH 8.9, for resolving gel), APS (freshly prepared) and distilled water in the ratio 1:1:4:1 and carefully dispensing the mixture by a pasture pipette between the glass slabs leaving sufficient space for the stacking gel (1.5 cm). After pouring the resolving gel solution, it was immediately over layered with water and allowed to polymerize for 1.5 - 2 h. After polymerization was complete, the water over layer was poured off and the gel was washed with water to remove any unpolymerized acrylamide.

The stacking gel (4%) solution was prepared by mixing the acrylamide stock solution (for stacking gel), Tris HCl (pH 6.7, for stacking gel), riboflavin (freshly prepared) and distilled water in the ratio 2:1:1:4. The mixture was poured over the resolving gel and the comb was inserted leaving a gap of approximately one cm below the well in the stacking gel. The gel was kept under bright sunlight or fluorescent light for polymerization. After solidification of the stacking gel, the comb was removed and the wells were washed thoroughly. Following casting, the gel with the glass slabs was fitted into the electrophoresis apparatus. Chilled tris-glycine running buffer (pH 8.4) was added sufficiently in both upper and lower reservoirs of the gel apparatus. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe. The gel was loaded with protein/enzyme samples and incubated at 4^0C supplying a constant current of 2.5 mA per well continuously for 3-4 h until the dye front reached the bottom of the gel. After electrophoresis, the gel was removed from the glass plates and then the stacking gel was cut off

from the resolving gel and finally proceeded for activity staining of superoxide dismutase.

26. Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Stock solutions:

Stock solution A: Acrylamide/Bis-acrylamide

Acrylamide 87.6 g

N`N`-bis-methylene acrylamide 2.4 g

The ingredients were dissolved in deionized water and the volume was adjusted to 300 ml. thereafter the solution was filtered and stored at 4ºC in the dark (30 days maximum).

Stock solution B: 10% (w/v) SDS

10 g SDS was dissolved in 90 ml water with gentle stirring and made to 100 ml with deionized water.

Stock solution C: 10% (w/v) APS

20 mg of APS was dissolved in 2 ml of deionized water.

Stock solution D: 1.5M Tris-HCl, (pH 8.8)

Tris base 27.23 g Deionized water 80 ml The ingredients were mixed and adjusted to pH 8.8 with 6N HCl. The final volume was made upto 150 ml with deionized water and store at 4^0C.

Stock solution E: 0.5M Tris-HCl, (pH 6.8)

Tris base6 g

Deionized water 60 ml

These chemicals were adjusted to pH 6.8 with 6N HCl and the total volume made upto 100 ml with deionized water and store at 4^0C.

Stock solution F: Sample buffer

Deionized water 3.55 ml

0.5M Tris-HCl (pH 6.8) 1.23 ml

Glycerol 2.5 ml 10% (w/v) SDS 2.0 ml

0.5% (w/v) Bromophenol blue 0.2 ml

Total volume 9.5 ml

Finally the solution was stored at room temperature.

ß-mercaptoethanol (50 µl) was added to 950 µl sample buffer prior to use and the sample was diluted to at least 1:2 with sample buffer and heated at 95^0C for 4 minutes.

Stock solution G: 10X Electrode (running) buffer, (pH 8.3)

Tris base 30.3 g

Glycine 144.0 g

SDS 10.0 g

The ingredients were dissolved and total volume made upto 1000 ml with deionized water and stored at 4^0C. Use: Diluted 50 ml 10X stock with 450 ml deionized water for each electrophoresis run.

SDS-PAGE protocol:

Protein samples were mixed with equal volume of sample buffer and heated for 5 min at 95^0C. Gel was made according to the method of Laemmli (1970). Separating gel (10%) was used for resolving the polypeptides whereas a 4% stacking gel was used to concentrate the polypeptides. Test samples as well as pure Fe-SOD (kindly provided by Dr. Syamal Roy, IICB, Kolkata) were loaded on the gel and electrophoresis was accomplished in electrode buffer at 30mA for 4h using a Bio-Rad, Mini PROTEAN Tetra Cell Electrophoresis system. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma) for overnight and destained. The gel was finally photographed.

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Research Article

Phytochemical analysis of two medicinal plants of North Bengal

Anindita Chakraborty[1*] and Bikramjit Raychaudhury[2]

[1]*Department of Physiology, A.B.N. Seal College, Cooch Behar, 736101, West Bengal.*

[2]*Department of Physiology, Ananda Chandra College, Jalpaiguri, 735101, West Bengal.*

**E-mail: aninditajal257438@gmail.com*

A B S T R A C T

Medicinal plants have been used as a source of remedies since ancient times in India. Traditional medicine systems consist of large numbers of plants with medicinal and pharmacological importance. Rouvolfia Serpentina and Moringa Olifera are two such important medicinal plants traditionally used in India for their immense therapeutic properties. In this present study plants leaf extracts are screened for the presence of major phytochemical groups which are plants primary and secondary metabolites and known for their therapeutic values. These compounds are reported to have antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protective and antimicrobial activities. Qualitative analysis showed the presence of metabolites such as glycosides, phenolic compounds, alkaloids, flabinoids, tannins, saponins, steroids for both the plants ensuring their potentiality as therapeutic agent.

Key Words*: Rouvolfia Serpentina, Moringa Olifera, medicinal plants, therapeutic properties, phytochemical*

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1. INTRODUCTION

The use of herbal medicine for the treatment of diseases and infections is as old as mankind. The World Health Organization supports the use of traditional medicine provided they are proven to be efficacious and safe (WHO, 1985). In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin, lesser side effect and cost

effective qualities (Report of seminar on Herbal Drug, 2001). Medicinal plants have been used as a source of remedies against many pathological conditions since ancient times in India. Traditional medicine systems consist of large numbers of plants with medicinal and pharmacological importance and hence represent an invaluable reservoir of new bioactive molecules.

The plant kingdom is a treasure house of potential therapeutic compounds and in the recent years there has been an increasing awareness about the importance of these compounds of medicinal plants and uses them as therapeutic agents because compounds from the plants are easily available, less expensive, safe, and efficient and proven to have side effects. Recently some plants have been selected for examination and proven therapeutically effective new drugs such as anticancer drugs (Dewick 1996), antimicrobial drugs (Phillipson and Wright 1996), antihepatotoxic compounds. Some organic compounds from medicinal plants which are by primary or rather secondary metabolites provide definite physiological action on the human body. These bioactive substances include tannins, alkaloids, carbohydrates, protein, glycosides terpenoids, steroids and flavonoids (Mann 1978; Edoga et al. 2005). They are widely used in the human therapy, as well as veterinary, agriculture, scientific research and many other different areas of human interest (Vasu et al. 2009). A large number of phytochemicals also have been shown to have inhibitory effects on all types of microorganisms in vitro and in animal models (Cowan 1999).

Among The secondary plant metabolites, phenol compounds such as flavonoid, phenolic acids, tocopherols etc are most ubiquitous groups of (Singh et al. 2007). Studies revealed that plant rich in phenolic compounds possess biological properties such as antiapoptotic, antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis cell proliferation activities antioxidant properties and (Brown and Rice-Evans 1998; Krings and Berger 2001; Han et al. 2007; Ali et al. 2008). In response to microbial infection plants synthesize a hydroxylated phenolic substance, flavonoids having activities against wide array of microorganisms in vitro, which may be due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Marjorie. 1996). Another metabolite, tannins interfere with protein synthesis by binding with proline rich protein. The plant extracts containing saponins, have anti inflammatory (Just et al. 1998) hemolytic (Okwu 2004) activities. Studies show that, Glycosides lower the blood pressure (Nyarko and Addy 1990). Steroids are very important compounds, having relationship with sex hormones (Okwu 2001).They also have antibacterial activities (Raquel 2007). Alkaloids have been used for centuries for their medicinal value. They have cytotoxic (Nobori et al. 1994), analgesic antispasmodic and antibacterial (Stray, 1998; Okwu and Okwu 2004) properties. The mechanism of action of some phytochemicals are shown below (Table 1)

Table 1: *Mechanism of action of some phytochemicals (Tiwari et al. 2011)*

Phytochemicals	Activity	Mechanism of action
Quinones	Antimicrobial	Binds to adhesins, complex with cell wall, inactivates enzymes
Flavonoids	Antimicrobial Antidiarrhoeal	Complex with cell wall, binds to adhesins Inhibits release of autocoids and prostaglandins, Inhibits contractions caused by spasmogens, Stimulates normalization of the deranged water transport across the mucosal cells, Inhibits GI release of Acetylcholine
Polyphenols and Tannins	Antimicrobial Antidiarrhoeal Anthelmintic	Binds to adhesins, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption, metal ion complexation Makes intestinal mucosa more resistant and reduces secretion, stimulates normalization of deranged water transport across the mucosal cells and reduction of the intestinal transit, blocks the binding of B subunit of heat-labile enterotoxin to GM1, resulting in the suppression of heat-labile enterotoxin-induced diarrhea, astringent action Increases supply of digestible proteins by animals by forming protein complexes in rumen, interferes with energy generation by uncoupling oxidative phosphorylation, causes a decrease in G.I. metabolism
Phytochemicals	**Activity**	**Mechanism of action**
Coumarins	Antiviral	Interaction with eukaryotic DNA
Terpenoids and essential oils	Antimicrobial Antidiarrhoeal	Membrane disruption Inhibits release of autocoids and

		Prostaglandins
Alkaloids	Antimicrobial Antidiarrhoeal Anthelmintic	Intercalates into cell wall and DNA of parasites Inhibits release of autocoids and prostaglandins Possess anti-oxidating effects, thus reduces nitrate generation which is useful for protein synthesis, suppresses transfer of sucrose from stomach to small intestine, diminishing the support of glucose to the helminthes, acts on CNS causing Paralysis
Lectins and Polypeptides	Antiviral	Blocks viral fusion or adsorption, forms disulfide bridges
Glycosides	Antidiarrhoeal	Inhibits release of autocoids and Prostaglandins
Saponins	Antidiarrhoeal Anticancer Anthelmintic	Inhibits histamine release in vitro Possesses membrane permeabilizing properties Leads to vacuolization and disintegration of teguments
Steroids	Antidiarrhoeal	Enhance intestinal absorption of Na+ and water

Plant products having phytomedicinal properties, can be derived from barks, leaves, flowers, roots, fruits, seeds of the plant (Criagg and David 2001). So for synthesis of complex chemical substances, knowledge of the chemical constituents of plants is essential (Mojab et al. 2003; Parekh and Chanda 2007; Parekh and Chanda 2008).

The present study investigates the fundamental scientific basis for the use of Rouvolfia Serpentina and Moringa Olifera plants leaf as therapeutic agent by defining the presence of crude phytochemical constituents .

2. MATERIALS AND METHODS

2.1. Collection of plant materials

The leaves of the plants were collected from different uncultivated farmlands of Jalpaiguri District, West Bengal. The sample leaves of two plants were identified by the authors. The leaves were air-dried and ground into uniform powder using a REMI Mixer grinder machine and kept in air tight container. The aqueous extract of each sample was

prepared by soaking 100 g of dried powdered samples in 200 ml of distilled water for 12 h. The extracts were filtered using Whatman filter paper No 42 (125 mm).

2.2. Phytochemical screening

Chemical tests were carried out on the aqueous extract and on the powdered specimens using standard procedures as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973) to identify the constituents phytochemicals.

2.3. Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

2.4. Test for phlobatannins

Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

2.5. Test for saponin

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

2.6. Test for flavonoids

5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2S0_4. A yellow coloration observed in each extract indicated the presence of flavonoids. The yellow coloration disappeared on standing. Few drops of 1% aluminium solution were added to a portion of each filtrate. A yellow coloration was observed indicating the presence of flavonoids. A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration was observed indicating a positive test for flavonoids.

2.7. Test for steriods and terpenoids

Libermann Burchard's test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. sulphuric acid was added to the solution. Formation of brown ring at the junction and green upper layer indicates the presence of phytosterols and formation of deep red colour indicates the presence of triterpenoids.

2.8. Test for cardiac glycosides (Keller-Killani test)

Five ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet

ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

2.9. Detection of alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

2.9.1. ***Mayer's Test*****:** Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow colour precipitate indicates the presence of alkaloids.

2.9.2. ***Wagner's Test*****:** Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

2.9.3. ***Hager's Test*****:** Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow colored precipitate.

2.10. Detection of carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

2.10.1. ***Molisch's Test*****:** Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

2.10.2. ***Benedict's Test*****:** Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

2.10.3. ***Fehling's Test*****:** Filtrates were hydrolysed with dilute HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

2.11. Detection of organic acids

2.11.1. ***Oxalic acid test*****:** To the test solution few drops of 1% $KMnO_4$ and dilute H_2SO_4 is added, if the colour disappears, shows the presence of organic acid.

2.11.2. ***Malic acid test*****:** To the test solution 2-3 drops of 40% $FeCl_3$ is added, appearance of yellowish colour proves the presence of organic acid.

3. RESULT

The leaf extract and the dried powder of leaf of Rouvolfia Serpentina and Moringa Olifera showed the presence of tannin, phlobatannin, saponin, flavonoids, sterols, alkaloids, carbohydrate, protein and organic acids. Both the leaf extract tested negative for the presence of triterpinoids, while only the leaf extract of Rouvolfia Serpentina tested negative for the presence of cardiac glycosides. The phytochemical constituents of Rouvolfia Serpentina and Moringa Olifera are represented in the following table (Table 2).

***Table 2**: Phytochemical constituents of Rouvolfia Serpentina and Moringa Olifera*

Sl. no.	Phytoconstituents	Rouvolfia Serpentina	Moringa Olifera
1.	Alkaloids	++	+
2.	Flavonoids	+	+
3.	Tannin	+	+
4.	phlobatannin	++	++
5.	Saponin	+	++
6.	Steroid	+	++
7.	Triterpinoid	-	-
8.	Cardiac Glycoside	-	++
9.	Organic acid	+	+
10.	Carbohydrate	+	+
11.	Protein	+	+

4. DISCUSSION

The result obtained in this study suggests that the selected plants leaves are source of many phytochemical compounds and bioactive constituents and these plants are proving to be an increasingly valuable reservoir of bioactive compounds of substantial medicinal merit. They are highly recommended for further quantitative analysis of phytochemicals present and their potentiality as therapeutic agent.

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In vitro Antifungal Activity of Plant Extracts against Pathogens of Clinical and Agricultural Importance and Phytochemical Analysis of the Active Compounds

Dipanwita Saha, Anindita Chakraborty, Ramashish Kumar, Bikramjit Raychaudhury

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Abstract This study was carried out with an objective to investigate the antifungal potentials of 14 plants known for medicinal properties against the human pathogen, *Candida albicans* and the tea pathogen, *L. theobromae.* Soxhlet extracts prepared from leaves in various organic solvents were first screened for their antifungal activity by agar diffusion method. While all tested plants showed antifungal activity, evident from distinct inhibition zones on PDA plates, extracts from *Clausena excavata, Ocimum sanctum, Piper betle, Polyalthia longifolia* and *Xanthium strumarium* exhibited higher activities and were selected for phytochemical analysis. *L. theobromae* was found to be more susceptible than *C. albicans.* Bioautography with the test pathogens revealed the presence of antifungal compounds which appeared as clear zones of inhibition against fungus growth on developed TLC plates. *X. strumarium* and *C. excavata* showed two antifungal zones each while each of the other 3 extracts produced single antifungal zone. Application of spray reagents on TLC revealed the chemical nature of all active compounds. Both compounds from *X. strumarium* were sesquiterpenes and those from *C. excavata* were furano-coumarins. Bioactive compounds from *O. sanctum*, *P. longifolia* and *P. betle* were found to be monoterpene, diterpene and phenolic respectively. The results form the basis for further characterization and development of newer fungicidal compounds.

Keywords Antifungal, Bioautography, *Clausena excavata, X. strumarium, P. betle.*

Dipanwita Saha*, Anindita Chakraborty, Ramashish Kumar
Department of Biotechnology, University of North Bengal, Siliguri 734013, India

Bikramjit Raychaudhury
Department of Physiology, Ananda Chandra College, Jalpaiguri, WB, India
e-mail : dsahanbu@yahoo.com
*Corresponding author

Introduction

Plant extracts has been traditionally used in India for treating various ailments and forms part of the cultural practice of this country. Scientific reports indicate that plants are an immense reservoir of valuable source of molecules with strong antimicrobial potential and considered as the prime source for the discovery of novel drugs. Different types of compounds produced by plants, particularly the secondary metabolites, may possess antifungal activity and some have successfully been utilized for clinical application (Atanasov

et al. 2015). In addition,many such antifungal compounds are now used in agriculture because synthetic chemical fungicides are unpopular for their residual toxicity, non targeted environmental impacts and direct or indirect effects on animal health system (Saha et al. 2012). Plant products are safer and eco-friendly in comparison to synthetic fungicides, as they have no deleterious effect on environment and on non target organisms in nature.

In recent years, *Candida* infections pose a serious threat to immunocompromised patients, especially those tested HIV positive and those who are receiving immunosuppressive drugs (Sanglard 2016, Campoy and Adrio 2017). Fungal infections lead to 1.5 to 2 million deaths per year, which is higher than either malaria or tuberculosis related deaths (Denning and Bromley 2015). Moreover, increasing resistances towards synthetic antifungal drugs are being reported (Cowen et al. 2014). *Lasiodiplodia theobromae*, a pathogen causing several crop diseases including diplodia disease in tea has also exhibited resistance against the fungicide thiopahanate methyl (Al-Jabri et al. 2017). Since tea is an important contributor to the economy of North East India, it is essential to look for effective alternatives for controlling diseases in tea plants.

The present study reports the antifungal activity of 14 plant extracts against the human pathogen, *Candida albicans* and the tea pathogen, *L.theobromae.* The chemical nature of the antifungal compounds present in the botanical extracts was determined following separation by thin layer chromatography and bioautography.

Materials and Methods

Selection and collection of plant materials

The plant materials were selected on the basis of literature reports of their traditional ethnomedicinal uses and bioactivity as well as availability in sub-Himalayan region of West Bengal. Altogether 14 plant

Table 1. List of plants used in this study.

Name of plants	Local/English name	Family	Traditional application and biological activity
Bidens pilosa Linn.	Spanish needle	Asteraceae	Leaf extract used for treatment of cough, laryngitis, headache, conjunctivitis, rheumatism, infection, digestive and stomach disorder including peptic ulcer
Clausena excavata Burm. f.	Agnijal	Rutaceae	Used in cold, malaria, abdominal pain, snake-bite, preliminary stage of AIDS and dermatopathy
Datura stramonium L.	Datura	Solanaceae	Smoke of leaves is used for asthma, causes sleepiness. Roots are good for tooth-ache
Datura innoxia Mill.	Safed dhatura, Indian-apple	Solanaceae	Leaves are used as repellent and vermicide, used in asthma, wound, malaria and Leishmaniasis. Seed are grind and cooked in mustard oil to cure scabies
Emblica officinialis Gaertn.	Amla	Phyllanthaceae	Fruits used to promote longevity, enhance digestion, strengthen heart, purify blood, stimulate hair growth and enhance intellect. Used against constipation, fever and cough
Eucalyptus globules Labill.	Eucalyptus	Myrtaceae	Leaf essential oil used as antiseptic, against coughs and colds, sore throats and other infections. Used as mouthwash toothpaste. Leaf extracts used in anti-bacterial, antioxidant and anti-inflammation deodorant
Lantana camara L. var aculeate Moldenke	*Raimuniya,* Guye genda	Verbenaceae	Used in bronchitis, stomach problems, rheumatism and to clean teeth
Leonurus sibiricus L.	Guma & Raktadron	Lamiaceae	Anti-inflammatory and anti-diarrhoea. Leaf extract used in hemorrhage, weakness
Measa indica (Roxb.) A. DC.	Ramjani	Primulaceae	Leaves used as an agent for clearing the throat/vocal cord for producing a melodious sound

Table 1. Continued.

Name of plants	Local/English name	Family	Traditional application and biological activity
Ocimum sanctum L.	Tulsi	Lamiaceae	Leaf juice with honey is given for 3-7 days for cough and cold. 1 : 1 ratio of tulsi leaf and neem leaf paste is very effective for diabetes
Polyalthia longifolia var *pendula*	Ashok tree	Annonaceae	Powder of stem bark mixed with curd and sugar and mixture is given orally thrice a day to cure diarrhoea. Stem bark is dried, powdered and given orally in the treatment of gout
Piper betle L.	Paan	Piperaceae	Leaf paste with *Acacia catchu* bark paste massaged on the skin of children in maggots. Herbal dye, antipyretic, antioxidant, anticancer, antiulcer, anti-inflammatory, pain reliever and immunomodulating
Syzygium cumini (L.) Skeels	*Jamun*	Myrtaceae	Hypoglycaemic, diuretics, analgesic, anti-inflammatory, antiplaque, antimicrobial antidiarrhoel, antioxidant and gastroprotective
Xanthium strumarium L.	Chotagokhru	Asteraceae	Dry fruits kept on dried stem of *Calotropis procera* are burnt and the smoke is inhaled

species were studied for their biological activity and phytochemical analysis. Table 1 shows a list of the plants along with their local names, families and traditional uses (Rastogi and Mehrotra 1995, Chatterjee and Pakrashi 1997). Most of the plant materials were coolected from local areas within and outside campus of University of North Bengal. Some of the plants were collected from forest areas of Sukna located in the Terai region of the Eastern Himalayas. Fresh disease free leaves of different plant species were collected. Voucher specimen of each species was deposited in the herbarium of Department of Botany, University of North Bengal.

Fungal pathogens

Two pathogens of clinical and agricultural importance were used in this study. A virulent strain of *Lasiodiplodia theobromae* (ITCC 5446.02) was earlier isolated in the laboratory from young tea plants showing diplodia disease from a nursery in the Darjeeling district of West Bengal and its identity was authenticated by IARI, New Delhi. *Candida albicans* (MTCC 183) which is an opportunistic human pathogen causes candidiasis especially in immunocompromised patients. This strain was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Soxhlet extraction

Fresh leaves of fourteen different plant species (Table 1) were thoroughly washed with distilled water and shade dried at room temperature for 5 to 10 days. The dried leaves (120 g) of each plant were ground to moderately fine powder (1mm) and extracted in a soxhlet apparatus using appropriate solvents (Table 2), for fifteen h at 35—45°C. The extract was concentrated to dryness under vacuum in a rotary evaporator (Eyela, Japan). The residue (15 g) obtained as a gummy solid mass was dissolved in proper solvent at 3 different concentrations (100 mg/ml, 10 mg/ml and 1 mg/ml) and used for further studies.

Agar cup bioassay

The plant extracts were screened for antifungal activity by agar cup diffusion method. Potato dextrose agar (PDA) medium was autoclaved at121°C for 15 min, cooled to 45 °C and 1ml of pure cell suspension or spore suspension (10^6 /ml) or of the test pathogen was mixed with 19 ml of molten medium and poured into sterile petriplates of 9 cm diameter. Agar cups were prepared with sterile cork-borer (4 mm diameter) in the PDA plates after solidification of the medium seeded with spores of the test fungi. Plant extracts (50 µl) were introduced into each well and the plates were incubated for 48-72 h at 28°C. The antifungal activity

Table 2. Screening of antifungal potential of crude soxhlet extracts of leaves from different plant species against *Candida albicans* and *Lasiodiplodia theobromae* by agar diffusion assay.* The inhibition zone includes the diameter of the agar cup (6 mm).

Name of plants	Solvent for extraction	Diameter of inhibition zone (mm) obtained at different concentrations of leaf extracts (mg/ml)* *C. albicans* 100	*C. albicans* 10	*C. albicans* 1	*L. theobromae* 100	*L. theobromae* 10	*L. theobromae* 1
Bidens pilosa	Ethyl acetate	21.3 ± 3.0	15.0 ± 1.7	9.3 ± 1.5	25.0 ± 2.6	16.6 ± 2.1	11.3 ± 2.5
Clausena excavata	Ethyl acetate	24.3 ± 3.5	17.3 ± 2.5	7.6 ± 0.6	34.6 ± 3.2	22.3 ± 3.0	14.3 ± 2.1
Datura stramonium	Benzene	17.0 ± 4.5	10.6 ± 1.2	0	26.3 ± 2.5	15.0 ± 2.6	0
Datura innoxia	Ethanol	18.6 ± 3.8	12.0 ± 2.6	8.3 ± 0.6	28.6 ± 3.2	18.3 ± 2.3	8.6 ± 1.1
Emblica officinalis	Ethyl acetate	13.3 ± 2.1	7.6 ± 0.5	0	17.6 ± 3.8	13.3 ± 2.1	0
Eucalyptus globulus	Ethanol	18.3 ± 1.5	9.3 ± 2.1	0	21.6 ± 3.2	14.6 ± 2.1	9.0 ± 1.0
Lantana camara	Ethanol	11.6 ± 1.1	0	0	15.6 ± 2.5	10.6 ± 1.5	0
Leonurus sibiricus	Ethanol	15.6 ± 2.1	10.6 ± 0.6	0	20.3 ± 2.3	14.0 ± 3.6	7.3 ± 0.6
Maesa indica	Dichloromethane	18.6 ± 2.9	14.3 ± 3.0	8.3 ± 0.6	25.6 ± 2.5	15.6 ± 2.5	10.3 ± 2.1
Ocimum sanctum	Ethyl acetate	22.0 ± 2.6	13.6 ± 2.1	9.6 ± 1.5	26.3 ± 1.5	18.3 ± 4.6	11.6 ± 3.0
Polyalthia longifolia	Ethyl acetate	31.3 ± 2.5	16.3 ± 2.3	11.0 ± 2.6	28.3 ± 2.3	23.3 ± 3.2	15.6 ± 3.0
Piper betle	Dichloromethane	29.6 ± 3.2	17.3 ± 1.5	12.6 ± 1.5	36.3 ± 2.9	24.6 ± 4.0	14.3 ± 1.5
Syzygium cumini	Ethyl acetate	15.3 ± 3.0	9.6 ± 1.5	0	22.3 ± 3.8	13.6 ± 0.6	11.0 ± 1.0
Xanthium strumarium	Benzene	24.6 ± 3.0	18.3 ± 2.1	9.6 ± 1.5	35.3 ± 3.5	24.3 ± 2.5	15.6 ± 3.0

was evaluated by measuring zones of inhibition of fungal growth around the plant extracts. Complete antifungal assay was carried out under strict aseptic conditions. The zones of inhibition were measured in mm and the experiment was carried out in triplicate. The average of 3 replications and standard deviation were computed by MS Excel 2007.

Thin layer chromatography

Thin layer chromatography was used both for detection of antifungal compounds and their phytochemical analysis. Antifungal activities were tested by bioautography following the method of Kumar et al. (2012). Phytochemical analysis was performed using ultraviolet light and different spray reagents (Wagner and Bladt 1996). Precoated silica gel 60 F254 aluminium TLC plate (Merck, India) was activated by heating at 70°C for 45 minutes prior to sample-loading. Each concentrated extract (10 mg/ml) was loaded on the activated TLC plate at 2 different spots (20 µl each) 2 cm apart and developed either in hexane : ethyl acetate : methanol (60 : 40 : l v/v) or in hexane : ethyl acetate (70 : 30 or 80 : 20 v/v) as solvent. The plates were air-dried until the solvent evaporated completely and subsequently cut symmetrically into 2 parts to separate the 2 developed chromatograms. These were used independently for phytochemical analysis and bioautography and the results were compared in order to determine the nature of antifungal compound.

Bioautography

For bioautography with *L. theobromae*, spore suspension (10^6 spores/ml) was prepared from 7 d old culture in Richard's medium and sprayed with an atomizer on dried TLC plates (Kumar et al. 2012). The plates were incubated in a humid chamber at 28°C for 2-5 days. For *C. albicans* developed chromatograms were placed in sterile petri plates. Molten PDA medium was mixed with phenol red (0.02%) and an inoculum of cell suspension of *Candida albicans,* at tolerable temperature. The mixture was poured evenly over developed TLC plates, covered with lid and incubated at 28°C for 24 h. Inhibition zones, which appeared as clear spots on a background of fungal growth, indicated the presence of antifungal compounds. R_f values of the inhibition zones and the zone diameters swere noted.

Phytochemical analysis

For phytochemical analysis, the developed chromatogram was viewed under UV light (254 and 365 nm) and sprayed with vanillin-sulfuric acid, anisaldehyde-sulfuric acid or Folin ciocalteu's reagent. The color of the developed spots, if any, was noted and

Table 3. Antifungal activity of crude soxhlet extracts of leaves from potential plant species against *Candida albicans* and *Lasiodiplodia theobromae* assessed by bioauto graphy technique and phytochemical detection of antifungal compounds.

Plants	No. of zones	R_f	Antifungal activity Zone of inhibition (mm) *C. albicans*	*L. theobromae*	Chemical nature of active compound
C. excavata	2	0.39	18	25	Furano-coumarin
		0.30	15	22	Furano-coumarin
O. sanctum	1	0.85	10	15	Monoterpene
P. betle	1	0.64	30	38	Phenolic
P. longifolia	1	0.73	15	27	Diterpene
X. strumarium	2	0.56	32	38	Sesquiterpene
		0.25	24	32	Sesquiterpene

the R_f was matched with that of the active compound. The result of chemical analysis was determined based on color of the spots (Wagner and Bladt 1996, Harborne 2005).

Results and Discussion

Screening of botanicals for antifungal activity

Agar cup bioassay showed that all 14 plants were active against both the tested fungal pathogens (Table 2). But overall *L. theobromae* was found to be more susceptible than *C. albicans*. *Piper betle* and *Xanthium struaium* was found to be the most active plants as it produced largest inhibition zones of 36.3 mm and 35.3 mm respectively at 100 mg/ml against *L. theobromae.* Other plants showing strong antifungal activity were *Polyalthia longifolia, Clausena excavata* and *Ocimum sanctum*. Besides *Datura innoxia* and *D. stramonium* also showed good antifungal activity and 100mg/ml which however reduced greatly at 1 mg/ml. *Emblica officinalis* and *Lantana camara* were less effective. Reports of antifungal activity of *Maesa indica* and *Leonurus sibiricus* is extremely rare (Yashoda et al. 2014). Considering the overall performances of the plant species, 5 plants, viz. *P. betle, O.sanctum, P.longifolia, C. excavata* and *X. strumarium* were selected for further phytochemical analysis.

Activity monitoring and phytochemical analysis of active compounds

Bioautography of the concentrated crude extracts prepared from *C.excavata, X. strumarium,P. longifolia, P.betle* and *O. sanctum* revealed antifungal activity against *C. albicans* and *L. theobromae* (Table 3). The occurrence of antifungal components was evident by the presence of clear zones of inhibition on TLC plates. *X. strumarium* and *C. excavata* showed 2 antifungal zones each while the other 3 plant extracts produced single antifungal zone each. The largest inhibition zone of 38 mm was produced by *X.strumarium* (R_f 0.56) and *P. betle* (R_f 0.64) extracts against *L.theobromae. X. strumarium* showed 2 antifungal compounds both of which produced green fluorescence under UV_{254}. The compounds produced reddish brown spots when sprayed with anisaldehyde-sulfuric acid and brown spots with vanillin-sulfuric acid which indicated presence of sesquiterpene derivatives. *X. strumarium* has been reported to contain an antimicrobial sesquiterpine lactone named xanthatin (Saha et al.2012). Another sesquiterpine lactone, deacetyl xanthumin, has been reported but this also structurally resembles xanthatin (Kim et al. 2002). Occurrence of a second antifungal compound in our extract indicates that this plant contains yet undetected bioactive compounds which warrants further study.

The single antifungal compound of *P. betel* produced green fluorescence under UV_{254} and deep blue and brown spots when sprayed with Folin ciocaltu's and vanillin-sulfuric acid respectively indicating phenolic compound. Row and Ho (2009) studied the chemical compositions of the crude oil by GC/MS analysis and identified 36 compounds including eugenol (36.2%) and chavibetol acetate (16.9%) both of which are antifungal phenolic compounds or their derivatives (Kumar et al. 2010b). In this study, only one large antifungal zone was observed possibly because the compounds failed to separate under the current experimental conditions.

P. longifolia (27 mm) and *C. excavata* (25 mm) also exhibited large inhibition zones against *L. theobromae*, however, zone diameters produced by *O. sanctum* (15 mm) were much lower than other

extracts. The two antifungal compounds from *C. excavata* showed intense quenching under UV_{254} but fluoresced with a bright blue color under UV_{365}. Both produced blackish blue spot upon spraying with anisaldehyde-sulfuric acid indicating presence of furano-coumarins. Literature review has revealed that root of *C. excavata* have diverse group of coumarins with broad range of biological activities (Wang et al. 2008). However, till date, very few studies have been done concerning the antifungal activity of phytochemicals of *C. excavata* leaves. We earlier reported a new compound excavarin A (Kumar et al. 2012) from dichloromethane extract. Characterization of this second compound with antifungal activity in the ethyl acetate extract is in progress.

The antifungal compound from *P.longifolia* appeared as a single blue spot on TLC plates upon spraying with anisaldehyde reagent and heating at 110°C which indicated the presence of diterpene. The occurrence of clerodane and halimane diterpenes in *P. longifolia* has been reported by several authors (Katkar et al. 2010, Bhattacharya et al. 2015). The antifungal compound from *O. sanctum* produced brown spots when sprayed with vanillin sulfuric acid indicating monoterpene derivative. Essential oils from *O.sanctum* leaves have been found to posses antifungal properties (Kumar et al. 2010 a). Eugenol, which is also a monoterpene has been reported to be the major compound (43%) in essential oil extract by GC-MS analysis (Devendran and Balasubramanian 2011). Thus it is most probable that the antifungal compound detected in this study is eugenol, but further studies are necessary to ascertain this.

Conclusion

The studies showed that extracts from several plants, especially, *X. strumarium* and *P. betle* has strong antifungal activity against *L. theobromae* and *C. albicans.* The phytochemical analysis coupled with bioautography revealed occurrences of several types of antifungal compounds which may form basis for further characterization and development of newer fungicidal compounds.

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IDENTIFICATION AND PURIFICATION OF FE CONTAINING SUPEROXIDE DISMUTASE FROM LEISHMANIA DONOVANI

Bikramjit Raychaudhury*[1], R. Jyoti[1], Kakuli Chakraborty[1], Anindita Chakraborty[2], Moushree Palroy[1] and Rajen Haldar[3]

[1]Department of Physiology, Ananda Chandra College, Jalpaiguri 735101.
[2]Department of Physiology, ABN Seal College, Coochbehar 736101.
[3]Department of Physiology, University Colleges of Science and Technology, University of Calcutta, Kolkata, India.

***Corresponding Author: Bikramjit Raychaudhury**

Department of Physiology, Ananda Chandra College, Jalpaiguri 735101.

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ABSTRACT

Leishmaniasis is a parasitic disease which infects as many as 400,000 people per year. Because the infective agent- a protozoan-inhabits phagolysosomes in host macrophages, the parasites are partially protected from chemotherapeutic agents. Thus, treated patients often relapse or experience toxic reactions to the drugs. In order to develop new leishmanicidal agents, studies have been conducted to understand the interactions of the parasite with the macrophage. Ordinarily, microorganisms which are taken up by macrophages are destroyed by oxygen-dependent and oxygen-independent antimicrobial systems. The oxygen-dependent antimicrobial activity of macrophages is dependent on the generation of superoxide (O_2^-) by the one-electron reduction of molecular oxygen. This O_2^- can then undergo a series of reactions to produce hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and perhaps, singlet oxygen ($^1O_2$), which may be responsible for destroying the ingested microorganism. Some microorganisms, such as Toxoplasma gondii, block the generation of superoxide by macrophages in order to survive phagocytosis. It is found that this was not the case for Leishmania. The uptake of Leishmania tropica promastigotes by mouse peritoneal macrophages was accompanied by the generation of high levels of superoxide and other activated forms of oxygen. The possibility that parasites might survive phagocytosis by efficient enzymatic decomposition of H_2O_2 and O_2^- has also been investigated. The parasites were found to lack or contain low levels of enzymes capable of decomposing H_2O_2 (glutathione peroxidase and catalase) but to contain relatively high levels of superoxide dismutase, which degrades O_2^-. Evidence was obtained that the leishmanial superoxide dismutase was substantially different from the superoxide dismutase found in its host and therefore made a potential target for the design of parasite-specific enzyme inhibitors. The leishmanial superoxide dismutase activity was found to be insensitive to inhibition by cyanide, but sensitive to inhibition by azide and peroxide, properties which suggest an Fe containing superoxide dismutase. In contrast, the mammalian host has only Cu/Zn-containing and Mn-containing superoxide dismutases. In order to further characterize the leishmanial superoxide dismutase, we have isolated superoxide dismutase from *Leishmania donovani*.

KEYWORDS: Leishmania donovani, Superoxide dismutase, Antioxidant.

INTRODUCTION

The superoxide radical is an intermediate reduction product of oxygen produced by a variety of biological reactions. The superoxide radical (O_2) and other reactive derivatives have received recent attention as agents of oxygen toxicity in cells. Most organisms, therefore, have defense systems, such as metallo-enzymes, to protect themselves from toxic oxygen species. Metallo-enzymes that catalyze the disproportionation of superoxide free radicals (O_2) to hydrogen peroxide (H_2O_2) and oxygen (O_2), are known as superoxide dismutases (SODs). SODs play an important role in the protection of cells from the oxidative damage of superoxide radicals. Cell damage may also be due to the superoxide itself or, indirectly, even more reactive oxygen species, such as hydroxyl radicals ($^{\cdot}OH$), formation of which, via the Fenton reaction, is favoured by excess superoxide[1] SODs have subunit molecular weights ranging from 16 kDa to 26 kDa and are divided into three classes on the basis of their active site metals: copper and zinc (Cu/Zn-SOD), manganese (Mn-SOD), and iron (Fe-SOD). Cu/Zn-SOD is found widely in the cytoplasm and certainly in the mitochondrial inter-membrane space of the eukaryotic cells and chloroplasts of plants. Mn-SOD is located in prokaryotes and in the mitochondria of eukaryotes[2] while Fe-SOD has been found in bacteria, blue-green algae and protozoa[3] Recent reports also indicated that the enzyme was present in higher land plants[4] In addition, Ni-SOD has been isolated from some microorganisms.[5]

NADPH oxidase, also referred to as phox, is expressed in professional phagocytes such as neutrophils and macrophages. It catalyzes the production of large amounts of $O_2^{\cdot-}$ (~10 mM inside the phagosome) upon activation. The neutrophil is particularly efficient and produces levels 2-3 times higher than those of macrophages. The important role of phox to the immune defense in exemplified by the fact that a defective enzyme results in chronic granulomatous disease (CGD)[6]. People with CGD have a genetic deficiency of phox components and suffers from recurrent infections. There are also other cellular sources of O_2, including xanthine oxidoreductase and homologues of the gp91phox subunit of phox. In addition, the mitochondria and certain metabolic pathways generate O_2.

Superoxide dismutases (SODs) represent a family of metalloenzymes, found in organisms ranging from bacteria to humans.[7] SODs exist in several different isoforms that differ in their structure and prosthetic ion/ions but all isoforms catalyze the dismutation of O_2^- to hydrogen peroxide (H_2O_2) and oxygen (O_2). Iron-SOD (Fe-SOD) and Manganese-SOD (Mn-SOD) are located in the cytoplasm of prokaryotes whereas Cu/Zn-SOD is located in the periplasm of gram-negative bacteria, anchored to the outer membrane, or secreted[8] Thus, Cu/Zn-SOD in bacteria has the potential to protect against reactive oxygen species (ROS) generated from external sources. Accordingly, it has also been shown to be a virulence mechanism of many bacteria. The amino acid sequences of Cu/Zn-SOD from different bacterial species show extensive variation[9] and is predicted to affect the architecture of the active site channel and subunit assembly and, hence, enzyme activity. Thus, the SOD activity may vary substantially between bacterial species.

MATERIALS AND METHODS

Reagents

Brain heart infusion broth was obtained from Acumedia Manufactures, Baltimore, Maryland, USA. Bactoagar was procured from DIFCO laboratories, Detroit, Michigan, USA. The culture media Medium 199, fetal bovine serum, penicillin-streptomycine, HEPES buffer and L-glutamine were purchased from GIBCO Laboratories, Grand Island, New York, USA. Alkaline phosphatase conjugated anti-rabbit and mice IgG (whole molecule) were from Sigma Immunochemicals. TRITC and FITC coupled anti rabbit and mice IgG were obtained from Bangalore Genei, India. Other reagents were of highest purity available from Sigma Chemical Company, St. Louis, Missouri, USA.

DEAE-52 was purchased from Whatman, and Sephadex G-75 from Amersham Pharmacia Biotech Corporation (Sweden). Molecular weight markers for gel electrophoresis were obtained Amersham Pharmacia Biotech Corporation (Sweden). The other chemicals were analytical reagents.

Parasite

Leishmania donovani strain MHOM/IN/AG/83 was obtained from kala-azar patient[10] and maintained by intracardial passage in every 8 weeks in Syrian golden hamster. Promastigotes were obtained by transforming amastigotes isolated from infected spleen[11] and maintained in medium -199 supplemented with 10% fetal calf serum *in vitro*. The strain was also maintained at 22° C in modified Rays medium.[12]

Purification of Fe-SOD from *Leishmania donovani*

Preparation of cell lysate

A total of 25 gm Leishmania cells were taken at this stage for purification of Fe-SOD. Cells were dissolved in x 2 volume ice-cold buffer A containing 50 mM potassium phosphate, 10mM TES, 1mM EDTA, 0.5 mM phenylmethylsulfonyl (in 0.1% ethanol) fluoride and 0.25 mM leupeptin at pH 7.6 and were sonicated by giving 5-6 strokes of 20 sec in an MSE sonicator at 4°C on a setting of 6 and centrifuged at 100,000g for 60 min in a Sorvall centrifuge. The supernatant cell free extract obtained was then used in subsequent steps of purification. .

$(NH4)_2SO_4$ precipitation

The cell-free extract was treated with $(NH4)_2SO_4$ in three steps. First, solid $(NH4)_2SO_4$ was added to the extract to 30% saturation at 4ºC, then the mixture was stirred for 15 min at 4ºC, and left at 4ºC for 60 min. The precipitate was removed by centrifugation at 9,000 rpm for 10 minutes. In the second step, the supernatant was treated with solid $(NH4)_2SO_4$ to 60% saturation at 4ºC, stirred for 15 min at 4ºC, and left at 4ºC for 60 min. The precipitate was removed by centrifugation at 9,000 rpm for 10 minutes. In the third step, the supernatant was treated with solid $(NH4)_2SO_4$ to 90% saturation at 4ºC, stirred for 15 min at 4ºC, and left at 4ºC for 60 min. The precipitate with SOD activity was centrifuged at 9000 rpm for 10 min (Hettich, Universal 30RF centrifuge) and then dissolved in a minimal volume of 20 mM potassium phosphate (pH 7.6 buffer B) and dialyzed overnight at 4ºC against the same buffer. The SOD solution was concentrated to 0.5 ml by a Amicon PM – 30 (30,000-molecular weight cut off).

Ion-exchange Chromatography

The concentrated enzyme solution was loaded on to a DEAE-52 column (2.6 cm x 60 cm) and equilibrated with buffer B for 5 h at 0.25 ml / min. The column was eluted with a 0–500 mM NaCl linear gradient and fractions of 3 ml were collected. The SOD active fractions were pooled, concentrated by Amicon PM–30 (30,000-molecular weight cut off) at 4ºC to 10 ml and dialyzed against 10 mM phosphate buffer (buffer C, pH 7.6) for 24 h (4ºC).

Gel Chromatography

The concentrated enzyme solution was applied on to a Sephadex G-75 column (2.6 cm x 100 cm), equilibrated with PBS at pH 7.2. The column was eluted with the

same buffer at a flow rate of 0.25 ml/min. The fraction with SOD activity was eluted with 55 ml PBS and dialyzed against 0.01M phosphate buffer pH 7.2 over night and concentrated with Amicon PM–30 (30,000-molecular weight cut off) and stored at -20ºC until used.

SOD activity assay

Superoxide dismutase activity was assayed after each purification step by measuring the inhibition of pyrogallol autoxidation rate.[13] The assay mixture contained 0.2mM pyrogallol in air equilibrated 50mM tris–cacodylic acid buffer, pH 8.2, and 1mM diethylene triamine penta acetic acid. The rate of autoxidation was obtained by monitoring the increase in absorbance at 420 nm in a Hitachi spectrophotometer, No U2000. SOD has the ability to inhibit the autoxidation and the extent of inhibition is taken as the measure of enzymic activity.

Molecular Weight Determination

The determination of the molecular weight by gel filtration was carried out on Sephadex G-200 column equilibrated with 0.05 M phosphate buffer pH 7.2, and calibrated with the following molecular weight standards: immunoglobulin G (160 000 D), human serum albumin (67 000 D), ß-lactoglobulin (35 000 D), cytochrome c (12 400 D), vitamin B12 (1355 D), cytidine (246 D).

Protein Estimation

Proteins were determined by the method of Bradford,[14] using bovine serum albumin as the standard.

Activity staining for SOD

Purified SOD was separated on a 10% non-denaturing polyacrylamide gel[15] for activity staining[16] Solution A was prepared by dissolving 10 mg nitroblue tetrazolium and 4 mg riboflavin in 10 ml glass distilled water. Solution B contained 600 µl TEMED in 10 ml glass distilled water. At the end of electrophoresis, gels were first incubated in Solution A for 20 min and then in Solution B for another 20 min to finally illuminate till the appearance of white bands against a blue background was observed.

This assay depends on the ability of SOD to inhibit the reduction of nitroblue tetrazolium to blue coloured formazan by O_2^- generated by reoxidation of photochemically reduced riboflavin. Thus regions containing active SOD appear colourless against a blue background. The band intensity is roughly proportional to the amount of SOD protein.[16]

SDS-Polyacrylamide gel electrophoresis

SDS-PAGE of different fractions was done as described by Laemmli.[15]

RESULTS AND DISCUSSION

Purification of SOD

After each purification step, the protein content and the enzyme activity were determined. According to our results, ammonium sulphate precipitation to 30% and 60% saturation eliminated 50% of the blast protein, without loss in SOD activity and the third precipitation step at 90% ammonium sulfate saturation resulted in a SOD preparation of higher specific activity.

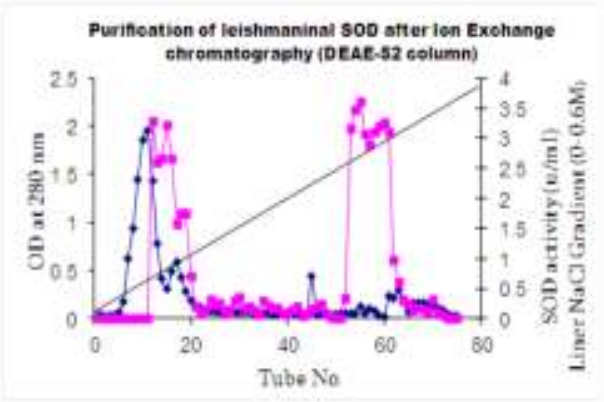

Fig. 1: Ion-exchange chromatography on a DEAE-52 column; protein concentration (-▲-), superoxide dismutase activity (–●–), concentration of NaCl (—).

After that, ion-exchange chromatography on DEAE-52 column with a concentration gradient of NaCl (0–0.6 M) was performed which resulted in elimination of 90% of the blast protein with a loss of SOD activity of about 57 %. Results of this chromatographic procedure are shown in Fig. 1.

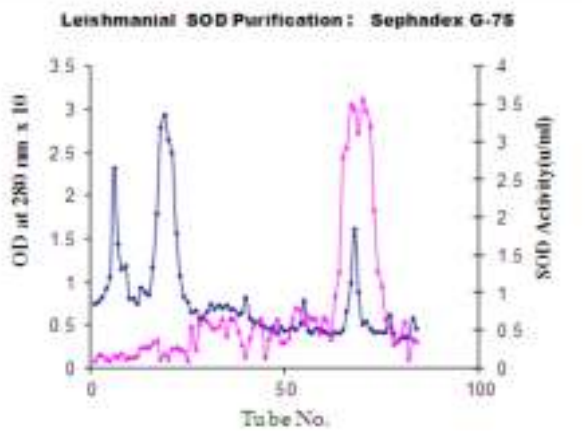

Fig. 2: Gel chromatography on a Sephadex G-75 column; protein concentration (-▲-), superoxide dismutase activity (–●–).

In Ion-exchange chromatography, the 28-44 eluted fraction detected at 280 nm presented an SOD activity and corresponds therefore to the SOD fractions. [Column Volume: 88 ml, Sample charged: 2 ml, Protein content of the sample: 0.47mg/ml, Active Fraction: Tube No. 65 to 72, Flow Rate: 1ml/min, Vol. of each tube: 2 ml].

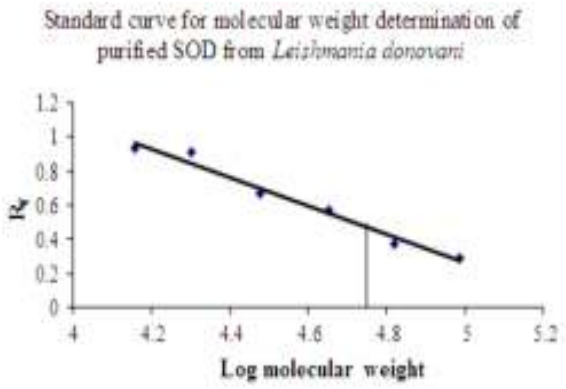

Fig 3: Plot for determination of molecular weight of leishmanial SOD.

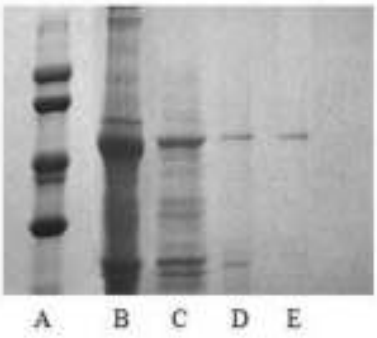

Figure 4: SDS PAGE : A–Protein markers, B–Leishmania cell-free extract, C–Pellet after ammonium sulfate precipitation at 90%saturation, D–Fractions with SOD activity after ion-exchange chromatography, E-Fractions with SOD activity after gel chromatography

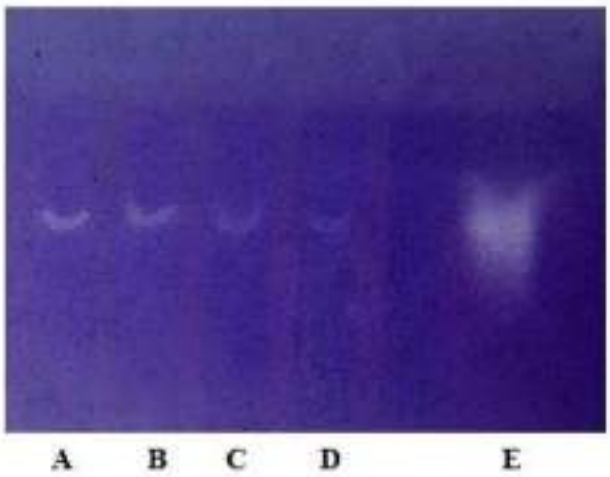

Fig 5: Activity staining of leishmanial SOD after separating by native PAGE in a 10% gel. 60 micrograms of protein was loaded in each lane. A-Leishmania cell-free extract, B-Pellet after ammonium sulfate precipitation at 90%saturation, C-Fractions with SOD activity after ion-exchange chromatography, D-Fractions with SOD activity after gel chromatography, E-Pure Fe SOD.

The SOD was then eluted from a Sephadex G-75 column. Figure 2 presents the results of the gel chromatography on Sephadex G-75 column. The pooled fraction had a volume of 20 ml and was concentrated to a 1.5 ml volume. The SOD fraction showed only one band of SDS-PAGE after gel chromatography. It can be concluded that the SOD was purified to homogeneity after three-step ammonium sulphate precipitation, ion exchange chromatography and gel chromatography. The final sample was applied on SDS-PAGE to determine its molecular weight (Fig 3). The molecular weight of SOD by SDS-PAGE was 37200 dalton. Figure 4 shows the electrophoretic pattern of SOD obtained during different steps of SOD purification. The total purification achieved by the procedure was 105 fold over the first soluble extract, with a yield of 15%. The specific activity of the purified enzyme was 9191 U/mg. Figure 5 shows the activity staining of leishmanial SOD after separating by native PAGE in a 10% gel. Activity staining of native polyacrylamide gels clearly showed the SOD activity obtained during different steps of SOD purification. SOD catalyzes dismutation of toxic superoxide radicals.[17] It is one of the key enzymes of oxygen defence system is known to be an essential factor in mediating normal cellular functions.[18] As a result, the enzyme has been found to be targeted for the treatment of several diseases.[19-21] SOD also plays a vital role during host–parasite interaction.

Its activity is elevated when Leishmania parasite infects host cells.[22] In a recent report, SOD has been demonstrated to be a key enzyme to play a vital role in the survival of intracellular parasites.[23] Importance of this enzyme in the host–parasite interaction was established by generating SOD-deficient *Leishmania donovani*. An earlier report suggested that the enzyme that was present in leishmanial glycosomes isolated by the classical sucrose gradient technique was of the Cu/Zn type.[16] In this present work, SOD was purified from *Leishmania donovani* promastigotes. The total purification achieved by the procedure was 105 fold over the first soluble extract, with a yield of 15%. The specific activity of the purified enzyme was 9191 U/mg. The molecular weight was estimated as 37200 dalton. SOD activity was observed in crude extracts of *Leishmania donovani* cells grown anaerobically. This finding indicates that these enzymes are being constitutively expressed during the growth of this organism in the absence of oxygen. Results elucidated the important roles of Fe-SOD in the cellular stress responses and anti-oxidative processes of the parasite *Leishmania donovani*. Future studies will be necessary to investigate functions of more antioxidant enzymes to gain a better understanding of the antioxidant mechanism in the Leishmania species under various stresses.

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