STUDIES ON THE ANTIOXIDATIVE AND OTHER BENEFICIAL PROPERTIES OF SOME PLANTS FROM WETLANDS OF NORTH BENGAL

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Submitted by

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DECLARATION

I solemnly declare that the thesis entitled "STUDIES ON THE ANTIOXIDATIVE AND OTHER BENEFICIAL PROPERTIES OF SOME PLANTS FROM WETLANDS OF NORTH BENGAL" has been prepared by me under the guidance of Professor Usha Chakraborty, Plant Biochemistry Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

This is to certify that Mr. Jayanta Choudhury has prepared the thesis entitled "STUDIES ON THE ANTIOXIDATIVE AND OTHER BENEFICIAL PROPERTIES OF SOME PLANTS FROM WETLANDS OF NORTH BENGAL"on the basis of his work carried out under my supervision at Plant Biochemistry Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

The change in the life style of human population during the last few decades has given rise of several harmful diseases which are mainly the result of free radicals. Free radicals are generated due to oxidative biochemical processes that are essential for existence of living cell. These free radicals are responsible for diseases like cancer, diabetes, atherosclerosis, arthritis, Alzheimer's disease, other neurodegenerative disorder etc.

The plants are known for their therapeutic nature from ancient past to the wise peoples of our country. During the development of our culture and civilization plants always were given supreme priority as well as respect for their utility in our ancient medical science Ayurveda. For the last few years lots of work has been done on the antioxidative and antidiabetic activity of different plants from India and abroad. Due to its wide and extremely diverse geographical distribution India is rich in plant diversity that can be explored for development of natural therapeutics to treat life style diseases.

Northern part, demarcated by the river Ganges, of the state West Bengal is known as North Bengal and is recognized for its richness in biodiversity. This part of land is also dominated by the presence of several wetlands which are rich for their biodiversity. Considering the importance of natural products and its application in pharmaceuticals the present study has been undertaken to document the available wetland plants with ethnobotanical uses from six districts of West Bengal. Furthermore, biological activities like antioxidant, antimicrobial and antidiabetic and anticancer have also been studied in selected plant species. Subsequently, characterization of active principles by HPLC, GC/MS and FTIR was done. Further, *in silico* molecular docking approach has been employed to understand the mechanism active constituents against the protein peroxisome proliferators activated receptor gamma (3DZY).

A review of literature has been compiled and presented on the ethnobotanical study of plants and their uses, compilation of various bioactive constituents present in the plants, their antioxidant, antimicrobial and antidiabetic activity. Subsequently an extensive survey was conducted between the common peoples of the districts of north Bengal regarding the uses of different plant species. Survey of the wetland plants reveals that the local community of North Bengal consume majority of the plants in

their daily diet. A total of 58 wetland plant species belonging to 42 families were recorded as ethnomedicinally important during the study. It has been also observed that some are sold in daily market regularly e.g *Amorphophalus paeoniifolius* (Dennst.), Nicolson, *Bacopa monnieri* (L.)Pennell., *Colocasia esculenta* (L.)Schott., *Diplazium esculentum* (Retz.)Sw., *Enydra fluctuans* Lour., *Euryale ferox* Salisb., *Glinus oppositifolius* (L.) A. DC., *Hygrophila auriculata* (Schumacher) Heine., *Ipomoea aquatica* Fossk., *Marsilea minuta* L., *Portulaca oleracea* L., *Trapa natans* L., *Nasturtium officinale* R.Br.

Finally, a total of fifteen plants were selected for study. All the plants were dried and mounted on herbarium sheet following the proper protocol and submitted to the NBU Herbarium for their authentication and subsequently the accession Nos. were also obtained against each of the samples.

After the proper identification plants were dried and crushed in to powder and extracted by using methanol as solvent. Flask extraction method was followed in this study and it was observed that the extractive yield percentage ranged from 8.32% to 12.52%. The highest extractive value was recorded in the sample *Marsilea minuta* and lowest was recorded in *Amaranthus spinosus.*

Different phytochemicals including primary and secondary metabolites were screened in the powdered plant samples. It was observed that saponins (12), alkaloids (09), phenolics and flavonoids (11) and tannin (12) were the predominant phytochemicals among the tested plants.

Plants used in this study were also tested for the presence of different biochemical components such as carbohydrate, protein and amino acid, phenol etc. The protein content was found to be significantly highest in the powdered sample of *A. spinosus* and lowest in *Pilea microphylla*. Similarly, significant differences were present in the free amino acid content of the 15 samples under study. Among the plant samples, *Glinus oppositifolius* contained the highest amount of amino acids whereas *Cryptocoryne retrospiralis* had the lowest. In case of phenol, the powdered sample of *Hydrocotyle sibthorpioides* had significantly ($p<0.05$) highest phenol content compared to all the other plant samples. And the lowest phenol content was recorded in *Portulaca oleracea*. Similarly, the total flavonoid content of *Amaranthus viridis* was highest and in *Portulaca oleracea* it is lowest. Significantly highest soluble sugar content was recorded in *Eclipta prostrata* and lowest concentration was detected in *Ipomoea aquatica*. Ascorbic acid content was found to be highest in *H. sibthorpioides*

and lowest in *Glinus oppositifolius*. On the other hand, the carotenoid content was found to be highest in *Amaranthus viridis* and lowest in *Ipomoea aquatica*. Total chlorophyll content was found to be highest in *Eclipta prostrata* and the least amount was observed in *Diplazium esculentum*.

After the phytochemical screening and estimation of biochemical component in plant powder the antioxidant activity of the plant methanolic extracts were evaluated. All the plant extracts showed varied degree of DPPH scavenging activity mostly ranged from 2.96-73.19 %. Among the plant extracts, the lowest IC50 value for DPPH was recorded for *H. sibthorpioides* (1.539 ± 0.065) and the highest IC50 value was recorded for *Enhydra fluctuans* (9.672 ± 0.082).

ABTS free radical scavenging activity of all the plants extracts was found to increase in a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml). All the extracts showed variable ABTS scavenging activity that mostly ranged from 04.96-76.09%.

The ferric reducing power of the positive control (ascorbic acid) was significantly higher than all extracts. The FRAP ability of extracts are given in ascending order *Pilea microphylla* > *Diplazium esculentum* > *Portulaca oleracea* > *Glinus oppositifolius* > *Marsilea minuta* > *Amaranthus spinosus* > *Hygrophila auriculata* > *Hydrocotyle sibthorpioides* > *Ipomoea aquatica* > *Amaranthus viridis* > *Phyla nodiflora* > *Enhydra fluctuans* > *Eclipta prostrata* > *Cryptocoryne retrospiralis* > *Barringtonia acutangula*.

All the plant extracts was tested for hydroxyl radical scavenging activity which was found to increase in a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml). All the extracts showed variable OH scavenging activity that mostly ranged from 8.34-81.23%.

All the plant extracts was tested for NO scavenging activity which was found to increase in a dose dependent manner for the range of concentrations tested. All the extracts showed variable NO scavenging activity that mostly ranged from 04.65-60.29%.

All the fifteen plant extracts was tested for superoxide radical scavenging activity which was found to increase in a dose dependent manner for the range of concentrations. All the extracts showed variable superoxide scavenging activity that mostly ranged from 20.53-65.14%. Among the plant extracts, the lowest IC50 value

was recorded for *H. sibthorpioides* (1.492 ± 0.085) and the highest IC50 value was recorded for *G. oppositifolius* (8.590 ± 0.062).

Metal chelating activity was also found to increase in a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml). All the extracts showed variable Metal chelating activity that mostly ranged from 12.23-84.67%.

In vitro α-amylase activity assay was also performed to determine whether any antidiabetic property is present in the plant samples under study. Dose dependent inhibition activity was also observed in this case. All the extracts showed variable α-amylase inhibitory activity that mostly ranged from 8.14-86.77%. Among the plant extracts, the lowest IC50 value was recorded for *C. retrospiralis* (0.69 ± 0.062) and the highest IC50 value was recorded for *H. auriculata* (6.293±0.144).

Based on the results of all previous experiments, finally two plant samples showing overall best performance were selected for all further tests. These plants samples were *C. retrospiralis and H. sibthorpioides*.

After the selection of two plants antibacterial and antifungal activity of the two plant extracts were evaluated against *Bacillus subtilis, Escherichia coli* and *Mycobacterium* sp. and three fungal pathogens. It was observed that both the isolates exhibited antimicrobial activity against *B. subtilis* and *E. coli*. No inhibition was observed against *Mycobacterium avium. C. retrospiralis* showed better antibacterial activity in comparison to control against the test organisms. The spore germination of the fungal pathogens was inhibited by the methanolic plant extracts. Inhibition was recorded higher in *C. retrospiralis* in comparison to *H. sibthorpioides* in all three plant pathogenic fungus.

Finally *in vivo* study was conducted to determine the antidiabetic activity of two plant extracts. Rats were induced to be hyperglycaemic by STZ injection. Results were recorded after 25 days of induction of diabetes. The body weight of the diabetic control was found to have decreased significantly in diabetic control (set II) when compared to the normal rats (set I). However, an oral administration of *C. retrospiralis* and *H. sibthorpioides* (200 & 400 mg/kg b.w.) and metformin to diabetic rats reversed the body weights changes to near normal. The hypoglycaemic effect of the extracts *C. retrospiralis* and *H. sibthorpioides* was recorded by measuring the fasting blood glucose levels in day 0, day 1, day 5, day 10, day 15, day 20 and day 25. It was observed that both the extracts could lower blood glucose level almost as effectively as metformin. Triglycerides, LDL- cholesterol, cholesterol levels were reduced and HDL

cholesterol level was increased significantly in standard drug (10 mg/kg) treated as well as extract (*C. retrospiralis*, *H. sibthorpioides*) treated groups. In case of liver enzyme such as SGPT and SGOT a significant increase was observed in diabetes induced rat. Although, increased level of SGPT and SGOT in the diabetic induced rats was decreased significantly with subsequent administration of the standard drug and the plant extracts in a dose dependent manner. Similar type of result was also observed in case of urea and creatinine.

Plants extracts were then characterized for their bioactive compounds. FTIR study revealed the presence of -O-H, –C=O and –C-C stretching in *C. retrospiralis.* Similar type of FTIR spectra was also recorded in *H. sibthorpioides*.

HPLC profiles of *C. retrospiralis and H. sibthorpioides* along with the other plant extracts were done and the analysis revealed the presence of a wide array of phenolic compounds. A total of 70 major peaks were detected across the plant extracts which were represented by individual peaks with different retention times in the chromatogram. Among the plant extracts, *Marsilea minuta* recorded the maximum number of phenolic compounds with the presence of 61 major peaks pointing out towards the presence of a wide range of phenolic compounds. In *H. sibthorpioides*, however only 15 peaks could be obtained and in *C. retrospiralis*, only 12 peaks could be recorded.

The methanolic extract of *C. retrospiralis* and *H. sibthorpioides* were further subjected to GC-MS for partial characterization of the compounds present in the fractions. *C. retrospiralis* methanolic extract were identified to contain nine different compounds, and *H. sibthorpioides* methanolic extracts were identified to contain ten different compounds.

In silico molecular docking with the compounds revealed by GC/MS showed that identified compounds were able to bind the catalytic site of Peroxisome proliferators activated receptor gamma (3DZY). Similarly docked conformation of different compounds *H. sibthorpioides* along with important amino acid residues of 3DZY was tested. The compound Corynan-17-ol,18,19-didehydro-10-methoxy acetate (ester) which was obtained from *H. sibthorpioides* possessed good binding affinity (-9.95 kcal/mol) by binding with amino acid residues ILE268, ALA 271, GLN275, TRP 305, ASN 306, LEU309, PHE 313, ARG 316, LEU 326, ALA 327, VAL 342, ILE 345, LEU 426, HIS 435.

Preface

Human society depends on nature and natural resources since the beginning of the civilization. As we all know human civilization primarily started its journey from constructing colonies to nearby water bodies. So they must have good knowledge of plants growing nearby water bodies, i.e. wetland plants. The plants growing in submerged, immersed or partially underwater or in nearby moist areas and grows well in moist soil are called wetland plants. In the present study we have tried to document the wetland plants and their uses by the local peoples of North Bengal. In this connection we have tried to evaluate their nutritional and and antioxidative properties and other medicinal values through characterization of the metabolites conferring these beneficial attributes.

In this context, I take this opportunity to thank all those who made this thesis possible. At the very outset, I would like to pay my utmost sense of gratitude to my supervisor, Prof. Usha Chakraborty, Plant Biochemistry Laboratory, Dept. of Botany, North Bengal University for her guidance, undivided attention, kind and valuable suggestions and wise counseling throughout the execution of this endeavour.

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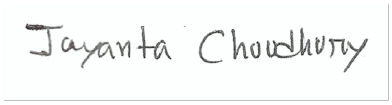

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1. INTRODUCTION

In the present era with fast moving lifestyle of human beings, they are affected by many lifestyle diseases. This is more so because, people have moved away from mother nature and natural resources and have become dependent on chemical medicines, synthetic food etc. Many of such diseases are directly related to oxidation process in cell, so antioxidants are needed to fight against oxidants. From various endogenous and exogenous sources Reactive oxygen species (ROS) are induced in cell. Antioxidant defence systems are present almost in all living systems but this system not always capable of complete prevention of the oxidation stress-induced damage in organisms (Khatua *et al.* 2013.)

Generally, normal levels of oxidants in cells are neutralised by cellular mechanism automatically but higher amount can damage cell in many ways. Physiological functions may deteriorate as a result of unbalanced antioxidant system. To rectify this unbalanced antioxidant system there are many synthetic antioxidants such as propyl gallate, tert-butyl hydroxyquinone, butylated hydroxyanisole (BHA) and butyl hydroxyl toluene (BHT) which are out in market with different brand names, but they produce ill effects/side effects after long term use. BHA and BHT cause liver damage and are also suspected to be carcinogenic. As a result, antioxidant containing natural products has created great interest for research since they can reduce the human cellular oxidative damage naturally (Sherwin *et al*. 1990). Special attention is also being given within science community in studying the natural antioxidants in foods. So it is quite obvious that nature becomes very relevant to treat these lifestyle diseases. Nature can heal almost every damage caused by nature. Earlier, medicines were produced from plant sources but with the advancement of science we are now a day's far away from nature and concentrating on synthetic drugs. Now time has come to look back to nature again.

Human society depends on nature and natural products since the beginning of the civilization. As we all know human civilization primarily started its journey from constructing colonies to nearby water bodies. So they must have good knowledge of plants growing nearby water bodies, i.e. wetland plants. The areas of land that are either permanently or temporarily covered by water are termed as wetland, it includes lakes, rivers, mangroves, peat lands, and also human-made wetland, such as fish

ponds, farm ponds, irrigated agricultural land, sewage farms, and canals. The plants growing in submerged, immersed or partially underwater or in nearby moist areas and grows well in moist soil are called wetland plants.

According to Lichvar *et al.* (2012) wetland plants are of following types –

- Obligate (OBL)- plants occurs almost always under natural conditions in wetlands.
- Facultative Wetland (FACW)- plants naturally occurs in wetlands but may also be present in non-wetlands.
- Facultative (FAC) likely to occur equally in wetlands and non-wetlands.
- Facultative Upland (FACU) plants occasionally found in wetlands, but are usually non-wetland plants.

Due to high diversity and richness of different plant species this area attracts itself for different studies related to wetland and its vegetation. Millions of people are directly and indirectly related to wetland such as medicine, food, fibre, clean water supply, educational and recreational benefits etc. According to Millennium Ecosystem Assessment wetlands cover 7% of the earth's surface and deliver 45% natural productivity of the world as medicine, food, fodder, fish production, veterinary support (National Wetland Atlas 2010).

North Bengal covers the northern districts of West Bengal. Districts of West Bengal north to the river Ganga. Malda, Dakshin Dinajpur, Uttar Dinajpur, Darjeeling, Jalpaiguri, Alipurduar and Coochbehar are the districts of North Bengal. These districts are enriched with different wetlands, except the hilly areas where wetlands are restricted only to some points, mainly at seasonal small ponds and nearby fountain areas. This area has different kind of wetlands- including both natural as well as manmade.

Many wetland plants are used as a normal human diet for thousands of years but in last several decades with the advancement of science the use of natural products is almost replaced with the chemical one. Natural foods have vanished from our diet, replaced by capsules, food supplement powders, health drinks etc. But recent studies show the adverse effect of these synthetic products in many ways with different side effects. On the other hand, the people still living in the vicinity of nature and using

natural foods and natural medicines are availing good health with less synthetic medical attention.

In the past decade the traditional medicine globally has regained its interest and attention. In China 40% of all health care covered by traditional healthcare system, 71% of total population use traditional medicine in Chile; in Cambodia it is 40 % of total population using this type of medicine. The rural people of India i.e. 65% of the population depends on medicinal plants, Ayurveda, ethno medicine for their primary health care need. Traditional, complementary and alternative medicines are becoming more popular in developed countries. For care and treatment of life-threatening diseases as AIDS and malaria, traditional medicines have been used in many countries. More than 60% of children with high fever in Mali, Nigeria, Zambia and Ghana are dependent for first line treatment on herbal medicine. In low-income countries traditional medicine is easily available and affordable (WHO 2004). North Bengal is a much diversified area with different communities living in different parts of this area. Some endemic communities like toto, lepcha etc. along with different tribe castes are also found in these areas.

The knowledge of early people regarding the plants and their therapeutic values were based on many years of trial and error experiments, observations and experience. And this knowledge of plant use in different ways were forwarded generation after generation orally in early stages and in some civilisation it is also written in different forms at later stages of civilization. In spite of this many communities are unwilling to circulate their knowledge to outer world in this regard. They keep their knowledge restricted to some specific families within the community. These medical men also have many names in different communities' e.g. Baidya, Janguru, Jhakri, Kabiraj, Hakim etc. Since ancient time world witnessed different medicinal practices in different parts.

Between 3500 B.C to 1800 B.C. earliest report of medicinal plant use by Indian people is found in Atharva Veda, the mother literature of Indian Ayurveda. Charak Samhita written somewhere between 100 to 200 B.C. is another very important literature regarding importance of medicinal plants in ancient Indian medicinal system. This Sanskrit literature describes the etiology, symptomology and therapeutics of many diseases along with human structure (Glucklich 2008). The importance of hygiene, diet prevention and medical education is also discussed in Charak Samhita

China is another very important civilization with great knowledge of Natural medicine. Chinese medical practice is more than 2,500 years old which includes different forms of botanical medicine, acupuncture, massage, exercise and dietary therapy etc (Shang 2007).

In Greece during 460-377 B.C. most probably by Hippocrates Unani medicine system originated. Arabs introduced this system in India. In India this system attains its maximum enrichment due to the availability of rich plant resources in different climatic conditions. Unani System of Medicine also uses various kinds of plants along with other ingredients to treat health problems like intestinal dysfunction, liver dysfunction, diuretic etc. (Kumar 2014).

All civilization had their knowledge related to the medicine source from nature and many of them noted their knowledge in written form. Since many literatures were lost, many unwritten knowledge seems to be lost forever, it is our duty to regain the knowledge for the betterment of our future generations. Phytochemicals are plant products helping us fight against various pathological conditions such as diabetes, blood pressure, tumor, inflammation, viral and microbial infections, nervous system disorders, parasitic infections, gastric ulcer, bone fracture, renal disease, urogenital disease etc. Among many phytochemicals mostly phenols, flavonoids are extensively studied in different parts of world **(**Dillard & German 2000). According to Harborne there are three major groups of plant phytochemicals:-

1. terpenoids,
2. phenolic metabolites
3. alkaloids and other nitrogen-containing compounds.

The terpenoids includes sesquiterpene lactones monoterpenoids, diterpenoids, triterpenoid, iridoids, sesquiterpenoids, saponins, steroid saponins, cardenolides, phytosterols, cucurbitacins, nortriterpenoids etc. The phenolics contain anthochlors, anthocyanins, benzofurans, coumarins, chromones, flavonoids, flavonol, lignans, phenols and phenolic acids, tannins etc. The alkaloids are represented by betalain, diterpenoid, indole, isoquinoline, monoterpene, sesquiterpene, steroidal compound etc. (Harborne 1990).

Clinical and experimental evidence shows the increased levels of ROS and reactive nitrogen species (RNS) generation in both types of diabetes and this proves the probable relationship between the oxidative stress and diabetes. Similar to other groups of plants, wetland plants are (Rosen *et al.* 2001, Johansen *et al.* 2005,

Matough *et al*. 2012). Ho *et al*. (2012) studied 31 medicinal wetland plants in Taiwan for the possible antioxidant activities of the methanol and water extracts of these plants. The experimental results suggested that the antioxidant parameter is not directly related to polyphenol quantity but proposed that the phytochemicals may play important role in the antioxidant activity of wetland plants.

Bright and Kanagappan (2016) evaluated 5 weeds from aquatic systems for their antioxidative properties. They measured the phenol, flavonoid contents as well as the DPPH radical scavenging activity. *Lemna minor* shows maximum DPPH scavenging activity and and *Pistia stratiotes* shows least scavenging activity. Maximum amount of phenol found in *Ceretophyllum demersum* and minimum in *Pistia stratiotes*. Flavonoid content in *L. minor* is maximum where as *Hydrila vericillata* contains maximum amount of flavonoid. A comparative antioxidant and phytochemical study of methanol extract of different plant parts of *Eichhornia crassipes* were done by Tyagi and Agarwal (2017). They evaluated the phenol, flavonoid content and DPPH radical scavenging activity of this wetland plant. Their study reveals the presence of highest amount of phenol and flavonoid content in leaf extract and the highest radical scavenging activity in petiole extract.

In recent years a number of research articles have been published in relation to ethnomedicinal and antioxidative evaluation of plants. However, much attention has not been received by the wetland plants of North Bengal region of West Bengal in this regard. Accordingly, considering the importance of wetland plants and their potential use in daily life of the inhabitants, the present study has been under taken in North Bengal region of West Bengal

The objectives of this study are as follows:-

- To collect and identify the locally available wetland plants.
- To prepare samples by drying followed by extraction.
- To document the medicinally used wetland plants.
- To perform the phytochemical screening of the active principles of plants.
- To determine the anti-oxidative activity of plant samples.
- To study the anti-microbial properties of plants.
- To study the anti-diabetic properties of plants.
- To select one or more plants showing maximum beneficial activities.

2. REVIEW OF LITERATURE

With the advancement of science and technology life became more luxurious and easy for mankind. While technology increased the availability of materials to humans, several diseases, known as life style diseases, also appeared in the arena of human civilization. Of these, diabetes and hypertension are extremely common and are predominantly caused by our busy life style and food habits. Generation of free radicals as by-product of biochemical reactions in our body are also responsible for several diseases. Plants have been an important source of medicine for thousands of years. India has a rich tradition of knowledge system in the use of plants for curative purposes. It also possesses the most diversified plant population due to a wide range of geographical span from great Himalayas in the North to Sunderbans in the East and vast coastal area in the South and West. So, from our ancient past the use of plants as therapeutic agents was a common practice. Wetlands are considered as the 'kidney of ecosystem'. Further, it is also the niche of several plants and animals. Though many wet land plants are consumed by ethnic communities and also used in therapeutics, but very little attention has been given by modern scientific community for scientific validation.

In this context, a brief review has been compiled and presented related to the present work with following subheadings:

- Relevance of ethno botanical study
- Phytochemicals present in plants
- Antioxidative activity of plants
- Antimicrobial activity of plats
- Antidiabetic activity of plants

2.1. Relevance of ethno botanical study

Ethno botany is the branch of ethno biology. Use of indigenous plants as medicine, food, shelter, clothing, hunting, and religious ceremonies by indigenous people when studied, the study is called as Ethno botany. It can also be described as the botanical knowledge of ethnic people of a particular area. John Harshberger in 1896 defined ethnobotany as "*the use of plants by aboriginal people*". "Ethno botany" means study of the relationship of botany with primitive human race or those who have particularly no written language (Debnath *et al.* 2015). In recent years the

attraction to ethno botany has increased many folds. Ethno botanical studies are the first steps in bio prospecting. These studies also help in conservation strategies of over utilized plants and in this way also helps the conservation of biodiversity. Ethno medicinal knowledge is associated with the indigenous or tribal people especially in a specific region of high biodiversity (Kumar 2015). A large number of people in the world still depend on traditional medicines for health care. Countries like India where different communities are present in different phytogeographical areas with rich biodiversity, people have great ethno botanical knowledge. But lack of extensive exploration in different parts of this country still provide great chance towards new discovery of many ethno botanical sources of medicine. Many workers have provided great contribution in the field of ethno botany and still many more are yet to come.

Ethno medicinal study of Khosian and other rural people of western region of South Africa revealed 38 new anecdotes. 13 species from seven genera from the family Menispermaceae was studied and identified as ethno medicinally important (De Wet and Van Wyk, 2008). Sajem and Gosai (2008) explored the traditional knowledge useful for the healing ailments of Lusai tribes of Northeast India. This exploration revealed 31 ethnomedicinally important plant species and their use in 41 types of illness.

An ethno medicinal study was carried out with the participation of 31 ethnic healers in Blue Nile State of South Sudan which revealed the presence of 53 very important ethno botanically important plant species. Majority of studied plants were wild. The studied plants were reported to be mainly useful in the treatment of infections, pain, digestive system disorders, respiratory troubles etc (Musa *et al*. 2011). Ninety ethno medicinally important plant species used by the Kani ethnic healers in Tirunelveli hills of Western Ghats, India were identified as being useful for treating ailment of 65 types. Majority of ethno medicinal preparations were prepared from leaves and administered orally. Jaundice, diabetes, skin diseases, abdominal disorders were mainly treated with these plants. Among these 90 species *Murraya koenigii*, *Syzygium cumini*, *Gymnema sylvestre*, *Terminalia chebula*, *Melia azedarach* are mostly used (Ayyanara and Ignacimuthub, 2011). Panda and Misra (2011) surveyed the 7 districts of South Odisha for gathering traditional ethno medicinal knowledge from the indigenous healers of that area. They reported 48 different wetland plant species useful in 47 different ailments and for the first time in this report they revealed 29 species with new ethno medicinal uses. Shankar and Mishra

(2012) recorded 24 hydrophytes of ethno medicinal use from the water bodies of Hazaribag district of Jharkhand, India. Their study revealed the use of aquatic plants by the tribal people of this area and can be very useful for future studies. Bhagyaleena and Gopalan (2012) reported 39 wetland species with potent ethno botanical activity from the wetlands of Palakkad district or Kerala.

Singh *et al.* (2013) explored the wetlands of Buxar District of Bihar, India, to study the ethno medicinally important wetland plants and gather information regarding their local name, application method of plant in diseases, and some other relevant data also. They identified 26 plant species important ethno botanically. Azad and Bhat (2013) studied ethno botany of 29 plant species used by the tribal as well as local people of Rajouri and Poonch districts of Jammu and Kashmir. Their study reveals that the people of these areas largely rely on this ethno botanical knowledge; but with the advancement of society the knowledge is getting deteriorated day by day as well as availability of important plants are also very less.

Mesfin *et al.* (2013) conducted a study on ethno medicinal plants from April to May, 2013 in Northern Ethiopia and identified 31 plant species from this area with maximum healing properties in treatment of 32 aliments of aboriginal people of this area. They gathered important ethno medicinal knowledge *e.g.* medicinal plant identification, disease treatment procedure, plant part used, preparation procedure, administration of preparations, ingredients etc.

Sarmah *et al.* (2013) reported 65 ethno medicinally important wetland plants from the Subansiri and Ranga river floodplains of Lakhimpur district, Assam, India. In a study by Alam (2014) 30 ethno medicinally important wetland plant species from East champaran Districts of Bihar were recorded. The data was collected during 2013-2014 calendar year. They collected data by interviewing ethnic medicine practitioners and also from some local people of the study area. Chhatarpur is a northern district of Madhya Pradesh, India. The main tribes of Chhatarpur district are Saur and Kondar having a traditional knowledge of 45 very important ethno medicinal plants useful in the treatment of cholera, leprosy, pneumonia, diarrhoea, botulism, dysentery, gastroenteritis, bronchitis, tonsillitis etc (Chaturvedi and Saxena, 2014). The use of 24 ethno botanical plants species from 23 genera, from Renalagadda Thanda, Kodangal mandal, Mahabubnagar district of Telengana, India was reported by Singh and Singh (2015). Information regarding the treatment process werealso recorded from the tribal communities of this area.Vijayakumara (2015)

explored the Nelliyampathy hills of Kerala, India during 2011-2013 and reported 85 ethno medicinally important plant species useful in the treatment of 19 different diseases in this area. In another study, Chowdhury and Das (2015) reported 52 ethno medicinal wetland plant species from Darjeeling and Jalpaiguri regions of West Bengal. Their study shows the intense knowledge of ethno medicines from wetland within the ethnic people and kabiraj, hakim, baidya of this area.

Savithramma *et al.* (2016) reported 48 ethno medicinally important plant species used by Yandi tribes of Chandragiri reserve forest area, Chittoor District, Andhra Pradesh, India, for the treatment of 53 different types of ailments of local people. They also compared their data with Dr. Duke's database of Phytochemical and Ethno botanicals. Murtem and Chaudhry (2016) reported ethno medicinal knowledge of Tagin, Hill Miri/ Nyshi and Galo community of upper Subansiri district of Arunachal Pradesh, India. The ethno medicinal study was done by collecting information from local people regarding the use of local forest plants for curing ailments. Purnima (2016) reported 65 ethno medicinally active plant species traditionally used for the treatment 35 ailments of local people Buxar district, Bihar India. The data was collected from Vaidhya and other resource person and compiled as a source of further study and repository for the future generation.

Bouiamrine *et al*. (2017) reported 69 commercialized ethno medicinal plants used by the Amazigh community of Morocco. Mainly digestive disorders, metabolic diseases skin disease were treated with these plants.

2.2. Phytochemicals present in plants

Phytochemical screening conducted by Karthishwaran (2010) of the methanolic extract of leaves of *Pergularia daemia* revealed the presence of flavonoids, tannins, carbohydrates, alkaloids, steroids, terpenoids, although Proteins were not detected in the extract. They also performed TLC, HPTLC, HPLC, FTIR for identification of phytochemicals. Aiyegoro and Okoh (2010) screened *Helichrysum longifolium* for the presence of tannins, flavonoids, saponins and steroids. The quantification of antioxidative group of compounds was also done and it was found that the total phenol content of aqueous leaf extract was 0.499 mg gallic acid equivalent/g. The flavonoid and proanthocyanidin contents were found to be 0.705 and 0.005 mg gallic acid equivalent/g respectively. Preliminary phytochemical screening of *Syzygium cumini* (L.) revealed the presence of major phytochemical compounds like flavonoids, alkaloids, steroids, glycosides, saponins, terpenoid,

phenols, cardiac glycosides, tannins (Gowri and Vasantha, 2010). Leaves of *S. cumini* were found to be rich in flavonoids, glycosides, alkaloids, steroids, tannins and saponins. The phytochemical compounds have been reported to confer many beneficial properties including antimicrobial properties. Flavonoids are known to have potent anti-allergic, anti-microbial, anti-inflammatory and anti-cancer activities. Tannins also possess medicinal properties like antiviral, antibacterial and anti-tumour activities and are also known to inhibit HIV replication. Saponins are useful in relieving hyper cholestrolaemia and hyperglycaemia and are also known to possess antifungal properties.

The screening of phytochemicals in different solvents also varied. Mojhi *et al*. (2011) screened four differrent solvent extracts of *Paedallium murex viz.* petroleum ether, ethanol, chloroform and water. Presence of carbohydrates, glycosides and steroids were detected in all the four extracts; alkaloids in petroleum ether; flavonoids in petroleum ether, ethanol and chloroform extracts were also detected. Saponins and tannins remained undetected in all the extracts. Phytochemical screening of leaves of *Vitex doniana* and *Mucuna pruriens* was performed by Agbafor *et al.* (2011). These two plants are known to be used for curing various ailments. The presence of saponins, tannins, anthraquinones, and flavonoids were observed in all the extract. All the extracts also showed antioxidant properties determined by their DPPH radical scavenging activity. Agbafor *et al*. (2011) reported highest inhibition by *Vitex doniana* which was not significantly different from that by vitamin C. The extracts also produced a significant decrease in liver MDA. According to Varadarajan *et al*. (2008), the secondary metabolites (phytochemicals) and other chemical constituents account for their medicinal value. Saponins, triterpene and steroids confer hypotensive and cardiodepressant properties. Anthraquinones possess astringent, purgative, anti-inflammatory, antitumor and bactericidal effects. Cardiac glycosides are useful in the prevention of congestive heart failure and cardiac arrhythmia. Flavonoids, tannins and phenolic compounds can act as primary antioxidants. Dhale (2011) studied the *Bauhinia variegata* leaf and bark extracts of petroleum ether, chloroform, alcohol. Results of the phytochemical analysis revealed the presence of alkaloids, glycoside, phenolics, saponins, flavonoids, tannins, lignin, and terpenoids. The antibacterial activities of the extracts detected could be attributed to the presence of the phytochemicals.

Bhandary (2012) revealed the presence of triterpenoids, steroids, tannins, carbohydrate, glycosides, flavonoids and vitamin C in pomegranate peel extract. However, the presence of triterpenoids, steroids, alkaloids, flavonoids, glycosides, saponins, tannins, carbohydrate and vitamin C in the whole fruit extract and triterpenoids, glycosides, saponins, alkaloids, steroids, tannins, carbohydrate and vitamin C in the seeds extract was reported. The presence of these compounds in the extracts was propounded to potent antioxidant capacity of pomegranate.

Diethyl ether, ethanolic and aqueous extracts of *Malva sylvestris* L. seeds revealed the presence of alkaloids, sterols, tannins and coumarins (Zohra *et al*. 2012). Stem extracts on the other hand revealed the presence of flavonoids, tannins, saponins, alkaloids, sterols, coumarins and anthocyanosides which may be responsible for the medicinal properties of the plant.

Phytochemical screening of the *Ephedra alata* plant extract reaveled the presence of cardiac flavonoids, glycosides, reducing sugars, alkaloids and phenolic compounds (Jaradat *et al*. 2015). Cardiac glycosides were detected in aqueous, methanolic and ethanolic extracts. Alkaloids detected in methanol, acetone, and ethanolic extracts. Reducing sugar detected in all used solvent extracts. Phenols detected in methanolic and ethanolic extracts. Flavonoid detected in aqueous, methanolic, and ethanolic extracts. Saponins, free amino acids, starch, volatile oil, tannins, steroids are not detected in any one of selected solvent extracts.

Various solvent extracts of the powder of *Terminalia catappa* was screened and phytochemicals tests confirmed the presence of sterols, triterpenes, carotenoids, emodols, coumarins, flavones aglycone, alkaloids, steroids, cardenolides, saponoins, cholesterol, amino acids, phlobatinins, cardiac glycosides and reducing compounds. In hexane extract, presence of carotenoids, steroids, triterpenes, coumarin and coumarin derivatives, steroid glycosides, cardenolides, saponins, flavanosides, cholesterol and cardiac glycosides. However, these compounds with the exception of cardiac glycosides were absent in the ethanolic extract. The chloroform extract on the other hand showed the presence of sterols, triterpenes, coumarins, alkaloids, saponins, cholesterol and cardiac glycosides. Phytochemical screening revealed the presence of sterols, triterpenes, coumarins, flavonoids, tannins, anthracenosides, steroid glycosides, cardenolides, cholesterol and cardiac glycosides in ethyl acetate extract. This pointed towards the fact that the presence of phytochemicals varied with the type of solvents used for the purpose of extraction (Jagessar and Allen, 2012).

Psidium guajava L. also known as amritphale is a small tree of Myrtaceae family, traditionally used for curing several diseases (inflammation, diabetes, hypertension, wounds, pain and fever). The leaves were powdered and subsequently extracted with different solvents *viz.* petroleum ether, chloroform, ethanol, water, hydroalcoholic. Phytochemical tests revealed the presence of flavonoids, tannins, triterpenoids, saponins, sterols, alkaloids and carbohydrates (Arya *et al*. 2012).

Adebiyi *et al*. (2012) selected two mosses - *Thidium gratum* and *Barbula indica* used as ethnomedicine and the extracts were screened for the presence of several phytochemicals like alkaloids, flavonoids, phenols, saponins and steroids. More or less all the phytochemicals were present in the two mosses except for the absence of phenol in *Barbula indica*. However, the percentage yield of phenols obtained in *B. indica* (0.055%) was lower than those obtained bywho worked on the phytochemical screening of some higher medicinal plants. But the quantity of phenols was similar to that of *Scoparia dulcis* (0.04%), *Sida acuta* (0.08%) and *Tridax procumbens* (0.06%).

Rani *et al*. (2013) collected the leaves of the plant *Annona reticulata*, extracted using different organic solvents with varying polarity *viz.* ethyl acetate (low), butanol (medium) and methanol (high). The phytochemical screening of the extracts showed the presence of alkaloids, tannins, terpenoids and coumarins. Ethyl Acetate and butanol extract possesses terpenoids, alkaloids and steroids. Methanolic extract posses terpenoid, steroid, tannins and coumarins.

In another study, Kulkarni *et al*. (2013) studied the presence of steroids, flavonoids, saponins in *Aspidium cicutarium*. They also performed the further investigation of the extracts by HPTLC to deduce the varied composition of methanolic and aqueous extracts. The chromatogram showed the presence of multiple peaks which indicated diverse phytocomposition of extracts. The methanolic and aqueous extracts of the rhizomes showed presence of sugars, tannins, steroids, flavonoids and saponins, whereas the methanolic extract showed the presence of anthroquinone glycosides, saponin glycosides and proteins.

Jyothiprabha and Venkatachalam (2016) studied the presence of different phytochemicals by qualitative analysis of several spices and confirmed the presence of alkaloids, carbohydrates, phenol, glycosides, terpanoids, flavonoids, saponins, proteins, steroids and tannins. Spices are important ingredients of foods, used since ancient times, as flavoring agent, food preservatives and also has been used in folk

medicines. The use of spices for medicinal purpose depends on the presence of phytochemicals they contains. The spices have been reported for anti-inflammatory, antidiarrheal, antimicrobial, antioxidant and insecticidal activities. They found the presence of steroids in ethanolic extracts of clove, pepper, saffron; which are of great importance in pharmacy as they possess compounds like sex hormones and can be used for drug production.

2.3. Antioxidative activity of plants

Free radicals and other reactive oxygen species, inorganic products of normal aerobic metabolism, are recognized as agents involved in the origin of diabetes, Parkinson's and Alzheimer's diseases, cancers as well as atherosclerosis. The reactive oxygen species are also responsible for aging in human. An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule (Yamagishi *et al*. 2011). Plants are considered the most promising source of antioxidant from ancient past. Phenolic acids, polyphenols and flavonoids present in plants, scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus act as antioxidant. Hence, the identification and development of phenolic compounds from different plant extracts have become a new and major area of health- and medical-related research. Dai *et al*. (2010) mainly focused on extraction, purification, analysis, quantification, antioxidant properties and anticancer effects of phenolics on *in vitro* and *in vivo* animal models as well as included recent human intervention. They provided an updated possible mechanism of action involve antioxidant, prooxidant and interference activities with cellular functions.

Ashafa *et al*. (2010) investigated the antioxidant potential of leaves from *Felicia muricata* herb was by using different solvents such as water, methanol, acetone and ethanol extracts which mainly used for the management of different human and livestock diseases in the Eastern Cape Province of South Africa. The extracts were rich in phenols, proanthocyanidins and flavonols but low in flavonoids. The methanol, acetone and ethanol extracts showed higher DPPH scavenging activities as compared to water extract but all the extracts showed high ABTS scavenging activity. Traditionally infusions, decoction and poultice of plant extract are prepared with water. Authors concluded that water and ethanol extracts of *Felicia muricata* show strong antioxidant properties.

Ginger (*Zingiber officinale*) contains several bioactive constituents which possess health promoting properties, especially used in Asia. Ghasemzadeh *et al*., (2010) checked the antioxidant activities of methanol extracts from the leaves, stems and rhizomes of two *Zingiber officinale* varieties such as Halia Bentong and Halia Bara by DPPH assay. This assay was used in an effort to compare and validate the medicinal potential of the subterranean part of the young ginger. Leaves contain higher amounts of phenolics and flavonoids than stem and rhizome. The rhizome on the other hand shows better result than leaves and stem in ferric reducing/antioxidant potential (FRAP) test. Halia Bara had higher antioxidant activities and total phenolic and flavonoid contents in comparison with Halia Bentong and leaves had higher inhibitory activity than or comparable to those of the young rhizomes at low concentration. *Sesbania grandiflora* leaves and flowers showed free radical scavenging capacity and antioxidant activities of the ethanol and 70% acetone extracts. Gowri *et al*. (2010) reported that the 70% acetone was more efficient for the extraction of bioactive compounds in terms of recovery percent, higher reducing power activity, highest peroxidation inhibiting activity and the values were comparable to that of α-tocopherol. The final result showed both solvent extracts of leaves exhibited a higher concentration of total phenolics, soluble sugars and total free amino acids than the respective solvent extracts of the flower.

Gupta *et al*. (2011) provided an updated assay on phytochemical analysis and *in vitro* antioxidant activity of dichloromethane, methanol and acetone extracts of *Abies pindrow* leaves because these leaves are traditionally used as an ayurvedic remedy for fever, hypoglycaemic, respiratory and inflammatory conditions. Final result provided evidence that *Abies pindrow* leaf extracts are a potential source of natural antioxidants because the methanol extract exhibited highest antioxidant activity while acetone extract showed the presence of relatively high total phenol and flavonoids contents. So it could serve as a base for future drugs. Nahak *et al*. (2011) reported that present scientific research has established by phytochemical analysis of many different active compounds in spices of Indian species that provided flavour, colour, and aroma of food and traditionally serve as a therapeutic agent in pharmacy according to Ancient Indian texts of Ayurveda, an Indian system of medicine. The medicinal effects validated by modern pharmacological and clinical experimental techniques have shown a promising effect in therapeutics. In an enormous potential Piperaceae family, Piperine is an alkaloid which is found naturally in plants such as

Piper nigrum and *Piper cubeba*. These are widely used in various herbal cough syrups and also used in anti-inflammatory, anti-malarial, anti-leukaemia treatment. Preliminary screening and confirmatory test were performed for identification of alkaloid. Finally, high antioxidant activity was found in *Piper cubeba* ethanol extracts in comparison to *Piper nigrum* extracts respectively.

Stanković (2011) made a comprehensive study of *in vitro* antioxidant activity as well as total phenolic content and flavonoid concentration of different extracts which was found in the whole herb of *Marrubium peregrinum* L. (Lamiaceae) by using spectrophotometric methods. The antioxidant activities were expressed as a percentage of DPPH radicals' inhibition and IC50 values (μg/mL). *M. Peregrinum* ethanolic extract showed highest phenolic compounds, flavonoid concentration and high antioxidant activity. Similarly, the linear correlation was confirmed between the values for the total phenolic content and antioxidant activity of this plant extracts because high contents of phenolic compounds indicated higher antioxidant activity. Orčić *et al*. (2011) developed a method for identification and determination of the most active antioxidant compounds in plant extract of *Hypericum perforatum* which had been used in traditional and modern medicine for a long time due to its high content of biologically active phenolics. Fast qualitative, semi quantitative identification and quick separation analysis was developed by LC-MS method and then significant antioxidant activity was determined by DPPH assay. Superoxide scavenging, lipid peroxidation and FRAP were also determined. The flavonoids, naphthodianthrones and phloroglucinols have been identified by these techniques. This experiment demonstrated that *H. perforatum* shows high antioxidant activity due to flavonoids and phenolic acids, while phloroglucinols and naphthodianthrones showed no significant activity by fractionation.

Different plant parts like leaves flowers and stems of *Teucrium polium* were used for the detection of flavonoids concentration, total phenolic content and *in vitro* antioxidant activity.The *in vitro* antioxidant activity was determined by using DPPH reagent and expressed as the concentration of each extract required to inhibit radical by 50% (IC50) values. The methanol extracts of *Teucrium polium* leaves contain the highest concentration of phenolic compounds and showed strong antioxidant activity. For the comparison study, Ginkgo and Green tea extracts were also used and *T. Polium* extracts showed greater activity with Ginkgo or Green tea. Separation assay to separate active phenolic compound from *T. polium* was also suggested by these

workers (Stankovic *et al*. 2012). Ferulic acid is a type of hydroxycinnamic acid. This is an abundant phenolic phytochemical which is commonly found in plant cell wall components. Previous reports are available regarding the anti-aging, anticancer, antioxidant potential of ferulic acid and this organic compound also reduced cholesterol, triglycerides and blood glucose levels. Ferulic acid was collected from ethyl acetate herbal extract of *Syzygium cumini* seed powder by HPLC technique. It was also reported that *Syzygium cumini* seeds and fruit have hepatoprotective, antidiabetic, anti-inflammatory, antihyperlipidemic, antibacterial, and diuretic properties. This experiment established that these plant and mainly seeds could be further used as a medicine with greatest pharmaceutical values (Shah *et al*. 2012).

In vitro chemical analyses of *Torilis leptophylla* (TLM) methanol extract and its derived fractions n-butanol (TLB),n-hexane (TLH), ethyl acetate (TLE), chloroform (TLC), and residual aqueous fraction (TLA)} were tested mainly for antioxidant activity. The hepatic injuries induced by carbon tetrachloride in the male Sprague-Dawley rat as test organism were also reduced to almost normal. Preliminary phytochemical screening test for various constituents in TLM was also conducted. The higher phenolic contents of TLM and lower flavonoid contents of TLE were found significantly higher than other solvent fractions comparably. On the other hand, phytochemical screening of TLM showed the presence of different phytochemical compounds such as alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, phlobatannins, tannins and terpenoids etc. The EC50 values based on the DPPH, ABTS and phosphomolybdate for TLB, hydroxyl radicals for TLC, superoxide radicals for TLM and hydrogen peroxide radicals for TLE were showed generally lower potential antioxidant properties.Its cytoprotective and free radical scavenging activity makes this plant *Torilis leptophylla* as an antioxidant agent (Saeed *et al*. 2012). Khan *et al*. (2012) investigated the therapeutic potential of nonpolar solvents such as hexane, SAHE; ethyl acetate, SAEE and chloroform, SACE and polar like methanol, SAME crude extracts of the whole *Sonchus asper* (SA) plant because this plant is traditionally used for the treatment of mainly various ailments associated with liver, lungs and kidneys. For this experiment, several parameters including free-radical ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2 and •OH) scavenging, iron chelating activity, scavenging of superoxide radicals, total flavonoids and total phenolic content (TPC) were tested. Results revealed that, SA extracts presented a remarkable capacity to scavenge all the tested reactive species with IC_{50} values. Finally the SAME was

also shown to have the highest TPCs while lowest IC_{50} values for the DPPH•, ABTS•+ radical scavenging capacities and iron chelating scavenging efficiency. On the other hand, SAME had best potential in scavenging of superoxide radicals and hydrogen peroxide as well as hydroxyl radicals.

Subramanian *et al*. (2013) tested the antioxidant activity of the acetone and methanol extracts of the stem and bark of traditionally used *Shorea roxburghii* plant in pharmacy. The total phenolic content and antioxidant activity of these plant part extracts were determined by DPPH, radical scavenging, ferric ion reducing power, hydroxyl radical, ABTS. Radical scavenging and hydrogen peroxide scavenging activities. In this experimental study, they also checked the reducing efficiency of the *S. roxburghii* towards silver spherical shaped nano particles by using surface plasmon resonance and transmission electron microscope. As a result, acetone and methanol extracts of *S. roxburghii* stem bark was found to be a potent antioxidant and formation of silver nano particles ascertains the role of the water soluble phenolic compounds present in *S. roxburghii*. The extract of this plant could be used as a green reducing agent for the synthesis of Ag nano particles because may potential applications in the treatment of the diseases caused by free radical. Kang *et al*. (2013) reported on the total phenolic content, total flavonoid contents, antioxidant activity and antimicrobial activity of ethanolic extracts of *Impatiens balsamina* L. (Balsaminaceae) stems and leaves which were harvested in Korea on March 10, 2011. Total phenolic and flavonoid contents of leaf extract were higher than those of stem extract in this experiment. Similarly, leaf extracts exhibited stronger free radical scavenging activity and higher inhibitory effects against microorganisms than that of the positive control and those stem extracts respectively. Leaf extracts showed the greatest antimicrobial activity against both Gram negative and positive strains at different harvest times. These leaves would be applicable as a natural source of antioxidant in food preservation because of their valuable bioactive resource.To investigate the antioxidant activity as well as the antibacterial activity of methanolic leaf and stem extracts of *Coscinium fenestratum*, the free radical scavenging activity was evaluated by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazo line-6-sulphonic acid) or ABTS. *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis* were used for the determination of anti microbial activity, aqueous, acetone, ethanol and methanloic extracts of stem and leaves. On the other side, total phenol and flavonoid content was

quantitatively estimated. The result showed the higher flavonoid contents and lower phenolic content is present in this stem and leaf extracts of *Coscinium fenestratum* (Goveas *et al*, 2013).

The *in vitro* and *in vivo* antioxidant activity of *Trichosanthis kirilowii* Maxim (Cucurbitaceae) the dry root tuber of which is commonly used as a traditional medicine in China. and the ethyl acetate (EtOAc), n-butanol, and the mixture of n-butanol and EtOAc fractions of *Radix trichosanthis* (RT), were tested. The wide range of saponin pharmacological properties has been identified previously but this may be the first reported to investigate the crude saponins from RT. *In vitro* antioxidant activity was detected by DPPH free radical, hydrogen peroxide scavenging, and reducing power assays. Pretreated saponins were used to evaluate the *in vivo* antioxidant potential by detection of superoxide dismutase (SOD), malonaldehyde (MDA), lactate dehydrogenase (LDH), and total antioxidant capacity (T-AOC) levels for established CCl4 induced acute cytotoxicity model. The result showed the reducing power of the EtOAc and the mixture of n-butanol and EtOAc extracts increased in a dose dependent manner because low dose exhibited in a time dependent manner with prolonged reaction time. This study shows thatEtOAc fraction mainly showed a dose dependent manner and the hydrogen peroxide scavenging activity is mainly demonstrated as a time dependent manner.The n-butanol extract *in vivo* study reported decrease in MDA and LDH levels and increased of SOD, T-AOC level. So the Conclusions of this experiment is saponins predated of RT in n-butanol fraction might be a potential antioxidant carrier and served as CCl_4-induced oxidative stress which has been found to be alleviated and may be associated with the time dependent manner of n-butanol saponins in a low dose (Chen *et al*. 2014). Upadhyay *et al*. (2014) investigated free radical scavenging potential of water and ethanol extracts of aerial parts and the root of *Phyllanthus fraternus* and antioxidant capacity was measured by DPPH. Ethanol extract of aerial part showed maximum inhibition activity. Lipid peroxidation (LPO) was measured by means of thiobarbituric acid-reactive substances (TBARS) by using lipid-rich media like egg-yolk homogenates with EC_{50}. The superoxide (SO) radical scavenging activity was also measured by using riboflavin-light-nitroblue tetrazolium assay. Total phenolic and flavonoid contents were also measured by the spectroscopic method which showed ethanolic extracts of aerial part is most active towards antioxidant potential. This result showed that this extract further can be used as the potent natural antioxidant.

Algerian medicinal plants such as *Echium pycnanthum*, *Haloxylon articulatum*, and *Solenostemma oleifolium* were used to evaluate the total phenolic, flavonoid and tannin contents. The antioxidant capacity was checked by 1,1-diphenyl-2-picrylhydrazyl, 2,20-azino-bis-3- ethylbenzo thiazoline-6-sulfonic acid, β-carotene, reducing, and chelating assays. The phenolic content in *H. articulatum* was superior to those in *E. pycnanthum* and *S. oleifolium*. The same tendency was observed for the relative amounts of flavonoids and condensed tannins in the three medicinal plants. *H. articulatum* shoots exhibited the strongest antioxidant activity, with the lowest IC_{50} values. This investigation confirmed that these herbs contain high *in vitro* antioxidant potency which can be successfully used for medicinal purposes in humans (Chaouche *et al*. 2014). A comparative study on the effect of *in vitro* free radical scavenging activity between the *Kedrostis foetidissima* leaf with various solvent extracts like aqueous, methanol, and acetone chloroform and petroleum ether was conducted. In this test, total phenols, flavonoids, tannins contents, antioxidant activity and free radical scavenging activity showed better values in methanolic for DPPH, hydroxyl, nitric oxide, superoxide, hydrogen peroxide (H_2O_2) radical in a concentration dependent manner. This experiment was also followed by chloroform, aqueous, acetone and petroleum ether extracts. The metal chelating activity and reducing power ability was also performed by the similar process. This result suggested that the methanolic extract of this leaf may serve as a potential source of antioxidants and can be used as a therapeutic agent in free radical induced diseases (Sasikumar *et al*, 2014).

Krishnaiah *et al*.(2015) investigated the antioxidant activity and total phenolic content of methanolic extract of *Morinda citrifolia* by using membrane separator and methanol extract of fruit was also separated into permeate and retentate by Poly-ethersulphone (PES). The antioxidant activity and total phenolic content were examined in specific temperature (30–70^0 C) and pressure (0.5–1.5 bar) range. The DPPH activity exhibited a gradual increase activity collection from membrane separation process and optimum magnitudes of DPPH radical scavenging activity and total phenolic content were found respectively. Sadeghi *et al*. (2015) evaluated that the antioxidant property of methanolic extracts of *Boerhaavia elegans* (Nyctaginaceae), a medicinal plant which is used broadly for the treatment of kidney disorders, urinary tract disorders and blood purification in Baluch tribe. The antioxidant power in DPPH and FRAP assay were shown in decreasing order such as methanolic extract > aqueous extract > ethyl acetate extract > chloroform extract. The

experimental results showed that *B. elegans* extract had potent antioxidant effects with high phenolic contents. In a study by Wong-paz *et al*. (2015) they conducted the colorimetric assay of total phenolic content (fPC) by heat-reflux system, antioxidant activities by DPPH, ABTS, lipid oxidation inhibition and HPLC characterization of the aqueous and ethanolic extracts of *Jatropha dioica*, *Flourensia cemua*, *Eucalyptus camaldulensis* and *Tumera diffusa*. Among this, three plant extracts showed strong antioxidant activities on scavenging of DPPH and lipid oxidation inhibition but in contrast, *J. dioica* extracts had the lowest potential antioxidant in three assays used. This high antioxidant activity can be used in the factories as antioxidant agents or for treatments in diseases. I*n vitro* antioxidant potential of methanolic extracts of 14 different medicinal plants, 8 of which are endemic species of Anatolia was evaluated by Ali *et al*. (2015). Scavenging activity was tested by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and the inhibitory effect on lipid peroxidation was examined by the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. Results showed significant differences such as *Crataegus microphylla*, *Salvia hypargeia*, *Cotinus coggygria*, *Origanum sipyleum* and *Rosa damascena* exhibited the highest DPPH scavenging activity, while *Centaurea nerimaniae*¸ *C. coggygria*, *Scorzonera tomentosa*, *R. damascena* and *Colchicum sanguicolle* showed strong antioxidant activity in the FTC and TBA. But *C. coggygria* and *R. damascena* exhibited potent antioxidant activity by the DPPH, FTC and TBA methods respectively.

Traditionally, Chinese fruits are a rich source of phenol, flavonoid, antioxidants, and have chemopreventive and chemotherapeutic properties against cancers and other diseases. Eleven Chinese fruits extracts were screened for their total phenol and flavonoid content. Folin-Ciocalteau and aluminium chloride methods were used to determine the amount of phenol, flavonoid in these 11 extract. To determine the antioxidant activities of these chinese fruit extract, DPPH radical scavenging activity, biological assay using *Saccharomyces cerevisiae*, metal chelating activity and ferric reducing antioxidant power (FRAP) were determined. The phenols and flavonoids contents of the hot water extracts were high whereas the endo polysaccharides lie in low range. The antioxidant properties of extracts of *Crataegus pinnatifida*, *Illicium verum*, *Ligustrum lucidum*, *Momordica grosvenori* and *Psoralea corylifolia* fruits were determined by the DPPH and FRAP methods (Jeong *et al*. 2016).

Odeja *et al*. (2016) focused on phytochemical screening, antioxidant and antimicrobial activities of hexane, ethylacetate and methanol crude extracts of *Acalypha ciliata* plant which was already used as a folk medicine for treatment of female sterility, dressing of sores and schistosomiasis. The results showed the presence of flavonoids, tannins, alkaloids, reducing sugar, anthraquinones, resins and glycosides of this plant extracts. The free radical scavenging capacity was also determined to evaluate the antioxidant activities of the extracts by using hydrogen peroxide. These pharmacological active compounds and antimicrobial effects could be used for treatment of bacterial infections and ailments in ethno medicine.

Contents of polyphenols, flavonoids and tannin as secondary metabolites and free radical scavenging activity on the basis of their dry weight of *Camellia sinensis* (tea plant) which are cultivated and commercially available in Ethiopian market were evaluated. Spectrophotometric assay were performed for the phytochemical analysis by using Folin–Dennis, Folin–Dennis/protein precipitation and aluminium chloride methods respectively. The free radical scavenging activity was determined by DPPH radical assay. The correlation between the antioxidant activity of total polyphenol, flavonoids and with tannin was calculated and maximum correlation value was found between polyphenol content and the free radical scavenging activity of the tea samples. Final results revealed that green tea had the higher polyphenolic contents and potent antioxidant activity (Bizuayehu *et al*. 2016).

Chrysophyllum cainito L. (kenitu or star apple) is widely used as the traditional remedy for inflammation, cancer, and diabetes mellitus. Different solvents with different concentration (96% of ethanol, 70% of ethanol, 50% of ethanol, 96% of acetonee, 70% of acetone, and 50% of acetone) were used for the extracts preparation. The antioxidant activities were calculated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, total phenolic content, and total flavonoid content. 70% of ethanol extracts showed highest antioxidant activity (Ningsiha *et al*. 2016).

Khan *et al*. (2017) reported the *in vitro* antioxidant ability, cytotoxicity and phytochemical components of *Ficus racemosa* in aqueous and ethanol extracts of leaves. Ethanolic extract possessed the highest phytochemical constituents and antioxidant activity as compared to aqueous extracts as tested by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay, nitric oxide scavenging assay, reducing power and superoxide radical scavenging assay respectively. Cytotoxicity analysis was determined against Dalton Lymphoma Ascites (DLA) cell line and the

IC50 value. This work showed that *F. racemosa* is a natural antioxidants source with anticancer agents and this antioxidant activity is dose-dependent which can be further used in pharmaceutical preparations for the treatment of diseases induced by oxidative stress.

Garcinia kydia Roxburgh is a plant of Clusiaceae family which is known to have antioxidant activity but lipoxygenase inhibition activity was unknown. The n-hexane, ethyl acetate and methanolic extract of this leaves with the help of Ferric Reducing Antioxidant Power method and anti-inflammatory activity was tested by inhibiting lipoxygenase. Similarly, total flavonoid content was checked by colorimetric methods using $AlCl_3$. The results showed the highest antioxidant activity of methanol extracts as compared to ethyl acetate and n-hexane leaves of *Garcinia kydia* Roxburgh. Ethyl acetate extract of *G. kydia* has flavonoids, antioxidant and lipoxygenase inhibition activity (Putri *et al*. 2017).

Mzid *et al*. (2017) analyzed the antioxidant and antimicrobial activities of ethanol and aqueous extracts of *Urtica urens*, a plant of Urticaceae family with important pharmacological properties commonly used as a medicinal plant. Polyphenol, flavonoid and tannin content of *U. urens* leaves were determined their antioxidant (DPPH, ABTS, b-carotene and FRAP) and the antibacterial (via the method of dilution tests) activities. Result concluded that ethanol was the most effective antibacterial agent with minimum inhibitory concentration. According to this work, *Urtica urens* leaves could be used as a natural source of antioxidant and antimicrobial agents.

Olamide *et al*. (2017) in a study evaluated the *in vitro* free radical scavenging activity of ethanol extract of *Grewia carpinifolia* leaf and stem. 1,1-Diphenyl-2-picryl hydroxyl (DPPH) quenching assay, 2,20-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) cation decolorization test, ferric reducing antioxidant power (FRAP) assay systems were performed for evaluating the antioxidant activity. Inhibition assay of lipid oxidation was measured by using Thiobarbituric acid active substances (TBARS) assay. The extracts were used at 0.2, 0.4, 0.6, 0.8 and 1 mg/mL Different concentrations of extracts was used to determined radical scavenging activity in terms of inhibition percentage. Then IC_{50} was also calculated for each radical. The result showed *Grewia carpinifolia* had a high radical scavenging activity in the various radical systems and antioxidant activity of *Grewia carpinifolia* extract may be due to the high level of flavonoids and phenols in the plant.

2.4. Antimicrobial activity of plants

Plants are rich in different phytochemicals which possess antimicrobial activity. In nature, plants are continuouosly being subjected to various kinds of abiotic and biotic stresses. Among these attacks by pathogens are very common.To combat with the bacterial and fungal pathogen attacks, plant synthesise different compounds with antimicrobial activity. There are several records of work done by earlier authors on extraction of these antimicrobial compounds from plants and testing them against microbes *in vitro* with a view to utilize them as source of medicines.

Abdel-Massih *et al*. (2010) conducted a study with a prime target to determine the antimicrobial activity of three selected plants - *Rosmarinus officinalis, Origanum majorana*, and *Trigonella foenum-graecum* against Extended Spectrum Beta Lactamase (ESBL) producing bacteria like *Escherichia coli* and *Klebsiella pneumonia*. Ethanol was used for the yield of crude extract preparation. These extracts were further sub fractionated by different solvents to obtain the petroleum ether, dichloromethane, ethyl acetate and aqueous fractions. The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) were also determined using broth micro dilution techniques. Final MICs ranged between 1.25 and 80 µg/µl. The majority of these microorganisms were inhibited by 80 and 40 µg/µl of the crude extracts. The petroleum ether fraction of *Origanum majorana* significantly inhibited 94% of the tested strains. Finally ethyl acetate extracts of all selected plants exhibited relatively low MICs and could be therefore described as strong antibacterial.

The aqueous and methanolic extracts of *Spinacea oleracea*, *Cucurbita pepo*, *Amorphophalus campanulatus* and *Colocasia esculenta* were evaluated for antimicrobial activity against different bacterial strains such as *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Enterobacter aerogenes, Bacillus subtilis, Bacillus sphericus*, *Bacillus thuringeninsis Enterobacter agglomerans, Klebsiella pneumonia, Salmonella enteritidis, S. cholerasius, S. aureus, Candida albicans, Penicillium chrysogenum, Enterobacter faecalis,* and *Cryptococcus meningitis*. The *in vitro* antimicrobial activity was performed by Agar well diffusion method on nutrient agar medium and Muller Hinton agar medium. The methanolic extracts of all the tested vegetables showed moderate to high activity against all the investigated microbial strains. But methanolic extract of *Spinacea oleracea* was most effective

among all extracts (32 mm inhibition zone against *E. coli*) and methanolic extracts were more effective than their aqueous extracts (Dubey *et al*. 2010).

Ördögh *et al*. (2010) reported that natural substances from plants are promising candidates to treat against maximum bacterial and fungal disease. Acne vulgaris was the most common skin disease in the world and the numbers of antibiotic resistant acne-inducing bacterial strains have increased in the past years; but natural plant products have shown good potential to treat this disease. *In vitro* biological activity of the juice, as well as water and methanol extracts of the pomace, of cultivated and wild fruits were investigated on 4 acne-inducing bacteria such as *Propionibacterium acnes*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Staphylococcus epidermidis*. The MIC values of juices and water and methanolic extracts of pomace were determined by broth micro dilution assays at pH 7 and at skin neutral pH 5.5. The total phenol content and radical scavenging capacity of the active juices and extracts were also calculated. Mainly Red and purple berries revealed a substantial antibacterial and antioxidant effect but there was no strong correlation between the antioxidant and antimicrobial properties. *Staphylococcus* strains were the most sensitive to the juices while *S. pyogenes*, to the methanol extracts. *P.acnes* performed very well among these bacteria and proved to be the most insensitive species in this study. The growth inhibition effect of *Ribes uva-crispa* (gooseberry) juice was stronger at acidic pH (MIC 0.40 mg/mL) than at neutral pH (MIC 5.30 mg/mL) but the antibacterial effect of the other fruits and berries showed no significant difference at the different pH values.

In recent years the success of chemotherapy lies in the continuous search for new drugs to counter the challenge posed by resistant strains. In India, methanol extracts of six plant species traditionally used for the treatment of various bacterial and fungal infections. The in vitro antimicrobial activity were checked mainly on *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger* by disc diffusion method. Methanol extracts of *Eugenia jambolana* and *Cassia auriculata* showed the highest toxicity against all bacteria. The plant extracts showed antibacterial activity but not antifungal activity against any of the fungi used in this experiment. The Minimum inhibitory concentration (MIC) assay was calculated for these two extracts against bacteria. *E. jambolana* revealed the highest antimicrobial activity at a minimum concentration (0.75 mg/mL) against *S. aureus*. The phytochemical analysis

carried out revealed the presence of coumarins, flavanoids, glycosides, phenols, tannins, saponins and steroids. But Alkaloids were not detected from any of the plant extracts. Final results provide justification for the use of these plants in folk medicine to treat various infectious diseases caused by these types of different bacteria. (Shihabudeen *et al*. 2010)

To investigate the antibacterial activity and phytochemical screening of methanol and petroleum ether leaf extracts of *Merremia emarginata* were used. The antibacterial activity of leaf extracts of *M. emarginata* were checked by agar well diffusion method. The methanolic extract of *M. emarginata* was more effective against *Bacillus cereus* and *E .coli*. But in aqueous extract of *M. emarginata* was more effective against *Staphylococcus aureus* and *Pseudomonas aeroginosa*. According to Elumalai *et al*. (2011) the results suggest that *M. emarginata* leaf can be used in treating diseases caused by these tested organisms.

Nanasombat *et al.* (2011) evaluated that cinnamon oil had highest antibacterial activity. To check the antimicrobial and antioxidant activities they were used essential oils of anise, bastard cardamom, cinnamon, dill, mace, zedoary, prikhom, and bitter ginger. The most sensitive bacteria were *Bacillus cereus* (0.5mg/mL minimum inhibitory concentration, MIC). Anise, cinnamon, dill, and prikhom showed strong antifungal activity against *Rhodotorula glutinis*, *Aspergillus ochraceus*, and *Fusarium moniliforme*. But among all these experiments, two oil combinations: i) cinnamon and mace oils and ii) cinnamon and prikhom oils showed a synergistic effect against *Staphylococcus aureus*, *Pseudomonas fluorescens*, and *Salmonella Rissen* (0.32–0.38 mg/mL fractional inhibitory concentration index, FICI). Similarly,Cinnamon, mace, and prikhom oils contained strong antioxidant activity with 0.29–5.66 mg/mL IC50, 61.46–68.52% antioxidant activity, 0.22–2.19 mM/mg reducing capacity, and 78.28–84.30% inhibition by 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene bleaching, ferric reducing (FRAP), and superoxide anion scavenging activity assays, respectively. These oils contained high amount of total phenolics (51.54–140.9 μg gallic acid equivalents/mg oil). Local herbs collected in UiTM Pahang Forest Reserve such as *Epipremnum* sp., *Zingiber* sp., *Tetracera indica*, *Tectaria crenata*, *Piper stylosum*, *Homalomena propinque*, *Goniothalamus* sp., *Elephantopus scaber*, *Mapania patiolale*, *Melastoma* sp., *Stemona tuberosa*, *Phullagathis rotundifolia*, *Thotea grandifolia* and *Smilax* sp. Methanol crude extracts were used to checked the antimicrobial activity against two gram positive bacteria *Bacillus subtilis* and

Staphylococcus aureus and one gram negative bacteria *Escherichia coli* by agar diffusion method. The inhibition zone (in diameter) of microbial growth showed that all extracts were active against gram-positive bacteria and gram negative bacteria. But *Stemona tuberosa* extract most active against the *E. coli* and *S. aureus* while *Piper stylosum* active against *B. subtilis* (Liliwirianis *et al*. 2011).The methanolic extracts of 12 medicinal plants were evaluated for its antibacterial activity against Gram-positive and Gram-negative bacteria by assay for minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC). The antibacterial activity was checked by an agar dilution method (according to the guidelines of Clinical and Laboratory Standard Institute). All the extracted compounds of the 8 medicinal plants (leaf or root) were active against both Gram-negative and Gram-positive bacteria. But the Gram-negative bacteria showed more potent action than Gram positive bacteria. The MIC concentrations were various ranged from0.6 μg/mL to 5000 μg/mL. The lowest MIC (0.6 μg/mL) and MBC (1.22 μg/mL) values were also obtained by these techniques (Kang *et al*. 2011).

Screenings of methanolic leaf extracts of *Cotinus coggygria*, *Adhatoda vesica*, *Argemone mexicana, Zanthoxylum armatum, Berberis asiatica, Corissa opaca, Euphorbia hirta, Cassia fistula* and *Ricinus communis* were tested against *Bacillus subtilis*, *Klebsiella pneumonia, Staphylococcus aureus*, *Escherichia coli, Enterobacter aerogenes*, *Proteus vulgaris* and *Pseudomonas aeruginosa* by disc diffusion method.The plant samples were collected from Uttarakhand. Leaf extracts of *Ricinus communis*, *Berberis* asiatica and *Carissa opaca* showed high (13 – 23) antimicrobial effect on all the bacterial strains while *E. hirta*, *Z. armatum* and *A. vesica* exhibits minimum (6 – 15) effects (Singh *et al.* 2012). Das *et al.*(2012) assayed that the antimicrobial potentiality of seven day-old fresh water extracts of *Triticum aestivum* L. at concentrations of 1.0 mg/mL on the growth of four bacterial strains such as two Gram-positive strains and two Gram-negative strains and one fungus, mostly foodborne including pathogens, was studied. It was found to be effective against all the tested organisms, with Gram-positive strains being more sensitive than Gram-negative strains. The each minimal inhibitory concentration (MIC) was studied by a gradient plate method. Among all strains, *Bacillus cereus* was found to be the most sensitive followed by *Staphylococcus aureus*, while Gram-negative *Escherichia coli* were found to be the least sensitive. The viable count technique were used for

evaluated the bacteriostatic / bactericidal effects of 1.0 mg/mL wheatgrass extract *B. cereus* was found to be the most sensitive, demonstrating 1 log cycle reduction.

The methanol, ethanol and aqueous extracts of *Phyllanthus niruri* (stone breaker) were evaluated for *in vitro* antimicrobial activity against medically important bacteria such as *Staphylococcus* sp., *Escherichia coli*, *Klebsiella* sp., *Pseudomonas* sp. by agar well diffusion method and disc diffusion method. The ethanolic and aqueous extracts showed minimum antimicrobial activity when compared to methanolic extracts. Now a days, the use of plant extracts with known antimicrobial properties, can be of great significance in therapeutic treatments (Selvamohan *et al*. 2012). Sen *et al*. (2012) reported that antimicrobial efficiency of *Melia azedarach* L. a medicinal plant (leaf extracts) were tested using different solvents like methanol, ethanol, petroleum ether and water against eight human pathogens both bacteria and fungi. These were *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and fungi: *Aspergillus niger*, *Aspergillus flavus, Fusarium oxisporum* and *Rhizopus stolonifer*. The antimicrobial efficiency were checked by using agar well diffusion method and Minimum inhibitory concentration. Plants showed significant activity against all pathogens. But the alcoholic extract of *M. azedarach* showed maximum zone of inhibition and minimum inhibitory concentration against all the microorganisms. Against all experimental strains Water extract of *M. azedarach* showed minimum zone of inhibition and the maximum zone showed in petroleum ether extract. It is observed in this experiment that the alcoholic extracts of this plant could be the future source of potent herbal medicines for the treatment of infections.

Different solvent extracts viz., petroleum ether, chloroform, ethyl acetate and methanol of the medicinal plant *Toona ciliata* (leaf and flower) were used for phytochemical analysis, antimicrobial activity and antioxidant activities. Antibacterial and antifungal activities were checked by disc diffusion assay against human and phyto-pathogens. Then MIC was carried out by Micro-broth dilution method for pathogenic bacteria and fungi and radical scavenging activity was also studied using DPPH and ABTS method. The experiments revealed the presence of carbohydrates, proteins, phytosterols, flavonoids, glycosides, tannins and phenolic compounds in ethyl acetate and methanolic extracts. Moderate activity was found against *Proteus mirabilis* and least activity against *Klebsiella pneumoniae*, *Salmonella typhi* and *Staphylococcus aureus* compared to tetracycline. The both extracts of exhibited

lowest MIC varied from 10-2.5 mg mL-1 against test human pathogenic and phyto-pathogenic bacteria as well as significant antifungal activity against *Microsporum canis* was observed in methanol extract with an MIC, 1.25 mg mL-1 compared to miconozole. The entire test showed significant DPPH and ABTS radical scavenging activity in comparison with BHT and plant *Toona ciliata* could be exploited for the isolation of bioactive compounds which could be a potential source for antimicrobials and antioxidants (Kavitha *et al*.2013). In a study conducted by Oliveira *et al*. (2013) antimicrobial activity against human and animal pathogenic microorganisms by using the aqueous extracts of currently utilized Amazonian medicinal plants were tested. Resuspended lyophilized aqueous extracts of different organs of Amazonian medicinal plants were mostly used by *in vitro* screening for antimicrobial activity. The ATCC and standardized microorganisms collected from Oswaldo Cruz Foundation/Brazil were grown in agar plate individually and homogeneously. Holes previously perforated in the gel were filled with diluted plant aqueous extracts. The inhibition halos were evaluated and controlled by the use of fluoroquinolone ciprofloxacin. *Hymenelobium petraeum* (Amazonian medicinal plant) showed inhibitory activity over *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonellar typhi*, *Acinetobacter baumannii* and *Candida albicans*, while *Vatairea guianensis* and *Symphonia globulifera* presented inhibitory activity exclusively for *Staphylococcus aureus*. Other than these, *Ptychopetalum olacoides* and *Pentaclethra macroloba* also inhibited the growth of *Klebsiella ozaenae* and *Acinetobacter baumannii*. These work revealed that the aqueous botanic extracts that showed activity against microroganisms of ATTC and Osvaldo Cruz strains had at least 40% of antimicrobial activity in comparison to commercial available antibiotic like ciprofloxacin, utilized as a control in this study. The *Hymenelobium petraeum* had the best performance, sometimes exhibiting higher activity than ciprofloxacin. But it is not well-defined by the physicians the exact indication of the majority of medicinal plants in the Amazon area in Brazil. Natives also utilize the plants according to their symptoms, mainly based on the traditional knowledge transmitted orally from generation to generation, among Amerindians, Afrodescendents and ethnic mixed populations. Still now a significant number of Amazonian medicinal plants are totally unknown related to their medicinal properties including mechanism of action and therapeutic effects, as very few information is available to scientific arena. A small

amount of data showed preliminary antimicrobial properties of the medicinal plants here accessed.

To check the possible *in vitro* interaction between natural plant extracts of *Nasturtium officinale* R. Br. and 2-phenylethyl isothiocyanate, a natural compound derived from gluconasturtiin, largely present in *Nasturtium* tissues, with a standard antibiotic and a synergy effect, a test was carried out against some isolates of extended-spectrum β-lactamases-*Escherichia coli*. Minimum inhibitory concentration and disc diffusion assay were applied to evaluate the antibacterial activity of methanol and aqueous watercress extracts and 2-phenylethyl isothiocyanate combination. The results showed that there is an increase in antibacterial activity of the antibiotic when it was combined with plant extracts and pure compounds. Finally the most interesting result was the combination between 2-phenylethyl isothiocyanate and the antibiotic. Synergistic effects of the antibiotic with watercress extracts and 2-phenylethyl isothiocyanate suggest the potential of these plants and their natural compounds to improve the performance of the antibiotics. These could be an interesting tool for antimicrobial therapy. It was concluded from the results that watercress has important pharmacological substances which can be used for developing new and effective antimicrobial agents **(**Freitas *et al*. 2013)

Plant derived medicines had a major significant contributions towards human health and plants and were a great source of novel drug compounds due to their antibacterial activity. Sensitivity to chemical substances varies from one strain of microorganisms to other. The active leaf extract of medicinally important plants obtained by aqueous and solvent extraction was tested against *E coli*, *Pseudomonas* and *Klebsiella*. The aqueous lemon leaf extracts showed a good inhibitory response against *E. coli* and ethanolic extract of *Eucalyptus* leaf showed best antimicrobial activity against *Klebsiella*. But *Pseudomonas* showed resistance to all the solvents except to ethanol extracts of *Tulsi* leaf (Zwetlana *et al.* 2014).

Amenu (2014) established that the antibiotics played the main role for the therapy of microbial (bacterial and fungal) infections. There is a continuous and urgent need to discover new antimicrobial compounds as chemotherapeutic agents with diverse chemical structures and novel mechanisms of action because there was a belief in the medical fraternity that these would lead to the eventual eradication of infectious diseases. A big concern is the development of resistance to the antibiotics in current clinical use. In recent days, so many drugs have become resistant to human

pathogenic bacteria which have been commonly reported from all over the world. In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this had necessitated a search for new antimicrobial substances from other sources including medicinal plants. Higher plants mainly produce thousands of diverse chemical compounds with different biological activities. The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms. So it is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. Due to the activation of efflux pumps in Gram-negative bacteria, the morbidity and mortality caused by bacterial infections significantly increased with resistance to commonly used antibiotics. Demetrio *et al*. (2015) reported that crude ethanol extracts of Philippine medicinal plants were evaluated for their antibacterial activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, extended spectrum b-lactamase-producing, carbapenem-resistant Enterobacteriaceae and metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Leaf extracts of *Psidium guajava*, *Phyllanthus niruri*, *Ehretia microphylla* and *Piper betle* showed antibacterial activity against the Gram-positive methicillin- resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. *P. betle* showed the highest antibacterial activity for these bacteria by the disk diffusion (16–33 mm inhibition diameter), minimum inhibitory concentration (19–156 mg/mL), minimum bactericidal concentration (312 mg/mL) assays. Similarly leaf extracts only showed remarkable antibacterial activity for all the Gram-negative multidrug-resistant bacteria (extended spectrum b-lactamase-producing, carbapenem-resistant Enterobacteriaceae and metallo-b-lactamase-producing) by the disk diffusion (17–21 mm inhibition diameter), minimum inhibitory concentration (312–625 mg/mL) and minimum bactericidal concentration (312–625 mg/mL) assays. Favorable antagonistic activities were also exhibited by the ethanol extracts of *Psidium guajava, Phyllanthus niruri* and *Ehretia microphylla*. Abdulkadir *et al*. (2015), aimed to investigate the phytochemical compositions and in-vitro antimicrobial activities of ethanolic extracts of *Moringa oleifera* Lam against isolates of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* by phytochemical screening, basic pharmacognostic procedures and agar well diffusion assay respectively. In this investigation alkaloids, flavonoids, saponins and tannins were detected in all extracts with the exception of root which was devoid of saponins and

the seeds which contained no tannins. The agar well diffusion assay showed antimicrobial activities of this extract against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The Minimum Inhibitory concentrations (MIC) values were (25 mg/mL and 50 mg/mL for the root), (100 mg/mL for the seed) and (50 mg/ mL and 100 mg/mL for the pod) against the tested organisms. *Moringa oleifera* leaf extracts was not active against *Candida albicans* but active against *Escherichia coli* and *Staphylococcus aureus*. Standard Ciprofloxacin and Ketoconazole used as controls and inhibited the test organisms by 100% at 50 mg/mL and 25 mg/mL concentrations respectively. The results concluded that the leaf extracts had the greatest antimicrobial activity against test bacteria (12 mm at 50 mg/mL) while bark extract had the least activity (8 mm at 50 mg/mL). However, only pod extract showed significant antifungal activity (10 mm at 50 mg/mL) whereas other extracts at the same concentration, showed no antifungal activity. Findings from this study revealed that ethanolic extracts of *Moringa oleifera* Lam exhibit significant antimicrobial activities on test pathogens and thus suggests need to refine and standardize these extracts as alternative source of antimicrobial medicines. Phytochemical screening, antimicrobial and antioxidant activities of the hexane, ethyl acetate and methanol crude extracts of *Senna occidentalis* (L.) leaves were carried out by Odeja *et al*. (2015). Phytochemical assay showed the presence of tannins, alkaloids, reducing sugar, phenols, anthraquinones, resins, saponins and glycosides. Bacterial strains like *Staphylococcus aureus, Eshericha coli*, *Bacillus subtilis*, *Pseudomonas aeruguinosa*, *Salmonella typhi, Klebsiellae pneumoniae*, and fungal strains such as *Penicillium notatum, Aspergillus niger*, *Rhizopus stolonifer* and *Candida albicans* were used to carried out antimicrobial screening. Besides, free radical scavenging capacity of hydrogen peroxide was also determined to evaluate the antioxidant activities of the extracts. Pharmacologically interesting active compounds having good antimicrobial and antioxidative efficiency were found in leaf extracts of *Senna occidentalis* (L.). Ethnomedicinally this plant could be used for the treatment of various infections.

Grujić *et al*. (2015) investigated the antimicrobial and antibiofilm activities as well as chemical analysis of *Vinca minor* L. extracts in different solvent (aquatic, acetone and ethyl acetate) collected in Balkan mountains (Dinaric Alps, Serbia). Minimum inhibitory concentration and minimal microbicidal concentration of microorganisms were also determined. This experiment showed that, strong antimicrobial activity was detected against Gram-positive bacteria, especially from

genus *Bacillus* but gram-negative bacteria were not sensitive within the tested extracts concentrations. On the other hand, ethyl acetate extract showed some antifungal effect on *Rhodotorula* and *Candida* but *Trichoderma viride* was sensitive to aquatic extract of the plant. The crystal violet assay technique was used for checking the antibiofilm activity. But only ethyl acetate extract was effective against formation of biofilm by *Proteus mirabilis* and biofilm inhibitory concentration (BIC50) was at 22.8 mg/mL. Phytochemical analysis were performed for determining the total amount of phenols, flavonoids and tannins as well as antioxidant activity monitoring capability to neutralize 2.2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and reduction potential. DPPH and reduction potential of the aquatic extract of *V. minor* was significantly stronger compared to the acetone and ethyl acetate extracts.

Dzotam *et al*. (2016) reported the antibacterial activity of methanol extracts of edible plants namely *Colocasia esculenta*, *Triumfetta pentandra*, *Hibiscus esculentus*, *Canarium schweinfurthii* and *Annona muricata* against multidrug resistant Gram-negative bacterial strains. The liquid broth micro dilution was used to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the extracts. The preliminary phytochemical screenings of the extracts were conducted according to the standard phytochemical methods and polyphenols, triterpenes and steroids, as well as other classes of chemicals were present in these extracts. The *Canarium schweinfurthii* extract showed the best activity with MIC values ranging from 64 to 1024 lg/mL against 89.5% of the bacteria strains.MIC values below or equal to 1024 lg/mL were also recorded with *Triumfetta pentandra*, *Annona muricata*, *Colocasia esculenta* and *Hibiscus esculentus* extracts respectively against 15/19 (78.9%), 11/19 (57.9%), 10/19 (52.6%) and 10/19 (52.6%) tested bacteria. The lowest MIC value of C. schweinfurthii extract was (64 mg/mL) against *Escherichia coli* AG100ATet. Finally, the results provide baseline information for the use of *C. esculenta*, *T. pentandra*, *H. esculentus*, and *C. schweinfurthii* and *A. muricata* in the treatment of bacterial infections on the basis of multidrug resistant phenotypes.

Dhanya *et al*. (2016) reported that seaweeds could exhibit various antimicro bial properties and indigenous bioactive compounds. The emergence of drug resistant strains had directed to the identification of prospective metabolites from seaweed and its endophytes, thereby exploiting the properties in resisting against bacterial diseases. The antimicrobial activity of *Ulva reticulata* extracts and metabolites, endophytes

were assessed against the human pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Bacillus subtilis*. They observed that the hexane extract of isolate VITDSJ2 was effective against all the tested pathogens but a significant inhibition was observed for *Staphylococcus aureus* and *Escherichia coli*. Further, Gas chromatography coupled with Mass spectroscopy (GC-MS) revealed the existence of phenol, 3, 5-bis (1, 1-dimethylethyl) in the crude hexane extract which was well-known to possess antibacterial activity. The effective isolate was closest neighbour of *Pseudomonas stutzeri* by phenotypic and genotypic methods. The compound in the crude extract of *Ulva reticulata* was identified as hentriacontane using GC-MS. The extracts obtained from dichloromethane did not shown significant activity in comparison with the hexane extracts. Science the metabolites of *s* and the bacterial secondary metabolites of the endophytes could be used in the treatment of bacterial infections. Wikaningtyas *et al.* (2016) assayed antibacterial activity of medicinal plants against methicillin resistant *Staphylococcus aureus* (MRSA), extended spectrum beta-lactamase and carbapenemase-resistant *Enterobacteriaceae*, which was the most prevalent caused of infections in inpatients. The antibacterial activities were calculated based on the minimum inhibitory concentration used Mueller–Hinton broth in a micro dilution technique. Antimicrobial potentialities were shown by the *Kaempferia pandurata* (Roxb) extract (256 mg/mL) and the *Senna alata* extract (512 mg/mL). Phytochemical screening of dried *S. alata* leaf extract showed the presence of flavonoids, alkaloids, saponins, quinones, tannins and sterols, while dried *K. pandurata* extract only showed the presence of flavonoids and sterols/triterpenoids. *K. pandurata* and *S. alata* had the potential to be established as antibacterial agents or as an antibiotic especially against MRSA strain. In another study Odeja *et al*. (2016) performed phytochemical screening, antioxidant and antimicrobial activities of hexane, ethylacetate and methanol crude extracts of *Acalypha ciliata* plant. According to folk medicine, this plant was used for treatment of female sterility, dressing of sores and schistosomiasis. To check the presence of flavonoids, tannins, alkaloids, reducing sugar, anthraquinones, resins and glycosides, phytochemical test were performed. *Staphylococcus aureus*, *Eshericha coli*, *Bacillus subtilis*, *Pseudomonas aeruguinosa*, *Salmonella typhi*, *Klebsiellae pneumoniae*, *Candida albicans*, *Aspergillus niger*, *Penicillium notatum* and *Rhizopus stolonifer* were used for antimicrobial assay. The free radical scavenging capacity using hydrogen peroxide was equally determined to evaluate the antioxidant activities of the

extracts. The result showed that *Acalypha ciliata* extracts had interesting pharmacological active compounds and antimicrobial effects, and as such could be used in ethno medicine for treatment of bacterial infections and ailments.

Ginovyan *et al*. (2017) very recently reported that antibiotic resistance has become one of the major problems facing humanity and need for new antimicrobials have been increased dramatically. So natural source mainly plants are considered as one of the most promising sources for new antimicrobials discovery. In recent work, Armenian folk medicine plant materials have been used to treat various microbial diseases since ancient times. The goal of this research was to evaluate antimicrobial efficiency of different parts of different wild plants species which are commonly used in Armenian traditional medicine. Different solvents like distilled water, methanol, chloroform, acetone, and hexane were used for the preparation of crude extracts for initial evaluation of antimicrobial properties of plant materials against bacterial by agar well diffusion technique. Minimum inhibitory and bactericidal/fungicidal concentrations of selected plant parts were also determined by broth micro dilution method. The results showed that the crude acetone and hexane extracts of *Hypericum alpestre* and acetone extract of *Sanguisorba officinalis* inhibited the growth of *P. aeruginosa* even at 64 μg mL^{-1} concentrations. Similarly chloroform and acetone extracts of *S. officinalis* exhibited cidal activity against *P. aeruginosa* till 256 μg mL^{-1}. In this this experimental work, acetone was the most effective solvent for solubilising antimicrobial compounds for almost all tested plant materials.The development of antibiotic resistant bacteria has occurred due to various circumstances, including inappropriate use of antibiotics in human and animal health and their prolonged use as growth promoters at sub-clinical doses in poultry and livestock production. Nine plant species were chosen with good activity against *Escherichia coli* based on earlier work in the *Phytomedicine Programme*. Acetone was used to prepare the extract and their minimal inhibitory concentration (MIC) values determined by uesd a microplate serial dilution technique against Gram-positive and Gram-negative bacteria. The numbers of bioactive compounds in each extract were determined by Bioautography. The extracts were active against all the pathogens with average MICs ranging from 0.02 to 0.52 mg/mL. *E. coli* was relatively sensitive, but *E. faecalis* and *S. typhimurium* were more resistant to the extracts. *Cremaspora triflora* and *Maesa lanceolata* leaf extracts had higher activity than the other extracts against Gram-positive and Gram-negative pathogens

respectively. Extracts of *Maesa lanceolata* and *Hypericum roeperianum* had the highest antibacterial activity (TAA) at 1417 and 963 mL/g respectively. All extracts with the exception of that of *Maesa lanceolata, Elaeodendron croceum* and *Calpurnia aurea* had showed relatively low cytotoxicity. *Cremaspora triflora* had the best selectivity index (SI) against *S. aureus* and *E. coli*. *Hypericum roeperianum* had a SI of 1.10 against *B. cereus*. Bioautography revealed 1–6 visible antimicrobial compounds that were generally non-polar. There was a weak positive, but non-significant correlation between the potency of the extracts and their cytotoxicity ($R= 0.45$, $\rho > 0.05$). The activity of the extracts on the test bacteria was in some cases not correlated with cytotoxicity, as shown by selectivity indices >1. This means that cellular toxicity was probably not due to compounds with antibacterial activity. Some of the extracts had a good potential for therapeutic use against the bacterial pathogens or for application in treating diarhoea. It does not appear that activity against *E. coli* is a good predictor of activity against Gram-negative rather than Gram-positive bacteria. Further investigation is in progress on *C. triflora* and *H. roeperianum*, both of which had promising activities and potential safety based on cytotoxicity (Elisha *et al.* 2017).

Sadiq *et al*. (2017) evaluated the antibacterial activity and mode of action of *Acacia nilotica* and the antibiogram patterns of foodborne and clinical strains of *Escherichia coli* and *Salmonella*. Morphological damages including cell integrity and cell membrane permeability, as well as changes in cell structures and growth patterns in kill-time experiments were observed to understand the mechanism of acacia against *E. coli* and *Salmonella*. The clinical isolates of *E. coli* and *Salmonella* were found resistant to more of the tested antibiotics, compared to food isolates. Minimum inhibitory concentration and minimum bactericidal concentration of acacia leaf extracts were between 1.56–3.12 mg/mL and 3.12–6.25 mg/mL, respectively. Also pods and bark extracts had higher values. The range was between 3.12–6.25 mg/mL and 6.25–12.5 mg/mL, respectively, against all tested pathogens. The release of electrolytes and essential cellular constituents (proteins and nucleic acids) indicated that acacia extracts damaged the cellular membrane of the pathogens. These changes resulted in simultaneous reduction in the growth of viable bacteria. This study indicated that *A. nilotica* could be a new source of new antimicrobials, effective against antibiotic-resistant strains of pathogens.

Mishra *et al.*, (2017) investigated the effectivity of tropical flowering plants of *Anogeissus acuminata*, *Azadirachta indica*, *Bauhinia variegata*, *Boerhaavia diffusa*, *Terminalia chebula, Punica granatum*, *Soymida febrifuga*, *Tinospora cordifolia* and *Tribulus terrestris* for possible use as an antibiotic against multidrug resistant (MDR) bacteria. Pathogenic bacteria were isolated from urine samples of patients admitted in the hospital. Because in present days, urinary tract infection (UTI) has become a more grievous problem, due to multidrug resistance of infecting Gram-positive (GP) and Gram-negative (GN) bacteria, sometimes even with multiple infections. Antibiograms of these isolated bacteria (GPs, *Enterococcus faecalis* and *Staphylococcus aureus*; and GNs, *Acinetobacter baumannii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae, Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* were ascertained by the disc-diffusion method. The antibacterial effectivity of plant extracts was monitored by the agar-well diffusion method. Methanol extracts of these plants were used, and 3 plant extracts, *A. acuminata*, *P. granatum* and *S. febrifuga* at least caused 25–29 mm as the maximum size of inhibition zone on bacterial lawns culture. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were also recorded. Final result showed the methanol extract of *A. acuminata* had 0.29 mg/mL as the lowest MIC value and 0.67 mg/mL as the lowest MBC value, against MDR *S. aureus*. But it had the highest MIC value.

2.5. Antidiabetic activity of plants

Increased level of blood and urine glucose is commonly known as diabetes. Search of alternative remedy for increased blood sugar from plants has attained great attraction in recent years. Several earlier workers have reported antidiabetic activity of plants and their probable use as therapeutic agent.

Twenty one days antihyperglycemic study of *Polyalthia longifolia* var. *angustifolia* stem bark methanol extract showed significant reduction in fasting blood glucose levels by 48.83 % at an extract concentration of 200 mg/kg body weight and 53.68 % at an extract concentration of 200 mg/kg body weight. They have also reported a low toxicity level (LD50 >3 g/kg) in methanolic extract of this plant bark. They performed oral glucose tolerance test, which shows positive result with the application of this stem bark extraction at different concentrations. Their study shows restoration of elevated triglyceride, total cholesterol level near normal level with the application of *Polyalthia longifolia* var. angustifolia stem bark methanol extract. The

estimated parameters e.g. SGPT,SGOT,ALP and total protein shows positive results in this experiment, indicating satisfying antihyperglycemic activity of this extract (Ghosh *et al*. 2010). .

Chakraborty *et al*. (2010) investigated the antihyperglycemic activity of *Cinnamomum tamala* leaves on blood sugar levels of experimental albino rats. Doses of *C. tamala* leaf extract of 125 mg/kg and 250 mg/kg body weight were applied to STZ (streptozotocin) induced diabetic experimental rats up to 21 days, and at the 250 mg/kg body weight concentration, this extract showing exceptionally better result. The blood glucose, urine glucose levels were decreased to normal range and the body weight also improved. Thiobarbituric acid reactive substances, glutathione, glycogen content were also changed to near normal in STZ induced rats after extract administration in experimental rats. A very in depth study by Sharma *et al.* (2010) shows the hypoglycemic activity of ethanol extract of *Ficus glomerata* leaves on alloxan monohydrate induced diabetes in male albino rats. They treated alloxan induced rats with different concentrations of *F. glomerata* leaves extract. At an extract concentration of 100 mg/kg body weight blood glucose level comes to 255.42 at 10^{th} day from its initial level of first day 291.76 and at the maximum concentration i.e. 500 mg/kg body weight the change in glucose level is very significant from 287.48 to 189.83. Serum urea, serum cholesterol, serum creatinine, serum protein and body weight were also close to normal after 10 days of experiment. Hassan *et al*. (2010) investigated the *in vivo* hypoglycemic activity of a cultivated medicinal plant *Gynura procumbens*. This plant belongs to the family Compositae and is extensively cultivated in Thailand, Malaysia, and Indonesia. Male Sprague-Dawley (SD) rats were used as experimental model. Diabetes was induced in these rats by intraperitoneal streptozotocin injection. Intestinal glucose uptake and abdominal skeletal muscle absorption of glucose showed positive results in stz induced rats with the administration of this plant extract at the end of this i.e. 14^{th} day of experiment. Immuno histochemical study of β cells of pancreas were also performed and the results were not very promising, and the researchers concluded that the plant extract reduces the glucose level of blood but does not have any good effect on the β cells of pancreas. They also used RIN-5F cells for *in vitro* study of this aqueous extract of this plant and intestinal glucose absorption and muscle glucose uptake tests were shown to have good results.

Akah *et al*. (2011) experimentally proved the effectiveness of *Gogronema latifoliuma*, an ethno medicinally important antidiabetic plant used by the Nigerian rural dwellers. The methanol extraction was first done by soxhlet apparatus and after that this extract was separated by column chromatography into methanol fraction, hexane fraction and chloroform fraction. Water extraction was done by cold maceration process. Blood glucose levels of alloxan induced rats were measured to calculate the effectiveness of these extracts in proper scientific manner. The results indicated that the intraperitoneal injections of methanol fraction were most effective among all extracts and fractions and the chloroform fraction were least effective. They calculated the LD50 of methanol fraction to be 900 mg/kg fraction (CF). The antidiabetic activity of *Albizzia odoratissima* Benth. bark methanol extract was investigated by Kumar *et al.* (2011). Methanolic bark extract of this plant was administered on alloxan induced diabetic rats at a dose of 250 and 500 mg/kg body weight. The blood glucose levels were significantly ($P<0.01$) reduced to near normal level. Decreased total protein, serum cholesterol, triglycerides, alkaline phosphate, SGPT and SGOT in alloxan induced diabetic rats treated with this extract indicated its antidiabetic potential. Heart, kidney, pancreas, spleen and liver were also tested and found almost protected from the adverse effect of alloxan in induced diabetic rats with the application of this extract. Traditional pharmacopeia was done by Vianna et al. (2011) in Cree of Eeyou Istchee, an area of Canada for the screening and validation of locally used antidiabetic plants. Among these plants *Sorbus decora* was selected. Male Sprague-Dawley and KK-Ay mice were used for *in vivo* study of the anti-diabetic activity of the plant ethanol plant extract (*Sorbus decora*).The promising result of this study confirmed the traditional knowledge of this plant as a potential antidiabetic medicine source. Antididiabetic activity of methanol extract of *Elaeodendron glaucum* Pers. a plant from the family Celastraceae was also tested by Lanjhiyana (2011). Alloxan induced diabetic adult male Charles-Foster (CF) albino rats were used to evaluate the antidiabetic properties of this plant extract. Positive results were found in study of Oral Glucose Tolerance Test, study on normoglycemic rats and study on Alloxan-induced diabetic rats. This evaluation process of 21 days shows potent hypoglycaemic of this plant extract. *Orthosiphon stamineus* leaf powder were extracted with different solvents like petroleum ether, chloroform, methanol and water (polarity wise) for the investigation of antidiabetic activity of different solvent extracts. Among these extracts, chloroform extract showed good antidiabetic property

($P < 0.05$). With the help of dry flash column chromatography method this chloroform extract again partitioned into five fractions and here also the chloroform fraction 2 (C*f*2) shows good result and decreasing blood glucose significantly in streptozotocin-induced diabetic rats. These Cf2 fractions were again fractioned into two sub fractions Cf2-A and Cf2-B and here the Cf2-B sub fraction shows more activity. Although the researchers concluded these results by saying that this Cf2-B neither has any direct neither effect on insulin secretion nor any direct effect on lowering blood glucose levels in experimental rats. The secret ethno botanical knowledge of coastal areas of southeast India regarding traditional uses of *Aegiceras corniculatum* (Linn) Blanco, an antidiabetic mangrove plant were experimentally established as a potent antidiabetic plant by Gurdeeban *et al*. in the year (2012). In this experiment diabetes was induced in experimental adult wistar rats by intraperitoneal injection of alloxan monohydrate. Leaf suspension of *A. corniculatum* was administered to the experimental rats orally with the help of an intragastric tube upto 60 days. After 60 days of treatment glucose-6phosphatase, fructose 1, 6 bisphosphatase and liver hexokinase were tested in both control set as well as treated sets of albino experimental rats. The result of this experiment shows moderate decrease in blood glucose level from 382 ± 34 to 105 ± 35. The fructose 1, 6-bisphosphatase, glucose-6 phosphatase activity were also reduced. Glycosylated haemoglobin and liver hexokinase also showed almost normal level. Hypoglycemic activity of *Carica papaya* leaves in intraperitoneal streptozocin induced diabetic rats was investigated by Juárez-Rojop *et al*. in the year 2012. Three different doses (0.75, 1.5 and 3 g/100 mL) of aqueous extract of *Carica papaya* were administered by mixing this with drinking water at a desired concentration. This extract reduces the blood glucose level ($p<0.05$), amino-transferases, cholesterol, and triglycerol in experimental diabetic ratssignificantly after 4 weeks of experiment. In this experiment islet regeneration manifestation was also reported. It also prevented hepatocyte disruption, glycogen and lipid accumulation. Alloxan –induced diabetic rats were used to evaluate the antidiabetic activity of ethanol root extract/fraction of *Anthocleista djalonensis*. 14 days administration of *A. djalonensis* root extract/fractions at a concentration ranging from 37mg/kg to 11 mg/kg to the alloxan induced rats revealed that this extract/fraction have significant capacity to reduce blood glucose level $P<0.001$. This results proves the authenticity of ethnic knowledge of west African people (Jude *et al*. 2012). *In vitro* antidiabetic activity of *Zingiber*

officinale rhizome water extract was evaluated by non-enzymatic and enzymatic glycation and Glucose diffusion inhibition method. Result shows positive, and attracts further investigation of this rhizome (Sattar *et al*. 2012). Different solvent extract of *Cynodon dactylon* leaf was assessed for its antidiabetic activity. Alloxan induced diabetic rats were treated with different extracts for 21 days. Urea, blood glucose, triglycerides and cholesterol levels were showing positive signs in methanol extracts as well as in petroleum ether and chloroform extract. Further investigation also suggested by this group of investigator to isolate potent compound from this plant (Ramya *et al*. 2014).

Tectona grandis Linn is a wood producing tree from the family Verbenaceae. *In vivo* antidiabetic activity of the bark of *T. grandis* was evaluated by Rajaram *et al*. (2013). Alloxan induced diabetic rats were treated with methanol bark extract for twenty days and results were compared with other tested group (Normal, Diabetic control) of rats as per standard *in vivo* antidiabetic experimental procedure. Two concentrations (150mg and 300mg) of this extract were applied and 300mg concentration was more potent. Protein, catalase, glycosylated haemoglobin, blood glucose, Glucose-6-phosphatase (G6P) urea, Serum creatinine, total cholesterol, Thiobarbituritic acid reactive substances (TBARS), Alanine Transaminase (ALT), Aspartate Transaminase (AST), Lactate Dehydrogenase (LDH) were assayed and found that *T. grandis* bark MEOH extract have potent antidiabetic activity in alloxan induced diabetic rats ($p<0.05$).

Cardiospermum halicacabum leaf methanol, n-hexane and ethanolic crude extract were evaluated for their *in vitro* antidiabetic activity. *In vitro* inhibitory Glucose diffusion method was applied to evaluate the efficiency of different extracts of this plant. 27 hours of experiment revealed that the potent concentration of extract was 50g/L and methanol extract has maximum activity among these three extracts. This experiment proves the authenticity of ethno medicinal claim of this plant as a potent antidiabetic source (Stalin *et al*. 2013). Ayurveda is a very rich source of knowledge regarding antidiabetic plants and their uses in Indian society. Recently many workers are trying to validate this knowledge through their modern experimental procedures. α-Amylase inhibitor inhibits the starch breakdown, and reduces the postprandial blood sugar level. Different *Azadiracta indica* extracts (petroleum ether, methanol, water, acetone, toluene ethanol) and extracted flavonoids were screened for α-amylase inhibitors with the help of starch-iodine test and 3, 5-

dinitrosalicylic acid assay. The result showing much higher efficiency of isolated flavonoids than crude extracts. IC 50 values were also analysed (Chitra *et al*. 2013). *Physalis minima* is a plant locally used by tribal people for the treatment of diabetes in different parts of Indian subcontinent. Sucharitha *et al*. (2013) studied the antihyperglycemic activity of hot water extract of different plant parts of *P. minima* on alloxan induced diabetic Wister strains of male albino rats. Toxicity study shows that the lethal dose must be higher than 1 g/kg body weight and at this concentration this plant extract has no side effect. The root and stem extract has no significant activity in reducing fasting glucose but the flower and leaf extracts are showing good positive results. This study sharply indicates that the plant used by ethnic people of this area is a potent antidiabetic medicine and further study is needed to identify the compound or group of compounds actually responsible for the antihyperglycemic activity of this plant extract.

Anacardium occidentale stem bark water extract were administered orally (400 mg/kg for 28 days) to evaluate its antidiabetic activity in both alloxan induced diabetic rats and normal rats. The study showed that this oral administration significantly controls ($p<0.05$) the levels of different parameter related to diabetic conditions (Sambo *et al.* 2014). Methanol extract and fractions of *Anthocleista vogelii* stem bark were experimentally evaluated for their antidiabetic activity by Osadebe *et al.* (2014). Gradient chromatographic separation was performed using ethyl acetate, chloroform, water and acetone. These fractions along with methanol extract were administered to investigate the efficiency of these in reducing diabetic effects in experimental rats. The result shows effectiveness of the plant extracts and fractions in this experiment. Acetone extract shows maximum activity and require further investigation to isolate and identify the compounds related to antidiabetic activity. Aleem *et al*. (2014) studied the antidiabetic activity of hydro alcoholic extracts of *Nardostachys jatamansi* rhizome. Two week experiment with diabetic rats reveals the efficiency of this plant extract in antihyperglycemic activity. Biochemical parameters, body weight and lipid profile were effectively changed in diabetic rats after two weak treatment with extract. Histopathological studies show that the regeneration of β-cells of pancreas in diabetic rats was also improved. *Rhizophora apiculata* leaf extracts were examined in vivo to get information regarding its antidiabetic potential. Both Insulin-dependent diabetes mellitus and non- Insulin-dependent diabetes mellitus were induced in male albino rats. Oral administration of ethanol extract,

dichloromethane (DCM-F) and aqueous basic fraction (AB-F) were applied for 21 days. At an interval of 7, 14, and 21 days blood glucose level were examined with the help of touch glucometer. Triglycerides, high-density lipoprotein cholesterol (HDL-C) and serum cholesterol were estimated at the completion of this experiment i.e. 21st days. Among these extracts dichloromethane fraction of *R. apiculata* shows much better result than the rest and require further investigation in this regard (Gurudeeban, 2015). *Musa sapientum* is a well known plant in different parts of world for its medicinal importance. Fruit peal of this plant was evaluated to establish its antidiabetic properties. Acetone extract at a dose of 200 and 400 mg/kg body weight were administered for 45 days. *M. sapientum* fruit peel acetone extract treated group shows increase of plasma insulin and decreased levels of fasting blood sugar as well as HbA1c in diabetic rats. Pancreas also histopathologically examined and result proves the antidiabetic properties of this extract. These results suggested that this extract can be used as a potent antidiabetic medicine source (Murthy, 2015).

Five Indian Ayurvedic and seven Australian Aboriginal plants were examined by Gulati *et al*. (2015) in search of possible mechanism of antidiabetic properties of these plants. Ethanol extract were applied on murine 3T3-L1 adipocytes to evaluate adipogenesis and glucose uptake modulation. Anti cancer activity of these extracts were examined with HeLa and A549 cell lines. Stimulated glucose uptake in adipocytes was found only in *Acacia kempeana* and *Santalum spicatum* among Australian plants and in *Curculigo orchioides* among Indian ayurvedic plants. *Euphorbia drumondii, Acacia tetragonophylla* and *Beyeria leshnaultii* from Australian group and from ayurvedic group *Curculigo orchioides*, *Pterocarpus marsupium* and *Andrographis paniculata* were adipogenesis positive. Against HeLa cells, *Acacia kempeana* and *Acacia tetragonophylla* showed specific and potent activity.

Oloyedea *et al*. (2015) investigated the antidiabetic activity of a plant (*Dioscoreophyllum cumminsii*) locally used for the treatment of diabetes in Nigeria. At a concentration of 50, 100 and 200 mg/kg body weight, extract of *D. cumminsii* was applied to the alloxan-induced diabetic rats. After 21 days of experiment result shows that the highest dose *i.e* 200 mg/kg body weight shows 72% reduction in blood glucose. This water extract of *D. Cumminsii* also increases the insulin production in alloxan induced diabetic rats. Significant restoration of levels of serum and liver high-density lipoprotein cholesterol (HDLc), cholesterol, triacylglycerides, low density

lipoprotein cholesterol, and very low-density lipoprotein cholesterol were also noticed. Oxidative stress and dyslipidemia were also prevented.

Poly herbal mixture of *Azadirachta indica*, *Bougainvillea spectabilis* and *Trigonella foenum-graecum*, were examined for their antidiabetic potential. The mixture contains (1:2:3) *A. indica* chloroform leaf extract, *B. spectabilis* aqueous leaf extract and *T. foenum-graecum* ethanol seed extract. This polyherbel mixture at a dose of 600 mg/kg body weight was administered orally for 28 days to streptozocin induced Sprague-dawley diabetic rats. Fasting glucose, body weight, total cholesterol and triglyceride levels were found statistically significant (Gupta *et al*. 2016). Chowtivannakul *et al*. (2016) investigated the antidiabetic property of *Leucaena leucocephala.* Limited scientific works were performed previously in this plant. Ethanol extract of seed of this plant were orally administered at a dose of 250 mg/kg body weight for 6 week. The experimental setup of streptozocin induced diabetic rats which orally received the extract shows significant reduction in fasting glucose level and the Akaline phosphatase, albumin, red blood cells, total protein and serum insulin levels were also become almost normal. Manikandan *et al.* (2016) investigated the *in vitro* antidiabetic activity of *Psidium guajava* leaves. The experimental setup includes the *in vitro* antidiabetic assay (alpha amylase and alpha glucosidase) of *Psidium guajava* leaves ethanol, chloroform, petroleum ether, aqueous, hexane extracts. The aqueous extract inhibited the alpha amylase activity 72.1% and alpha glucosidase enzymes 74.8% .The ethanol extract inhibited the alpha amylase 97.5% and alpha glucosidase enzymes 91.8%. They have suggested further study to isolate active compound from these extract.

In vivo antidiabetic assay of ethanol extract of *Alternanthera ficodia* were performed by Rajan *et al*. (2016). Oral dose of this extract at a concentration of 400 mg/kg body weight were applied to the streptozocin induced diabetic rats for 15 days. This extract treated diabetic group shows significant reduction of blood glucose levels.

Turnera diffusa is a very common Mexican plant used traditionally by local people as a potent antidiabetic agent. Methanol extract were fractionated and the fractions were studied through in vitro assay of the fractions. Experiment with *in vivo* murine model reveals hypoglycemic and antidiabetic activities of this plant extracts/fractions (Parra-Naranjo *et al*. 2017).

In vivo and *in vitro* study of *Crassocephalum crepidioides* hydromethanolic extract was performed by Bahar *et al*. (2017). Wistar albino rat were used for oral glucose tolerance test as well as alloxan-induced diabetic test. Histopathological studies of pancreatic specimens from extract treated diabetic rats were executed at the end of the experiment. Pancreatic β-cell culture and α-amylase inhibition technique were employed to evaluate the in vitro antidiabetic potential of this plant. Result shows significant effect of this extract in both in vivo and in vitro experiment. They conclude at the end of their study by saying that the extract can protect β-cell from the adverse effect of diabetes and can protect us from diabetes as well.

Methanol extract of three plants were evaluated for their antidiabetic activity against streptozocin induced diabetes in rats. Methanol extract of *Schrebera swietenoides* (aerial parts), *Barleria montana* (root) and *Rotula aquatic* (aerial parts) were applied orally at a dose of 100, 200,400 mg/kg body weight. *Barleria montana* among the three plants at a dose of 400 mg/kg body weight significantly reduces the blood glucose levels within 4^{th} and 8^{th} hours **(**Balakrishnaiah 2017).

Available literature thus indicates that obtaining medicines from plants is a premier area of research today globally. Almost all tested plants have some or other medicinal properties. Proper scientific validation of preliminary tests, specially in *in vivo* studies are needed. It is interesting that many of the plants have a number of properties and such plants with multiple characteristics should be targeted.

3. MATERIAL AND METHODS

3.1. Ethnobotanical survey and selection of plant materials

3.1.1. Survey of wetland plants

Locally available wetland plants in the districts of North Bengal region of West Bengal were collected. The plants were selected mostly on the basis of their importance in this area. Useful wetland plants were collected through survey based field observations. Several field trips to different wetlands of North Bengal were conducted to collect the plant samples from December 2012 to January 2015. Traditional medical practitioners of this region like Kaviraj, Jahakri, Janguru and locales with knowledge related to the use of wetland plants were interviewed to collect information regarding the importance of these plants. This study was also based on information gathered from the seller and consumers of the plants in market as well as from the people living in the vicinity of these wetland studied. The sellers and consumers were interviewed and discussed with questionnaires for collecting the information on the uses of these plants and the daily need, market value, seasonal variation of availability in markets. The collected data is based on first hand information and after that various literature were also discussed. The collected plants were dried and herbarium specimens were prepared which later on identified with the help of floras, herbaria as well as in consultation with Taxonomy experts. Final authentication was done at Taxonomy & Environmental Biology Laboratory, Department of Botany, University of North Bengal. Herbarium sheets of selected plants were submitted to the North Bengal University Herbarium, Department of Botany, University of North Bengal, India and accession numbers were obtained.

3.1.2. Selection of plant material for experimentation

Out of the surveyed plants with medicinal and other beneficial properties, 15 plants were finally selected for studying the potentiality of these plants as reported.

3.2. Preparation of plant extract

3.2.1. Preparation of dried plant powder

The collected wetland plants were primarily washed thoroughly (4-5 times) with normal tap water and then with distilled water and dried using blotting paper. The cleaned plant materials were covered with thin blotting paper and allowed to dry under the shade for about 10 to 21 days. Special care was taken to stop spoilage due to fungal degradation of plant material. The dried plant materials were ground to

obtain fine powder using mixer grinder (Jaipan, Super Deluxe, India) and was stored in glass bottles at 4°C till further use.

3.2.2. Preparation of methanol (MEOH) extract

With slight modification, the method described by Okwori *et al.* (2006) was used for the preparation of methanol extract. Powdered samples and methanol were mixed in the ratio of 1:10 (sample:solvent) and vigorously shaken for 20 min and then kept for 72h at room temperature while stirring for 40 min at an interval of 24h. The mixture was then filtered and the supernatant were concentrated in rotary evaporator, at 40°C, lyophilized and stored at -20°C until further use.

3.3. Phytochemical screening

3.3.1. Test for phenol

Presence of phenol in the methanolic extract was determined by following the method of Martinez and Valencia (2003). Briefly, 1 mL extract was mixed equal volume of 1% $FeCl_3$ and observed for the formation of blue or green colour which indicated the presence of phenols.

3.3.2. Test for flavonoid

The presence of flavonoid in the samples was done following the method of Evans (2002). Briefly, 2g of the sample was mixed with 10 mL of acetone and the acetone was evaporated by keeping the flask in a hot water bath for 5 min. After that the sample was extracted using 10 mL of warm double distilled water. The solution was mixed, filtered while hot and allowed to cool at room temperature. 5mL filtrate was then mixed with equal volume of 20% NaOH and observerd for the change in color of the solution to yellow indicating the presence of flavonoid**.**

3.3.3. Test for tannin

Presence of tannin in powered plant samples were determined by following the method of Jigna and Sumitra (2007) where 200 mg crude plant powder was mixed with 10 mL of double distilled water and incubated at room temperature for 10 min followed by filtration. 1mL of 5% $FeCl_3$ was added to 2 mL of the filtrate, the formation of yellow brown precipitate indicated the presence of tannin.

3.3.4. Test for alkaloid

200 mg of powdered sample was mixed with 10 mL methanol for 1h at room temperature and after filtration 2 mL of 1% HCL was added to equal volume of the filtrate. Then few drops of Mayer's / Wagner's reagent were added to the filtrate.

Apperance of creamish / brown / red / orange precipitate indicated the presence of alkaloid **(**Trease and Evans, 1989).

3.3.5. Test for cardiac glycosides

The method described by Trease and Evans (1989) was followed for the qualitative screening of cardiac glycosides. 2 mL methanolic filtrate was mixed with 1 mL glacial acetic acid, added 3-4 drops of 5% $FeCl_3$ and then 1 mL of concentrated H_2SO_4 was added carefully. Appearance of brown ring at the interface indicated the presence of cardiac glycosides.

3.3.6. Test for carbohydrates

Qualitative analysis of carbohydrates was done following the protocol of Evans (2002). Plant sample (0.5 g) was boiled in 30 mL of double distilled water and filtered. 2mL of Molish's reagent (5% α-naphthol in absolute ethanol) was then added to 2 mL of aqueous extract and shaken vigorously followed by addition of concentrated H_2SO_4. Presence of carbohydrate was confirmed by the formation of reddish-ring at the junction of two liquids.

3.3.7. Test for reducing sugars

Qualitative analysis of reducing sugars was done following the protocol of Evans (2002). Aqueous extract of the sample was prepared by boiling 500 mg sample in 30 mL of double distilled water followed by filtration. 1 mL of the filtrate was then mixed with 2 mL of Fehling's solution and boiled for 5 min. Appearnce of brick red precipitation at the bottom of test tube indicated the presence of reducing sugars.

3.3.8. Test for protein

About 1 g of the plant sample was mixed thoroughly with 10 mL of double distilled water following the method of Pullaiah (2006). Then 2 mL of the filtrate was mixed with 1 mL of 40% NaOH and 1-2 drops of $CuSO_4$. Change in the color of solution to violet indicated the presence of proteins.

3.3.9. Test for saponin

0.5 mL aqueous filtrate prepared as above was mixed with 5 mL of double distilled water and shaken vigorously for about 30 seconds following the protocol of Trease and Evans (1989). The presence of saponins was indicated by the formation and persistent frothing.

3.3.10. Test for terpenoid

For the test of terpenoid 2 mL of plant extract was mixed with 5 mL of chloroform and 2 mL of acetic anhydride. Then, 1mL of concentrated H_2SO_4 was

added carefully along the wall of the test tube, and the formation of reddish brown ring at the interface indicated the presence of terpenoid (Harborne, 1973).

3.3.11. Test for steroid

The methanolic filtrate (5 mL) was treated with 0.5 mL of anhydrous CH_3COOH followed by cooling on an ice bath for 15 mins. To the cold solution 0.5 mL of chloroform and 1 mL of concentrated H_2SO_4 was added. Presence of steroid was confirmed by the formation of reddish-brown ring at the junction (Gokhale and Kokate, 2008).

3.3.12. Test for anthraquinone

0.5g of powdered sample was added to 5 mL of chloroform, shaken for 5 min and filtered using Whatman No.1 filter paper. 3 mL filtrate was mixed with equal volume of 10% ammonia solution and shaken properly. Development of red / pink /violet color in the aqueous layer after shaking indicated the presence of free anthraquinone (Evans, 2002).

3.3.13. Test for amino acid

Few drops of ninhydrin reagent was added to 0.5 mL methanolic plant extract and then heated in a water bath. The presence of amino acid was indicated by the formation of purple colour (Kumar *et al*. 2009).

3.3.14. Test for resins

0.5 mL of extract was evaporated and dissolved in 2 mL of petroleum ether. After that, 2mL of 2% cupper acetate solution was then added, vigorously mixed and allowed to separate. Green colour of the solution indicated the presence of resin (Trease and Evans, 1983).

3.3.15. Test for glycosides

Methanolic extract (0.5 mL) was added to 2 mL of 50% hydrochloric acid and placed on a water bath for 2 hrs. 1 mL pyridine, few drops of 1% sodium nitroprusside solution and 5% sodium hydroxide solution were then added to the hydrolysed mixture. Appearance of pink to red colour indicated the presence of glycosides (Kumar *et al*. 2009).

3.3.16. Test for triterpenoids

0.5 mL of methanolic plant extract was evaporated and the residue was dissolved in 1mL chloroform. 1mL acetic anhydride was then added and after cooling, conc. H_2SO_4 was added. Appearance of violet colour confirmed the presence of triterpenoids (Kumar *et al*. 2009).

3.4. Quantification of biochemical components

3.4.1. Extraction and estimation of total phenols

3.4.1.1. Extraction

With minor modification the method given by Mahadevan and Sridhar (1982) was employed for the extraction of phenol. 1g of the powdered material was immersed in a beaker containing 10mL of boiling absolute alcohol and kept in dark for 10 mins. The sample was allowed to cool at room temperature and then crushed in motor pestle using 80% alcohol and filtered using Whatman No.1 filter paper. The residue was re-extracted with 80% alcohol making the final volume up to 10mL. The total procedure was carried out in diffuse light.

3.4.1.2. Estimation

The extract (1mL) was mixed with 1mL of 1N Folin ciocalteu's phenol reagent followed by 2mL of 20% Na_2CO_3 solution and was kept in boiling water bath for 1min. Under running tap water the reaction mixture was cooled and after that diluted with distilled water to make the final volume up to 25mL. The absorbance was taken at 650nm in a colorimeter and the concentration of total phenols was expressed as mg gallic acid (GAE) equivalents/ g dry weight sample (dw), using the standard curve of gallic acid (Bray and Thorpe, 1954).

3.4.2. Quantification of total soluble protein

3.4.2.1. Extraction

From dried powdered material the extraction of protein was done using the method of Chakraborty *et al.* (1995). 1g powdered sample was homogenized in a refrigerated motor and pestle with 5mL of 50 mM sodium phosphate buffer (pH-7.2) and polyvinyl-pyrrolidone under ice cold condition. The mixture was then centrifuged at 10,000 rpm at -4^0C for 15min. For further estimation the supernatant was collected and used.

3.4.2.2. Estimation

According to the method of Lowry *et al.* (1951) estimation of protein content in the extract was done. In short, 1mL of the extract was mixed with 5mL of freshly prepared alkaline reagent (2% Na_2CO_3 in 0.1N NaOH, to which 1mL each of 1% $CuSO_4$ and 2% Na^+ - K^+ tartarate was added just before use) and incubated for 15min. After that, 0.5mL of 1N Folin Ciocalteau's phenol reagent was added to the mixture which was further incubated for 20 min at room temoerature. The absorbance was measured at 690 nm in spectrophotometer against a proper blank. Quantification of

total protein was done by using a standard curve of Bovine serum albumin and expressed as mg Bovine serum albumin equivalent (BSAE)/ g dry weight sample (dw).

3.4.3. Extraction and quantification of flavonoid

3.4.3.1. Extraction

The extraction procedure of flavonoid, described by Mahadevan and Sridhar (1982) was followed with minor modification. 1g dried powdered material was immersed in boiling methanol (10mL) for 5-10 min and then filtered. The final volume was made upto 10 mL.

3.4.3.2. Quantification of flavonoid

The flavonoid content was estimated using the method of Sultana *et al*. (2009). The extract (1 mL) was mixed with 4mL of distilled water and 300µL of 5% $NaNO_2$ and allowed to incubate for 5min at room temperature. After 5min of incubation 300 µL of 10% $AlCl_3.6H_2O$ was added. Next, at 6^{th} min 2mL of NaOH, and 2.4 mL of distilled water was added and mixed properly. Absorbance of the reaction mixture was recorded at 510 nm in UV-VIS spectrophotometer (Model 118 systronics) against a proper blank solution. The total flavonoid content was expressed as mg quercetin equivalents (QE)/ g dry weight sample (dw) using the standard curve of quercetin.

3.4.4. Extraction and quantification of Amino acid

Free amino acids were estimated with standardized protocol with slight modifications (Moore and Stein 1948). 0.5 g leaf tissue was extracted in ethanol and filtered using Whatman No. 1 filter paper. To 1 ml of the filtrate, 1 ml of ninhydrin reagent was added and boiled in a water bath for 20 min. The absorbance was read at 570 nm and the free amino acid content was estimated using a standard curve of L-proline.

3.4.5. Extraction and estimation of carbohydrates

3.4.5.1. Extraction of total and reducing sugar

Total and reducing sugar was extracted following the method of Harborne (1973). 1 g of tissue was extracted in 10 ml of 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath. The residue was reextracted with ethanol and the process was repeated 3 times. Then the residue was dissolved in dH_2O and the

final volume was made up to 5 ml which was then centrifuged at 5000 rpm for 10 min. The supernatant was collected and used for estimation.

3.4.5.2. Estimation of total soluble sugar

Estimation of total sugar was done by Anthrone reagent following the method of Plummer (1978). To 1 ml of test solution, 4 ml of Anthrone reagent (0.2% Anthrone in conc. H_2SO_4) was added. The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10 mins. Then the reaction mixture was cooled under running tap water and absorbance was measured in a colorimeter at a 50 wavelength of 620 nm and sugar content was quantified using a standard curve of D-glucose.

3.4.5.3. Estimation of reducing sugar

Reducing sugar was estimated by Nelson-Somogyi method as described by Plummer (1978). 1 ml of the test solution was mixed with 1 ml of alkaline copper tartarate solution (2 g $CuSO_4$, 12 g Na_2CO_3 anhydrous, 8 g Na-K tartarate, 90 g Na_2SO_4 anhydrous in 500 ml of dH_2O) and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water and 1 ml Nelson's Arsenomolybdate reagent was added along with 2 ml of dH_2O and mixed vigorously. A blue colour was developed, the absorbance of which was the measured in a colorimeter at 515 nm and reducing sugar content was quantified using a standard curve of D-glucose.

3.4.6. Extraction and estimation of chlorophylls

3.4.6.1. Extraction of chlorophylls

From the samples chlorophyll was extracted following the method of Harborne (1998) with minor changes. 1g of the dried powder was crushed with 80% acetone in a mortar pestle and filtered through Whatman No.1 filter paper in a dark room. The residue was re-extracted with 80% acetone until it became completely colorless attaining final volume upto 10mL.

3.4.6.2. Estimation of chlorophylls

Total chlorophyll, chlorophyll a and b were estimated using the method of Harborne (1998). The crude or diluted filtrate was taken directly in the cuvettes and the absorbance was measured at 663nm and 645nm respectively in a UV-VIS spectrophotometer against a blank of 80% acetone. Using the formula given by Arnon (1949), the chlorophyll content was calculated.

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} dry weight

Chlorophyll a = $(12.7\ A_{663} - A_{645})$ mg g^{-1} dry weight

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} dry weight

3.4.7 Determination of carotenoid content

3.4.7.1. Extraction

Following the method of Lichtenthaler (1987), the carotenoid was extracted from the samples. About 1g of the powdered material was soaked and crushed with 100% methanol in dark condition and filtered using Whatman filter paper No.1. The final volume was made upto 10mL with methanol and used for further experiment.

3.4.7.2. Estimation

Carotenoid content was estimated by the procedure of Lichtenthaler (1987). The desired amount of crude or diluted filtrate was taken directly into the cuvate and the absorbance was taken at 480nm, 645nm and 663nm wavelength against a blank in UV-VIS spectrophotometer 118 systronics. The amount of carotenoid was calculated using the standard formula as follows:

$$A_{480} - (0.114 \times A_{663}) - 0.638\ (A_{645})\ \mu g\ g^{-1} \text{ dry weight}$$

3.4.8. Quantification of Ascorbic Acid (Vit C)

3.4.8.1. Extraction

Mukherjee and Choudhuri (1983) described method for the extraction of Ascorbic acid from the powdered samples. The samples were crushed in pre-chilled motor and pestle on ice using 6% trichloroacetic acid (10mL) and filtered at chilled condition (0°C). The final volume of the filtrate was made upto 10mL by adding trichloroacetic acid and used for further estimation of ascorbic acid.

3.4.8.2. Estimation

For this estimation, 4mL of the extract, 2mL of 2% Dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% Thiourea (70% ethanol) was added one after another and mixed thoroughly. The reaction mixture was then kept in boiling water for 15min and cooled at chilled condition in ice. To the reaction mixture, 5mL of 80% (v/v) sulphuric acid (H_2SO_4) was added at 0°C. The absorbance was taken at 530nm against a blank in UV-VIS spectrophotometer. The concentration was determined from the standard curve of ascorbic acid and expressed as mg ascorbic acid equivalent (AAE)/g dry weight sample (Mukherjee and Choudhuri 1983).

3.5. Determination of anti-oxidative activities of extracts

3.5.1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH (2,2-diphenylpicrylhydrazyl) assay measures the free radical scavenging capacity of the extracts under investigation (Blois 1958). Different concentrations of plant extracts (0.1 mL) were put in the test tube and 2.9 mL of a methanol solution of DPPH (0.1 mM) was added. The mixture was kept in the dark at room temperature for 30 min and absorbance was measured at 517 nm against a blank. The same procedure was used for the vitamin C (1 mg/mL) used as standard. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

Where, A_o is the absorbance of the blank and A_s the absorbance of the sample.

3.5.2 Scavenging effect of the $ABTS^+$ radical

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay was based on method of Re *et.al.*(1999) with slight modifications. ABTS radical cation ($ABTS^+$) was produced by the reaction of a 7 mM ABTS solution with potassium persulphate (2.45 mM). The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. The mixture was stored in the dark at room temperature for 12 h before use. After addition of 25 µL of extract sample or vitamin C used as standard to 2 mL of diluted $ABTS^+$ solution, absorbance was measured at 734 nm after exactly 6 min. The decrease in absorption was used for calculating scavenging effect values. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

3.5.3. Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity was determined according to the Griess Illosvoy reaction (Garratt 1964). The reaction mixture contained 2 mL of sodium nitroprusside (10 mM) in 0.5 mL phosphate buffer (0.5 M; pH 7.4). Various concentrations of the extracts (0.5 mL) were added in a final volume of 3 mL. After incubation for 60 min at 37°C, Griess reagent [N-(1-Naphthyl) ethylenediamine (0.1%) and sulphanilic acid (1%) in H_3PO_4 (5%)] was added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-(1-Naphthyl) ethylenediamine was measured spectrophotometrically

at 540 nm. Ascorbic acid was used as a positive control. The scavenging ability (%) of the nitric oxide was calculated using the formula:

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

Where A_o is the absorbance of the blank and A_s the absorbance of the sample.

3.5.4. Superoxide anion radical scavenging activity

Determination of superoxide anion radicals scavenging activity was based on the method described by Nishikimi *et al.* (1972) with minor changes. The superoxide anions were generated in a non-enzymatic system Phenazine methosulfate-Nicotinamide adenine dinucleotide (reduced form) (PMS-NADH), through the reaction of PMS, NADH and oxygen which was detected by the reaction with 2,2'-di-p- nitrophenyl -5,5'- diphenyl –(3,3'- dimethoxy-4,4'-diphenyline) di-tetrazolium chloride (nitro blue tetrazolium-NBT). The reaction mixture contained 1mL of sample/reference standard at differrrent concentration (10-50µg/mL), 1mL of NBT (312µM prepared in potassium phosphate buffer pH 7.4,0.2M) and 1mL of NADH (936 µM in phosphate buffer pH 7.4). Finally, the reaction was accelerated by adding 200µL of PMS solution (120 µM) to the mixture. After 5min incubation at 25°C the absorbance was taken at 560nm against proper blank sample containing phosphate buffer. The control was prepared with all the reagents except the plant extract. As a reference standard L-ascorbic acid was used. Superoxide anion radical scavenging percentage was measured using the equation as follows:

$$\text{\% Superoxide anion scavenged} = (A_0 - A_1)/A_0 \times 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the extract/standard. IC_{50} values of all extracts and ascorbic acid were also calculated.

3.5.5. Total antioxidant activity by ferric reducing antioxidant power assay (FRAP)

The FRAP was determined using the method of Benzie & Strain (1996) with slight modifications. The fresh FRAP reagent consisted of 500 mL of acetate buffer (300 mM; pH 3.6), 50 mL of 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) (10 mM), and 50 mL of $FeCl_3 \cdot 6H_2O$ (50 mM). The colorimetric measurement was performed at 593 nm and the measurement was monitored up to 12 min on 75 µL of each extract and 2 mL of FRAP reagent. The vitamin C was used to draw a standard curve and the butylated hydroxy toluene (BHT) was used for the comparison. The results were expressed as mg equivalent vitamin C/g of dried extract (mg eq VitC/g DE).

3.5.6. Metal chelating activity

With slight modification the method of Dinis *et al,* (1994) was followed to estimate the ability of the extract to chelate ferrous ions. The extract was added to a solution of 2 mM $FeCl_2$ (0.05 mL and the reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The mixture was then shaken vigorously and left to stand at room temperature for 10 min. Spectrophotometrically the absorbance of the solution was measured at 562 nm. The percentage inhibition of ferrozine-Fe2+ complex formation was calculated as Chelating rate (%) = (A0 - A1) / A0 × 100, where *A*0 was the absorbance of the control, and *A*1 of the mixture containing the extract or the absorbance of a standard solution.

3.5.7. Hydroxyl radical scavenging activity

The scavenging activity of the extracts on hydroxyl radical was measured according to a method of Yu. *et al*. (2004) with slight modifications. To 1.5 mL of each diluted extract, 60 µL of $FeCl_3$ (1 mM), 90 µL of 1,10-phenanthroline (1 mM), 2.4 mL of phosphate buffer (0.2 M; pH 7.8) and 150 µL of H_2O_2 (0.17 M) were added respectively. The mixture was then homogenized using a vortex and incubated at room temperature for 5 min. The absorbance was read at 560 nm against the blank. The percentage of the radical scavenging activity of each extract was calculated from the equation below:

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

where A_o is the absorbance of the blank and A_s the absorbance of the sample.

3.6. Sample preparation for HPLC analysis of phenolics

Extraction of total phenolics from the dried samples for HPLC analysis was done following the method described by Pari & Latha (2005) with minor changes. Two gram of powdered sample was soaked overnight in10mL of absolute methanol in dark. After 12h of soaking, the suspension was filtered and the filtrate was completely evaporated using a rotary evaporator at 40°C and lyophilized. The lyophilized extract was re-dissolved in 1mL of HPLC grade methanol and filtered through Millipore membrane filter (0.45µm).

3.7. Fingerprint analysis of total phenolics

HPLC fingerprint analysis of total phenolics was done following the protocol of Pari *et al*. (2007). The analysis was done using High Performance Liquid Chromatograph (Shimadzu) equipped with LC 10ATVP pumps, UV-Vis detector and C18 column. The HPLC program used a flow rate of 1 ml/min, binary gradient elution of

HPLC grade acetonitrile–water–acetic acid (5:93:2, v/v/v) [solvent A] and acetonitrile–water–acetic acid (40:58:2, v/v/v) [solvent B], starting with solvent B from 0 to 100% over a period of 50 min were applied. An injection volume of 20 µl of the filtrate was used and the separation of phenolics was monitored at 278 nm.

3.8. Testing of *in vitro* hypoglycemic activities

3.8.1. *In vitro* α-amylase inhibition activity

In vitro α-amylase inhibition activity of the plant extracts was tested following the method of Bernfield (1955) with minor changes. Plant extracts and positive control acarbose (500µL) of different concentrations was allowed to react with 500µL of freshly prepared α-amylase (3.246mg in 100mL of cold dH_2O) for 20min at room temperature. Then, 1% starch (1g in 0.1M, pH 4.7 acetate buffer) was added to the solution and incubated further for 15min at room temperature. In order to stop the reaction, 1mL DNSA solution (prepared in 1% NaOH) was added to the reaction mixture and kept in boiling H_2O for 5min. The mixture was cooled under running tap water. The final volume was made upto 4mL by adding 1.8mL of dH_2O and absorbance was recorded at 540nm against appropriate blank solution. Control solution was prepared with all the reagents except the plant samples. Percentage of α-amylase inhibition activityof the extracts was calculated according to the formula:

$$\% \alpha \text{ - amylase inhibition} = (A0 - A1)/A0 \times 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the extract/standard. IC_{50} values for all the extracts and acarbose were also calculated.

3.9. Testing of antimicrobial activities

3.9.1. Sample preparation

To determine the antimicrobial activity the extracts were prepared using the method described by Okwori *et al.* (2006) and Coban and Konuklugil (2005). The solvent used here was 50% methanol and sterile distilled water (sdH_2O) in the ratio of 1:10 (w/v). After, extraction the solvents was evaporated using a rotary evaporator under reduced pressure and further lyophilized for complete solvent removal. The extracts were stored in sterilized glass vials at -20°C until further analysis. To avoid any contamination and alterations of chemical constituents, the extracts were used within 2-3days of preparation (Singh *et al*., 2012).

3.9.2. Preparation of media

3.9.2.1. Potato Dextrose Agar (PDA)

For maintaining fungal culture potato dextrose agar was prepared using fresh potato decoction (400g/L). Then the decoction was filtered through muslin cloth and final volume was adjusted to 1L. To this, dextrose (20g/L) and agar (20g/L) was added and heated until the uniform mixture was obtained. The media was autoclaved at 15lbs for 15min at 121ºC. For the media preparation distilled water was used.

3.9.2.2. Nutrient Broth (NB) and nutrient agar (NA)

Bacterial cultures were grown and maintained both in nutrient broth and agar media. Both the media were prepared taking 13g of available media (Himedia) in 1L of distilled water and warmed the media until dissolved completely. The NA media contains peptone (5g/L), NaCl (5g/L), Beef extract (1.5g/L), Agar (15g/L)Yeast extract (1.5g/L) and pH 7.2±0.2 (at 25ºC) whereas in NB all the ingredients are same as of NA except agar. The nutrient media were then sterilized at 15lbs for 15min at 121ºC.

3.9.3. Antibacterial activities

3.9.3.1. Disc diffusion method

Agar disc-diffusion method of Murray (1995) was followed to evaluate the antibacterial activities of the crude plant extracts. The bacterial strains were maintained and tested on nutrient agar (NA). The media was sterilized at 15lbs (121°C) for 15min prior to pouring it into the sterilized petridishes. A final inoculum of 100µL suspension containing 10^{8}CFU/mL of each bacterium was mixed with the sterilized nutrient agar media and allowed to solidify in the laminar air flow. Crude extracts were sterilized in disposable Milliporefilter (0.22µm) prior to use. After 15min, the plates were impregnated with sterile Whatman No.1 filter paper dics (6mm) containing desired concentrations viz. 500 mg/mL, 250 mg/mL and 100mg/mL of methanolic extract (ME) of the plants. The solvents were completely evaporated from the disc in the laminar air flow before aseptically placing it on the agar surface. Negative control (sterile hot water), solvent control (50% MeOH) and positive control plates were also prepared. Positive control discs (7mm) of A= Azithromycin (15 mcg/disc), S=Streptomycin (10 mcg/disc) were used in the study. The plates were then incubated at 37°C for 24 h after which the diameter of inhibition zones was noted. All the assays were performed in triplicates.

3.9.4. Antifungal activities

3.9.4.1. Spore gerimation bioassay

The spore germination bioassay against the tested fungal spores was performed following the method of Trivedi and Sinha (1976). Spore suspension was prepared by filtering the fungal culture from the broth through muslin cloth. The suspension was centrifuged for 5min at 1000 rpm to separate the debris from the spores and was washed with sdH_2O for about 3-4 times. The spores were collected and suspended in 1mL of sdH_2O till further use. The methanolic and hot water extracts of concentration 500 mg/mL (100µL) were placed at the two ends of each clean, grease free slide and allowed to dry inside a laminar air flow. After drying, about 10 µL of spore suspension of test fungus was placed on top of the dried extract spots. Similarly the slides for positive control (Griseofulvin 1mg/mL), solvent control (50% MeOH) and negative control (sdH_2O) were prepared. All the slides were kept on the glass rods in a petri plates with 5mL of sdH_2O and incubated for 24h. Precautions were taken to avoid the drying of the spores. Following the incubation, the spores (or the spots) were stained with lactophenol cotton blue, fixed and observed under the microscope. The microscopic observation was done from about 5-8 microscopic fields and a total of 500 spores were counted for each case. Further, the percentage of germination was calculated using the formulae:

% of spore germination = Number of spores germinated/Total number of spore counted × 100

3.10. Testing of hypoglycemic activities

3.10.1. Preparation of crude extract for *in vivo* experiments

The dried plant extract used for analyzing hypoglycemic activities was extracted following the method of Coban and Konuklugil (2005) with slight modification. Prior, to the use the extract was mixed with normal drinking H_2O to make the stock solution.

3.10.2. *In vivo* test

3.10.2.1. Animals

Swiss albino male rats (150-200g) were procured from the Ghosh Enterprise, Kol-55. The animals were acclimatized to the experimental room at the temperature of 25 ± 2^0C and 12h light and dark cycles for one week (Niyonzima & Vllietinck, 1993). The animals were then grouped and kept in polypropylene cages with a

maximum of two animals and were fed with standard food pellets (Hindustan Lever, Kolkata, and India.) alternating with some soaked cereals (Black gram seeds) and water.

3.10.2.2. Acute toxicity study

The extracts, *C. retrospiralis* aqueous extract and *H. sibthorpioides* aqueous extract were studied for acute toxicity prior to the experimentation on animals according to OECD (Organization for Economic Cooperation and Development) guidelines (test 423: Acute oral toxicity- Acute toxic class method; 2002) (OECDiLibrary, 2002). The rats were dosed once with 2000mg/kg b.w. and monitored for 14 days for general clinical or toxicological signs and symptoms as well as mortality (Sundarranjan *et al.* 2011).

3.10.2.2.1. Permission

All procedures employed were reviewed and approved by the Animal's ethical Committee of University.

3.10.2.3. Induction of experimental diabetes in test animals

The animals were deprived of food and water for 14h prior to the induction of diabetes to the experimental rats (Siddique *et al.* 1987).

Freshly prepared streptozotocin in citrate buffer (0.1M, pH 4.5) was administered intraperitoneally (i.p) at a single dose of 65 mg/kg body weight and in a volume of 1mL/kg. Diabetes development was confirmed by measuring the blood sugar level after 48h of administration. From the tail tip blood was collected and the glucose levels were determined by glucose meter (Accu-Chek, Active Glucose meter) by glucose oxidase-peroxidase method using strips. Rats with blood glucose levels above 200mg/dL were considered to be diabetic and used for further experimentations.

3.10.2.4. Treatment of diabetic animals

For the treatment of diabetic animals, an experiment was conducted for 25days with 42 rats distributed into six groups (n=6) in the following manner:

Group-I: Normal control (treated with normal water).

Group-II: Streptozotocin –induced diabetic control (treated with normal water).

Group-III: Diabetic rats treated with metformin (10mg/kg b.w).

Group-IV: Diabetic rats orally administered with aqueous extract of *Cryptocoryne retrospiralis* (CR) (200mg/kg b.w.) once daily.

Group-V: Diabetic rats orally administered with aqueous extract of *Cryptocoryne retrospiralis* (CR) (400mg/kg b.w) once daily.

Group-VI: Diabetic rats orally administered aqueous extract of *Hydrocotyle sibthorpioides* (HS)(200mg/kg b.w) once daily.

Group-VII: Diabetic rats orally administered with aqueous extract of *Hydrocotyle sibthorpioides* (HS)(400mg/kg b.w) once daily.

3.10.2.5. Analytical procedure

3.10.2.5.1. Measurement of body weight

The total body weight of all the experimental rats were recorded on day 1, day 5, day 10, day 15, day 20 and day 25 of the treatment.

3.10.2.5.2. Estimation of blood sugar level

Blood sugar level was also recorded on day 0, day5, day 10, day 15,day 20 and day 25 of the treatment. Blood was collected from the tail tip and the glucose levels were determined using glucose meter (Accu-Chek, Active Glucose meter) by glucose oxidase-peroxidase method using strips.

3.10.2.5.3. Collection of serum

In a sterile centrifuge glass tube (without anticoagulant) blood was collected and allowed to stand in room temperature for 20-30min.Then, it was centrifuged at 1500g for 10min at 20°C. The serum gets separated from the blood as an upper transparent liquid over the clotted blood. Then the serum was collected without disturbing the residue and used for studying further biochemical parameters.

3.10.2.5.4. Study of serum biochemical parameters

3.10.2.5.4.1. Lipid profile analysis

Estimation of Total Cholesterol in the serum

The total cholesterol level in the serum samples of experimental rats was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany). The cholesterol was estimated by dynamic extended stability with lipid clearing agent (Allian *et al.,* 1974; Roeschlau *et al.,* 1974). Prior to experimentation, the working reagent was prepared by mixing the providedrol reagents (i.e, mixture of cholesterol esterase, cholesterol oxidase, peroxidase, sodium phenolate, 4-aminoantipyrine, phosphate buffer (pH 6.5 ± 0.1) and lipid clearing agent) with appropriate amount of sterilized distilled water. 2 ml of the working reagent was then mixed with 40 µl of test sample and allowed

to incubate for 37 ºC for 10 min. The reaction mixture was aspirated and the absorbance was recorded at 505 nm. The cholesterol content was calculated using the formula provided with the kit:

Cholesterol mg dL^{-1} = Absorbance of test / Absorbance of standard× Conc. of standard(mg dL^{-1})

Estimation of Triglycerides in the serum

The content of triglycerides in the serum samples of experimental rats was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany). Triglycerides were estimated by dynamic extended stability with lipid clearing agent (McGowan *et al*., 1983; Fossati and Prencipe, 1982; Trinder 1969). Briefly, supplied triglycerides reagent (consisting of ATP, Mg^{2+}, 4- aminoantipyrine, 3-5 DHBS, peroxidase, glycerol kinase, glycerol phosphate oxidase (GPO), lipoprotein lipase, buffer (pH 7.0 ± 0.1 at 20 ºC) was mixed with appropriate amount of sterilized distilled water and allowed to stand for 10 min at room temperature. 2 ml of the working reagent was then mixed with 20 µl of the serum sample and incubated for 10 min at 37 ºC and the absorbance was recorded at 505nm. The triglycerides content was calculated using the formula provided with the kit:

Triglycerides mg dL^{-1} = Absorbance of test / Absorbance of standard× Conc. of standard(mg dL^{-1})

Estimation of HDL-cholesterol and LDL cholesterol in the serum

The estimation of HDL-cholesterol was done following the phosphotungstic acid method using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) as described by Burstein et al. 1970. The cholesterol working reagent (consisting the mixture of cholesterol esterase, cholesterol oxidase, peroxidase, sodium phenolate, 4-aminoantipyrine, phosphate buffer (pH 6.5 ± 0.1) and lipid clearing agent) was used to determine the concentration of HDL cholesterol. Briefly, 500 µl of sample was mixed thoroughly with 1 ml of precipitating reagent and allowed to stand for 10 min at room temperature (15-30ºC). The reaction mixture was centrifuged at 4000 rpm for 10 min to obtain the clear supernatant. The clear supernatant was then mixed well with cholesterol working reagent and incubated for 10 min at 37 ºC. The

absorbance was then read at 505 nm. The HDL and LDL (Freidewald's Formula) cholesterol content was calculated using the formula provided with the kit.

HDL cholesterol (mg dL^{-1}) = Abs.of test/ Abs. of standard × Conc. of standard (mg dL^{-1}) × dilution factor

LDL Cholesterol (mg/dL) = (Total Cholesterol) – (Triglycerides/5) – (HDL Cholesterol)

3.10.2.5.4.2. Liver function test

Analysis of SGPT/ALT (Alanine aminotransferase) level

The ALT level in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to IFCC method (International Federation of Clinical Chemistry method, 1980). Briefly, 1000 µl of the working reagent (composed of L-Alanine, NADH, lactate dehydrogenase, 2-oxoglutarate and tris buffer) was mixed thoroughly with 100 µL of the test sample and the absorbance was immediately recorded at 340 nm at an interval of 1 min.

The ALT activity was calculated using the formula provided with the kit:

First, the mean absorbance change obtained was converted into International Units (IU) of activity using the general formula provided in the kit as follows:

IU/L = (ΔA/min) x T.V. x 10^3/ S.V. x Absorptivity x P

Where:

T.V. = Total reaction volume in µL

S.V. = Sample volume in µL

Absorptivity = millimolar absorptivity of NADH at 340 nm= 6.22

P = cuvette lightpath = 1cm

Activity of ALT at 37 ºC (IU/L) = (ΔA/min) x Factor (1768)

Analysis of SGOT/ AST (Aspartate aminotransferase) level

The AST level in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to IFCC method (International Federation of Clinical Chemistry method, 1980). The working reagent (2-oxoglutarate, L-aspartate, malate dehydrogenase, lactate dehydrogenase, NADH, tris buffer and EDTA). For estimation, 1000 µl of the

working reagent was mixed thoroughly with 100 µl of the test sample and aspirated. The absorbance was immediately recorded at 340 nm at an interval of 1 min.

The AST activity was calculated using the formula provided with the kit:

First, the mean absorbance change obtained was converted into International Units (IU) of activity using the general formula provided in the kit as follows:

IU/L = (ΔA/min) x T.V. x 10^3/ S.V. x Absorptivity x P

Where:

T.V. = Total reaction volume in µL

S.V. = Sample volume in µL

Absorptivity = millimolar absorptivity of NADH at 340nm = 6.22

P = cuvette lightpath= 1cm

Activity of AST at 37 ºC (IU/L) = (ΔA/min) x Factor (1768)

3.10.2.5.4.3. Kidney function test

Estimation of urea level

The estimation of urea level in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to GLDH-Urease method, as described by Talke and Schubert (1965); Triffany *et al.*(1972). The urea working reagent (mixture of α-ketoglutarate, NADH, urease, glutamate dehydrogenase (GLDH), ADP, Tris buffer (pH 7.9 ± 0.1) was used for estimation of urea level. For estimation, 2000 µl of the working reagent was mixed with 20 µl of the test samples and aspirated and the absorbance was immediately recorded at 340 nm at an interval of 1 min. The rate of decrease in absorbance was directly proportional to urea concentration.

The concentration of urea was calculated using the formula provided with the kit:

Urea (mg/dL^{-1}) = ΔA of test / ΔA of standard × Conc. of standard (mg/dL^{-1})

Where, ΔA (absorbance change) = A_2 (final) - A_1 (initial)

Estimation of creatinine level

The creatinine level was estimated in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to the modified Jaffe's method described by Slot (1965) and Bartel *et al.* (1972). The working reagent was prepared by mixing equal amount of

picric acid and sodium hydroxide reagent provided in the kit and allowed to stand for 15 min. For estimation, 2000 µl of the working reagent was mixed with 200 µl of sample and the initial absorbance (A_1) was read at 20 seconds after mixing followed by final absorbance (A_2) 80 seconds after mixing at a wavelength of 505 nm. The absorbance of the orange–yellow colour was directly proportional to the creatinine concentration.

The creatinine concentration was calculated using the formula provided with the kit:

Creatinine (mg/dL^{-1}) = ΔA of test / ΔA of standard × Conc. of standard (mg/dL^{-1})

Where, ΔA (absorbance change) = A_2 (final) - A_1 (initial)

3.11. Cell culture and Cytotoxic effect of plant extract

From the cell line repository at NCCS, Pune, human hepatocarcinoma cell line ($HepG_2$) was purchased and maintained in Ham's F-12 medium, with 10% Foetal Bovine Serum in a humidified CO_2-Incubator at 5% CO_2 level and 37° C. confluent cells were trypsinized with 0.25% trypsin-EDTA and seeded in a 96-well microtiter plate (104 cells per well) and incubated under the same conditions for 24 hours. In a rotary evaporator the methanolic extract of these plant samples were dried and redissolved in DMSO to obtain a concentration of 500 mg/ml. Further these extract was diluted for experiment again (Acharya *et al*. 2017). The percentage of DMSO at all circumstances was not greater than 1%. The cells seeded in the 96-well plate were treated with different concentrations of the extract. After 24 hours of incubating of the plates, cytotoxicity was measured with iMark™ Microplate Absorbance Reader (BIO-RAD, USA) employing WST-I reagent (TaKaRa) according to manufacturer protocol.

3.12. Characterization of active compounds

Since the methanolic extracts of *Cryptocoryne retrospiralis* and *Hydrocotyle sibthorpioides* was found to possess significant better pharmacological activity compared with the other extracts, an attempt has been made to partially characterize the various components present in these extracts through FTIR spectroscopy and Gas Chromatography mass spectrometry (GC-MS).

3.12.1. Fourier Transform Infrared Spectroscopy (FTIR)

The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when Fourier transformed, gives rise to

the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated on-line computer. For analysis, samples were sent to Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Madras. The Perkin Elmer Spectrum1 FT-IR instrument consists of globar and mercury vapor lamp as sources, an interferometer chamber comprising of KBr and mylar beam splitters followed by a sample chamber and detector. Entire region of 450-4000 cm-1 is covered by this instrument. The spectrometer works under purged conditions. Solid samples are dispersed in KBr or polyethylene pellets depending on the region of interest. This instrument has a typical resolution of 1.0 cm-1. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible.

Instrument details

Model:	Spectrum one : FT-IR Spectrometer
Scan Range :	MIR 450-4000 cm-1
Resolution :	1.0 cm-1
sample required	50 mg, solid or liquid.

3.12.2. Gas Chromatography mass spectrometry (GC-MS)

Two plant samples, *viz. Cryptocoryne retrospiralis* and *Hydrocotyle sibthorpioides* were further analysed through GC-MS to identify the various bioactive constituents. For analysis, samples were sent to SAIF, Indian Institute of Technology, Madras. The analysis was performed using a JEOL GCMATE II GC-MS spectrometer. Initially, the instrument was kept at temperature of 110 ºC and maintained as such for 2 min, after which the oven temperature was increased to 280 ºC at the rate of 5 ºC /min and maintained further at this temperature for 9 min. The helium flow rate was maintained at 1 ml/min and the injection port temperature was kept at 250 ºC. The samples were injected in split mode at 10:1. The ionization voltage was 70 eV. Mass spectral scan range was at the rate of 45-450 (m/z). National Institute of Standards and Technology (NIST) Ver.2.1 MS data library was used to identify the compounds present in the plant extracts. The spectrum obtained through GC-MS of each compounds were compared with the NIST data library for the identification.

3.13. *In silico* molecular docking studies

Molecular docking

The coordinates of Peroxisome proliferators activated receptor gamma (3DZY.pdb), one of the diabetic molecular targets, were obtained from the RCSB protein data bank (www.rcsb.org). The biologically active compounds were docked into the active pocket of the enzyme by using docking program Autodock 4.0 (Huey *et al*. 2004, Huey *et al*.2007 and Morris *et al*. 2009).

Initially the structures of the compounds have been generated by ChemSketch software [ACD/ChemSketch, 2009] and the hydrogen atoms were added to the enzyme. The Lamarckian genetic algorithm (LGA) was applied to look out for the best conformers. A grid map with 80x80x80 points and 0.375 Å spacing was used in Autogrid program to evaluate the binding energies between the biologically active compounds and 3DZY. The grid centre was set at the catalytic site of the enzyme and the default settings were used. For each compound ten docking poses were saved and ranked by binging energy. The lowest energy docking pose was selected for analyzing the type of interactions.The binding site was analyzed with molegro molecular viewer software (Thomsen and Christensen 2006).

3.14. Statistical analysis

All the data was subjected to statistical analyses and the results were expressed as mean ± standard deviation (SD). Analyses like one way and two way analysis of variance followed by Duncan's multiple range test (DMRT). IBM SPSS statistic version 21 was used for the analysis.

4. RESULTS

4.1. Ethnobotanical survey of plants from the wetlands of North Bengal

Detailed surveys of the wetland plants reveal that the local community of North Bengal consume majority of the wetland plants in their daily diet (Table 1). Some of these plants are used as medicine to cure various ailments (Table 2), some plants are utilised as ethnoveterinary medicine (Table 3), whereas, a few plants are also used in pisciculture for as feed (Table 4). Among these 36 species, some are regularly sold in daily market e.g *Amorphophalus paeoniifolius* (Dennst.) Nicolson, *Bacopa monnieri* (L.) Pennell, *Colocasia esculenta* (L.) Schott. *Diplazium esculentum* (Retz.) Sw. *Enydra fluctuans* Lour, *Euryale ferox* Salisb. *Glinus oppositifolius* (L.) A. DC., *Hygrophila auriculata* (Schumacher) Heine, *Ipomoea aquatica* Fossk, *Marsilea minuta* L, *Portulaca oleracea* L, *Trapa natans* L. *Nasturtium officinale* R.Br. In Darjeeling hills, wetland plants are found in wet areas of hills, especially in Maneybhanjang, Mirik, Rimbik, Bijanbari etc. during their growing season (i.e., rainy season). *Diplazium esculentum* (Retz.) Sw., a wetland pteridophyte, is also a very important wetland plant sold in almost all vegetable market throughout the year. *Hygrophila auriculata* (Schumacher) is another very useful plant which locally called as *Kule Khanra* (in Bengali). Among these plants, the seeds of *Euryale ferox* Salisb. which is locally called as *Makhna* is a very high priced wetland product. *Trapa natans* L. is also sold in market locally as *Panifol*. Now a days many parts of these area are being under cultivation with two plants viz. *Euryale ferox* Salisb. and *Trapa natans* L., however, this cultivation process is disbalancing the natural wetland system by inhibiting the growth of natural vegetation and disturbing the habitat of wetland animals as well. It very interesting to note that not all of the available edible wetland plants are consumed by all communities and only some of them find use as vegetables, specifically by some communities (Table 1).

A total of 58 wetland plant species belonging to 42 families were recorded as ethnomedicinally important during the study (Table 2). Wetland plants are used in different human ailments like skin diseases including wounds, acne, stomach problems, diarrhoea, dysentery, blood impurity etc. It is evident from the study that the knowledge regarding use of plant are more or less present in almost all community, but there is variation in the usage pattern of the plant among the

communities. In this study 10 species from 8 families were identified as potential ethnoveterinary medicine used by various communities, and 23 species from 15 families were recorded as important wetland plant in pisciculture.

4.2. Selection of wetland plants for further studies

Fifteen wetland plants were selected among the surveyed plants based up on their reported medicinal properties. The plants were collected from the different locations spread across the six Districts of North Bengal. The locations of the site of collection of plant samples are recorded in (Table 5); alongwith the Google Earth maps of the locations (Figure 1 A-H). The plants were mainly collected from and adjacent to the major wetland places *viz.* Rasik Beel, Korola River, Rajmata Dighi, Dhobi Kuwa, Magurmari River, Kulic River, Bangarah Khari, Balurghat Khari and Chatra Beel. The collected plants before performing any experimentation were submitted to the NBU Herbarium for their authentication and subsequently the Accession Nos. were also obtained against each of the samples (Table 6). Also the systematic position of the plants were determined which is being listed in (Table 7). The digital photography of the plants was also performed that is being illustrated in Figure 2. Brief description of the plants are given below.

A. *Cryptocoryne retrospiralis* (Roxb.) Kunth.

Herbaceous, prefers marshy areas, rhizome short, petiole and blade distinct, lamina green to dark violet, leaf margins undulated, linear to lanceolate in shape, acuminate, inflorescence with a long peduncle, spathe expanded, glabrous, spadix with 5-6 pistillate flowers.

B. *Barringtonia acutangula* (L). Gaertn.

Shrubby to small tree in habit, elliptic to oblong leaves, pubescent, infloresence densely flowered arranged in terminal raceme, fruit and seed ovoid in shape.

Table 1: Edible wetland plants used by different communities of North Bengal

Scientific name	Family	Local Name	Community and area	Parts used	Mode of Use
Acmella oleracea (L.) R. K. Jansen	Asteraceae	Rasun sak	Mech-Jalpaiguri	Young leaf	Cooked as vegetable
Alocasia macrorrhiza (L.) G.Don.	Araceae	Man kachu	Santal-Malda	Leaves, Rhizome	Curry of Leaves and Rhizomes
Alternanthera sessilis (L.) R.Br.ex DC.	Amaranthaceae	Senchi sak	Santal-Malda, Munda-Jalpaiguri	Leaves, apical bud	Vegetable
Amaranthus spinosus L.	Amaranthaceae	Katakhuria	Santal-D.dinajpur	Shoot	Vegetable
Amaranthus viridis L.	Amaranthaceae	Notesak	Santal-Malda	Shoot	Vegetable
Amorphophalus paeoniifolius (Dennst.) Nicolson	Araceae	Ool	Munda-Jalpaiguri, Rava-Coochbehar	Rhizomes, Apical shoot	Curry,Boil and eaten with Green chili, lemon,salt and kasundi.
Argemone mexicana L.	Papaveraceae	Sialkata	Munda-Jalpaiguri	Stems	Vegetable
Bacopa monnieri (L.) Pennell	SC. retrospiralisophulariaceae	Bramhmi/Bamni	Santal-Malda	Shoot	Vegetable coked with Ghee
Chenopodium album L.	Chenopodiaceae	Bothua sak	Santal-D.dinajpur	Tender Shoot	Cooked as Vegetable
Colocasia esculenta (L.) Schott.	Araceae	Kachu	Munda-Jalpaiguri	Leaves,and rhizomes	Curry
C. retrospiralisyptocoryne retrospiralis(Roxb.) Kunth	Araceae	Kerkerali	Mech-Jalpaiguri	Root stock, Petiole	Eaten as cooked vegetable
Diplazium esculentum (Retz.) Sw.	Polypodiaceae	Dheki sak	Santal-Malda	Immature fronds	Cooked as Vegetable
Eclipta prostrata (L.) L.	Asteraceae	Kesut	Munda-Jalpaiguri	Young leaf	Cooked as vegetable

Enydra fluctuans Lour.	Asteraceae	Helancha	Munda-Jalpaiguri	Tender Shoot	Cooked as Vegetable
Euryale ferox Salisb.	Nymphaeaceae	Makhna	Santal-Malda	Seeds	Puffed seeds are eaten raw or with milk.
Glinus oppositifolius (L.) A. DC.	Molluginaceae	Gimma	Santal-Malda Munda-Jalpaiguri	Tender Shoot	Cooked as Vegetable
Hygrophila auriculata (Schumacher) Heine	Acanthaceae	Kulekhara	Santal-Malda, Rava-Coochbehar	Tender Shoot	Cooked as Vegetable
Ipomoea aquatica Fossk.	Convolvulaceae	Kolmi	Santal-Malda	Tender Shoot	Cooked as Vegetable
Lasia spinosa (L.) Thwaiys	Araceae	Kata kachu	Munda-Jalpaiguri	Petioles	In curry
Leucas Indica (L.) R. Br. ex Vatke	Lamiaceae	Dolphi	Mech-Jalpaiguri	Tender Shoot	In curry
Marsilea minuta L.	Marsileaceae	Susni	Santal-Malda	Fronds	Cooked as Vegetable
Monochoria hastata (L.) Solms	Pontaderiaceae	Panpana	Mech-Jalpaiguri	Young leaf	New shoots are eaten as vegetables by poor
Monochoria vaginalis (Burm.f.) C.Presl	Pontaderiaceae	Panpana	Munda-Jalpaiguri, Rava-Coochbehar	Young leaf	New shoots are eaten as vegetables by poor.
Nasturtium officinale R.Br.	Brassicaceae	Simrayo	Limbu-Dar jeeling	Tender shoot	Cooked leafy vegetable
Nelumbo nucifera Gaertn.	Nelumbonaceae	Poddoful	Munda - Jalpaiguri, Santal - D.Dinajpur	Rhizome, seed	Rhizome, seed, petiole edible. Leaf used as cooling pad on burning parts of body.
Nymphaea nauchali Burm.f	Nymphaeaceae	Sapla	Santal - Malda, Munda-Jalpaiguri	Young Petioles, seeds, Rhizome	Puffed seeds are eaten raw, petiole and Rhizome as curry

Nymphaea pubescens Willd	Nymphaeaceae	Bhat	Santal-Malda, Munda-Jalpaiguri	Petioles, seeds	Puffed seeds are eaten raw
Ottelia alismoides (L.) Pers	Hydrocharitaceae	Panikola	Munda-Jalpaiguri	Seeds	Eaten raw
Oxalis corniculata L.	Oxalidaceae	Amrul	Mech-Jalpaiguri	Leaves	Cooked as vegetable
Portulaca oleracea L.	Portulaceae	Nuniasak	Santal-Malda	Shoot	Cooked leafy vegetable
Ricinus communis L.	Euphorbiaceae	Reri	Oraon-Malda	Young fruits	Cooked as vegetable
Rumex maritime L.	Polygonaceae	Ban palang	Rava-Coochbehar	Tender shoot	Curry
Solanum nigrum L.	Solanaceae	Kakmachi	Munda-Jalpaiguri	Leaves	Cooked as vegetable
Trapa natans L.	Lytheraceae	Panifol	Santal - D.Dinajpur	Fruit	Fruit edible
Typhonium trilobatum (L.)	Araceae	Khanman	Santal-Malda	Leaves with Petioles	Cooked as vegetable
Xanthium indicum L.	Asteraceae	Bon Okra	Mech-Jalpaiguri	Immature plant	Curry

Table 2. Ethnomedicinal Wetland plants used by different communities of North Bengal

Name	Family	Local Name	Use	Community and area
Acorus calamus L.	Acoraceae	Boch	Rhizome useful in throat infection,leaf is used to treat small acne.	Santal-Malda
Amaranthus spinosus L.	Amaranthaceae	Katakhuria	Blood Purifier	Oraon-Malda
Anisomeles indica (L.) Kuntze	Lamiaceae	Gobhura	Insect bite, externally used for ring worm.	Mech-Jalpaiguri
Bacopa monnieri (L.) Wettst.	Plantaginaceae	Bamni sak	Raw eaten for gastritis, semi coocked with oil used as memory improving medicine.	Mech-Jalpaiguri. Oraon-Malda. Santal-U. Dinajpur
Barringtonia acutangula (L.) Gaertn.	Lecythidaceae	Hijol	Fruits, leaves, bark and seeds are used to treat fungal infection of foot.	Santal-Malda, Munda-Jalpaiguri
Biophytum sensitivum (L.) DC.	Oxalidaceae	Jhalai	Diuretic, Mouth infection.	Santal-Malda
Centella asiatica (L.) Urb.	Apiaceae	Thank uni	Abdomen pain, Chronic *Entamoeba* infection.	Santal-Coochbehar
Ceratopteris thalictroides (L.) Brongn	Parkeriaceae	Jhanji	Itching of skin diseases minor cut and wounds	Oraon-Malda
Chrozophora rottleri (Geiseler) A.Juss. ex Spreng.	Euphorbiaceae	Khudi Ojkra	Leaf used in Liver dysfunction, and blood purification	Mech-Jalpaiguri. Santal-Malda
Cleome viscosa L.	Cleomaceae	Hurhuria	Anti helminthic	Santal-Malda
Croton bonplandianus Baill.	Euphorbiaceae	Bontulsi	Reduce Wound infection, helps blood clotting after any minor cut on skin.	Oraon-Malda. Santal-Malda
Cryptocoryne retrospiralis (Roxb.) Kunth	Araceae	pachful	Useful in diarrhoea treatment	Santal-Malda
Cynodon dactylon (L.) Pers.	Poaceae	Durba ghas	Nasal drops controls nasal bleeding, diarrhoea, reduce abortion chances.	Mech-Jalpaiguri Limbu-Darjeeling
Cyperus rotundus L.	Cyperaceae	Mutha ghas	Rhizome paste boiling with mustard oil, useful in rheumatic arthritis	Rava-Coochbehar

Datura stramonium L.	Solanaceae	Dhutra	Leaf paste used in Rheumatic pain of joints	Oraon-Malda
Drymaria cordata (L.) Willd. ex Schult.	Caryophyllaceae	Avijolo	Increasevitality after prolonged illness.	Limbu,Lepcha-Darjee ling
Eclipta prostrata (L.) L.	Asteraceae	Keshut	Promotes hair growth and maintains hair color, Leaf juice is good for liver	Santal-Malda
Eichhornia crassipes (Mart.) Solms	Pontaderiaceae	Kachuripana	Used to treat feet infection	Santal-Malda
Enydra fluctuans DC.	Compositae	Helencha	Very useful treatment of constipation,reduces blood sugar when taken raw leaf juice at morning	Santal-Malda Mech-Jalpaiguri
Eupatorium odoratum L.	Asteraceae	Jarmani lota	Antidiarrhetic,stops rectal bleeding	Mech-Jalpaiguri
Euphorbia hirta L.	Euphorbiaceae	Bara dudhia	Female disorders, dysentery	Santal-Coochbehar
Euryale ferox Salisb.	Nymphaeaceae	Makhna	Leaf powder antiseptic	Santal-U.dinajpur
Glinus oppositifolius (L.) A. DC.	Molluginaceae	Gimma	Taken as juice to treat jundice	Santal-Malda
Heliotropium indicum L.	Boraginaceae	Hatishur.	Leaf paste on abdomen reduces abdominal pain	Mech-Jalpaiguri
Helminthostachys zeylanica (L.) Hook.	Ophioglossaceae	Akirgach	Rhizome paste with salt and boil rice used to treat piles. Rhizome paste also applied to cure nerve pain.	Santal-D.Dinajpur
*Houttuynia cordata*Thunb.	Saururaceae	Mach machinda	Dried leaf powder used to treat ulcer.	Santal-D.Dinajpur
Hydrocotyle sibthorpioides Lam.	Araliaceae	Khudithankuni	Gastro problem, abdomen pain	Limbu,Lepcha-Darjee ling
Hydrolea zeylanica (L.) Vahl	Hydroleaceae	Kasschara	Paste of leaf used for migraine pain	Mech-Jalpaiguri

Hygrophila auriculata (Schumach.) Heine	Acanthaceae	Kulekhara	Urinogenital tract infection and lubrication, used to reduce abortion, purify blood,and usefull in anemia, joint pain.	Santal-U.Dinajpur Mech-Jalpaiguri
Ipomoea aquatica Forssk.	Convolvulaceae	Jolkolmi	Laxative in piles patients, anthelmintic,anti-inflammatory	Oraon-Malda
Ipomoea carnea Jacq.	Convolvulaceae	B-Dal kolmi	Leaf paste curing lower feet pain.	Santal-Malda
Jatropha gossypiifolia L.	Euphorbiaceae	Lal Bheranda	AntimiC. retrospiralisobial, anti-inflammatory in case of tooth problem	Oraon-Malda. Santal-Malda
Ludwigia adscendens (L.) H.Hara	Onagraceae	Kesardam	Leaf extract used in ulcers and itching skin diseases.	Oraon-Malda
Lygodium flexuosum (L.)	Lygodaceae	Lota dheki	Rheumatism.	Mech-Jalpaiguri
Marsilea minuta L.	Marsileaceae	Sushni	Reduce hyperte nsion, Headaches, sleeping abnormalities, Migraine headache, Sinus.	Santal,Oraon-D.Dinajpur Munda, Mech-Jalpaiguri
Monochoria hastata (L.) Solms	Pontaderiaceae	Panpana	Mature leaf paste mixed with slightly heated honey and one or two drops of lemon cures gallbladder disorder.	Mech-Jalpaiguri. Santal-Malda
Monochoria vaginalis (Burm.f.) C.Presl	Pontaderiaceae	Panpana	Mature leaf paste mixed with slightly heated honey and one or two drops of lemon cures gallbladder disorder.	Mech-Jalpaiguri, Oraon-Malda. Santal-Malda
Nasturtium officinale R.Br.	Brassicaceae	Simrayo	Useful in treatment of TB alongwith normal medicine.	Limbu-Dar jeeling
Nelumbo nucifera Gaertn.	Nelumbaceae	Poddo ful	Rhizome, seed, petiole edible.leaf used as cooling wrap on burning parts of body.	Santal-Malda, Mech -Jalpaiguri
Nicotiana plumbaginifolia Viv.	Solanaceae	Bontamak	Ash of whole plant used to treat external itching.	Oraon-Malda. Santal-Malda
Nymphaea nouchali Burm.f.	Nymphaeaceae	Nil saluk	Rhizome taken raw to treat abdomen swelling	Santal,Oraon-Malda

Nymphaea rubra Roxb. ex Andrews	Nymphaeaceae	Lal saluk	Leaf powder used to treat in fection caused by over use of water.	Rava-Coochbehar
Nymphoides indica(L.) Kuntze	Menyanthaceae	Chandmala	Applied on forehead to get relief from headache	Oraon-D.Dinajpur
Oldenlandia corymbosa L.	Rubiaceae	Khetpapra	Nervous depression,Jundice,Liver diseases,anti vomiting	Mech-Jalpaiguri, Coochbehar
Ottelia alismoides (L.) Pers.(panikola)	Hydrocharitaceae	Jol kola	Fruit after slight boiling in warm wateris useful curing piles	Mech-Jalpaiguri
Oxalis corniculata L.	Oxalidaceae	Amrula	Leaf juice useful in cataract	Mech-Jalpaiguri
Phyla nodiflora(L)Greene	Verbenaceae	Juktajolful	Used to treat helminth problem.	Santal-Coochbehar
Pilea microphylla (L) Liebm	Urticaceae	Chotpatta	Whole plant along with salt taken in indigestion	Mech-Jalpaiguri
Pistia stratiotes L.	Araceae	Tokapana	Leaf paste with salt is used in eczema and skin diseases	Santal-Malda
Polygonum plebeium R.Br.	Polygonaceae	Chemtisak	Useful in rheu-matism	Santal-Malda
Portulaca oleracea L.	Portulaceae	Nuniasak	Raw juice with black salt used to treat indigestion .	Santal-Malda
Ricinus communis L.	Euphorbiaceae	Rerrhi	Seed oil is useful in Rheumatic nee pain	Santal-U.Dinajpur
Schumannianthus dichotomus (Roxb.) Gagnep.	Marantaceae	Sheetalpati	Leaf paste applied on burning wound, it reduces the	Mech-Jalpaiguri. Santal-Malda
Scoparia dulcis L.	Plantaginaceae	Bondhania	Leaf paste is useful in stomach disorders , in blood sugar ,hypertension,	Oraon-Malda. Santal-U.Dinajpur
Senna sophera (L.) Roxb.	Leguminosae	Kalkasunde	Used to treat constipation	Oraon-Tapan
Trapa natans L.	Lytheraceae	Panifol	Fruit usefull in Constipation.	Munda –Malda
Trapa natans var. B *bispinosa* (Roxb.) Makino	Lytheraceae	Jongli Panifol	Fruit edible and useful in Constipation,	Santal-D. dinajpur.Santal-Malda
Xanthium strumarium L.	Asteraceae	Bonokra	Help in digession,improves voice, Antirheumatic	Santal-Malda

Table 3: Ethnoveterinary uses of wetland plants by the local people of North Bengal

Name	Family	Local name	Community and area	Use
Oxalis corniculata L.	Oxalidaceae	Amrula	Santal-Malda	Juice of leaf is used to cure eye disease in cow, goat.
Acorus calamus L. .	Araceae	Boch	Mech –Jalpaiguri, Oraon-Malda, Santal-U.Dinajpur	Corn powder is used externally with sesame oil Oil to treat skin disease and ecto parasite
Croton bonplandianus Baill.	Euphorbiaceae	Bontulsi	Santal-Malda, Rava-Coochbehar	Young leaves used to stop eound bleeding in goats
Leucas aspera (Willd.) Link.	Lamiaceae	Dulphi	Mech –Jalpaiguri, Oraon-Malda.	Apical plant part C. retrospiralisashed and juice applyed to deworm cow externally
Ricinus communis L.	Euphorbiaceae	Rerrhi	Santal-Malda, Rava-Coochbehar, Oraon-Malda, Santal-U.Dinajpur	The paste of apical buds are used to treat the bacterial infection of mamary gland of goat ,cow etc,seed oil used in ecto-parasite infection.Some people also use the mature leaf paste applied on head during fever.Seed oil is also used in poultry to treat foot foot infection.
Solanum anguivi Lam.	Solanaceae	Bon baigun	Mech –Jalpaiguri, Rava-Coochbehar, Oraon-Malda.	Deworming of endo-parasite is done by leaf paste mixed with boilled rice powder salt and little sugar.
Achyranthes aspera L	Amaranthaceae	Apang	Rava-Coochbehar, Santal-U.Dinajpur	Whole plant paste orally administered to reduce the difficulty during delivery.Fruit are collected and dried and given to poultry to treat helminth.
Chenopodium album L.	Amaranthaceae	Bothua	Mech –Jalpaiguri,	It is very useful in oral infection,and leaf paste

			Oraon-Malda.	is also used to treat external infection.It is also used in poultry as a food supliment and for healthy growth of foul.
Cynodon dactylon (L.) Pers.	Poaceae	Dubba ghas	Santal- Malda	Whole plant paste used to treat new as well as old wound of goat and cow.
Rumex dentatus L.	Polygonaceae	Langersakam	Santal-Malda	The leaf paste is used to treat old wounds and also used to control constipation of cow.

Table 4: Plants used as fish feed

Name	Family	Local Name
Marsilea minuta L.	Marsileaceae	Sushni
Nymphaea nouchali Burm.f.	Nymphaeaceae	Nil saluk
Ludwigia adscendens (L.) H.Hara	Onagraceae	Kesardam
Ipomoea aquatica Forssk.	Convolvulaceae	Jolkolmi
Nymphoides indica (L.) Kuntze	Menyanthaceae	Chandmala
Ottelia alismoides (L.) Pers.	Hydrocharitaceae	Jol kola
Pistia stratiotes L.	Araceae	Tokapana/Khudi pana
Eichhornia crsassipes(Mart.) Solms	Pontaderiaceae	Kachuripana
Myriophyllum tuberculatum Roxb	Haloragaceae	Jhangi
Aldrovanda vesiculosa L.	Droseraceae	Malacca Jhangi
Utricularia aurea Lour.	Lentibulariaceae	Jhannji
Vallisneria spiralis L.	Hydrocharitaceae	Pata seola
Nechamandra alternifolia (Roxb. ex Wight) Thwaites	Hydrocharitaceae	Rasna jhangi
Lemna trisulca L	Araceae	Khudi pana
Hydrilla verticillata (L.f.) Royle	Hydrocharitaceae	koisal
Aponogeton natans (L.) Engl. & K.Krause	Aponogetonaceae	Ghenchu
Aponogeton crispus Thunb.	Aponogetonaceae	Ghenchu
Potamogeton nodosus Poir.	Potamogetonaceae	Not found
Sagittaria sagittifolia L.	Alismataceae	Choto kut
Spirodela polyrrhiza (L.) Schleid.	Araceae	Motor pana
Najas graminea Delile	Hydrocharitaceae	Kanta jhangi
Wolffia arrhiza (L.) Horkel ex Wimm	Araceae	Kutti pana
Ceratophyllum demersum L	Ceratophyllaceae	Jhangie

Figure 1: Location map of the collection and survey sites. (A): Rasik Beel **(B)** Korola River & Rajmata Dighi.

Figure 1: Location map of the collection and survey sites. (C): Dhobi Kuwa **(D)** Magurmari River

Figure 1: Location map of the collection and survey sites. (E): Kulic River **(F)** Bangarah Khari

Figure 1: Location map of the collection and survey sites. (G): Balurghat khari **(H)** Chatra Beel

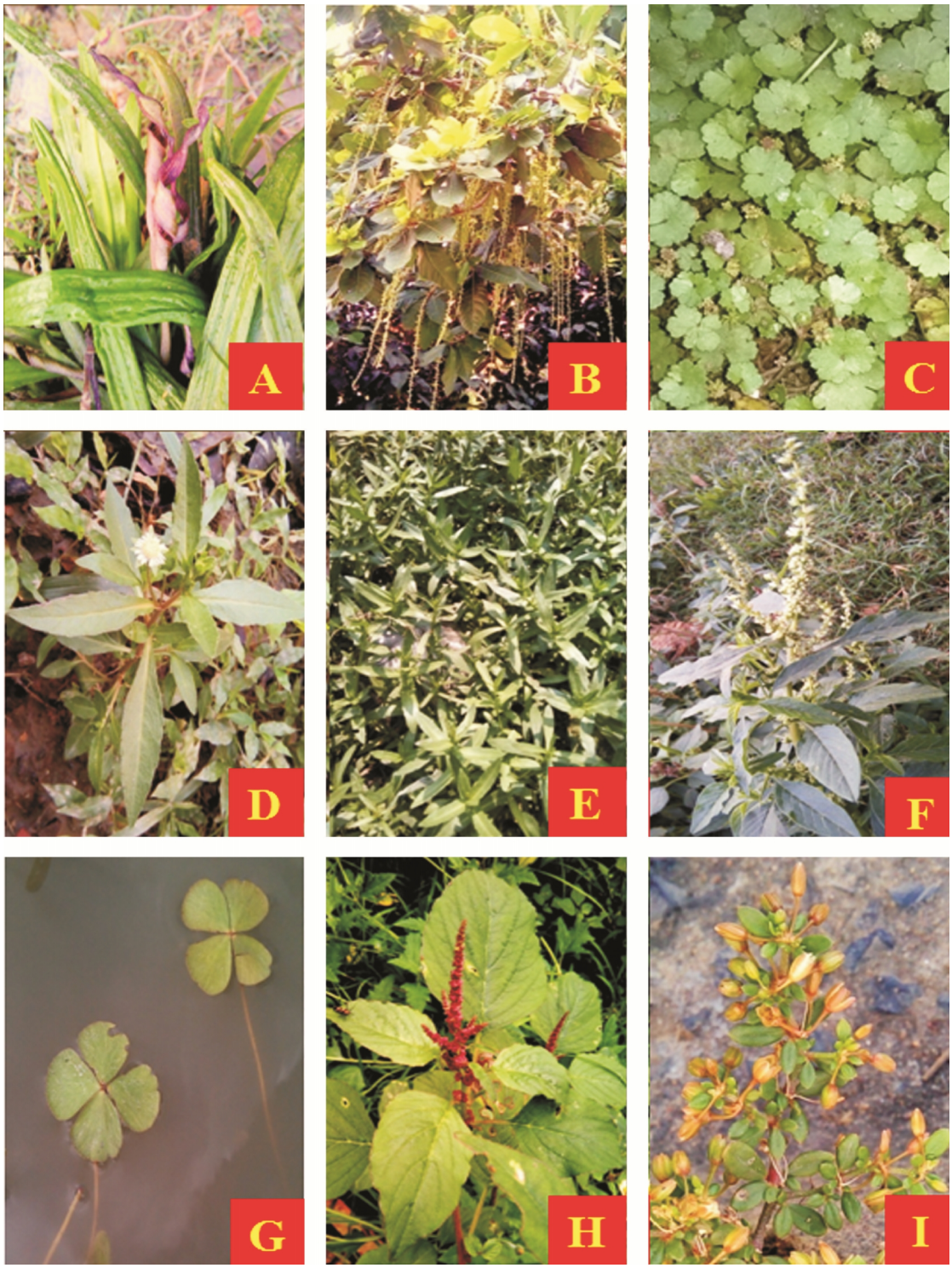

Figure 2. Plant samples collected for study. **A:** *Cryptocoryne retrospiralis*, **B:** *Barringtonia acutangula,* **C:** *Hydrocotyle sibthorpioides*, **D:** *Eclipta prostrata*, **E:** *Enydra fluctuans*, **F:** *Amaranthus spinosus*, **G:** *Marsilea minutia,* **H:** *Amaranthus viridis*, **I:** *Glinus oppositifolius*

Figure 2. Plant samples collected for study. **J:** *Portulacca oleracea*, **K:** *Hygrophila auriculata*, **L:** *Phyla nodiflora*, **M:** *Pilea microphylla*, **N:** *Diplazium esculentum*, **O:** *Ipomoea aquatica*

Table 5: Table showing the locations of the collection and survey sites

Area	Survey and Collection sites	GIS location	Elevation
Coochbehar	Rasik Beel	26º24' 36.90" N 89º43' 35.52'E	53m
Jalpaiguri	Korola river	26º32' 10.20" N 88º43' 01.84"E	83m
	Rajmata Dighi	26º32' 14.89" N 88º43' 18.69"E	85m
Darjeeling	Dhobiqua	26º59' 18.74" N 88º07' 11.62"E	1959 m
	Magurmari River	26º42 ' 29.62" N 88º21' 27.15"E	131m
Uttar Dinajpur	Kulic River	25º38' 14.40" N 88º07' 14.05"E	37 m
Dakshin Dinajpur	Bangarah Khari	25º24' 58.12" N 88º31' 58.61"E	30m
	Balurghat khari	25º13' 34.54" N 88º46' 52.29"E	27m
Malda	Chatra Beel	24º59' 06.68" N 88º07' 58.74"E	23m

Table 6: Identification of the plant samples along with the accession no. of the deposited samples

Name	Code	Accession No.
Cryptocoryne retrospiralis (Roxb.) Kunth.	CR	09825
Barringtonia acutangula (L).Gaertn.	BA	09818
Hydrocotyle sibthorpioides Lam.	HS	09826
Eclipta prostrata (L.) L.	EP	09830
Enhydra fluctuans DC	EF	09823
Amaranthus spinosus L.	AS	09828
Marsilea minuta L.	MM	09821
Amaranthus viridis L.	AV	09829
Glinus oppositifolius (L) Aug.DC	GO	09831
Portulaca oleracea L.	PO	09819
Hygrophila auriculata (Schumach) Heine	HA	09822
Phyla nodiflora (L) Greene	PN	09827
Pilea microphylla (L) Liebm	PM	09817
Diplazium esculentum (Retz)Sw	DE	09820
Ipomoea aquatica Frossk.	IA	09824

C. *Hydrocotyle sibthorpioides* Lam.

Small prostrate herb, stem weak, slender, creeping, diffusely branched, leaves reniform, adaxial surface glabrous, abaxial surface sparsely strigose, inflorescence solitary umbel at the nodes, fruit broadly globose.

D. *Eclipta prostrata* (L.) L.

Semi erect to prostrate in habit, annual herb, stems often rooting from the nodes, leaves in opposite phyllotaxy, elliptic, flower heads small, white, heterogamous, almost sessile, fruits laterally winged, compressed, pappus absent.

E. *Enhydra fluctuans* DC.

Herbaceous, profusely branched, aquatic herb, leaves opposite, oblong or linear, acuminate, margins dentate, capitulum inflorescence, terminal or axillary heterogamous, pale yellowish green, achenes black, glabrous.

F. *Amaranthus spinosus* L.

Stem glabrous or pubescent, erect, leaves ovate to lanceolate, acuminate, spines present at the base of petioles, inflorescence terminal spikes, usually with all male flowers toward apex, seeds brownish black

Table 7: Systematic position of the collected plant samples

Code	Class	Order	Family
CR	Liliopsida	Arales	Araceae
BA	Magnoliopsida	Ericales	Lecythidaceae
HS	Magnoliopsida	Apiales	Araliaceae
EP	Magnoliopsida	Asterales	Compositae
EF	Magnoliopsida	Asterales	Compositae
AS	Magnoliopsida	Caryophyllales	Amaranthaceae
MM	Filicopsida	Hydropteridales	Marsileaceae
AV	Magnoliopsida	Caryophyllales	Amaranthaceae
GO	Magnoliopsida	Caryophyllales	Molluginaceae
PO	Magnoliopsida	Caryophyllales	Portulaceae
HA	Magnoliopsida	Lamiales	Acanthaceae
PN	Magnoliopsida	Lamiales	Verbenaceae
PM	Magnoliopsida	Rosales	Urticaceae
DE	Polypodiopsida	Polypodiales	Athyriaceae
IA	Magnoliopsida	Solanales	Convolvulaceae

G. *Marsilea minuta* L.

Rhizomes creeping, roots arising from nodes and internodes, longer shoots sparsely pubescent, short shoots often densely pubescent with pale reddish brown hairs.

H. *Amaranthus viridis* L.

Erect, branched, annual herb, stem with deeply grooved leaves, ovate, cuneate at base flowers pale-green, present in small clusters, terminally panicled spikes, fruits with lenticular seeds, black and shining.

I. *Glinus oppositifolius* (L) Aug. DC.

Diffuse or prostrate herb, leaves in whorls, obovate-spathulate, apex obtuse or sometimes acute, sub-sessile, flowers axillary, fruit capsule, loculicidal, enclosed in erect calyx.

J. *Portulaca oleracea* L.

Stems glabrous, succulent, purplish red in colour, often prostrate, leaves also succulent, alternate, obovate with an obtuse apex, flowers yellow in colour, produced in a group at terminal part of stem.

K. *Hygrophila auriculata* (Schumach) Heine

Erect, marshy undershrub, leaves sessile, oblong to oblanceolate, straight yellowish spines in each node, flowers bright blue to bluish purple, showy, capsule linear oblong.

L. *Phyla nodiflora* (L) Greene.

Annual C. retrospiraliseeping herb, leaves cuneate to spathulate, margin serrate, flowers pale pink, small, sessile, numerous in dense globose, long peduncled axillary heads, peduncles axillary, bracteates, corolla tube slender, bilipped.

M. *Pilea microphylla* (L) Liebm.

Herbaceous in habit, prefers marshy places, grows on stones or solid substrates, slender, branched, stem transparent, leaves ovate-orbicular, flowers monoecious, produced in small umbellate clusters, fruit ellipsoid.

N. *Diplazium esculentum* (Retz) Sw.

Rhizome erect, scaly, brown, narrow, leaves lanceolate, lamina 1-pinnate or 2-pinnate, apex acuminate, 12-15 pairs of pinnae, alternate, lamina glabrous or hairy, sori mostly linear, curved from midribs to laminar margin.

O. *Ipomoea aquatica* Frossk.

Annual herb, with long stem, rooted at nodes, stem hollow, leaves elliptic, hastate, flowers large, corolla pink, purple or white, capsule ovoid, seed glabrous to greyish pubescent.

4.3. Phytochemical screening of powdered plant samples

In the present study, different phytochemicals including primary and secondary metabolites were screened in the powdered plant samples. In all the plant samples except *C. retrospiralis*, *A. viridis* and *I. aquatica* saponins were detected. Presence of alkaloids was detected in 9 plant samples except *H. sibthorpioides*, *A. spinosus*, *M. minuta*, *A. viridis*, *P. oleracea* and *I. aquatica*. The phenolics and flavonoids were detected in all the plant samples except *P. oleracea*. In *B.acutangula*, *E. prostrata* and *G. oppositifolius*, the phytochemical test for steroids showed positive

result. In *C. retrospiralis*, *A. viridis*, *P. oleracea*, *P. nodiflora*, *P. microphylla* and *I. aquatica*, carbohydrates were not detected. The phytochemical screening showed positive results for the presence of anthroquinones only in case of *B.acutangula*, *P. nodiflora*, *D.esculentum*; but in other samples anthroquinones were not detected. Only *C. retrospiralis* and *D.esculentum* showed negative results for amino acid. Reducing sugars were not traced in *C. retrospiralis*, *P. oleracea* and *P. microphylla*. Also, phytochemical screening of resins revealed their presence only in case of *B.acutangula*, *E. prostrata* and *I. aquatica*. On the other hand, cardiac glycosides were present in *A.spinosus*, *A. viridis*, *G. oppositifolius, P. oleracea* and tannins were detected in all the samples except *A.spinosus*, *D.esculentum* and *I. aquatica*. Likewise, phytochemical screening of glycosides revealed their presence in *C. retrospiralis*, *H. sibthorpioides*, *A.spinosus*, *A. viridis*, *G.oppositifolius*, *P. oleracea* and *P. nodiflora*. However, protein was not detected in *P. nodiflora* and *P. microphylla*; and terpenoids were detected in *C. retrospiralis*, *B.acutangula*, *E. prostrata*, *E. fluctuans*, *P. oleracea*, *H. auriculata* (Table 8).

4.4. Determination of methanolic extractive values

Samples were extracted in methanol as described in material and methods section and their extractive values determined. Extraction yield percentage ranged from 8.32% to 12.52%. The highest extractive value was recorded in the sample *M. minuta* and lowest was recorded in *A.spinosus* (Table 9). The descending order of the extractive values of all the samples is as follows: *M. minuta* > *E. prostrata* > *P. microphylla* > *H. sibthorpioides* > *P. oleracea* > *I. aquatica* > *E. fluctuans* > *G. oppositifolius* > *D. esculentum* > *C. retrospiralis* > *P. nodiflora* > *B. acutangula* > *A. viridis* > *H. auriculata* > *A. spinosus*.

Table 8: Phytochemical screening of plants used in study

Plants	Glycosides	Steroids	Antraquinones	Amino Acid	Alkaloid	Saponins	Reducing sugar	Resin	Cardiac glycosides	Terpenoid	Tannins	Carbohydrate	Protein	Phenols	Flavonoids	Triterpenoids
CR	+	−	−	−	+	−	−	−	−	+	+	−	+	+	+	+
BA	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
HS	+	−	−	+	−	+	+	−	−	−	+	+	+	+	+	−
EP	−	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+
EF	−	−	−	+	+	+	+	−	−	+	+	+	+	+	+	−
AS	+	−	−	+	−	+	+	−	+	−	−	+	+	+	+	+
MM	−	−	−	+	−	+	+	−	−	−	+	+	+	+	+	−
AV	+	−	−	+	−	−	+	−	+	−	+	−	+	+	+	+
GO	+	+	−	+	+	+	+	−	+	−	+	+	+	+	+	+
PO	+	−	−	+	−	+	−	−	+	+	+	−	+	−	−	+
HA	−	−	−	+	+	+	+	−	−	+	+	+	+	+	+	−
PN	+	−	+	+	+	+	+	−	−	−	+	−	−	+	+	+
PM	−	−	−	+	+	+	−	−	−	−	+	−	−	+	+	−
DE	−	−	+	−	+	+	+	−	−	+	−	+	+	+	+	+
IA	−	−	−	+	−	−	+	+	−	−	−	−	+	+	+	+

'+' = detected, '-'= not detected. All the data are the mean of three replicates.

Table 9: Methanolic extractive values of the studied plants

Plant sample	Yield (%, w/w)*
C. retrospiralis	10.44±0.035
B.acutangula,	09.81±0.054
H. sibthorpioides	11.79±0.084
E. prostrata	12.07±0.091
E. fluctuans	11.16±0.044
A.spinosus	08.32±0.050
M. minuta	12.52±0.041
A.viridis	09.02±0.597
G. oppositifolius	10.93±0.039
P.oleracea	11.60±0.053
H. auriculata	08.67±0.026
P.nodiflora	10.16±0.080
P. microphylla	11.92±0.059
D. esculentum	10.61±0.032
I.aquatica	11.21±0.040

*On dry weight basis

4.5. Determination of different biochemical components in the plant samples

4.5.1. Protein and free amino acid content

The protein content was found to be significantly highest in the powdered sample of *A. spinosus* and lowest in *P. microphylla* (Table 10). The descending order of the protein content in the studied plants is as follows: *A. spinosus* > *A.viridis* > *E. fluctuans* > *D. esculentum* > *M. minuta* > *E. prostrata* > *G. oppositifolius* > *P. oleracea* > *P. nodiflora* > *I. aquatica* > *H. auriculata* > *H. sibthorpioides* > *B. acutangula* > *C. retrospiralis* > *P. microphylla*.

Similarly, significant differences were present in the free amino acid content of the 15 samples under study. Among the plant samples, *G. oppositifolius* contained the highest amount of amino acids whereas *C. retrospiralis* had the lowest (Table 10). The descending order in which the free amino acid content was present in the plant samples is as follows: *G. oppositifolius* > *B. acutangula* > *H. sibthorpioides* > *M. minuta* > *A. spinosus* > *P. oleracea* > *Ipomoea aquatica* > *E. prostrata* > *P.nodiflora* > *E. fluctuans* > *A.viridis* > *H. auriculata* > *P. microphylla* > *D. esculentum* > *C. retrospiralis*.

Table 10: Protein and free amino acid contents in plant samples

Plant sample	Protein (in mg BSA equivalent/g dwt)	Free amino acids (in mg glycine equivalent/g dwt)
C. retrospiralis	05.00 ± 0.41^{b}	0.07 ± 0.001^{a}
B. acutangula	10.50 ± 0.91^{c}	0.58 ± 0.430^{k}
H. sibthorpioides	13.00 ± 1.01^{d}	0.48 ± 0.400^{j}
E. prostrata	24.50 ± 1.83^{j}	0.34 ± 0.190^{f}
E. fluctuans	37.50 ± 2.28^{m}	0.33 ± 0.290^{f}
A. spinosus	70.00 ± 2.78^{o}	0.41 ± 0.320^{h}
M. minuta	25.50 ± 2.04^{k}	0.42 ± 0.210^{i}
A.viridis	62.50 ± 2.89^{n}	0.31 ± 0.170^{e}
G. oppositifolius	23.50 ± 1.43^{i}	0.82 ± 0.430^{l}
P. oleracea	21.00 ± 1.12^{h}	0.39 ± 0.210^{g}
H. auriculata	14.00 ± 1.02^{e}	0.29 ± 0.150^{d}
P.nodiflora	19.00 ± 1.16^{g}	0.34 ± 0.170^{f}
P. microphylla	01.50 ± 0.067^{a}	0.24 ± 0.120^{c}
D. esculentum	26.50 ± 1.41^{l}	0.15 ± 0.010^{b}
I. aquatica	17.50 ± 1.08^{f}	0.39 ± 0.020^{g}

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

Table 10 A: ANOVA of data presented in table (Protein content)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15385.797	14	1098.986	134350.308	.000
Within Groups	.245	30	.008		
Total	15386.043	44			

Table 10 B: ANOVA of data presented in table (Free amino acids)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.286	14	.092	2756.267	.000
Within Groups	.001	30	.000		
Total	1.287	44			

4.5.2. Total phenol and flavonoid content

The powdered sample of *H. sibthorpioides* had significantly ($p<0.05$) highest phenol content compared to all the other plant samples. And the lowest phenol content was recorded in *P. oleracea* (Table 11). Values were expressed as mg Gallic acid (+) equivalent/ gdw (mgGE/gdwt). The descending order at which the phenol content was present in these samples is as follows: *H. sibthorpioides* > *B. acutangula* > *C. retrospiralis* > *E. prostrata* > *I.aquatica* > *A.viridis* > *D. esculentum* > *A. spinosus* > *P.nodiflora* > *E. fluctuans* > *G. oppositifolius* > *M. minuta* > *H. auriculata* > *P. microphylla* > *P. oleracea*.

The total flavonoid content of *A.viridis* was highest and in *P. oleracea* it is lowest. Values were expressed as mg equivalent/g dwt. The descending order at which the flavonoid content was present is as follows *A.viridis* > *P. microphylla* > *A. spinosus* > *E. fluctuans* > *E. prostrata* > *H. sibthorpioides* > *D. esculentum* > *I. aquatica* > *B. acutangula* > *C. retrospiralis* > *H. auriculata* > *P.nodiflora* > *M. minuta* > *G. oppositifolius* > *P. oleracea*. (Table11).

Table 11: Estimation of total phenol and flavonoid content in crude plant samples

Plant sample	Phenol (in mg gallic acid equivalent/g dwt)	Flavonoid (in mg quercetin equivalent/g dwt)
C. retrospiralis	0.086 ± 0.07 ^{g}	0.137 ± 0.11 ^{d}
B. acutangula	0.145 ± 0.11 ^{h}	0.199 ± 0.16 ^{e}
H. sibthorpioides	0.168 ± 0.14 ^{i}	0.241 ± 0.19 ^{g}
E. prostrata	0.069 ± 0.04 ^{f}	0.261 ± 0.21 ^{h}
E. fluctuans	0.034 ± 0.02 ^{c}	0.293 ± 0.23 ^{i}
A. spinosus	0.042 ± 0.03 ^{d}	0.321 ± 0.29 ^{j}
M. minuta	0.018 ± 0.01 ^{b}	0.127 ± 0.09 ^{b}
A.viridis	0.067 ± 0.04 ^{f}	0.407 ± 0.31 ^{l}
G. oppositifolius	0.018 ± 0.01 ^{b}	0.123 ± 0.10 ^{b}
P. oleracea	0.009 ± 0.01 ^{a}	0.105 ± 0.01 ^{a}
H. auriculata	0.017 ± 0.02 ^{b}	0.135 ± 0.09 ^{cd}
P.nodiflora	0.035 ± 0.02 ^{c}	0.132 ± 0.11 ^{c}
P. microphylla	0.017 ± 0.01 ^{b}	0.369 ± 0.25 ^{k}
D. esculentum	0.054 ± 0.04 ^{e}	0.238 ± 0.21 ^{g}
I. aquatica	0.068 ± 0.05 ^{f}	0.231 ± 0.17 ^{f}

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

Table 11 A: ANOVA of data presented in table (Phenol)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	93901.250	14	6707.232	585.759	.000
Within Groups	343.515	30	11.451		
Total	94244.765	44			

Table11 B: ANOVA of data presented in table (Flavonoids)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	383363.467	14	27383.105	6255.024	.000
Within Groups	131.333	30	4.378		
Total	383494.800	44			

4.5.3. Total soluble and reducing sugar content

Significantly highest soluble sugar content was recorded in *E. prostrata* and lowest concentration was detected in *I. aquatica* (Table 12). The descending order at which the total soluble content was present is as follows *E. prostrata* > *A. spinosus* > *M. minuta* > *B. acutangula* > *H. sibthorpioides* > *H. auriculata*> *E. fluctuans* >*D.*

esculentum > *G. oppositifolius* > *P.nodiflora* > *P. oleracea* > *A.viridis* > *P. microphylla* > *C. retrospiralis* > *I. aquatica*.

On the other hand, the highest amount of reducing sugar is present in *P. oleracea* and it is lowest in *C. retrospiralis* (Table 12). The descending order at which the reducing suger content was present is as follows *P. oleracea* > *D. esculentum* > *B. acutangula* > *H. auriculata* > *I. aquatica* > *E. prostrata* > *A. spinosus* > *H. sibthorpioides* > *M. minuta* > *A.viridis* > *E. fluctuans* > *P.nodiflora* > *G. oppositifolius* > *P. microphylla* > *C. retrospiralis*.

Table 12: Estimation of total and reducing sugar content in crude plant samples

Plant sample	Total sugar (in mg glucose equivalent/g dwt)	Reducing sugar (in mg glucose equivalent/g dwt)
C. retrospiralis	2.75 ± 0.12 ^{ab}	0.032 ± 0.005 ^{a}
B. acutangula	33.00 ± 1.53 ^{i}	0.351 ± 0.008 ^{l}
H. sibthorpioides	24.00 ± 1.01 ^{h}	0.131 ± 0.001 ^{g}
E. prostrata	41.00 ± 1.04 ^{l}	0.210 ± 0.002 ^{i}
E. fluctuans	14.00 ± 1.06 ^{f}	0.108 ± 0.001 ^{e}
A. spinosus	39.00 ± 1.01 ^{k}	0.161 ± 0.001 ^{h}
M. minuta	35.00 ± 1.01 ^{j}	0.130 ± 0.001 ^{g}
A.viridis	04.00 ± 0.052 ^{bc}	0.116 ± 0.002 ^{f}
G. oppositifolius	07.00 ± 0.035 ^{d}	0.101 ± 0.004 ^{c}
P. oleracea	04.00 ± 0.323 ^{c}	0.860 ± 0.008 ^{n}
H. auriculata	15.40 ± 1.98 ^{g}	0.287 ± 0.003 ^{k}
P.nodiflora	06.20 ± 0.025 ^{d}	0.105 ± 0.004 ^{d}
P. microphylla	03.80 ± 0.02 ^{bc}	0.071 ± 0.002 ^{b}
D. esculentum	11.00 ± 0.025 ^{e}	0.435 ± 0.011 ^{m}
I. aquatica	02.00 ± 0.03 ^{a}	0.237 ± 0.007 ^{j}

Diferent superscipts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

Table 12 A: ANOVA of data presented in table (Total sugar)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	61760.454	14	4411.461	7836.096	.000
Within Groups	16.889	30	.563		
Total	61777.343	44			

Table 12 B: ANOVA of data presented in table (Reducing sugar)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.828	14	.131	48969.129	.000
Within Groups	.000	30	.000		
Total	1.828	44			

4.5.4. Ascorbic acid and carotenoid content

Ascorbic acid content was found to be highest in *H. sibthorpioides* and lowest in *G. oppositifolius* (Table 13). The ascorbic acid content in *H. sibthorpioides* was approximately 8 times high as compared to that in *G. oppositifolius*. The descending order in which the ascorbic acid was present in the plant samples are as follows *H. sibthorpioides > I. aquatica > H. auriculata > M. minuta > D. esculentum > C. retrospiralis > E. fluctuans > A.viridis > P.nodiflora > A. spinosus > B. acutangula > P. oleracea > E. prostrata > P. microphylla > G. oppositifolius*.

On the other hand, the carotenoid content was found to be highest in *A.viridis* and lowest in *I. aquatica* (Table 13). The carotenoid content in *A.viridis* was approximately 3 times high as compared to that in *I. aquatica*. The descending order in which the carotenoid content was present in the plant samples are as follows: *A.viridis > M. minuta > G. oppositifolius > A. spinosus > H. auriculata > E. fluctuans > B. acutangula > P. microphylla > E. prostrata > P.nodiflora > H. sibthorpioides > P. oleracea > C. retrospiralis > D. esculentum > I. aquatica*.

4.5.5. Total chlorophyll, Chlorophyll-a and b content

Total chlorophyll content was found to be highest in *E. prostrata* and the least amount was observed in *D. esculentum*; which was approximately 4 times higher. The amount of total chlorophylls in the studied samples in their descending order is as follows: *E. prostrata > B. acutangula > H. auriculata > E. fluctuans > A. spinosus > P. microphylla > M. minuta > A.viridis > P.nodiflora > H. sibthorpioides > G. oppositifolius > I. aquatica > C. retrospiralis > P. oleracea > D. esculentum*. Similarly, the amount of Chl-a and b was also determined for all the samples which are being recorded in (Table 14).

Table 13: Estimation of ascorbic acid and carotenoid content in crude plant samples

Plant sample	Ascorbic acid (in mg/g dwt)	Carotenoid (in mg/g dwt)
C. retrospiralis	0.434 ± 0.006 [a]	0.284 ± 0.003 [c]
B. acutangula	0.305 ± 0.005 [k]	0.400 ± 0.005 [i]
H. sibthorpioides	0.879 ± 0.004 [j]	0.340 ± 0.007 [e]
E. prostrata	0.204 ± 0.007 [f]	0.388 ± 0.001 [g]
E. fluctuans	0.424 ± 0.005 [f]	0.459 ± 0.003 [j]
A. spinosus	0.311 ± 0.022 [h]	0.485 ± 0.001 [l]
M. minuta	0.478 ± 0.031 [i]	0.517 ± 0.003 [n]
A.viridis	0.389 ± 0.033 [e]	0.569 ± 0.011 [o]
G. oppositifolius	0.105 ± 0.021 [l]	0.504 ± 0.001 [m]
P. oleracea	0.290 ± 0.009 [g]	0.335 ± 0.019 [d]
H. auriculata	0.650 ± 0.011 [d]	0.471 ± 0.021 [k]
P.nodiflora	0.351 ± 0.021 [f]	0.372 ± 0.016 [f]
P. microphylla	0.112 ± 0.005 [c]	0.398 ± 0.017 [h]
D. esculentum	0.459 ± 0.007 [b]	0.279 ± 0.009 [b]
I. aquatica	0.679 ± 0.003 [g]	0.193 ± 0.006 [a]

Diferent superscipts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

Table 13 A: ANOVA of data presented in table (ascorbic acid)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.826	14	.130	2877.348	.000
Within Groups	.001	30	.000		
Total	1.828	44			

Table 13 B: ANOVA of data presented in table (carotenoids)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.445	14	.032	8315.953	.000
Within Groups	.000	30	.000		
Total	.445	44			

Table 14: Chlorophyll (Total, Chl a and Chl b) content of plant samples

Plant sample	TOTAL Chl	Chl a	Chl b
C. retrospiralis	0.923±0.003^{c}	0.662±0.005^{h}	0.261±0.022^{b}
B. acutangula	2.254±0.005^{n}	1.503±0.002^{l}	0.751±0.004^{h}
H. sibthorpioides	1.251±0.015^{f}	0.884±0.003^{i}	0.367±0.005^{d}
E. prostrata	2.341±0.021^{o}	1.563±0.018^{m}	0.778±0.006^{i}
E. fluctuans	1.623±0.023^{l}	1.041±0.021^{j}	0.582±0.015^{f}
A. spinosus	1.530±0.019^{k}	0.522±0.019^{d}	1.008±0.018^{l}
M. minuta	1.424±0.020^{i}	0.499±0.004^{b}	0.925±0.019^{k}
A.viridis	1.318±0.019^{h}	0.521±0.002^{d}	0.797±0.005^{j}
G. oppositifolius	1.210±0.021^{e}	0.531±0.003^{e}	0.679±0.003^{g}
P. oleracea	0.894±0.005^{b}	0.552±0.003^{g}	0.342±0.014^{c}
H. auriculata	1.634±0.022^{m}	1.054±0.020^{k}	0.580±0.016^{f}
P.nodiflora	1.272±0.020^{g}	0.519±0.002^{c}	0.753±0.017^{h}
P. microphylla	1.434±0.019^{j}	0.498±0.003^{b}	0.486±0.019^{e}
D. esculentum	0.648±0.004^{a}	0.452±0.005^{a}	0.196±0.022^{a}
I. aquatica	1.031±0.025^{d}	0.545±0.006^{f}	0.487±0.002^{e}

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT (P<0.05).

Table 14 A: ANOVA of data presented in table (Total Chlorophyll)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.017	14	.644	226424.176	.000
Within Groups	.000	30	.000		
Total	9.017	44			

Table 14 B: ANOVA of data presented in table (Chlorophyll a)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.794	14	.414	433115.980	.000
Within Groups	.000	30	.000		
Total	5.794	44			

Table 14 C: ANOVA of data presented in table (Chlorophyll b)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.453	14	.175	164295.884	.000
Within Groups	.000	30	.000		
Total	2.454	44			

4.6. Determination of *in vitro* antioxidative potential of methanolic extracts

4.6.1. DPPH free radical scavenging activity

DPPH radical scavenging activity of all the plants extracts was found to increase in a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml) [Figure 3 (A- D)]. All the extracts showed variable DPPH scavenging activity that mostly ranged from 2.96-73.19 %. However, the DPPH scavenging activity of ascorbic acid as standard for the same set of concentrations ranged from 48.93-93.03%. The IC50 values of all the plant samples were also calculated and represented in (Table 15). The IC50 value of ascorbic acid standard was the lowest and calculated to be 0.110 ± 0.056. Among the plant extracts, the lowest IC50 value was recorded for *H. sibthorpioides* (1.539 ± 0.065) and the highest IC50 value was recorded for *E. fluctuans* (9.672 ± 0.082). The ascending order of IC50 values for the tested plants is in the following order: *H. sibthorpioides* > *B. acutangula* > *H. auriculata* > *C. retrospiralis* > *I. aquatica* > *P. oleracea* > *D. esculentum* > *A.viridis* > *E. prostrata* > *P.nodiflora* > *A. spinosus* > *G. oppositifolius* > *P. microphylla* > *M. minuta* > *E. fluctuans*.

Table 15: IC50 values of DPPH radical scavenging activity exhibited by the methanol extracts of different plant samples at different concentrations

Sample	IC50 (mg/ml)
C. retrospiralis	2.781 ± 0.047 ^{d}
B. acutangula	2.203 ± 0.04 ^{c}
H. sibthorpioides	1.539 ± 0.065 ^{b}
E. prostrata	4.140 ± 0.068 ^{i}
E. fluctuans	9.672 ± 0.082 ^{n}
A. spinosus	4.586 ± 0.091 ^{j}
M. minuta	7.709 ± 0.095 ^{m}
A.viridis	4.093 ± 0.093 ^{h}
G. oppositifolius	5.711 ± 0.123 ^{k}
P. oleracea	3.429 ± 0.035 ^{f}
H. auriculata	2.633 ± 0.019 ^{d}
P.nodiflora	4.570 ± 0.078 ^{j}
P. microphylla	6.591 ± 0.047 ^{l}
D. esculentum	3.890 ± 0.073 ^{g}
I. aquatica	2.947 ± 0.068 ^{e}
Ascorbic acid	0.110 ± 0.056 ^{a}

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

Table 15 A: ANOVA of data presented in table.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	111494384.256	12	9291198.688	2833.790	.000
Within Groups	85246.667	26	3278.718		
Total	111579630.923	38			

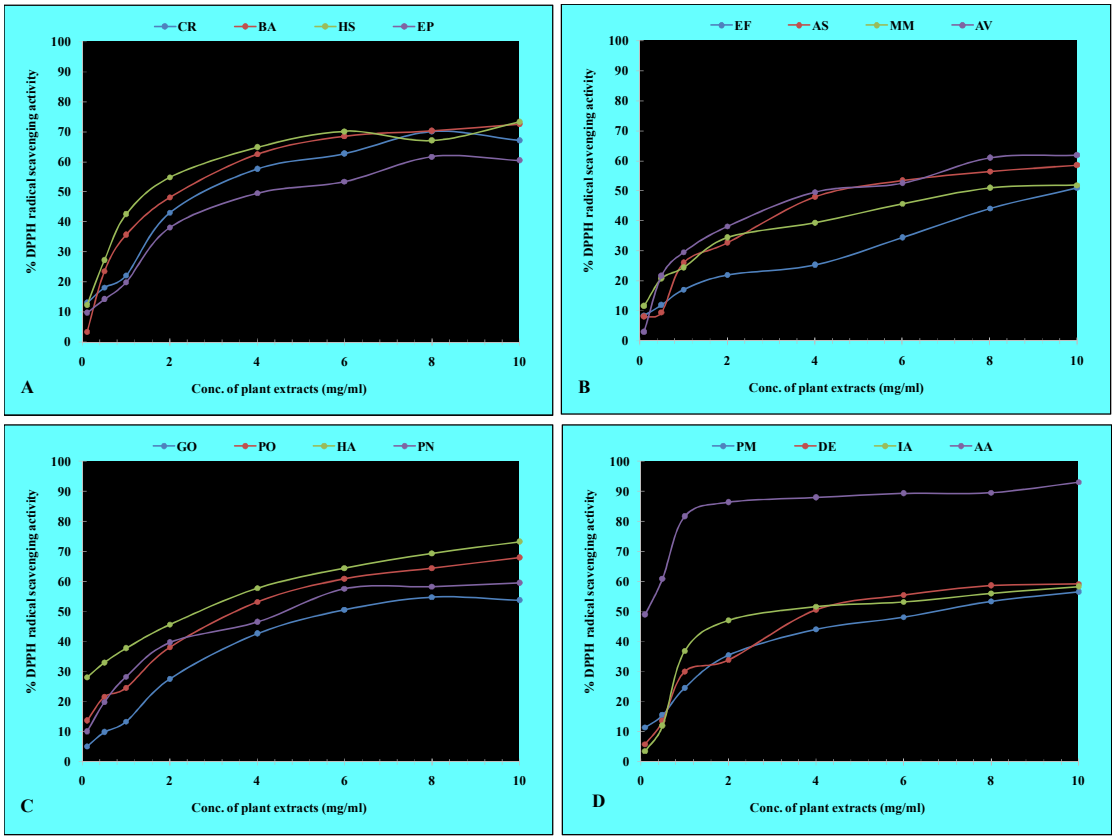

Figure 3: DPPH free radical scavenging activity (expressed as %) exhibited by different concentrations of methanolic extracts of 15 plants, along with ascorbic acid used as positive control. (A) CR - *C. retrospiralis*, BA - *B. acutangula*, HS- *H. sibthorpioides*, EP- *E. prostrata*, (B) EF- *E. fluctuans*, AS- *A. spinosus*, MM- *M. minuta*, AV- *A.viridis*, (C) GO- *G. oppositifolius*, PO- *P. oleracea*, HA- *H. auriculata*, PN- *P. nodiflora*, (D) PM- *P. microphylla*, DE- *D. esculentum*, IA- *I. aquatica*, AA-Ascorbic Acid.

4.6.2. ABTS free radical scavenging activity

ABTS free radical scavenging activity of all the plants extracts was found to increase in a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml) [Figure 4 (A- D)]. All the extracts showed variable ABTS scavenging activity that mostly ranged from 04.96-76.09%. However, the ABTS scavenging activity of ascorbic acid as standard for the same set of concentrations ranged from 57.55-95.29%. The IC50 values of all the plant samples were also calculated and

represented in Table 16. The IC50 value of ascorbic acid standard was the lowest and calculated to be 0.193±0.072. Among the plant extracts, the lowest IC50 value was recorded for *H. sibthorpioides* (0.805±0.088b) and the highest IC50 value was recorded for *E. fluctuans* (9.281±0.070). In this experiment, 2 plant samples *viz. G. oppositifolius* and *P. microphylla* was unable to scavenge 50% ABTS and as a result no IC50 values could not be calculated in case of these samples. The ascending order of IC50 values of the tested plants in which the IC50 values ould be calculated is in the following order: *H. sibthorpioides* > *C. retrospiralis* > *B. acutangula* > *E. prostrata* > *D. esculentum* > *H. auriculata* > *I. aquatica* > *A. spinosus* > *P.nodiflora* > *M. minuta* > *P. oleracea* > *A.viridis* > *E. fluctuans*.

Table 16: IC50 values of ABTS radical scavenging activity exhibited by the methanol extracts of different plant samples at different concentrations

Sample	IC50 mg/ml
C. retrospiralis	2.676 ± 0.046 [c]
B. acutangula	4.030 ± 0.063 [d]
H. sibthorpioides	0.805 ± 0.088 [b]
E. prostrata	4.323 ± 0.074 [e]
E. fluctuans	9.281 ± 0.070 [n]
A. spinosus	5.850 ± 0.093 [i]
M. minuta	6.434 ± 0.080 [k]
A.viridis	8.409 ± 0.067 [m]
G. oppositifolius	ND
P. oleracea	7.203 ± 0.069 [l]
H. auriculata	5.140 ± 0.057 [g]
P.nodiflora	6.021 ± 0.082 [j]
P. microphylla	ND
D. esculentum	4.620 ± 0.031 [f]
I. aquatica	5.589 ± 0.054 [h]
Ascorbic acid	0.193 ± 0.072 [a]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT (P<0.05).

Table 16 A: ANOVA of data presented in table.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	184500326.103	12	15375027.175	5592.848	.000
Within Groups	71475.333	26	2749.051		
Total	184571801.436	38			

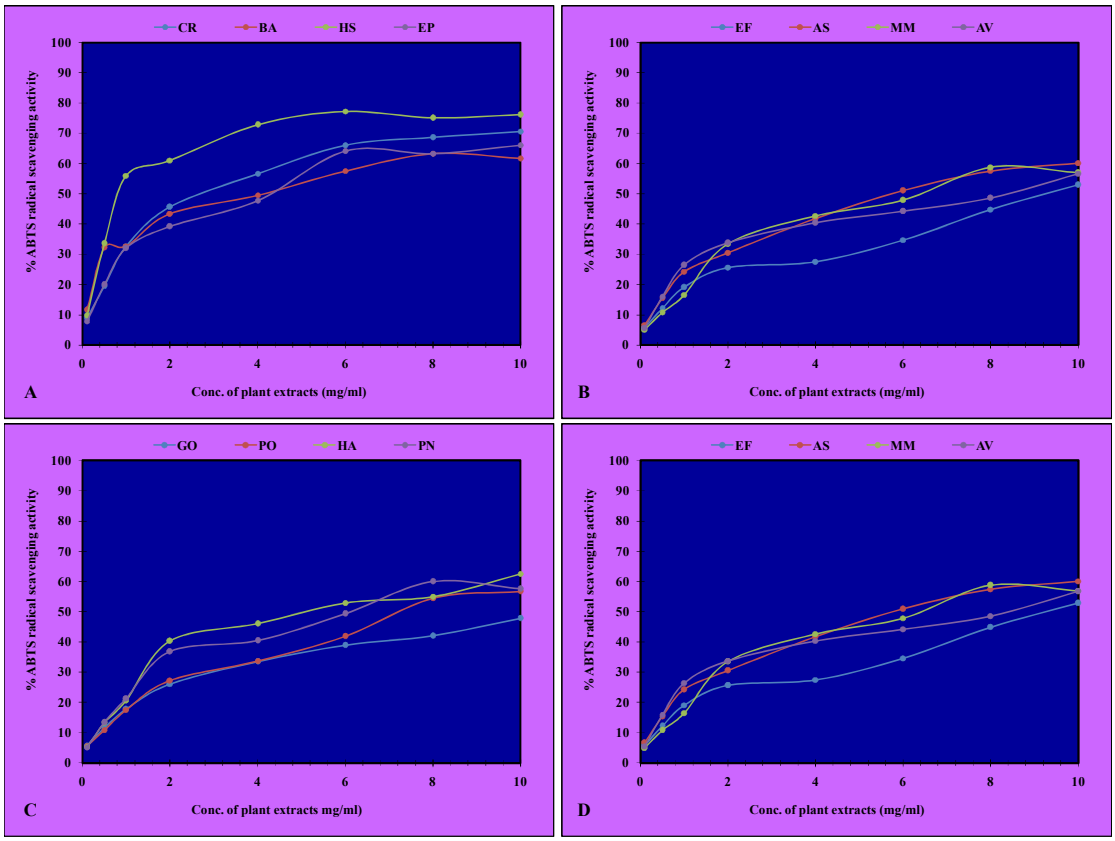

Figure 4: ABTS radical scavenging activity (expressed as %) exhibited by different concentrations of methanolic extracts of 15 plants, along with ascorbic acid used as positive control. (A) CR - *C. retrospiralis*, BA - *B. acutangula*, HS- *H. sibthorpioides*, EP- *E. prostrata*, (B) EF- *E. fluctuans*, AS- *A. spinosus*, MM- *M. minuta*, AV- *A.viridis*, (C) GO- *G. oppositifolius*, PO- *P. oleracea*, HA- *H. auriculata*, PN- *P. nodiflora*, (D) PM- *P. microphylla*, DE- *D. esculentum*, IA- *I. aquatica*, AA-Ascorbic Acid.

4.6.3. FRAP activity

The ferric reducing power of the positive control (ascorbic acid) was significantly higher than all extracts. The FRAP ability of extracts are given in ascending order *P. microphylla* > *D. esculentum* > *P. oleracea* > *G. oppositifolius* > *M. minuta* > *A. spinosus* > *H. auriculata* > *H. sibthorpioides* > *I. aquatica* > *A.viridis* > *P.nodiflora* > *E. fluctuans* > *E. prostrata* > *C. retrospiralis* > *B. Acutangula* (Table 17).

Table 17: FRAP assay of the MEOH extract of different plants at different concentrations

Plant sample	Conc. from SC (mM Fe^{2+} eq.)
C. retrospiralis	0.444 ± 0.004 [j]
B. acutangula	0.467 ± 0.005 [k]
H. sibthorpioides	0.170 ± 0.002 [ef]
E. prostrata	0.302 ± 0.001 [i]
E. fluctuans	0.223 ± 0.002 [h]
A. spinosus	0.156 ± 0.001 [e]
M. minuta	0.121 ± 0.001 [d]
A.viridis	0.175 ± 0.002 [f]
G. oppositifolius	0.110 ± 0.001 [c]
P. oleracea	0.096 ± 0.001 [b]
H. auriculata	0.161 ± 0.001 [e]
P.nodiflora	0.212 ± 0.005 [g]
P. microphylla	0.069 ± 0.006 [a]
D. esculentum	0.095 ± 0.001 [b]
I. aquatica	0.170 ± 0.001 [f]
Ascorbic acid	0.745 ± 0.011 [l]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

Table 17 A: ANOVA of data presented in table.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.604	14	0.043	4171.995	.000
Within Groups	0.000	30	0.000		
Total	0.604	44			

4.6.4. Hydroxyl radical scavenging activity

All the plant extracts was tested for hydroxyl radical scavenging activity which was found to increase in a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml) [Figure 5 (A- D)]. All the extracts showed variable OH scavenging activity that mostly ranged from 8.34-81.23%. However, the OH scavenging activity of ascorbic acid as standard for the same set of concentrations ranged from 40.05-87.56%. The IC50 values of all the plant samples were also calculated and represented in (Table 18). The IC50 value of ascorbic acid standard

was the lowest and calculated to be 0.790±0.046. Among the plant extracts, the lowest IC50 value was recorded for *C. retrospiralis* (1.783±0.055[b]) and the highest IC50 value was recorded for *M. minuta* (8.408±1.006). In this experiment, 6 plant samples *viz. B. acutangula*, *E. prostrata*, *E. fluctuans*, *H. auriculata*, *P.nodiflora*, *P. microphylla* were unable to scavenge 50% hydroxyl radical and as a result no IC50 values could not be calculated in case of these samples. The ascending order of IC50 values of the tested plants in which the IC50 values could be calculated is in the following order: *C. retrospiralis* > *P. oleracea* > *I. aquatica* > *D. esculentum* > *A. spinosus* > *H. sibthorpioides* > *G. oppositifolius* > *A.viridis* > *M. minuta*.

Table 18: IC_{50} values of OH^- scavenging activity exhibited by the methanol extracts of different plant samples at different concentrations

Sample	IC50 mg/ML
C. retrospiralis	1.783 ± 0.055 [b]
B. acutangula	ND
H. sibthorpioides	7.621 ± 1.037 [g]
E. prostrata	ND
E. fluctuans	ND
A. spinosus	7.215 ± 1.091 [f]
M. minuta	8.408 ± 1.006 [i]
A.viridis	7.856 ± 1.027 [h]
G. oppositifolius	7.623 ± 1.019 [g]
P. oleracea	3.441 ± 0.079 [c]
H. auriculata	ND
P.nodiflora	ND
P. microphylla	ND
D. esculentum	5.935 ± 0.050 [e]
I. aquatica	5.280 ± 0.073 [d]
Ascorbic acid	0.790 ± 0.046 [a]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT (P<0.05).

Table 18 A: ANOVA of data presented in table.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	195911935.467	9	21767992.830	8615.528	.000
Within Groups	50532.000	20	2526.600		
Total	195962467.467	29			

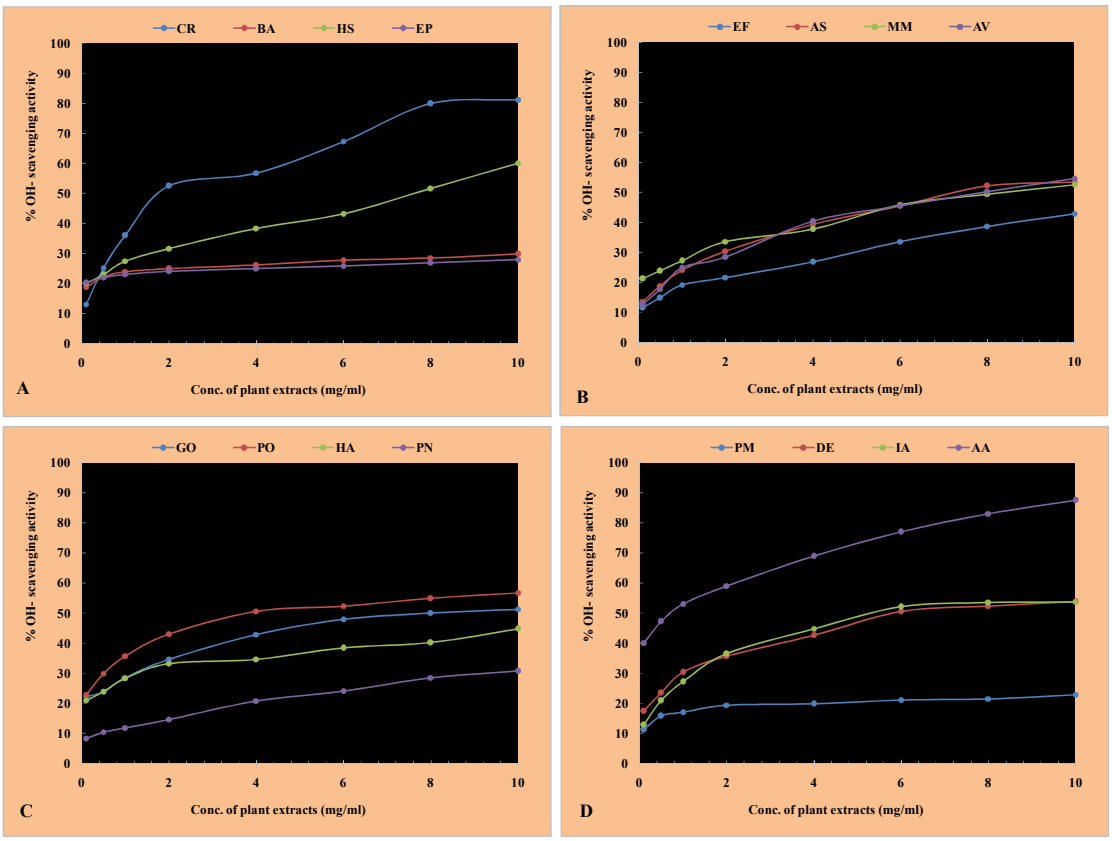

Figure 5: OH^- scavenging activity (expressed as %) exhibited by different concentrations of methanolic extracts of 15 plants, along with ascorbic acid used as positive control. (A) CR - *C. retrospiralis*, BA - *B. acutangula*, HS- *H. sibthorpioides*, EP- *E. prostrata*, (B) EF- *E. fluctuans*, AS- *A. spinosus*, MM- *M. minuta*, AV- *A.viridis*, (C) GO- *G. oppositifolius*, PO- *P. oleracea*, HA- *H. auriculata*, PN- *P. nodiflora*, (D) PM- *P. microphylla*, DE- *D. esculentum*, IA- *I. aquatica*, AA-Ascorbic Acid.

4.6.5. Nitric oxide (NO) radical scavenging activity

All the plant extracts was tested for NO scavenging activity which was found to increase in a dose dependent manner for the range of concentrations tested [Figure 6 (A- D)]. All the extracts showed variable NO scavenging activity that mostly ranged from 04.65-60.29%. However, the Nitric oxide scavenging activity of ascorbic acid as standard for the same set of concentrations ranged from 17.37-79.21%. The IC50 values of all the plant samples were also calculated and represented in (Table 19). The IC50 value of ascorbic acid standard was the lowest and calculated to be 1.632 ± 1.10. Among the plant extracts, the lowest IC50 value was recorded for *C. retrospiralis* (3.350±0.090) and the highest IC50 value was recorded for *E. fluctuans* (9.352±0.054). In this experiment, 2 plant samples *A. spinosus* and *P. nodiflora* were unable to scavenge 50% Nitric oxide and as a result no IC50 values could not be calculated in case of these samples. The ascending order of IC50 values of the tested

plants in which the IC50 values could be calculated is in the following order: *C. retrospiralis* > *A.viridis* > *E. prostrata* > *H. sibthorpioides* > *D. esculentum* > *H. auriculata* > *P. oleracea* > *M. minuta* > *B. acutangula* > *I. aquatica* > *G. oppositifolius* > *P. microphylla* > *E. fluctuans*.

Table 19: IC_{50} values of NO scavenging activity exhibited by the methanol extracts of different plant samples at different concentrations

Sample	IC50 mg/ml
C. retrospiralis	3.350 ± 0.090 [b]
B. acutangula	8.473 ± 1.033 [h]
H. sibthorpioides	6.081 ± 0.057 [d]
E. prostrata	6.009 ± 0.070 [d]
E. fluctuans	9.352 ± 0.054 [k]
A. spinosus	ND
M. minuta	7.941 ± 0.084 [g]
A.viridis	5.560 ± 0.060 [c]
G. oppositifolius	8.793 ± 0.047 [i]
P. oleracea	7.830 ± 0.095 [f]
H. auriculata	7.635 ± 1.053 [f]
P.nodiflora	ND
P. microphylla	9.272 ± 1.121 [j]
D. esculentum	6.695 ± 0.092 [e]
I. aquatica	8.577 ± 1.07 [hi]
Ascorbic acid	1.632 ± 1.10 [a]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

Table 19 A: ANOVA of data presented in table

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	195513264.667	13	15039481.897	1922.253	.000
Within Groups	219068.667	28	7823.881		
Total	195732333.333	41			

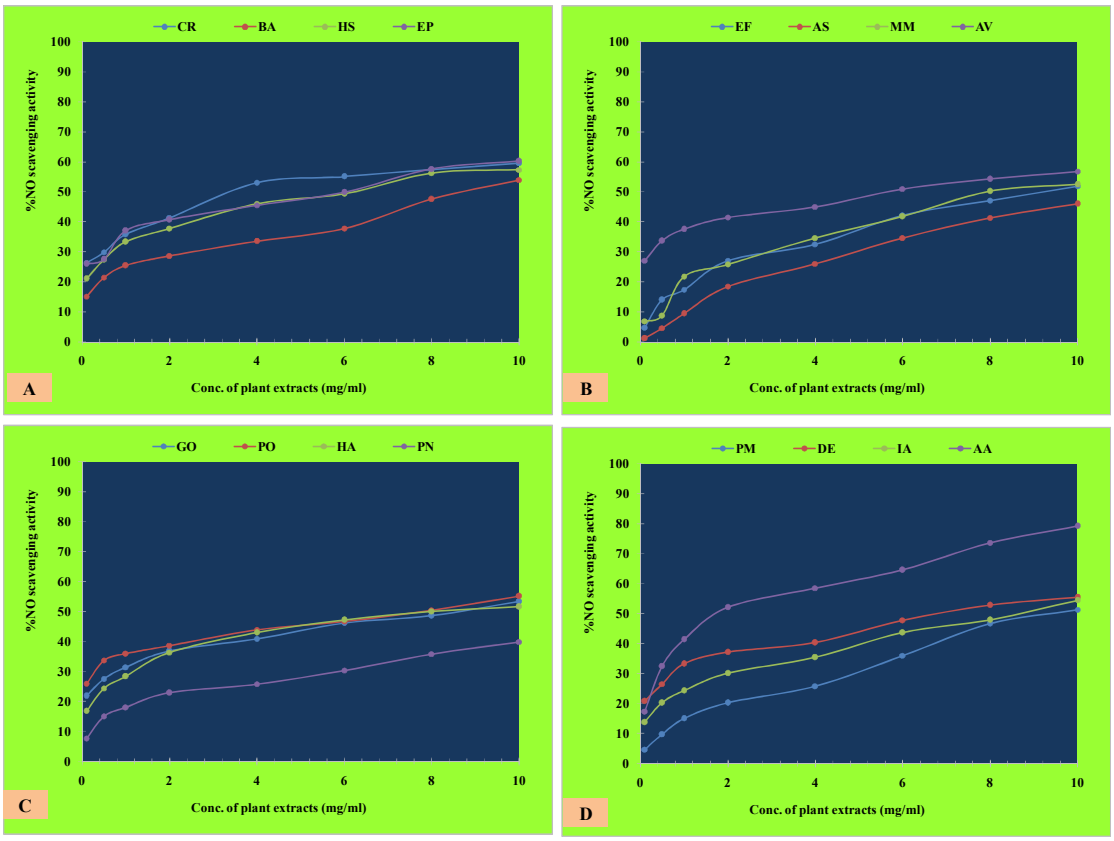

Figure 6: NO scavenging activity (expressed as %) exhibited by different concentrations of methanolic extracts of 15 plants, along with ascorbic acid used as positive control. (A) CR - *C. retrospiralis*, BA - *B. acutangula*, HS- *H. sibthorpioides*, EP- *E. prostrata*, (B) EF- *E. fluctuans*, AS- *A. spinosus*, MM- *M. minuta*, AV- *A.viridis*, (C) GO- *G. oppositifolius*, PO- *P. oleracea*, HA- *H. auriculata*, PN- *P. nodiflora*, (D) PM- *P. microphylla*, DE- *D. esculentum*, IA- *I. aquatica*, AA-Ascorbic Acid.

4.6.6. Superoxide radical scavenging activity

All the plant extracts was tested for superoxide radical scavenging activity which was found to increase in a dose dependent manner for the range of concentrations tested [Figure 7 (A- D)]. All the extracts showed variable superoxide scavenging activity that mostly ranged from 20.53-65.14%. However, the superoxide scavenging activity of ascorbic acid as standard for the same set of concentrations ranged from 35.01-98.10%. The IC50 values of all the plant samples were also calculated and represented in (Table 20). The IC50 value of ascorbic acid standard was the lowest and calculated to be 0.610 ± 0.054. Among the plant extracts, the lowest IC50 value was recorded for *H. sibthorpioides* (1.492 ± 0.085) and the highest IC50 value was recorded for GO (8.590 ± 0.062). The ascending order of IC50 values of the tested plants in which the IC50 values could be calculated is in the following order: *H.*

sibthorpioides > *C. retrospiralis* > *D. esculentum* > *M. minuta* > *A.viridis* > *P. oleracea* > *H. auriculata* > *A. spinosus* > *P. microphylla* > *G. oppositifolius*.

Table 20: IC50 values of Superoxide scavenging activity exhibited by the methanol extracts of different plant samples at different concentrations

Sample	IC50 mg/ml
C. retrospiralis	1.580 ± 0.097 [b]
B. acutangula	ND
H. sibthorpioides	1.492 ± 0.085 [b]
E. prostrata	ND
E. fluctuans	ND
A. spinosus	7.693 ± 0.105 [g]
M. minuta	4.695 ± 0.069 [d]
A.viridis	5.133 ± 0.073 [de]
G. oppositifolius	8.590 ± 0.062 [h]
P. oleracea	5.621 ± 0.059 [e]
H. auriculata	5.957 ± 0.088 [f]
P.nodiflora	ND
P. microphylla	7.694 ± 0.092 [g]
D. esculentum	2.249 ± 0.071 [c]
I. aquatica	ND
Ascorbic acid	0.610 ± 0.054 [a]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT (P<0.05).

Table 20 A: ANOVA of data presented in table.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	239161353.636	10	23916135.364	189.362	.000
Within Groups	2778569.333	22	126298.606		
Total	241939922.970	32			

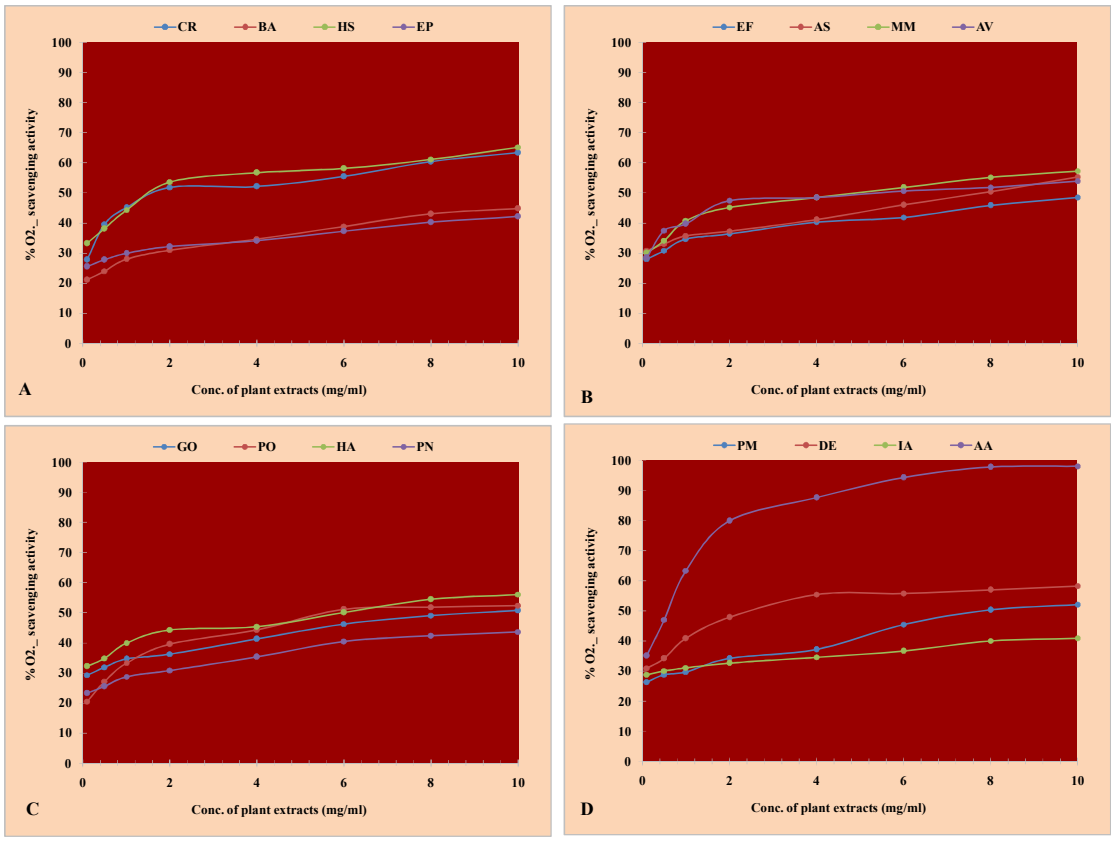

Figure 7: Superoxide scavenging activity (expressed as %) exhibited by different concentrations of methanolic extracts of 15 plants, along with ascorbic acid used as positive control. (A) CR - *C. retrospiralis*, BA - *B. acutangula*, HS- *H. sibthorpioides*, EP- *E. prostrata*, (B) EF- *E. fluctuans*, AS- *A. spinosus*, MM- *M. minuta*, AV- *A.viridis*, (C) GO- *G. oppositifolius*, PO- *P. oleracea*, HA- *H. auriculata*, PN- *P. nodiflora*, (D) PM- *P. microphylla*, DE- *D. esculentum*, IA- *I. aquatica*, AA-Ascorbic Acid.

4.6.7. Metal chelating activity

Metal chelating activity was found to increase in a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml) [Figure 8 (A- D)]. All the extracts showed variable Metal chelating activity that mostly ranged from 12.23-84.67%. However, the Metal chelating activity of ascorbic acid used as standard for the same set of concentrations ranged from 34.5-98.10%. The IC50 values of all the plant samples were also calculated and represented in (Table 21). The IC50 value of ascorbic acid standard was the lowest and calculated to be 0.110 ± 0.056. Among the plant extracts, the lowest IC50 value was recorded for *P. oleracea* (0.510±0.075) and the highest IC50 value was recorded for *B. acutangula* (6.240±0.442). In this experiment, 2 plant samples *viz. E. prostrata* and *P. microphylla* were unable to produce 50% Metal chelating activity and as a result no IC50 values could not be calculated in case of these samples. The ascending order of IC50 values of the tested

plants in which the IC50 values could be calculated is in the following order: *P. oleracea* > *M. minuta* > *I. aquatica* > *C. retrospiralis* > *E. fluctuans* > *D. esculentum* > *H. sibthorpioides* > *P.nodiflora* > *A. spinosus* > *H. auriculata* > *G. oppositifolius* > *A.viridis* > *B. acutangula*.

Table 21: IC50 values of Metal chelating activity exhibited by the methanol extracts of different plant samples at different concentrations

Sample	IC50 mg/ML
C. retrospiralis	0.890 ± 0.117 [c]
B. acutangula	6.240 ± 0.442 [k]
H. sibthorpioides	1.193 ± 0.106 [e]
E. prostrata	ND
E. fluctuans	0.994 ± 0.103 [d]
A. spinosus	2.360 ± 0.119 [g]
M. minuta	0.590 ± 0.111 [b]
A.viridis	4.342 ± 0.167 [j]
G. oppositifolius	4.023 ± 0.098 [i]
P. oleracea	0.510 ± 0.075 [b]
H. auriculata	2.835 ± 0.101 [h]
P.nodiflora	2.047 ± 0.149 [f]
P. microphylla	ND
D. esculentum	1.092 ± 0.122 [e]
I. aquatica	0.824 ± 0.102 [c]
Ascorbic acid	0.310 ± 0.029 [a]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT (P<0.05).

Table 21 A: ANOVA of data presented in table.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	120781681.143	13	9290898.549	3045.222	.000
Within Groups	85427.333	28	3050.976		
Total	120867108.476	41			

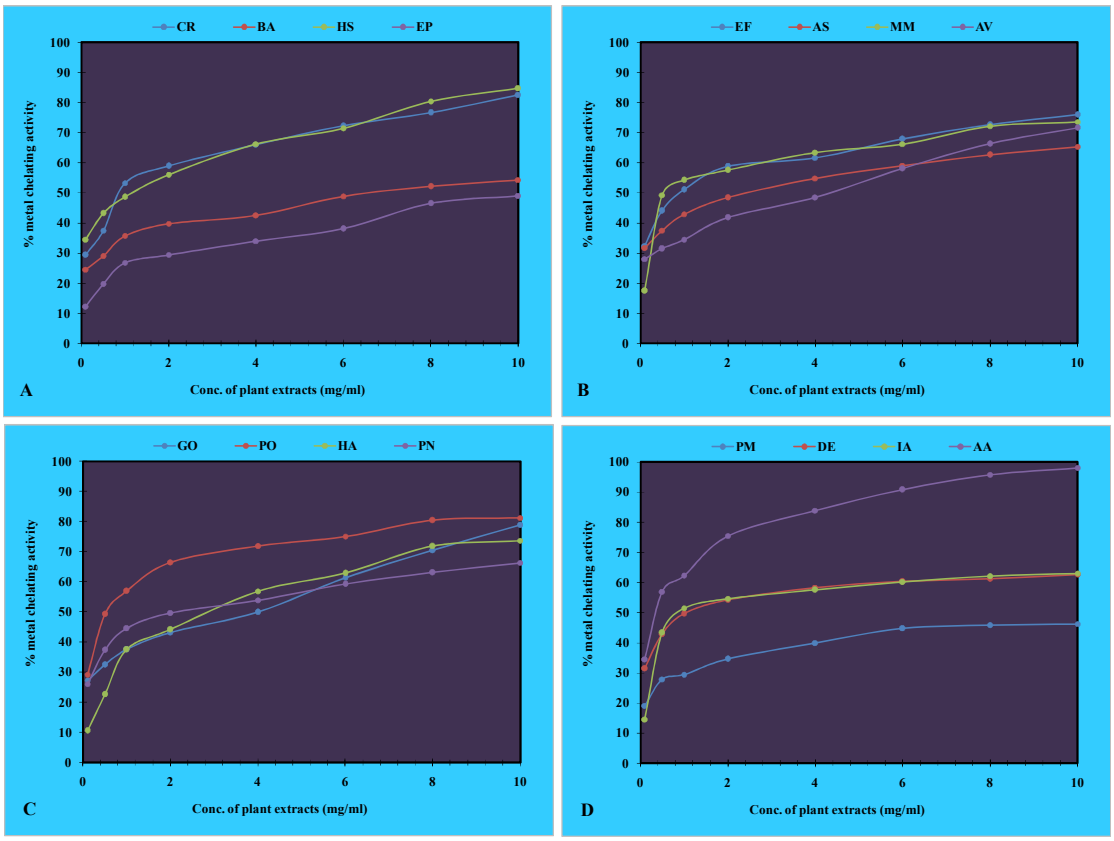

Figure 8: Metal chelating activity (expressed as %) exhibited by different concentrations of methanolic extracts of 15 plants, along with ascorbic acid used as positive control. (A) CR - *C. retrospiralis*, BA - *B. acutangula*, HS- *H. sibthorpioides*, EP- *E. prostrata*, (B) EF- *E. fluctuans*, AS- *A. spinosus*, MM- *M. minuta*, AV- *A.viridis*, (C) GO- *G. oppositifolius*, PO- *P. oleracea*, HA- *H. auriculata*, PN- *P. nodiflora*, (D) PM- *P. microphylla*, DE- *D. esculentum*, IA- *I. aquatica*, AA-Ascorbic Acid.

4.7. *In vitro* α-amylase inhibitory activity

In vitro α-amylase activity assay was also performed to determine whether any antidiabetic property is present in the plant samples under study. Likewise to the antioxidative activity, the enzyme inhibitory activity was found to increasein a dose dependent manner for the range of concentrations tested (0.1, 0.5, 1, 2, 4, 6, 8, 10 mg/ml) [Figure 9 (A- D)]. All the extracts showed variable α-amylase inhibitory activity that mostly ranged from 8.14-86.77%. However, the α-amylase inhibitory activity of acarbose used as standard for the same set of concentrations ranged from 31.15-99.49%. The IC50 values of all the plat samples were also calculated and represented in (Table 22). The IC50 value of acarbose standard was the lowest and calculated to be 0.310 ± 0.056. Among the plant extracts, the lowest IC50 value was recorded for *C. retrospiralis* (0.69 ± 0.062) and the highest IC50 value was recorded

for *H. auriculata* (6.293±0.144). In this experiment, 2 plant samples *viz. M. minuta* and *P. microphylla* were unable to scavenge to the level of 50% enzyme activity and as a result no IC50 values could not be calculated in case of these samples. The ascending order of IC50 values of the tested plants in which the IC50 values could be calculated is in the following order: *C. retrospiralis* > *H. sibthorpioides* > *G. oppositifolius* > *P. oleracea* > *B. acutangula* > *I. aquatica* > *A. spinosus* > *D. esculentum* > *E. prostrata* > *A.viridis* > *E. fluctuans* > *P.nodiflora* > *H. auriculata*.

Table 22: IC_{50} values of α amylase activity exhibited by the methanol extracts of different plant samples at different concentrations

Samples	IC50 mg/ML
C. retrospiralis	0.690 ± 0.062 [b]
B. acutangula	2.496 ± 0.103 [f]
H. sibthorpioides	1.140 ± 0.158 [c]
E. prostrata	4.273 ± 0.111 [j]
E. fluctuans	5.320 ± 0.150 [l]
A. spinosus	3.389 ± 0.032 [h]
M. minuta	ND
A.viridis	4.700 ± 0.102 [k]
G. oppositifolius	1.632 ± 0.099 [d]
P. oleracea	2.214 ± 0.175 [e]
H. auriculata	6.293 ± 0.144 [m]
P.nodiflora	6.198 ± 0.078 [m]
P. microphylla	ND
D. esculentum	4.035 ± 0.063 [i]
I. aquatica	3.117 ± 0.049 [g]
Acarbose	0.310 ± 0.056 [a]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT (P<0.05).

Table 22 A: ANOVA of data presented in table.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	151.840	13	11.680	3.511E3	.000
Within Groups	.093	28	.003		
Total	151.933	41			

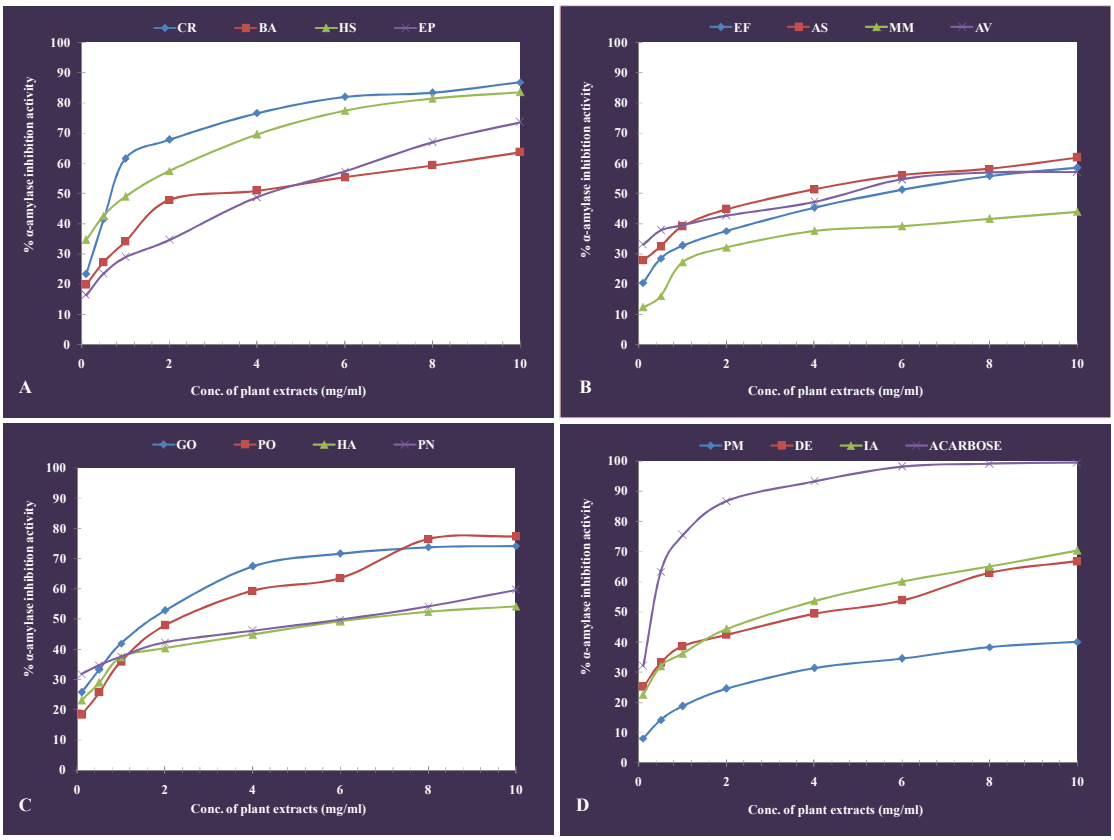

Figure 9: α amylase activity (expressed as %) exhibited by different concentrations of methanolic extracts of 15 plants, along with Acarbose used as positive control. (A) CR - *C. retrospiralis*, BA - *B. acutangula*, HS- *H. sibthorpioides*, EP- *E. prostrata*, (B) EF- *E. fluctuans*, AS- *A. spinosus*, MM- *M. minuta*, AV- *A.viridis*, (C) GO- *G. oppositifolius*, PO- *P. oleracea*, HA- *H. auriculata*, PN- *P. nodiflora*, (D) PM- *P. microphylla*, DE- *D. esculentum*, IA- *I. aquatica*, AA-Ascorbic Acid.

4.8. Selection of two plants for further studies

Based on the results of all previous experiments, finally two plant samples showing overall best performance were selected for all further tests. These plants samples were *Cryptocoryne retrospiralis* and *Hydrocotyle sibthorpioides.*These two plants samples have shown promising antioxidative and antidiabetic activities in *in vitro* experiments. Also the plant extracts were found to be rich in secondary metabolites like phenolics and flavonoids that could contribute to some other beneficial properties. The two plant extracts were further analysed for their antibacterial and antifungal properties. Alongwith this antidiabetic properties of these two extracts were confirmed by *in vivo* experiments on rat model. Also, the cytotoxicity of the extracts were evaluated on $HepG_2$ cell lines and the chemical

characterization was performed with the aid of FTIR, GC-MS, HPLC and so on, the results of which are as recorded in the coming sections.

4.9. Antimicrobial activities of the extracts

4.9.1. Antibacterial activity

Antibacterial acivity of the methanolic extracts of *C. retrospiralis* and *H. sibthorpioides* was evaluated against the test pathogen. *B. subtilis, E. coli* (MTCC 452) and *Mycobacterium avium* (MTCC 1723). All the strains were obtained from Plant Biochemistry laboratory, North Bengal University. Both the plant extracts showed antimicrobial activity against *B. subtilis* and *E. coli*, but the extracts could not show any antibacterial activity against *Mycobacterium avium* (Table 23).

Table 23: Antibacterial activity of plant extract against four pathogenic strains

Extract	**Bacteria**	**Inhibition zones in mm**				
		Concentration of extracts (mg/ml)				
		100	**250**	**500**	**Azi**	**Strp**
CR	***Bacillus subtilis***	2±0.35	8±0.25	15±0.33	38±0,18	22±0.36
	E.coli	6±0.37	11±0.29	14±0.24	32±0.32	25±0.39
	Mycobacterium avium	-	-	-	-	-
HS	***Bacillus subtilis***	9±0.21	13±0.37	16±0.23	38±0.27	22±0.35
	E.coli	7±0.19	9±0.32	12±0.26	32±0.30	25±0.36
	Mycobacterium avium	-	-	-	-	-

Azi-Azithromycin (15 mcg/disc); Strep-Streptomycin (10 mcg/disc)

4.9.2. Antifungal activities of the extracts

Antifungal activity of the plant extracts was determined by spore germination assay. In this study three phytopathogenic fungi namely *Alternaria alternata* (NCBI ACC NO. KT818507), *Curvularia lunuta* (NCBI ACC NO. KT697995) and *Fusarium oxysporum* (NCBI ACC NO. KF952602) were used. The fungal cultures

were received from the culture depository of Immuno-Phytopathology Laboratory, Department of Botany, and University of North Bengal. Three different concentrations (500, 250 and 100 mg/mL) of plant extracts were used to study the germination inhibitory effect. Although 250 and 100 mg/mL concentrations were not able to inhibit the germination of any of the fungi studied, thus, the result of percent germination (% inhibition) of only 500 mg/mL has been presented in the (Table 24).

Table 24: Spore germination bioassay of crude plant extracts and controls against different fungal species

Samples	**Conc. (µg/mL)**	**% spore germination**		
		Alternaria alternata	***Curvularia lunuta***	***Fusarium oxysporum***
C. retrospiralis	500	15.76 ± 0.43 (84.24)	19.56 ± 1.21 (80.43)	20.02 ± 1.34 (79.98)
H. sibthorpioides	500	23.33 ± 0.93 (76.67)	25.46 ± 1.41 (74.54)	34.81 ± 1.21 (65.19)
Positive control (Griseofulvin)	500	21.57 ± 0.75 (78.43)	0.05 ± 0.001 (99.98)	1.14 ± 0.87 (98.86)
Solvent control (MEOH)	-	96.40 ± 0.89	98.45 ± 1.26	99.11 ± 0.97

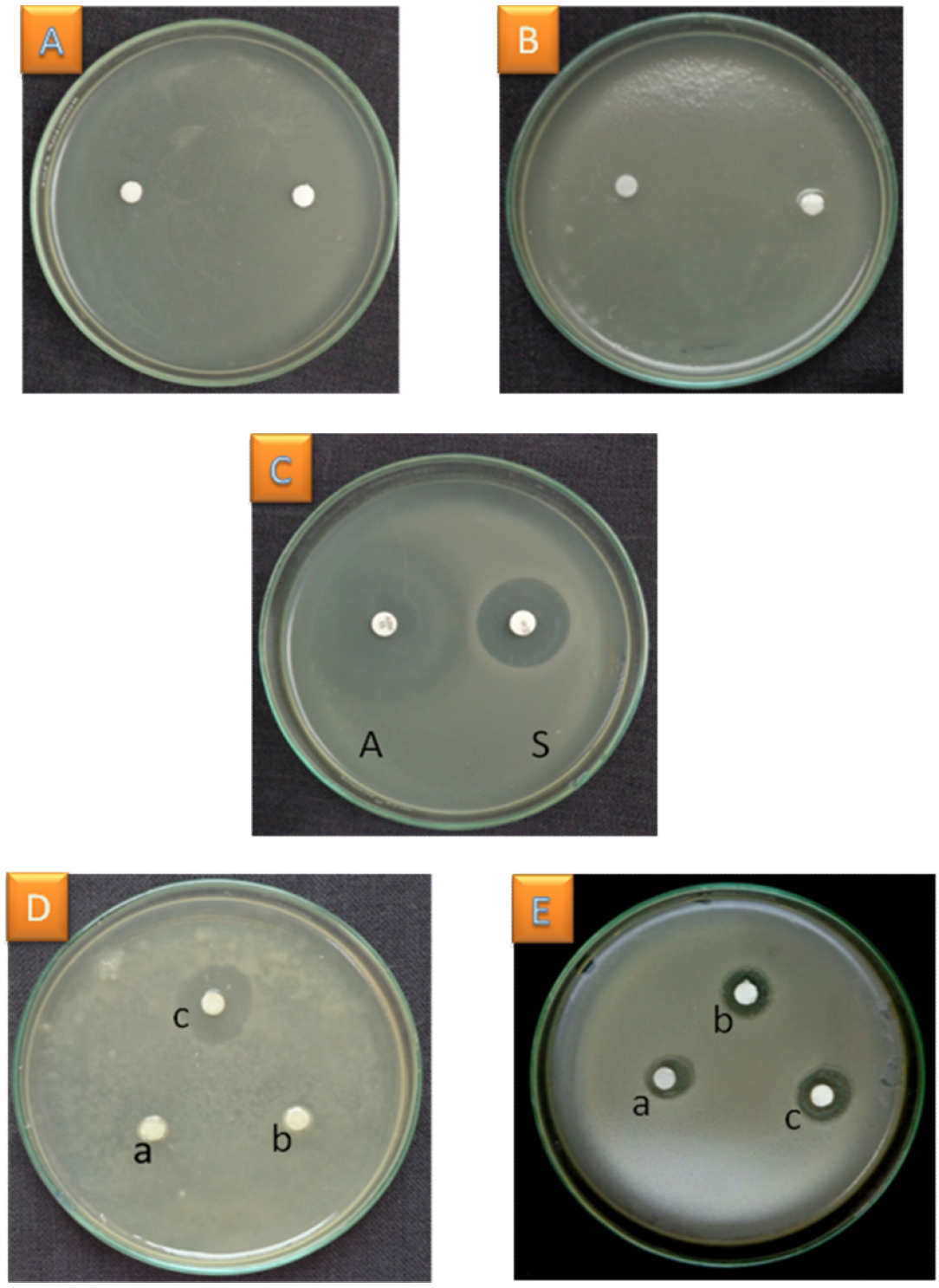

Figure 10: Antibacterial activity of plant extract against *Bacillus subtilis*. **A:** Control-Negative, **B:** Solvent, **C:** Control-Positive (A= Azithromycin, S=Streptomycin), **D:** *C. retrospiralis*, **E:** *H. sibthorpioides* (a: 100 mg/ml; b: 250 mg/ml; c: 500 mg/ml)

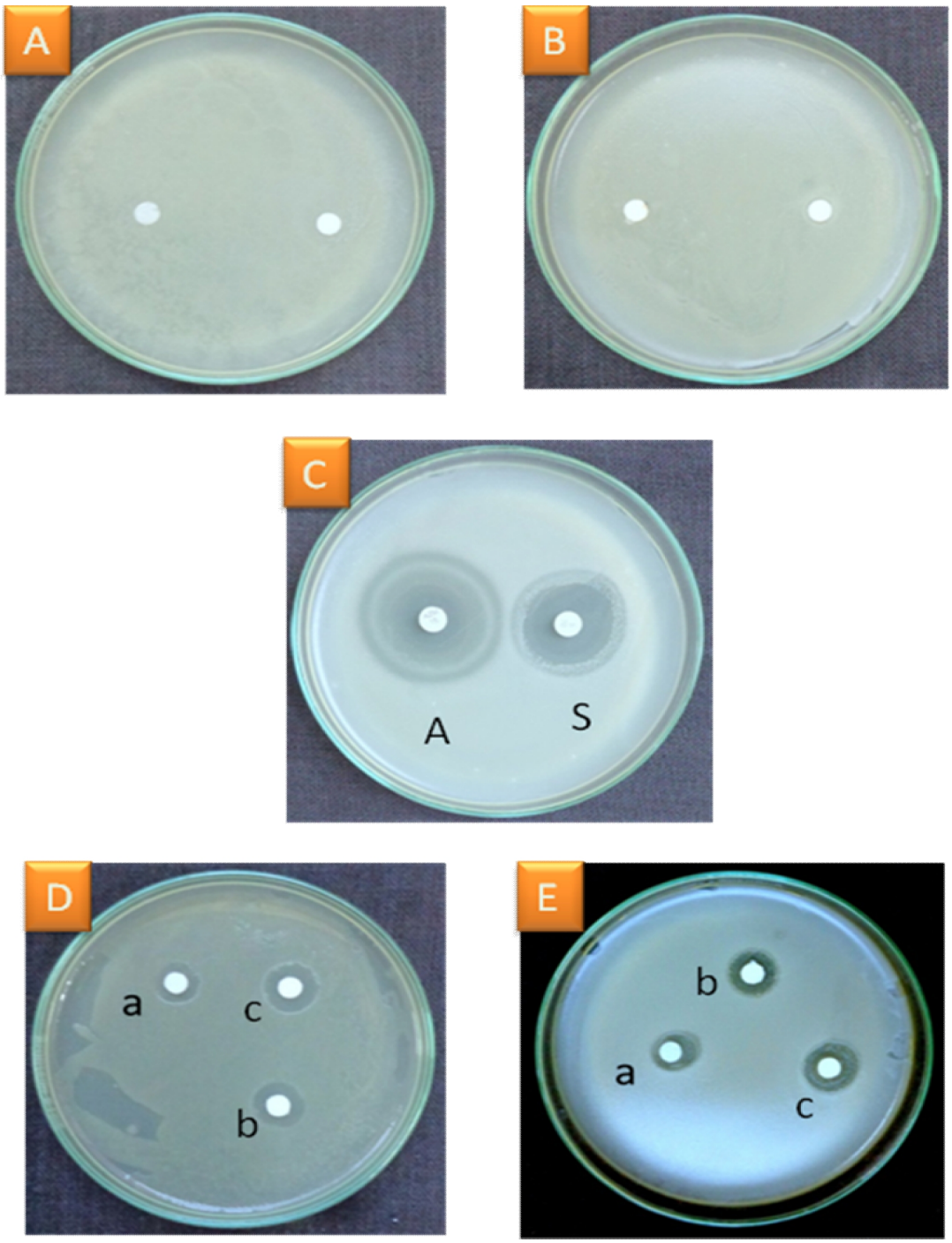

Figure 11: Antibacterial activity of plant extract against *Escherichia coli*. **A:** Control-Negative, **B:** Solvent, **C:** Control-Positive (A= Azithromycin, S=Streptomycin), **D:** *C. retrospiralis*, **E:** *H. sibthorpioides* (a: 100 mg/ml; b: 250 mg/ml; c: 500 mg/ml)

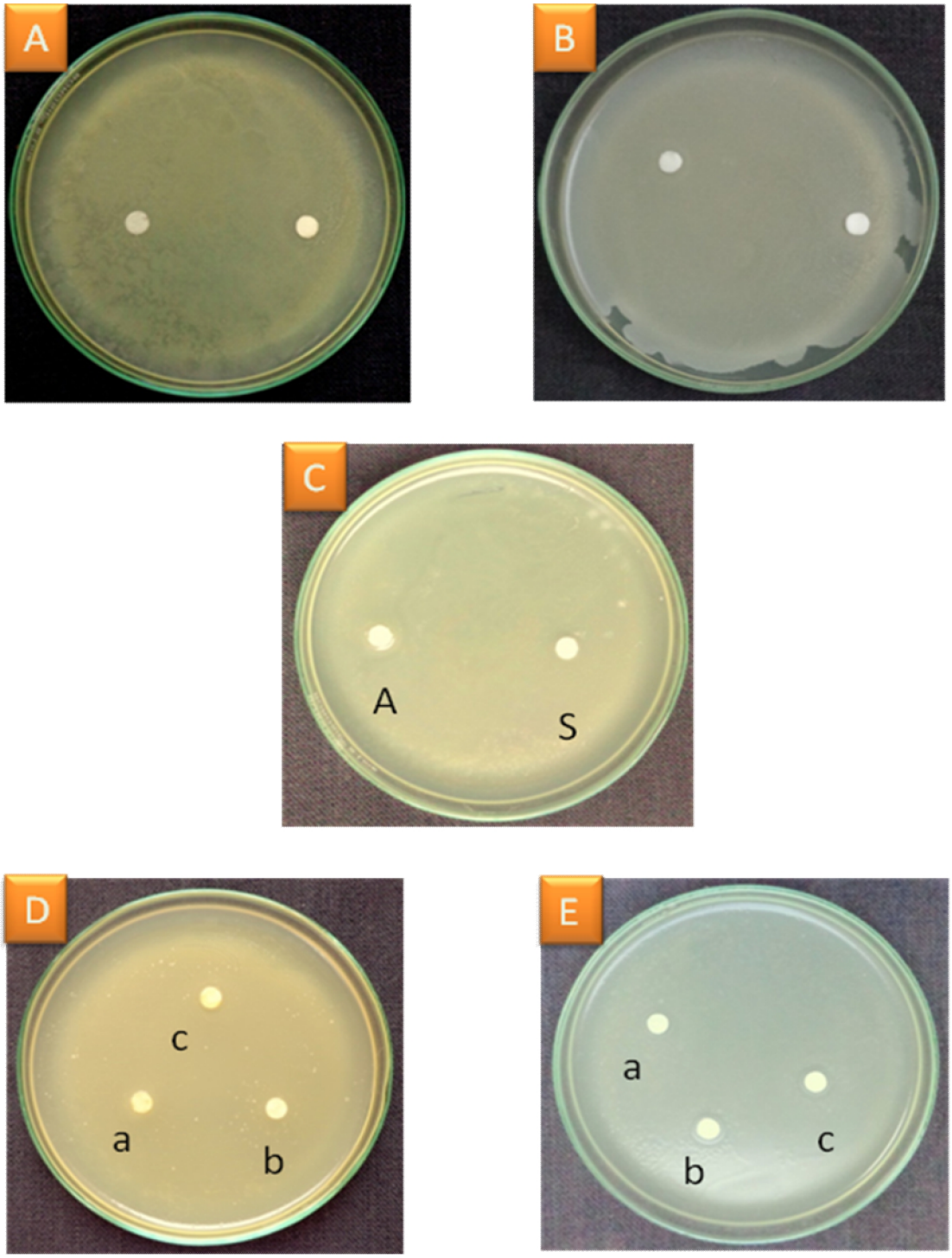

Figure 12: Antibacterial activity of plant extract against *Mycobacterium avium* **A:** Control- Negative, **B:** Solvent, **C:** Control-Positive (A= Azithromycin, S=Streptomycin), **D:** *C. retrospiralis*, **E:** *H. sibthorpioides* (a: 100 mg/ml; b: 250 mg/ml; c: 500 mg/ml)

4.10. *In vivo* study of antidiabetic property of the plant extracts

4.10.1. Acute and subchronic oral toxicity study

Oral administration of *C. retrospiralis* and *H. sibthorpioides* methanol extract at a dose of 2000 mg/kg prior to the subsequent *in vivo* studies did not produce any signs of toxicity. No changes were recorded in their behavioural observation and general appearances change for toxicity within 14 hours after the application of extracts (Table 25-27). Mortality was not observed till the end of 25 days of experimentation. At the end of the toxicity test, after 25 days the relative organ weight of the experimental rats remains unchanged (Table 28-29). For further studies 400 mg/kg b.w. and 200 mg/kg b.w. were chosen. *C. retrospiralis* at the doses (200 and 400mg/kg b.w.) produced +9.93% and +11.95% increases in body weight respectively compared to diabetic control −28% lose body weight on 25th day. On the other hand, *H. sibthorpioides* at the doses of 200 mg/kg b.w. and 400 mg/kg b.w. produced +10.75% and +11.87% increases in body weights respectively compared to diabetic control.

Table 25: Behavioural observations and general appearance

Observation	Control group I		Control group II		Control group III	
	6 hour	14 hour	6 hour	14 hour	6 hour	14 hour
Behavioural patterns	Normal	Normal	Normal	Normal	Normal	Normal
Coma	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Diarrhoea	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal	Normal	Normal
Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal
Salvation	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed

Table 26: Behavioral observations and general appearance in plant extract (*C. retrospiralis*) treated experimental groups

Observation	Treated group I		Treated group II*		Treated group III	
	6 hour	14 hour	6 hour	14 hour	6 hour	14 hour
Behavioural patterns	Rapid Heart rate	Normal	Rapid Heart rate	Normal	Rapid Heart rate	Normal
Coma	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Diarrhoea	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal	Normal	Normal
Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal
Salvation	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed

*= One rat died, may be due to injury during feeding procedure.

Table 27: General appearance and behavioral observations for Plant extract (*H. sibthorpioides*) treated experimental groups

Observation	Treated group I		Treated group II		Treated group III	
	6 hour	14 hour	6 hour	14 hour	6 hour	14 hour
Behavioural patterns	Rapid Heart rate	Normal	Rapid Heart rate	Normal	Rapid Heart rate	Normal
Coma	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Diarrhoea	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal	Normal	Normal
Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal
Salvation	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed

Table 28: Relative weight of selected organs after 25 days of sub chronic oral administration of *C. retrospiralis* extract

Organs	**Group- I Control g/100g bw**	**Group - II(*C. retrospiralis*) Treatment g/100g bw**	**Group - III(*C. retrospiralis*) Treatment g/100g bw**	**Group –IV(*C. retrospiralis*) Treatment g/100g bw**
Kidney	0.561±0.001[a]	0.557±0.002[a]	0.558±0.003[a]	0.562±0.002[a]
Heart	0.336±0.002[a]	0.333±0.001[a]	0.335±0.003[a]	0.332±0.001[a]
Liver	2.502±0.002[a]	2.509±0.001[a]	2.506±0.001[a]	2.505±0.002[a]
Lung	0.401±0.001[a]	0.399±0.003[a]	0.402±0.001[a]	0.403±0.001[a]
Testis	1.284±0.003[a]	1.276±0.004[a]	1.288±0.002[a]	1.278±0.004[a]

Diferent superscripts indicate significant differences of mean within the rows as determined by one way ANOVA followed by DMRT (P<0.05)

Table 29: Relative weight of selected organs after 25 days of sub chronic oral administration of *H. sibthorpioides* extract

Organs	**Group - I Control g/100g bw**	**Group - II(*H. sibthorpioides*) Treatment g/100g bw**	**Group - III(*H. sibthorpioides*) Treatment g/100g bw**	**Group –IV(*H. sibthorpioides*) Treatment g/100g bw**
Kidney	0.561±0.002[a]	0.559±0.003[a]	0.556±0.003[a]	0.561±0.001[a]
Heart	0.336±0.002[a]	0.338±0.001[a]	0.332±0.001[a]	0.336±0.002[a]
Liver	2.502±0.004[a]	2.510±0.005[a]	2.507±0.004[a]	2.502±0.002[a]
Lung	0.401±0.001[a]	0.402±0.001[a]	0.406±0.001[a]	0.401±0.003[a]
Testis/2	1.284±0.002[a]	1.295±0.004[a]	1.293±0.001[a]	1.296±0.004[a]

Diferent superscripts indicate significant differences of mean within the rows as determined by one way ANOVA followed by DMRT (P<0.05).

4.10.2. Effect of plant extracts on STZ induced hyperglycaemic rats

4.10.2.1. Changes in body weight

At the end of the experimental period, i.e. 25days, the body weight of the diabetic control was found to have decreased significantly in diabetic control (set II) when compared to the normal rats (Group I). However, an oral administration of *C. retrospiralis* and *H. sibthorpioides* (200 & 400 mg/kg b.w.) and metformin to diabetic rats reversed the body weights changes to near normal (Table 30).

Table 30. Changes in body weight of experimental rats

Days after induction of diabetis	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
0	156 ± 9.74	160 ± 10.60	158 ± 8.50	161 ± 7.50	159 ± 11.50	158 ± 9.70	160 ± 9.24
5	161 ± 10.93^{bc}	154 ± 8.53^{a}	161 ± 11.62^{c}	163 ± 7.98^{e}	162 ± 10.17^{d}	160 ± 8.64^{b}	164 ± 10.77^{f}
10	166 ± 10.53^{cd}	144 ± 8.62^{a}	165 ± 7.53^{bc}	166 ± 6.25^{c}	166 ± 11.53^{c}	164 ± 10.17^{b}	169 ± 8.53^{d}
15	172 ± 7.35^{e}	133 ± 9.77^{a}	168 ± 6.83^{b}	169 ± 8.78^{bc}	170 ± 9.34^{cd}	168 ± 10.53^{b}	171 ± 9.35^{d}
20	178 ± 9.53^{d}	121 ± 11.28^{a}	173 ± 6.94^{b}	172 ± 8.45^{b}	174 ± 5.32^{c}	171 ± 11.40^{b}	175 ± 10.68^{c}
25	184 ± 6.32^{e}	115 ± 7.55^{a}	180 ± 9.68^{d}	177 ± 10.56^{c}	178 ± 8.22^{bc}	175 ± 8.65^{b}	179 ± 7.65^{d}
% Difference at the end of 25th day with respect to 1st day	+17%	−28%	+13.92 %	+9.93 %	+11.1 %	+10.7 %	+11.8 %

Group I= Control, Group II= Control + STZ, Group III= Control + STZ + Metformin, Group IV= Control + STZ + *C. retrospiralis* (200mg/kg), Group V= Control + STZ + *C. retrospiralis* (400mg/kg), Group VI= Control + STZ + *H. sibthorpioides* (200mg/kg), Group VII= Control + STZ + *H. sibthorpioides* (400mg/kg)
Diferent superscripts indicate significant differences of mean within the rows as determined by one way ANOVA followed by DMRT ($P<0.05$).

4.10.2.2. Changes in blood sugar level

The hypoglycemic effect of the extracts *C. retrospiralis* and *H. sibthorpioides* was recorded by measuring the fasting blood glucose levels in day 0 ,day 1, day 5, day 10, day 15 , day 20 and day 25 (Table 31), The fasting blood glucose levels of diabetic control rats were increased significantly after the injection of streptozotocin, compared with normal control rats. Before the administration of respective drugs, the fasting blood glucose levels of the six diabetic groups (DC, DC + Metformin, DC *C. retrospiralis* 200 mg/kg, *C. retrospiralis* 400 mg/kg, *H. sibthorpioides* 200 mg/kg and *H. sibthorpioides* 400 mg/kg) were almost similar. Metformin (10mg/kg) revealed 71.65% reductions in blood glucose levels at the end of experimental period when compared to diabetic control. In *C. retrospiralis* 200(200mg/kg) and *C. retrospiralis*

400(400mg/kg) treated groups reduction in blood gloucose levels were recordes as 26.31% and 28.34% respectively. *H. sibthorpioides* 200 (200mg/kg) and *H. sibthorpioides* 400 (400mg/kg) shows relatively better hypoglycaemic efficiency and reduces the elevated blood glucose levels 28.31% and 39.37% respectively.

Table 31: Effect of different plant extracts on blood glucose level in streptozocin induced diabetic rats. Fast blood glucose level (mg/dL)

Groups	**Days after induction of diabetes**							
	0	**2**	**5**	**10**	**15**	**20**	**25**	**% change**
Control	82 ± 6.23^{b}	84 ± 5.18^{a}	82 ± 7.69^{a}	84 ± 5.77a	82 ± 7.28a	82 ± 6.19^{a}	83 ± 7.52^{a}	- 1.22 %
Control + STZ	79 ± 6.15^{a}	334 ± 22.52^{b}	387 ± 31.49^{e}	398 ± 35.69^{g}	412 ± 37.61^{f}	428 ± 40.21^{g}	456 ± 38.10^{g}	+ 36.52 %
Control + STZ + Metformin (10mg/kg)	84 ± 6.61^{c}	321 ± 29.11^{b}	315 ± 26.47^{c}	243 ± 18.16^{b}	165 ± 10.18^{b}	92 ± 6.50^{b}	91 ± 7.83^{b}	-71.65 %
Control + STZ + *C. retrospiralis* (200mg/kg)	84 ± 8.02d^{e}	318 ± 26.8^{b}	331 ± 30.73^{d}	298 ± 26.03^{e}	287 ± 21.59^{d}	283 ± 25.73^{f}	280 ± 24.3^{f}	-11.94 %
Control + STZ + *C. retrospiralis* (400mg/kg)	86 ± 7.55^{e}	321 ± 29.98^{b}	320 ± 29.6^{cd}	320 ± 31.39^{f}	288 ± 27.91^{d}	244 ± 18.95^{d}	230 ± 16.19^{d}	-28.34 %
Control + STZ + *H. sibthorpioides* (200mg/kg)	83 ± 7.23^{cd}	332 ± 31.44^{b}	314 ± 21.12^{c}	294 ± 25.10^{d}	268 ± 18.42^{c}	253 ± 19.64^{e}	238 ± 21.06^{e}	-28.31 %
Control + STZ + *H. sibthorpioides* (400mg/kg)	81 ± 5.03^{b}	320 ± 25.15^{b}	305 ± 30.03^{b}	285 ± 26.88^{c}	269 ± 25.50^{c}	214 ± 15.5^{c}	194 ± 18.05^{c}	-39.37 %

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

4.10.2.3. Changes in lipid profile

In lipid profile study, parameters like triglycerides, LDL- cholesterol, HDL-cholesterol, cholesterol were monitored in seven different groups of both normal and diabetic treated rats. Observations revealed that the triglycerides, LDL- cholesterol and Cholesterol level were increased and the HDL- cholesterol level was decreased in diabetic rats. Triglycerides, LDL- cholesterol, cholesterol levels were reduced and HDL cholesterol level was increased significantly in standard drug (10 mg/kg) treated as well as extract (*C. retrospiralis*, *H. sibthorpioides*) treated groups (Table 32).

Table 32: Effect of different plant extracts on lipid profile level in streptozocin induced diabetic rats

Groups	Triglycerides (mg/dL)	LDL cholesterol (mg/dL)	HDL cholesterol	Total cholesterol (mg/dL)
Control	91.33 ± 06.21^{a}	30.35 ± 04.16^{a}	49.66 ± 03.95^{g}	98.29 ± 08.30^{a}
Control + STZ	174.16 ± 14.7^{g}	128.27 ± 9.18^{f}	25.5 ± 2.08^{a}	188.61 ± 14.6^{g}
Control + STZ + Metformin	103.35 ± 8.63^{c}	58.05 ± 2.29^{b}	38.35 ± 3.17^{f}	117.33 ± 10.4^{b}
Control + STZ + *C. retrospiralis* (200mg/kg)	121.83 ± 10.8^{f}	85.12 ± 3.15^{e}	29.33 ± 2.5^{b}	138.82 ± 11.4^{f}
Control + STZ + *C. retrospiralis* (400mg/kg)	115.5 ± 10.33^{d}	79.79 ± 2.09^{d}	30.25 ± 2.91^{c}	133.14 ± 12.5^{d}
Control + STZ + *H. sibthorpioides* (200mg/kg)	117.05 ± 11.5^{e}	80.65 ± 3.88^{d}	31.64 ± 2.51^{d}	135.7 ± 10.69^{e}
Control + STZ + *H. sibthorpioides* (400mg/kg)	98.61 ± 7.76^{b}	68.71 ± 2.60^{c}	35.5 ± 3.47^{e}	123.94 ± 8.75^{c}

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

4.10.2.4. Liver enzymes (SGPT and SGOT level)

In this study, liver enzymes (SGPT and SGOT) of both normal and STZ-induced diabetics rats were assessed. It was observed that in diabetic rats the levels of SGPT and SGOT was an increased compared to normal control rat. Although, increased level of SGPT and SGOT in the diabetic induced rats was decreased significantly with subsequent administration of the standard drug and the plant extracts (*C. retrospiralis*, *H. sibthorpioides*) in a dose dependent manner (Table 33).

Table 33: Effect of different plant extracts on liver enzymes (SGPT and SGOT level) in normal and streptozotocin induced diabetic rats

Groups	SGPT (U/dL)	SGOT (U/dL)
Control	46.21 ± 2.39 [a]	69.43 ± 3.54 [a]
Control + STZ	81.46 ± 3.59 [g]	139.37 ± 6.57 [g]
Control + STZ + Metformin	49.11 ± 3.89 [b]	74.37 ± 3.76 [b]
Control + STZ + *C. retrospiralis* (200mg/kg)	57.44 ± 4.02 [e]	97.87 ± 3.93 [f]
Control + STZ + *C. retrospiralis* (400mg/kg)	52.06 ± 4.15 [c]	95.22 ± 3.91 [e]
Control + STZ + *H. sibthorpioides* (200mg/kg)	61.37 ± 4.45 [f]	87.41 ± 3.98 [d]
Control + STZ + *H. sibthorpioides* (400mg/kg)	55.09 ± 3.31 [d]	82.55 ± 3.67 [c]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

4.10.2.5. Kidney functions (urea and creatinine level)

In diabetic rats the serum urea and creati levels were elevated. This elevated level of serum urea and creatinine were reduced significantly in groups treated with normal drugs as well as in extract treated groups (Table 34). Although the untreated diabetic group maintain its high level of serum urea and creatinine with respect to normal and treated groups.

Table 34: Effect of different plant extracts on serum urea and creatinine level of normal and streptozotocin induced diabetic rats

Groups	Urea(mg/dL)	Creatinine (mg/dL)
Control	16.96 ± 1.08 [a]	0.29 ± 0.07 [a]
Control + STZ	56.16 ± 4.98 [f]	1.97 ± 0.14 [g]
Control + STZ + Metformin	21.62 ± 3.47 [b]	0.35 ± 0.07 [b]
Control + STZ + *C. retrospiralis* (200mg/kg)	31.46 ± 4.66 [d]	0.57 ± 0.1 [f]
Control + STZ + *C. retrospiralis* (400mg/kg)	25.53 ± 2.27 [c]	0.49 ± 0.09 [d]
Control + STZ + *H. sibthorpioides* (200mg/kg)	35.92 ± 3.05 [e]	0.52 ± 0.06 [e]
Control + STZ + *H. sibthorpioides* (400mg/kg)	31.38 ± 3.19 [d]	0.41 ± 0.04 [c]

Diferent superscripts indicate significant differences of mean within the column as determined by one way ANOVA followed by DMRT ($P<0.05$).

4.11. Partial characterization of the Methanolic extract

4.11.1. FTIR analysis of the plant extracts

FTIR spectra over the range from 4000 to 650 cm-1 have been measured at room temperature (25 ^{0}C) using Perkin Elmer FTIR spectrometer. The main stretching vibrations of *C. retrospiralis* appear at 3394cm^{-1} (broad), 2924 cm^{-1}2852 cm^{-1}, 2265 cm^{-1}2065cm^{-1}, 1640cm^{-1}, 1407cm^{-1} and1237 cm^{-1}. Absorption bands in the 3600-3200 cm^{-1} regions are from O-H stretching vibrations (Figure 13). So the broad band appears at 3394 cm-1 is coming from -O-H stretching frequency. The C-H (alkane) stretching frequency generally appears below 3000 cm^{-1} so the stretching frequencies appears at 2924 and 2852 cm^{-1} are attributed to the alkyl C-H of *C. retrospiralis*. The band at 1640 is attributed to –C=O (carbonyl) stretching frequency. There is another band which appears at 1407 cm-1 attributed as –C-C stretching frequency of the *C. retrospiralis.*

Similarly we got same type of FTIR spectra of *H. sibthorpioides* (Figure 14). The main stretching vibrations of *H. sibthorpioides* appear at 3397cm-1 (broad), 2926 cm^{-1}2855 cm^{-1}, 2265 cm^{-1}, 2065 cm^{-1}, 1648cm^{-1} and 1411cm^{-1}.The broad band appears at 3397cm-1is coming from -O-H stretching frequency. The C-H (alkane) stretching frequency generally appears below 3000 cm^{-1} so the stretching frequencies appears at 2926 and 2855 cm-1 are attributed to the alkyl C-H of *H. sibthorpioides*. The band at 1648is attributed to –C=O (carbonyl) stretching frequency.There is another band

which appears at 1411 cm^{-1} attributed as –C-C stretching frequency of the *H. sibthorpioides*. From the FTIR spectra it confirmed that *C. retrospiralis* and *H. sibthorpioides* contain similar types of functional groups. From the FTIR spectra it was confirmed that *C. retrospiralis* and *H. sibthorpioides* contain similar types of functional groups.

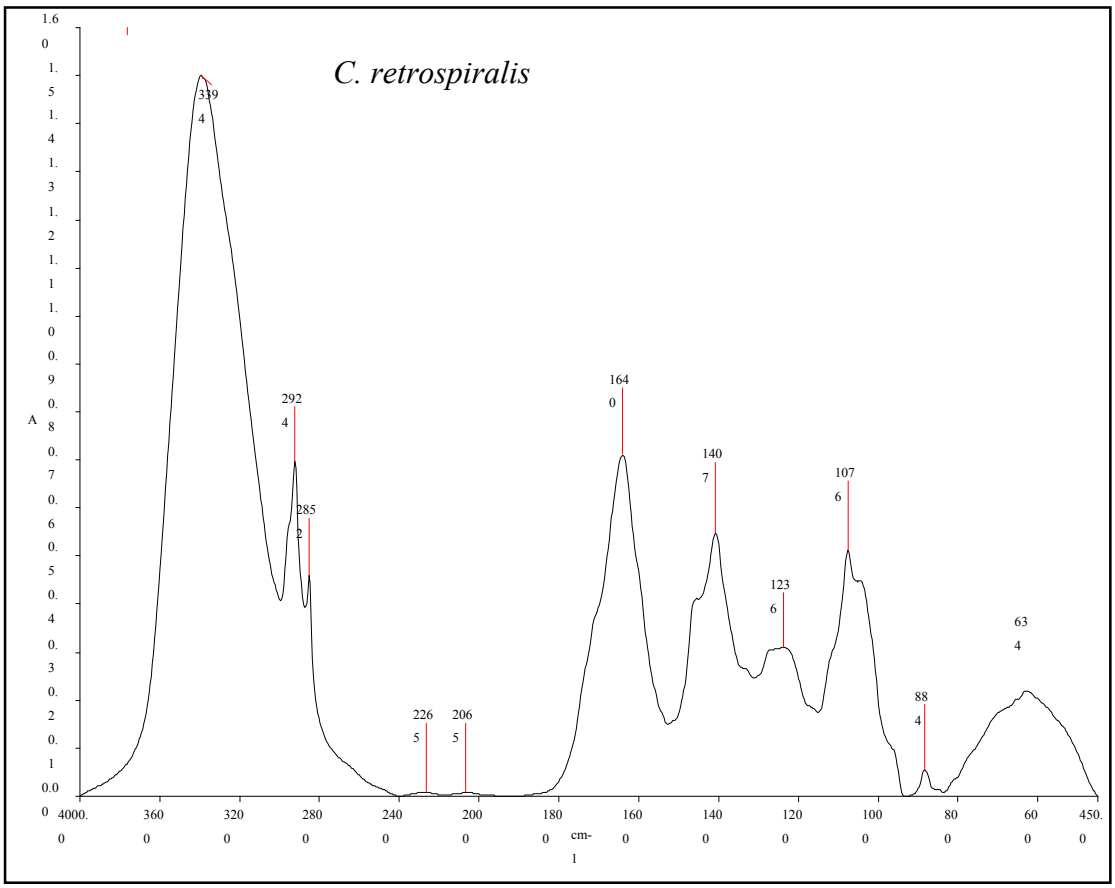

Figure 13: FTIR spectra of C *C. retrospiralis* methanolic extract.

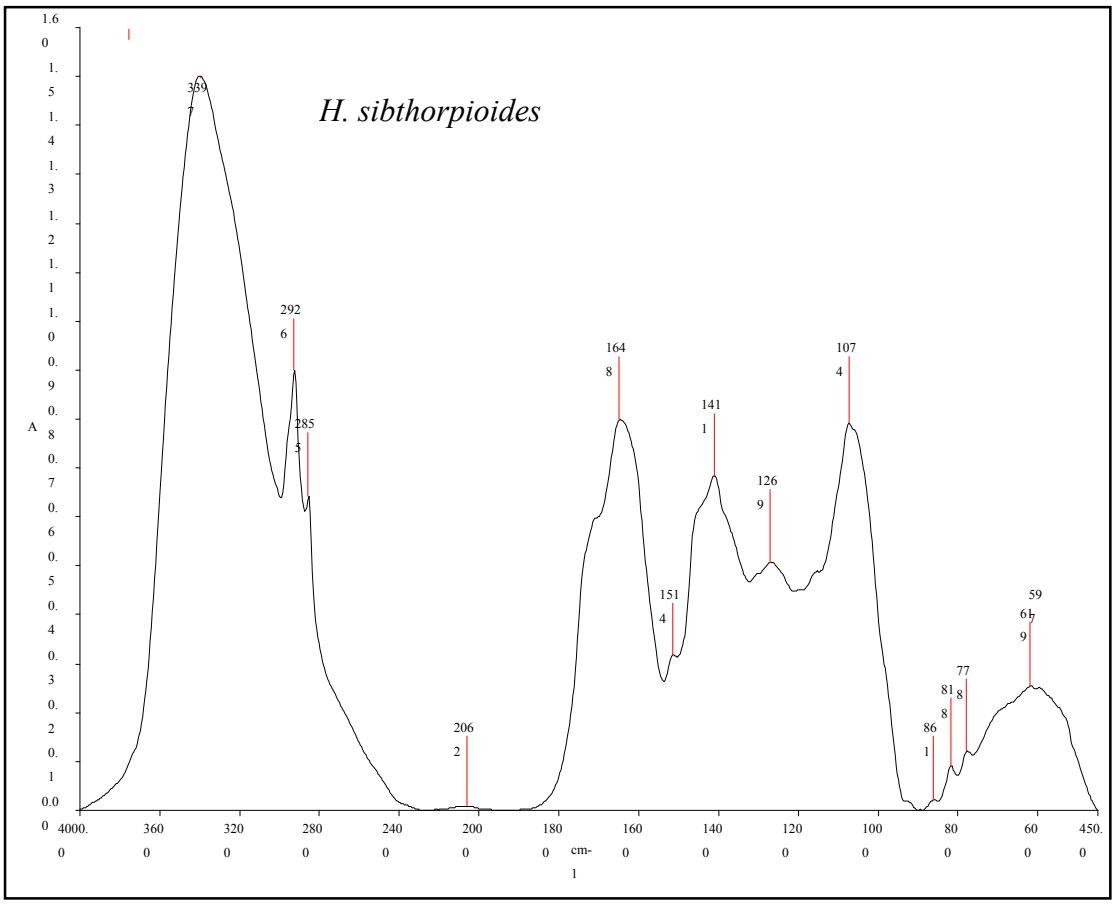

Figure 14: FTIR spectra of *H. sibthorpioides* methanolic extract.

4.11.2 HPLC analysis of phenolic compounds of the plant extracts

HPLC profile of *Cryptocoryne retrospiralis* and *Hydrocotyle sibthorpioides* along with the other plant extracts were done and the analysis revealed the presence of a wide array of phenolic compounds (Table 35; Fig. 15-29). A total of 70 major peaks were detected across the plant extracts which were represented by individual peaks with different retention times in the chromatogram. Among the plant extracts, *M. minuta* recorded the maximum number of phenolic compounds with the presence of 61 major peaks pointing out towards the presence of a wide range of phenolic compounds. In *H. sibthorpioides*, however only 15 peaks could be obtained and in *C. retrospiralis*, only 12 peaks could be recorded. This indicated towards the fact that the phenolic compounds in *H. sibthorpioides* and *C. retrospiralis* seems to be less probable in conferring the antioxidative, antidiabetic and antimicrobial properties of the two plant extracts. Therefore, the HPLC analysis clearly revealed the role of other putative compounds in conferring the desired properties of *H. sibthorpioides* and *C. retrospiralis* (Table 35).

Table 35: HPLC profile of phenolics of the 15 plant extracts with peak area and retention time of the individual phenols. A total of 70 major peaks were detected in the HPLC profile. Absence of corresponding peaks for the individual extracts is shown with coloured cells.

Peak No.	R.T. (in min)	Peak area of the plant extracts														
		MM	HS	CR	PO	IA	GO	AV	BA	EF	PM	EP	DE	HA	AS	PN
1	4.54	298.45				137.61	22.78			38.66			122.12			
2	4.91	486.18		265.16												
3	5.36	329.70							18.20							
4	5.73	1948.3	383.41			109.41				158.38						
5	5.85				34.77			122.25	49.77							
6	5.99	80.38		52.12					58.09	16.91						
7	6.30	255.61		93.52		69.40		32.92		38.56						
9	6.49	78.24							188.44							
11	6.76	302.28					87.42		167.91							
12	6.96	171.74							286.36							
16	7.17	190.00		267.24	48.89	69.43	148.68	32.07	794.15							
17	7.34	654.69	39.55	360.21												
18	7.60	411.06				81.42		33.04		41.28						
19	7.74	66.99	182.91													
20	7.82	125.03	1320.2				309.70									
21	8.01	646.02				44.92		36.78								
23	8.33	852.35	109.97	106.09		381.71	38.82		96.42			263.22				
24	8.57	2110.5	324.80	54.79	85.42	762.19	39.33	92.59	1095.1	930.62	57.21	44.39		8.61		
25	8.68	448.67											35.40			
26	8.79	111.86		190.50		149.24			136.15	39.92						
27	8.87		264.41	111.63			714.20		268.32							
29	9.10	857.66	79.55				625.44		46.28			351.69				
31	9.55	158.37						35.58	149.82					9.43		
32	9.64	666.08														
33	9.81	554.87				98.15	262.05							9.77		

34	**10.00**	**50.20**				**88.30**	**150.16**		**174.76**	**50.88**			**23.12**	**10.07**		
36	**10.30**		**95.26**									**108.28**	**20.45**	**10.28**	**51.46**	**9.63**
37	**10.42**						**126.61**						**462.92**			
39	**10.87**		**87.81**		**131.91**	**388.24**	**949.02**	**74.33**		**94.10**	**458.14**		**361.86**	**10.83**	**17.19**	
45	**12.32**				**39.46**	**51.21**		**42.49**								
46	**12.64**									**141.33**						
58	**15.24**		**133.96**									**50.44**				
59	**15.43**	**60.63**	**48.24**		**97.63**	**65.42**	**35.58**	**80.34**			**104.42**		**25.51**	**59.99**	**22.40**	**10.29**
63	**16.30**												**76.82**			
65	**16.88**	**48.06**	**44.77**		**41.17**	**71.38**		**56.01**			**47.80**			**58.97**	**63.44**	
68	**17.51**		**40.54**	**58.90**		**54.97**	**45.26**	**51.81**			**54.20**			**40.30**		
69	**17.69**		**59.93**	**221.88**	**221.87**	**182.71**	**138.90**	**203.32**	**35.63**		**141.43**	**76.92**	**15.35**	**89.61**		**58.66**
70	**17.85**			**86.67**	**84.87**	**95.88**	**64.24**	**112.99**					**17.71**			

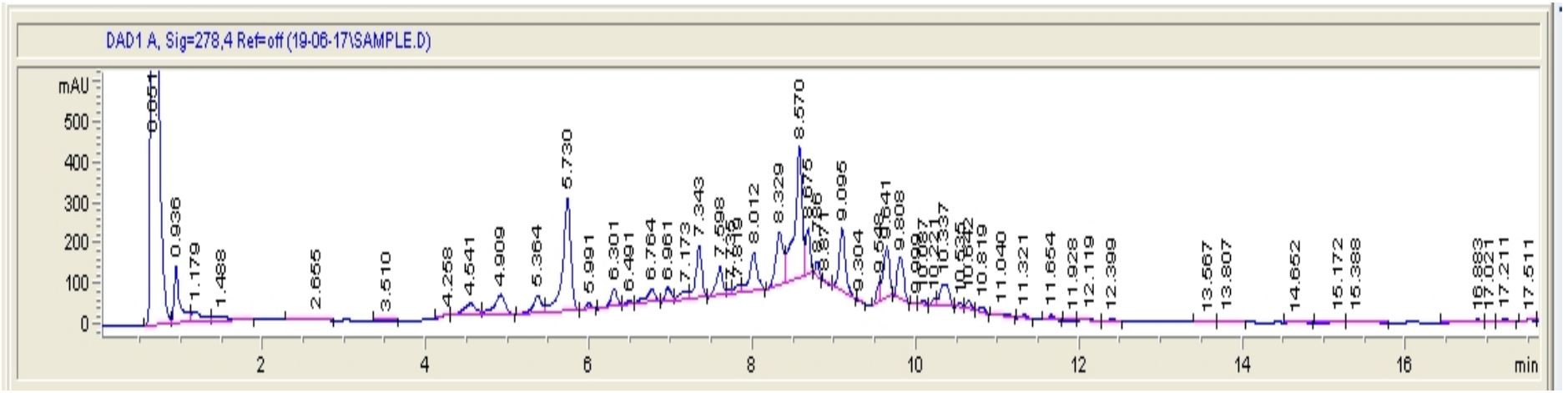

Figure 15. HPLC chromatogram of *M. minuta* with major peaks highlighted with corresponding retention time (in mins)

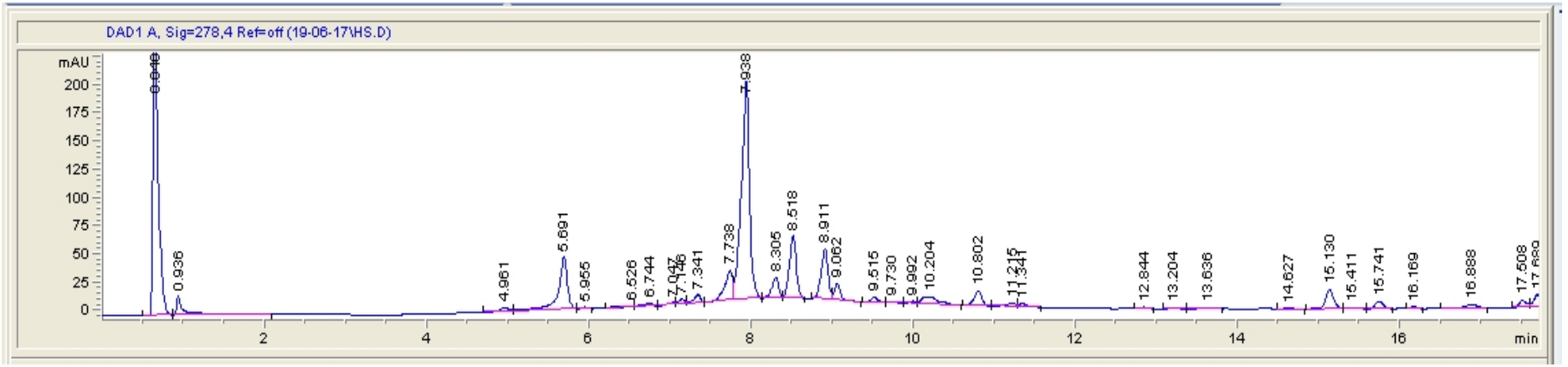

Figure 16. HPLC chromatogram of *H. sibthorpioides* with major peaks highlighted with corresponding retention time (in mins)

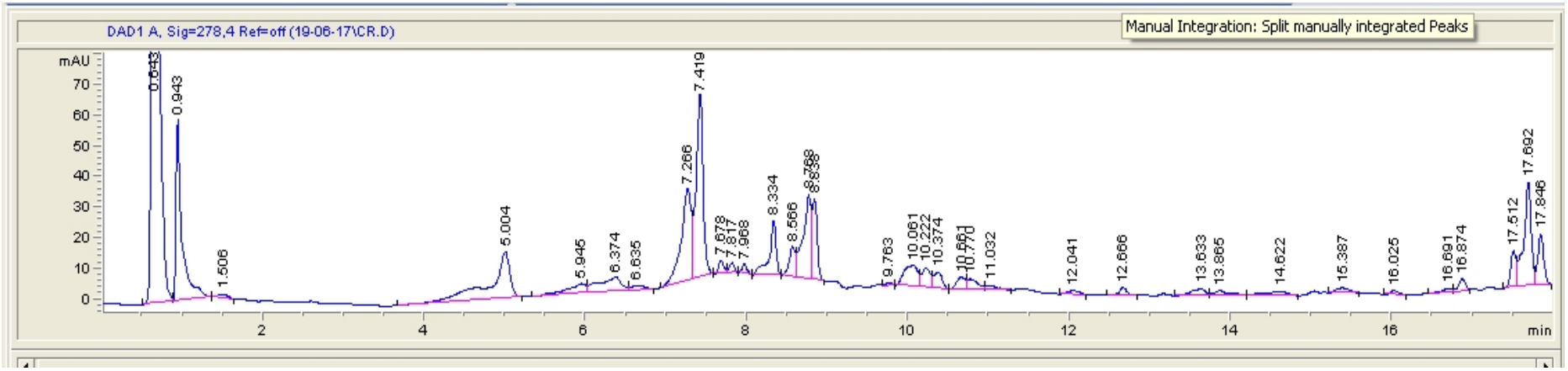

Figure 17. HPLC chromatogram of *C. retrospiralis* with major peaks highlighted with corresponding retention time (in mins)

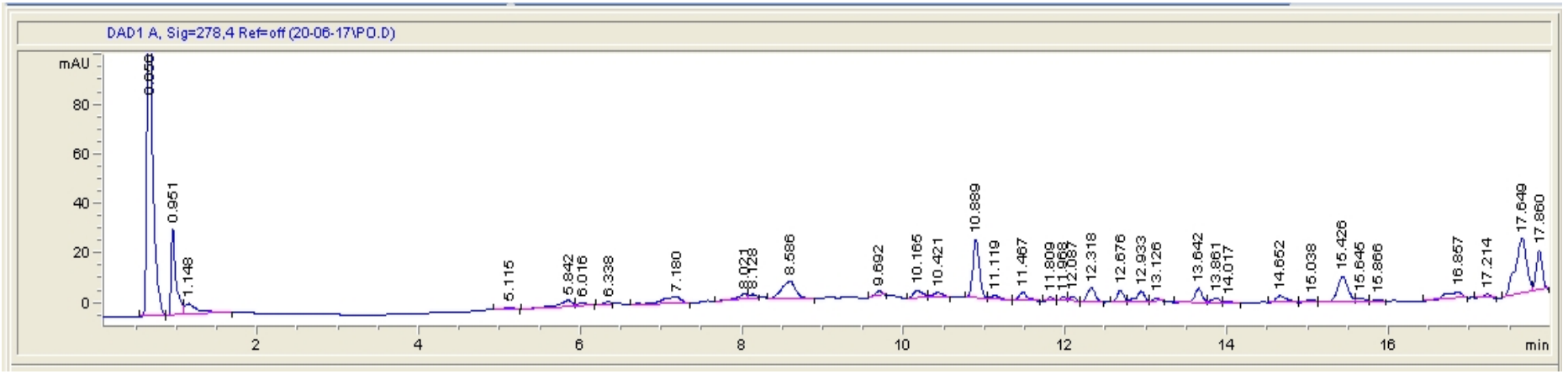

Figure 18. HPLC chromatogram of *P. oleracea* with major peaks highlighted with corresponding retention time (in mins)

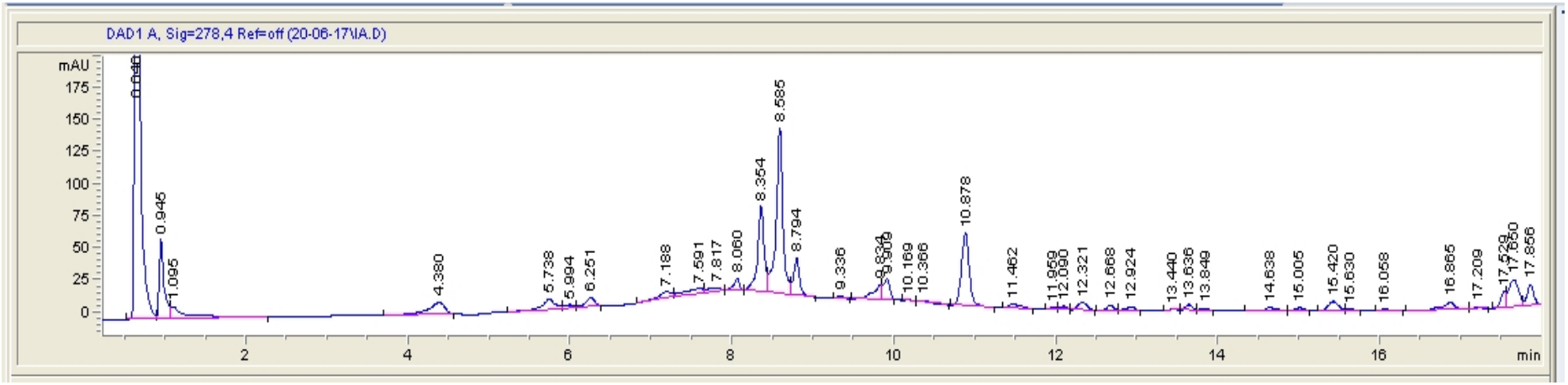

Figure 19. HPLC chromatogram of *I. aquatica* with major peaks highlighted with corresponding retention time (in mins)

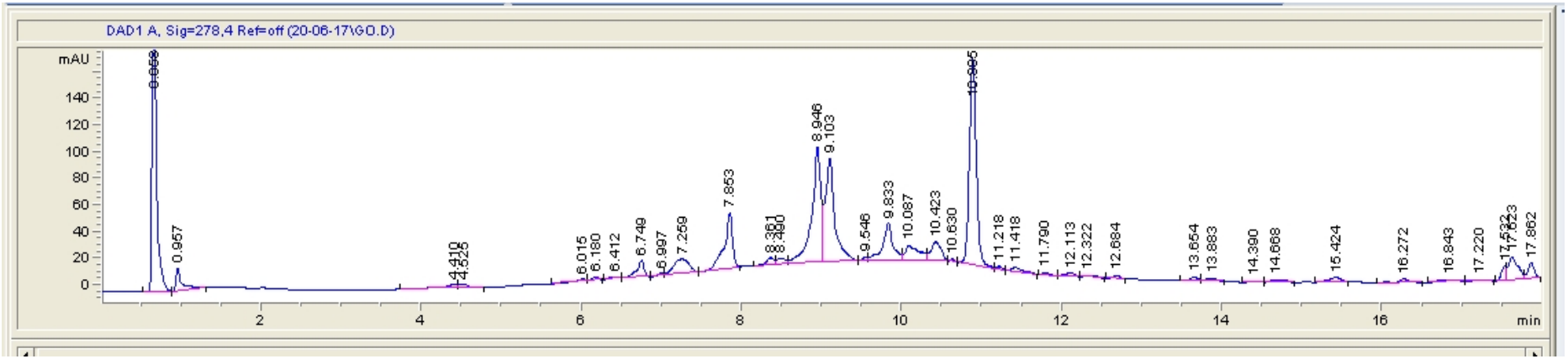

Figure 20. HPLC chromatogram of *G. oppositifolius* with major peaks highlighted with corresponding retention time (in mins)

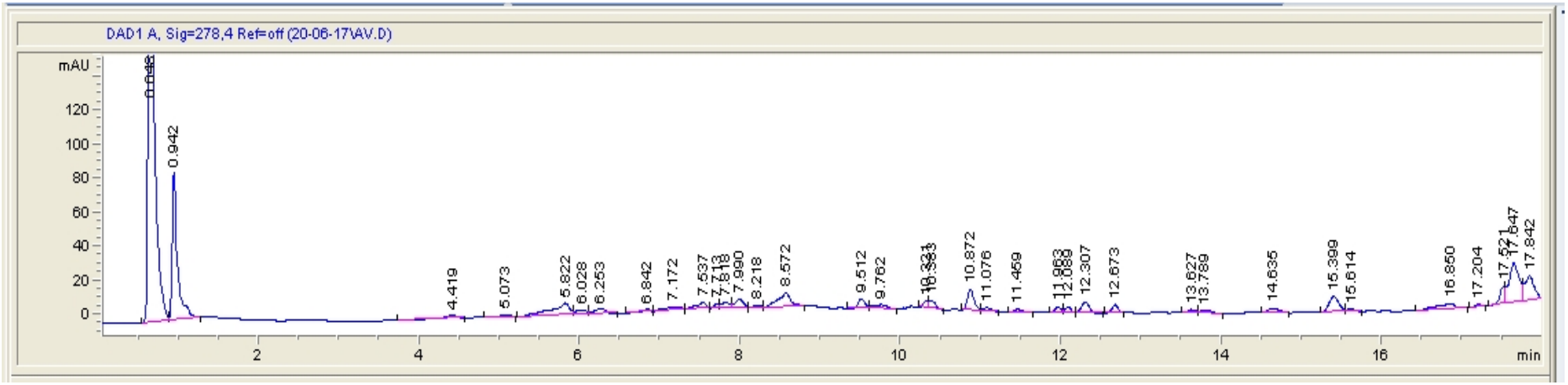

Figure 21. HPLC chromatogram of *A. viridis* with major peaks highlighted with corresponding retention time (in mins)

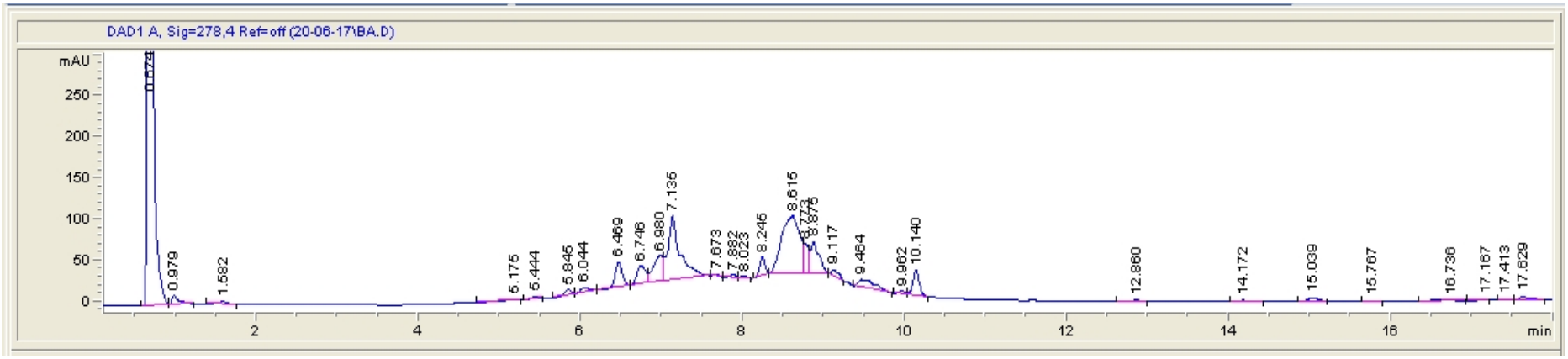

Figure 22. HPLC chromatogram of *B. acutangula* with major peaks highlighted with corresponding retention time (in mins)

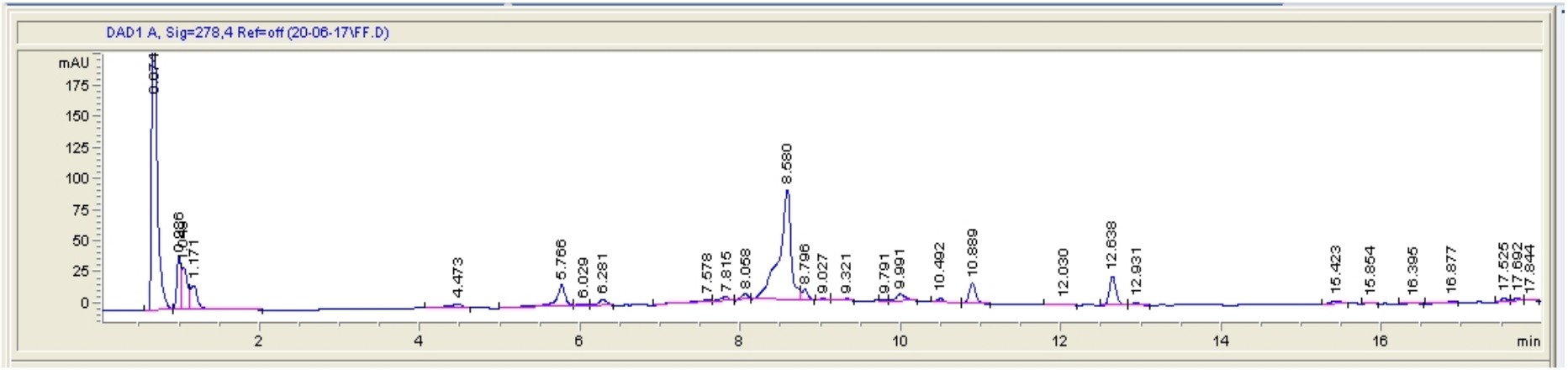

Figure 23. HPLC chromatogram of *E. fluctuans* with major peaks highlighted with corresponding retention time (in mins)

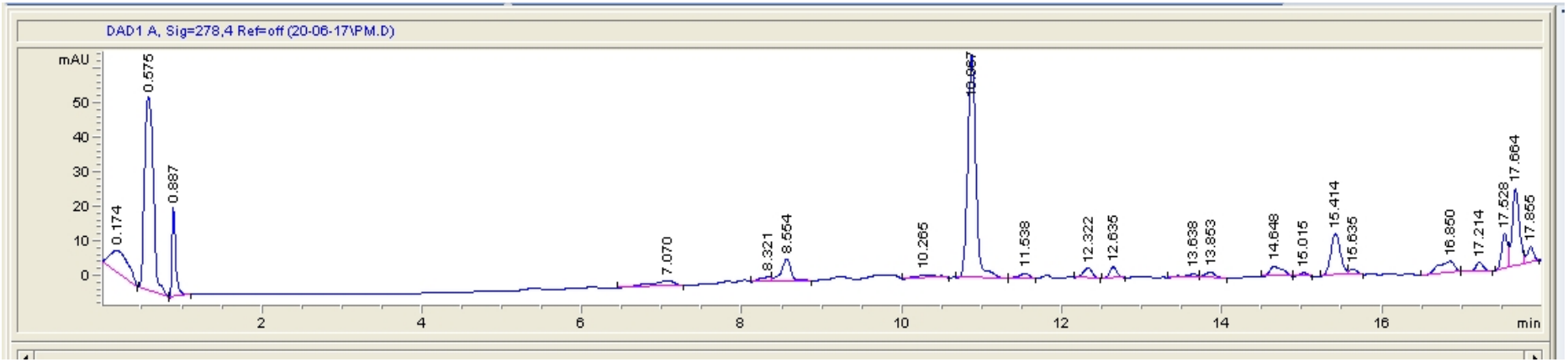

Figure 24. HPLC chromatogram of *P. microphylla* with major peaks highlighted with corresponding retention time (in mins)

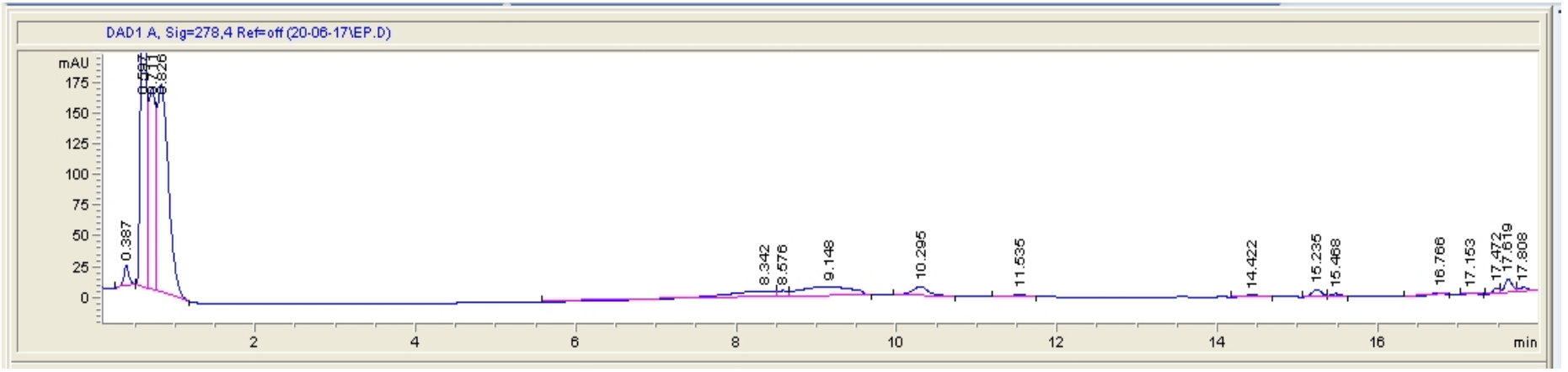

Figure 25. HPLC chromatogram of *E. prostrata* with major peaks highlighted with corresponding retention time (in mins)

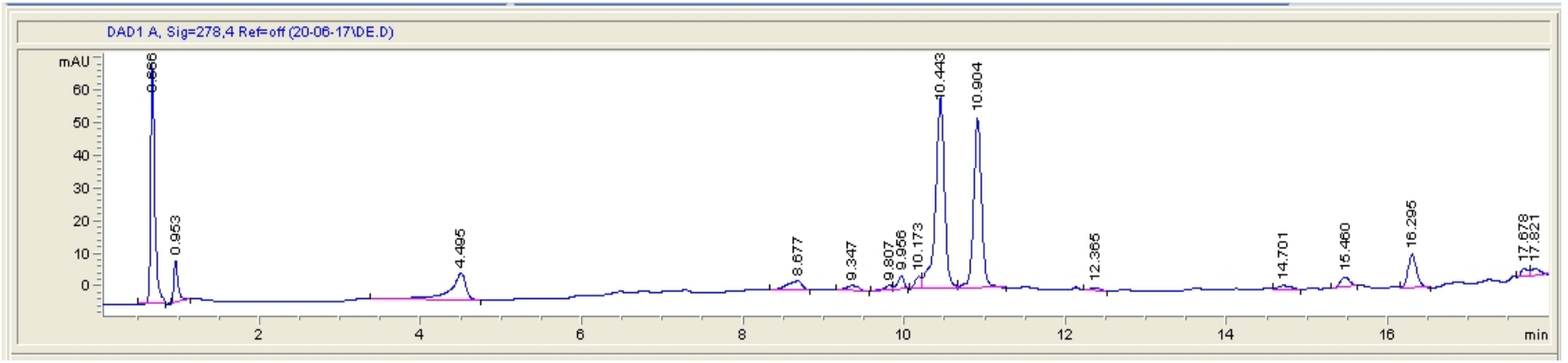

Figure 26. HPLC chromatogram of *D.esculentum* with major peaks highlighted with corresponding retention time (in mins)

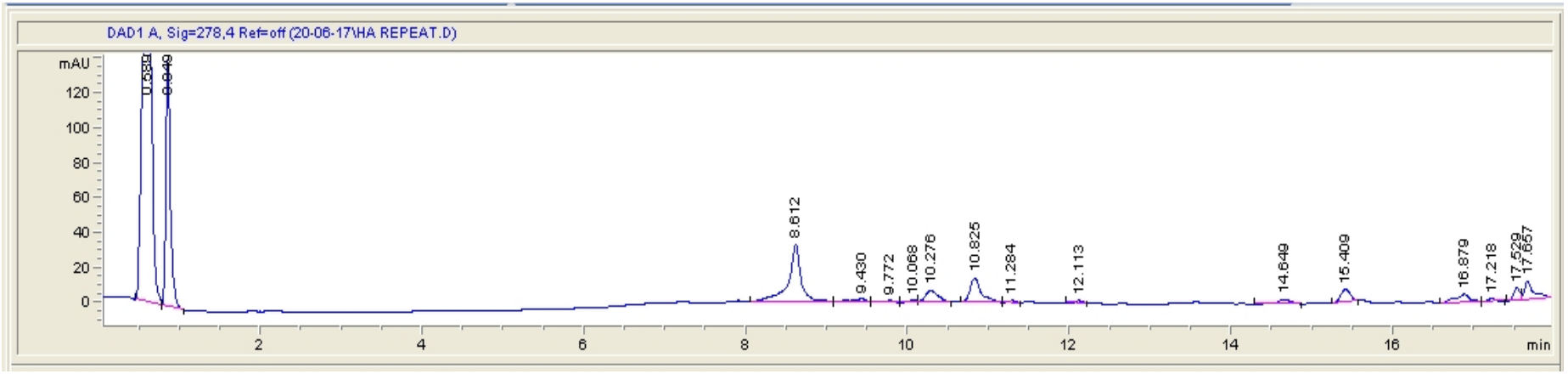

Figure 27. HPLC chromatogram of *H. auriculata* with major peaks highlighted with corresponding retention time (in mins)

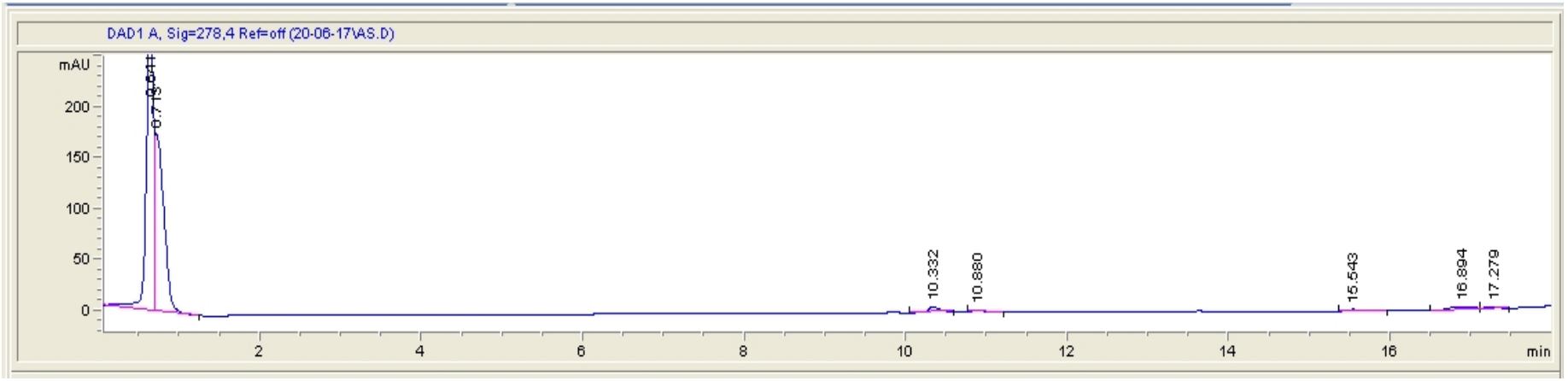

Figure 28. HPLC chromatogram of *A.spinosus* with major peaks highlighted with corresponding retention time (in mins)

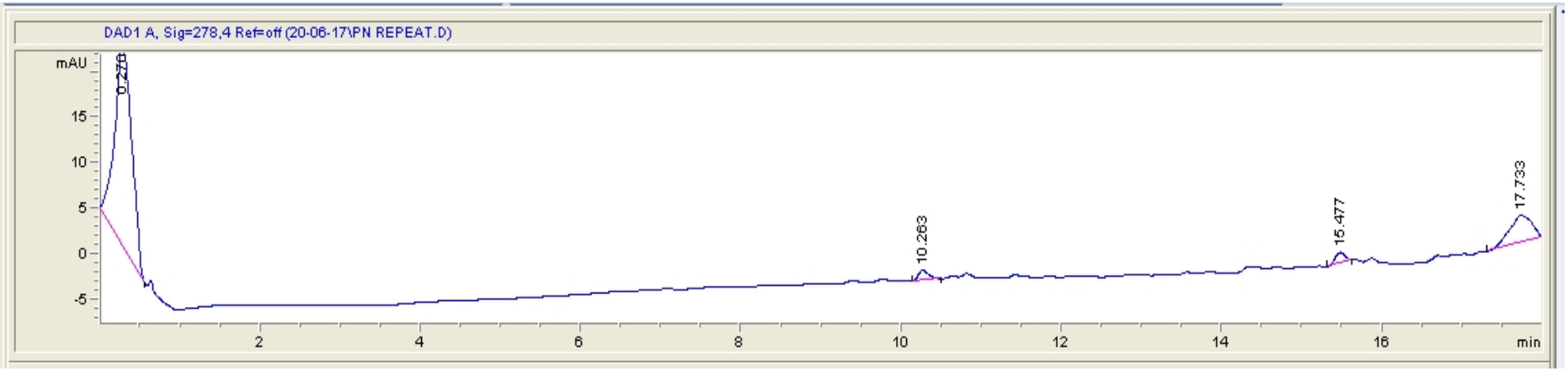

Figure 29: HPLC chromatogram of *P. nodiflora* with major peaks highlighted with corresponding retention time (in mins)

4.11.3. Gas Chromatography - Mass Spectrophotometry (GC-MS) of the plant extracts

The methanolic extract of *C. retrospiralis* and *H. sibthorpioides* were further subjected to GC-MS for partial characterization of the compounds present in the fractions. Numerous compounds were identified and the variation of these compounds in the plants has been presented in Tables 36 & 37. Further, GC-MS total ion chromatogram and mass spectra for all the identified compounds with its respective structures are given in Figure 30 & 31. *C. retrospiralis* methanolic extract were identified to contain nine different compounds (Table 36), and *H. sibthorpioides* methanolic extracts were identified to contain ten different compounds (Table 37).

Table 36. Compounds identified in the methanolic extract of *C. retrospiralis*

Sl no	**Compound name**	**Molecular formula**	**Molecular Weight**	**RT**
1	1,3- Diazocane -2 thione	$C_6H1_2N_2S$	144.24	8.78
2	E 11 - Methyl-12-tetradecan-1-ol acetate	$C_{17}H_{32}O_2$	268.43	16.07
3	Hexadecanoic acid- methyl ester	$C_{17}H_{34}O_2$	270.45	17.02
4	Phytol	$C_{20}H_{40}O$	296.53	18.93
5	9, 12- Octadecadienoic acid	$C_{18}H_{32}O_2$	280.45	19.38
6	Isopropyl Stearate	$C_{20}H_{20}O_2$	312.53	20.28
7	6,9,12-Octadecatrienoic acid, Phenylmethyl ester	$C_{18}H_{30}O_2$	278.43	23.83
8	Testosterone Enanthate	$C_{26}H_{40}O_3$	400.59	25.93
9	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	17.75

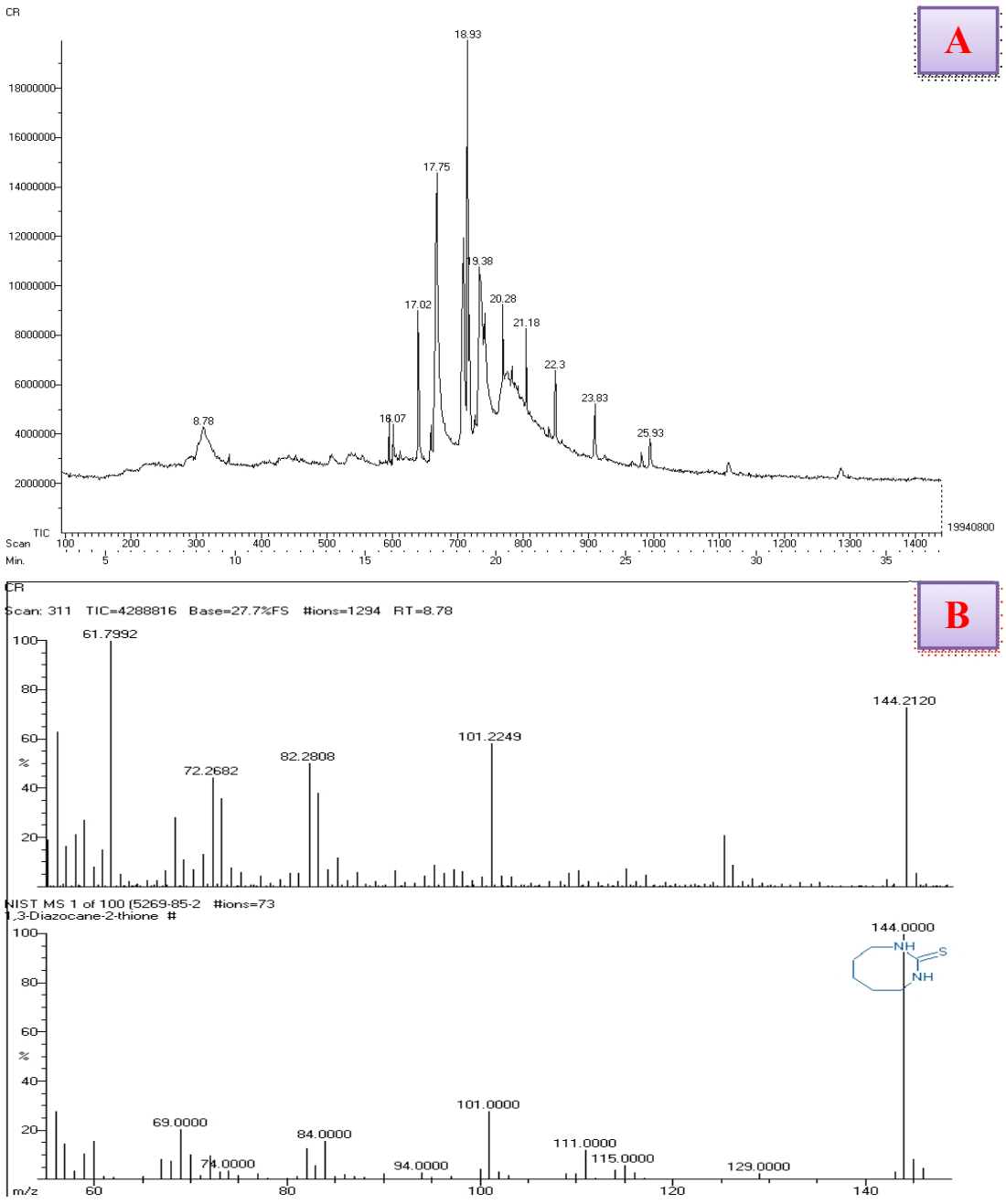

Figure 30: A-B . A- GC-MS Total ion chromatogram (TIC) of MEOH extract of *C. retrospiralis*, **B-** Compound identified in CR-MEOH extract: 1,3- Diazocane -2 thione.

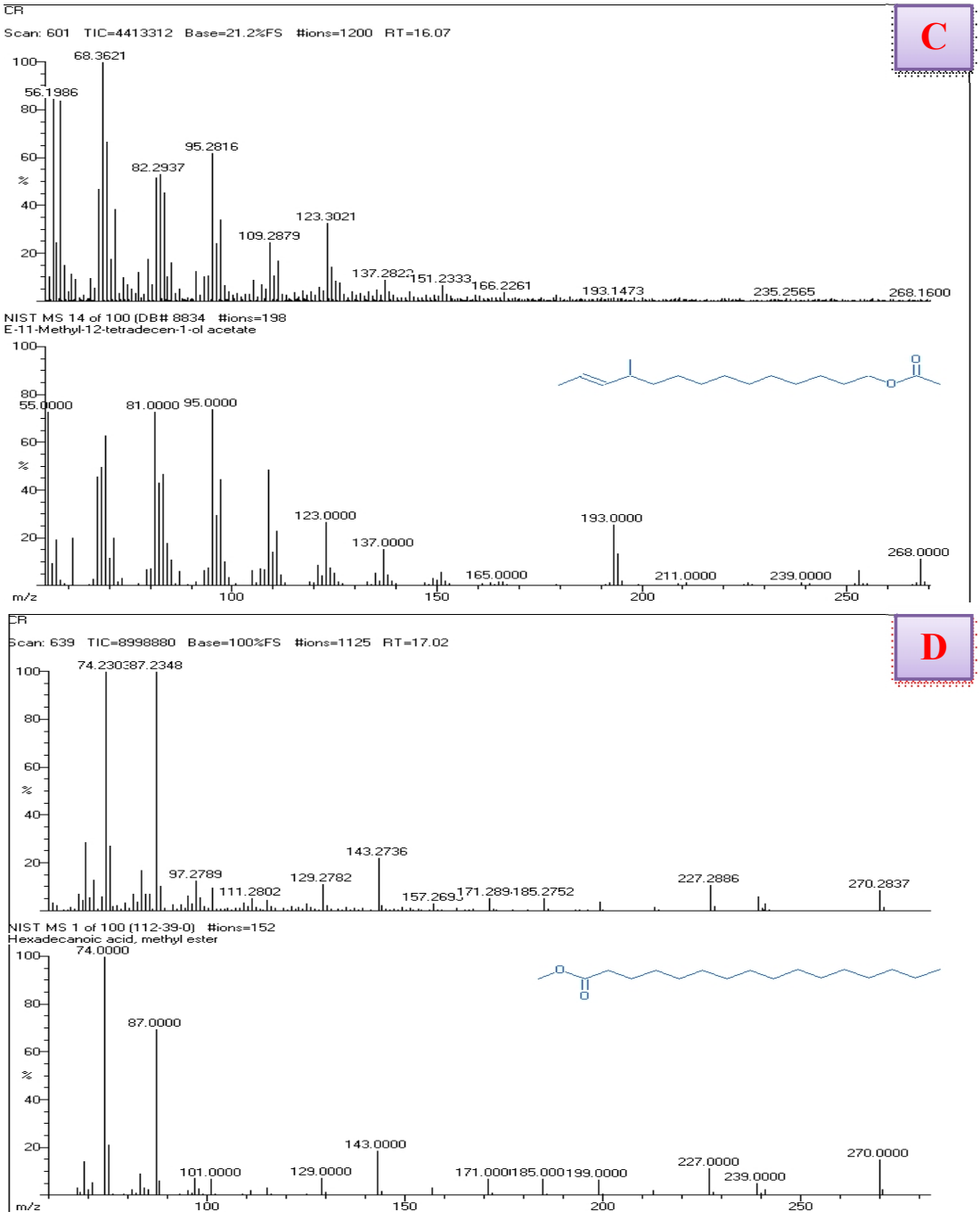

Figure 30: C-D. Compounds identified in CR-MEOH extract: **C.** 11-Methyl-12-tetradecan-1-ol acetate, **D.** Hexadecanoic acid- methyl ester

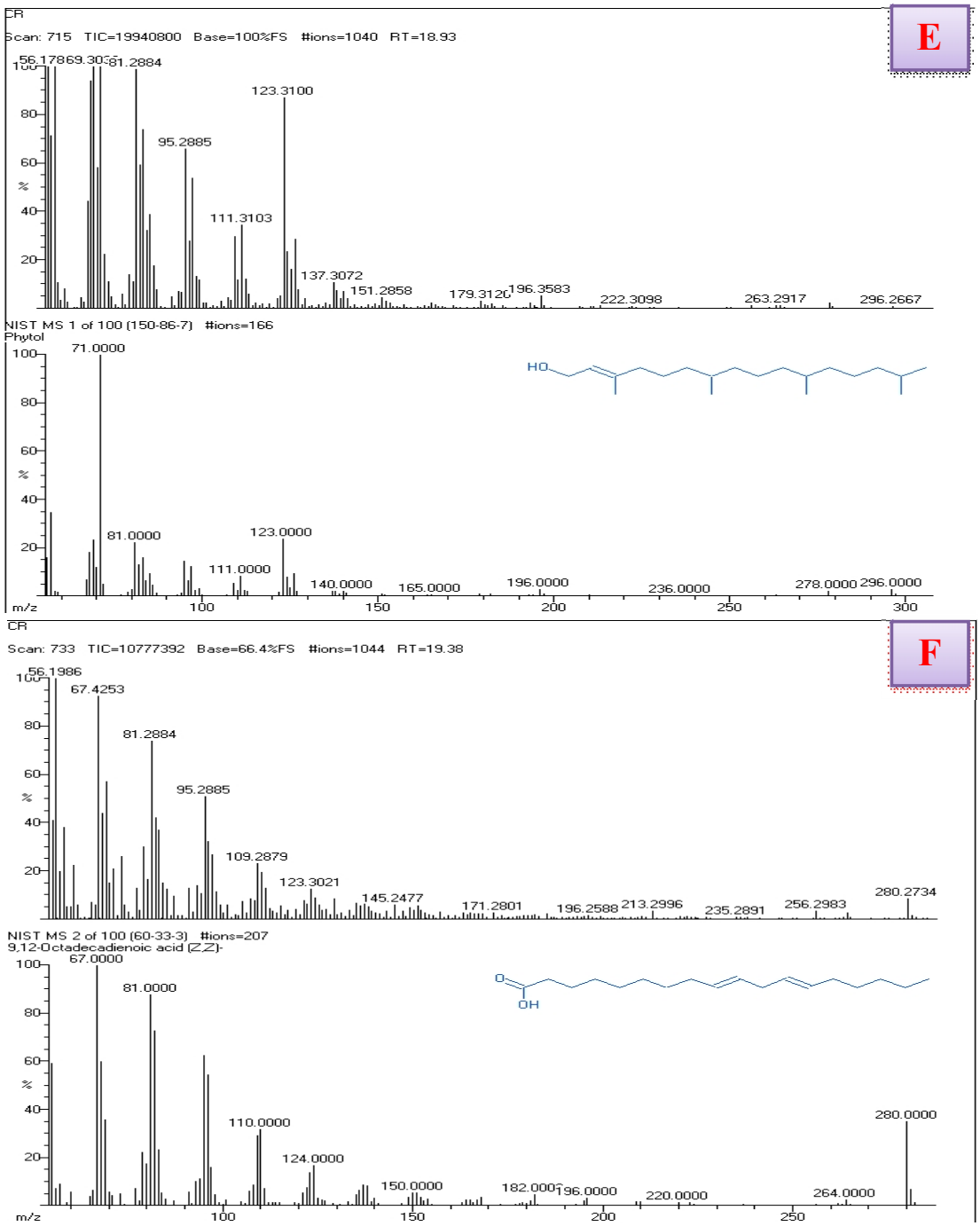

Figure 30: E-F. Compounds identified in CR-MEOH extract: **E.** Phytol**, F.** 9,12-Octadecadienoic acid

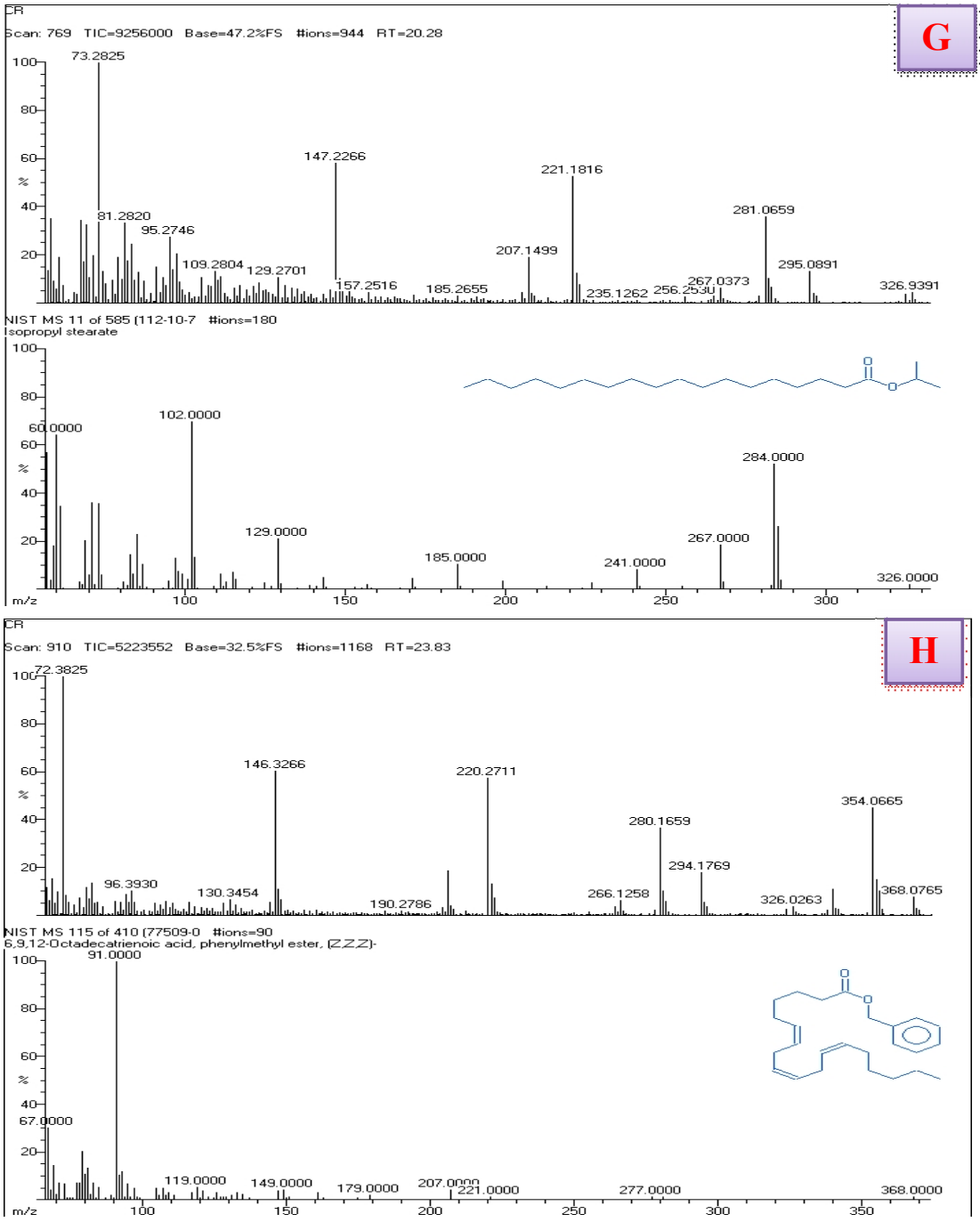

Figure 30 G-H: Compound identified in CR-MEOH extract: **G.** Isopropyl stearate, **H.** 6,9,12-Octadecatrienoic acid, phenylmethyl ester.

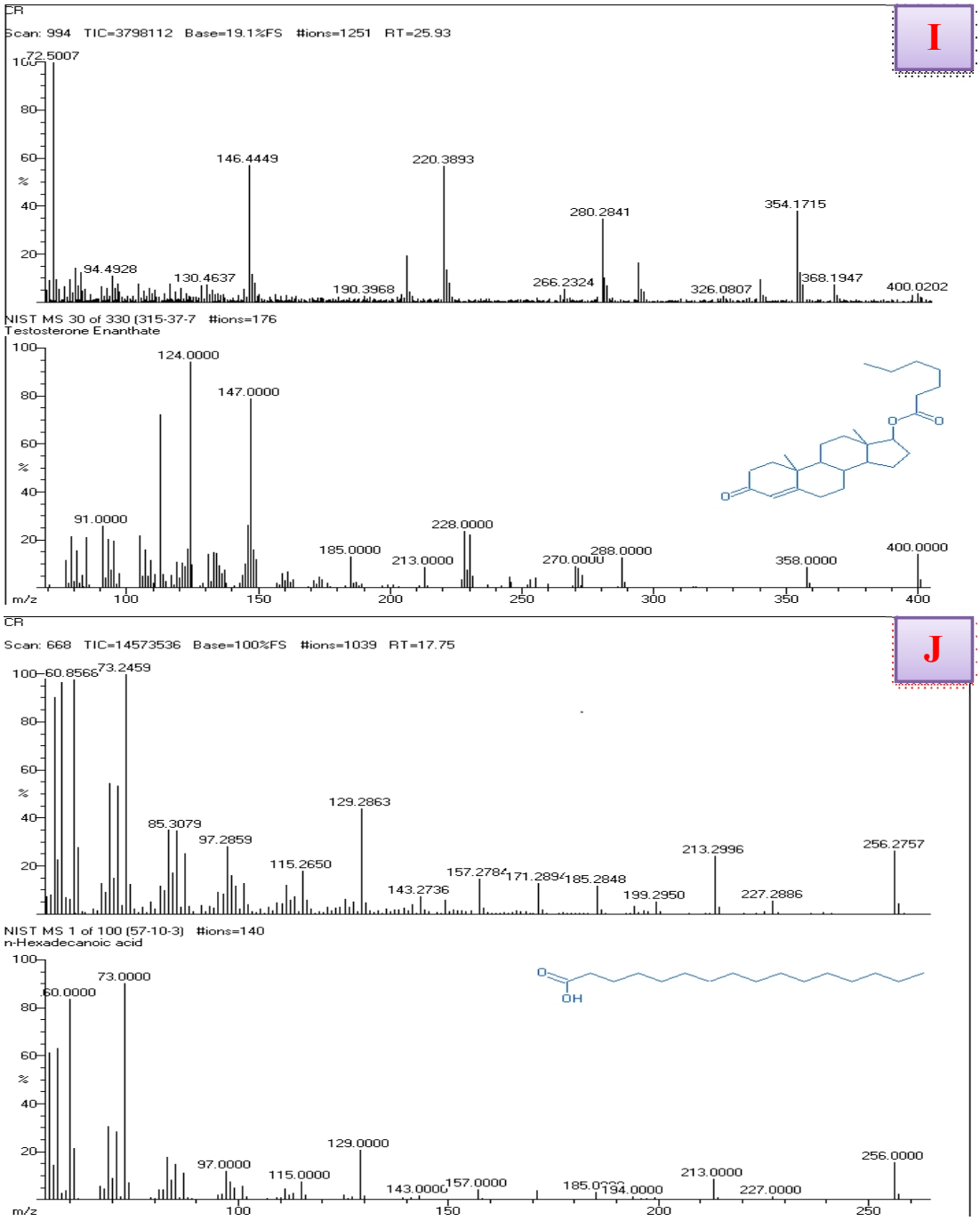

Figure 30: I-J. Compound identified in CR-MEOH extract: **I.** Testosterone enanthate, **J.** n-Hexadecanoic acid

Table 36: Compounds identified in the Methanolic extract of *H. sibthorpioides*

Sl no	Compound name	Molecular formula	Molecular Weight	RT
1	3 Eicosyne	C20H20	280.53	16.1
2	Morphinan- 3,14-diol, 4,5-epoxy(5 á)	C16H19NO3	273.33	17.47
3	9,12,15-Octadecatrienoic acid, 2,3-dihydroxy propyl ester	$C_{20}H_{34}O_4$	338.48	19.33
4	Corynan-17- ol,18,19-didehydro-10-methoxy acetate	$C_{22}H_{28}N_{20}O_3$	368.47	20.48
5	Curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo, methyl ester	$C_{20}H_{22}N_2O_4$	354.40	22.30
6	10-Octadecanoic acid, methyl ester	$C_{19}H_{36}O_2$	296.49	18.78
7	Z,E-3,13-Octadecadien -1 ol	$C_{18}H_{34}O$	266.46	16.55
8	Thujopsene	$C_{15}H_{24}$	204.35	11.15
9	3-Cyclohexen-1-ol, 4- methyl-1-1(1-methylethyl)	$C_{10}H_{18}O$	154.25	12.4
10	Hexadecanoic acid- methyl ester	$C_{17}H_{34}O_2$	270.45	17.05

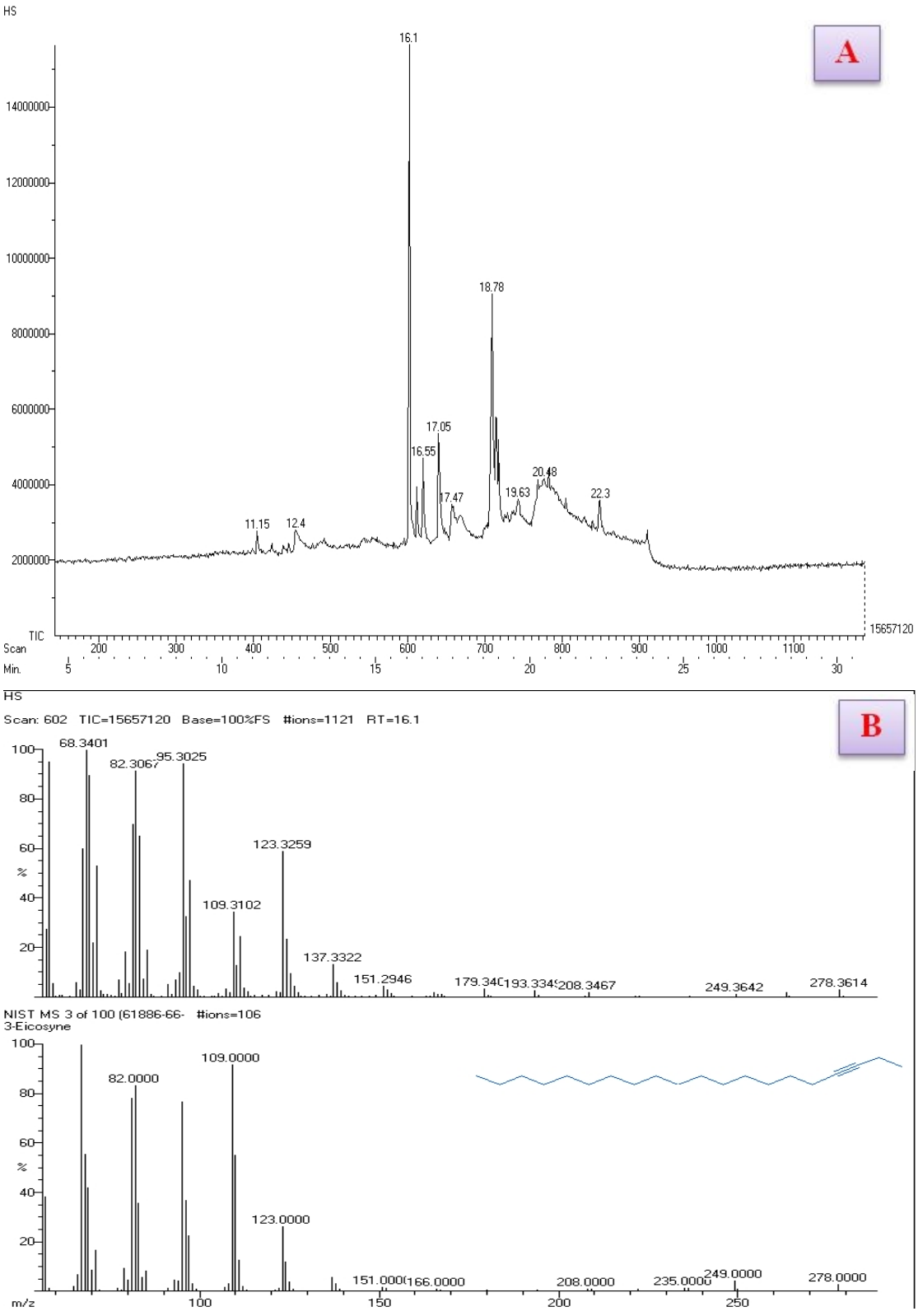

Figure 31. A-B. A. GC-MS Total ion chromatogram (TIC) of *H. sibthorpioides*, **B.** Compound identified in HS-MEOH extract: 3- Eicosyne

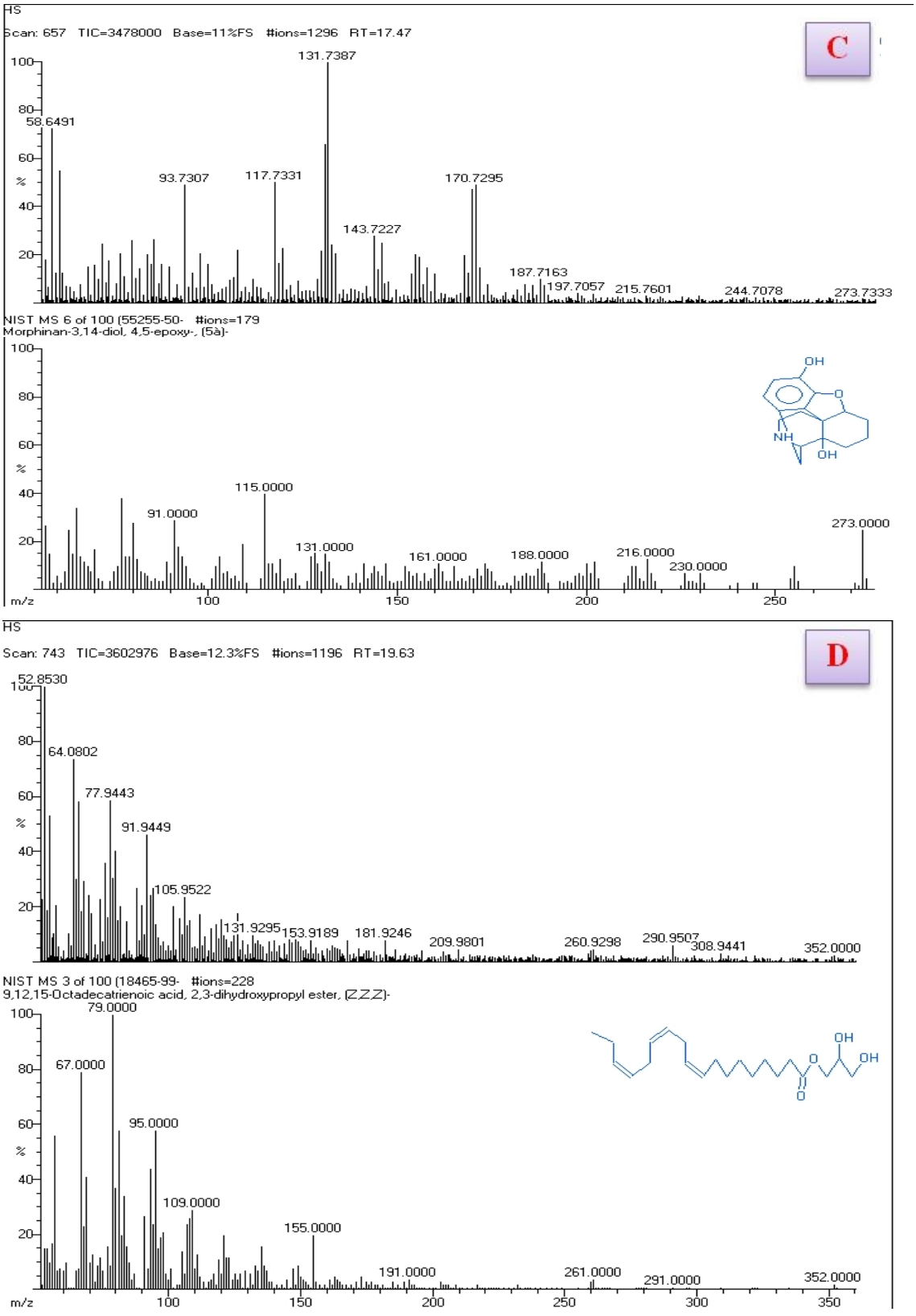

Figure 31.C-D. Compound identified in HS-MEOH extract: **C.** Morphinan- 3,14-diol, 4,5-epoxy(5 á) **D.** 9,12,15-Octadecatrienoic acid, 2,3-dihydroxy propyl ester (ZZZ)

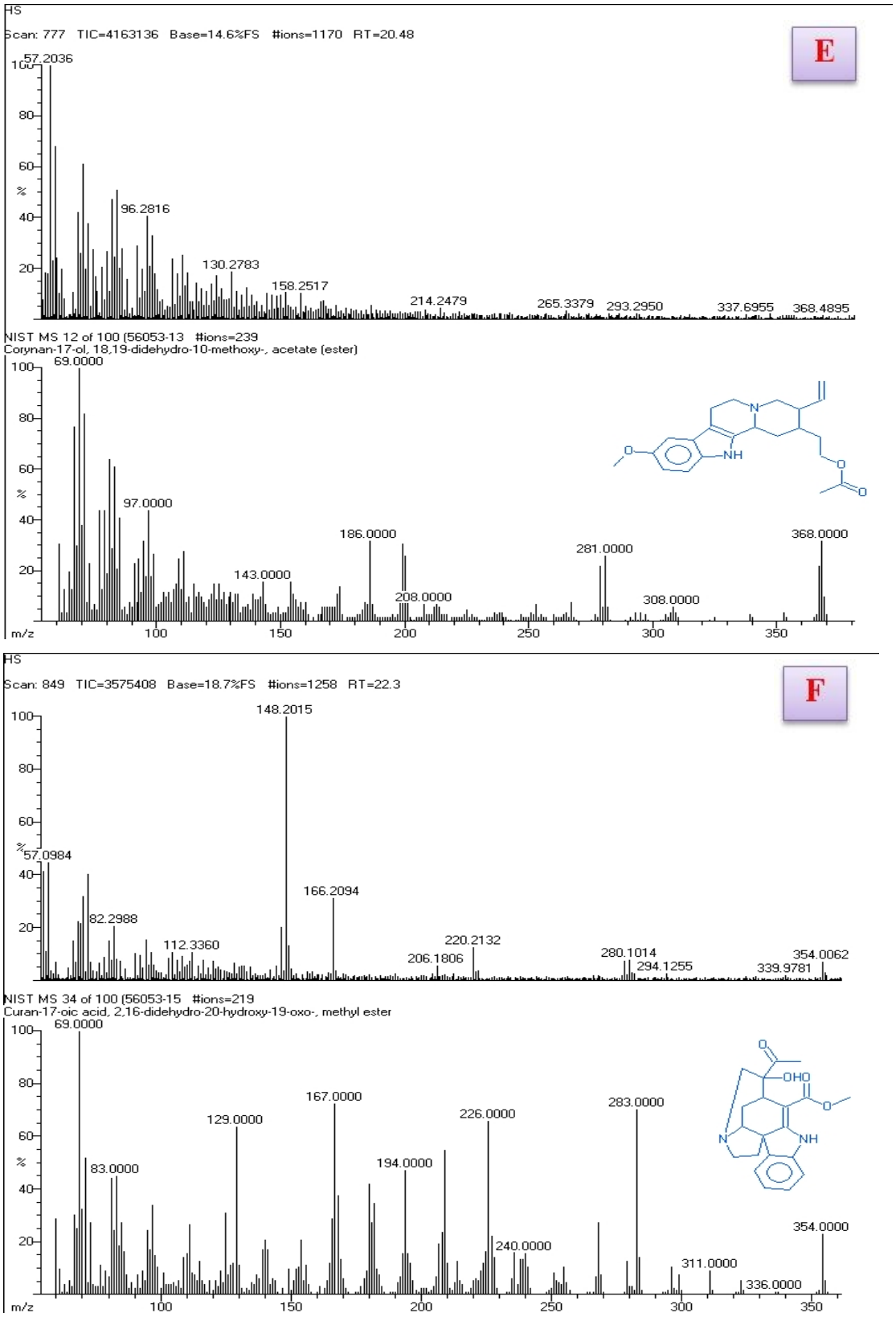

Figure 31.E-F. Compound identified in HS-MEOH extract: **E.** Corynan-17- ol,18,19-didehydro-10-methoxy acetate (ester), **F.** Curan-17-oic acid, 2, 16-didehydro-20-hydroxy-19-oxo, methyl ester

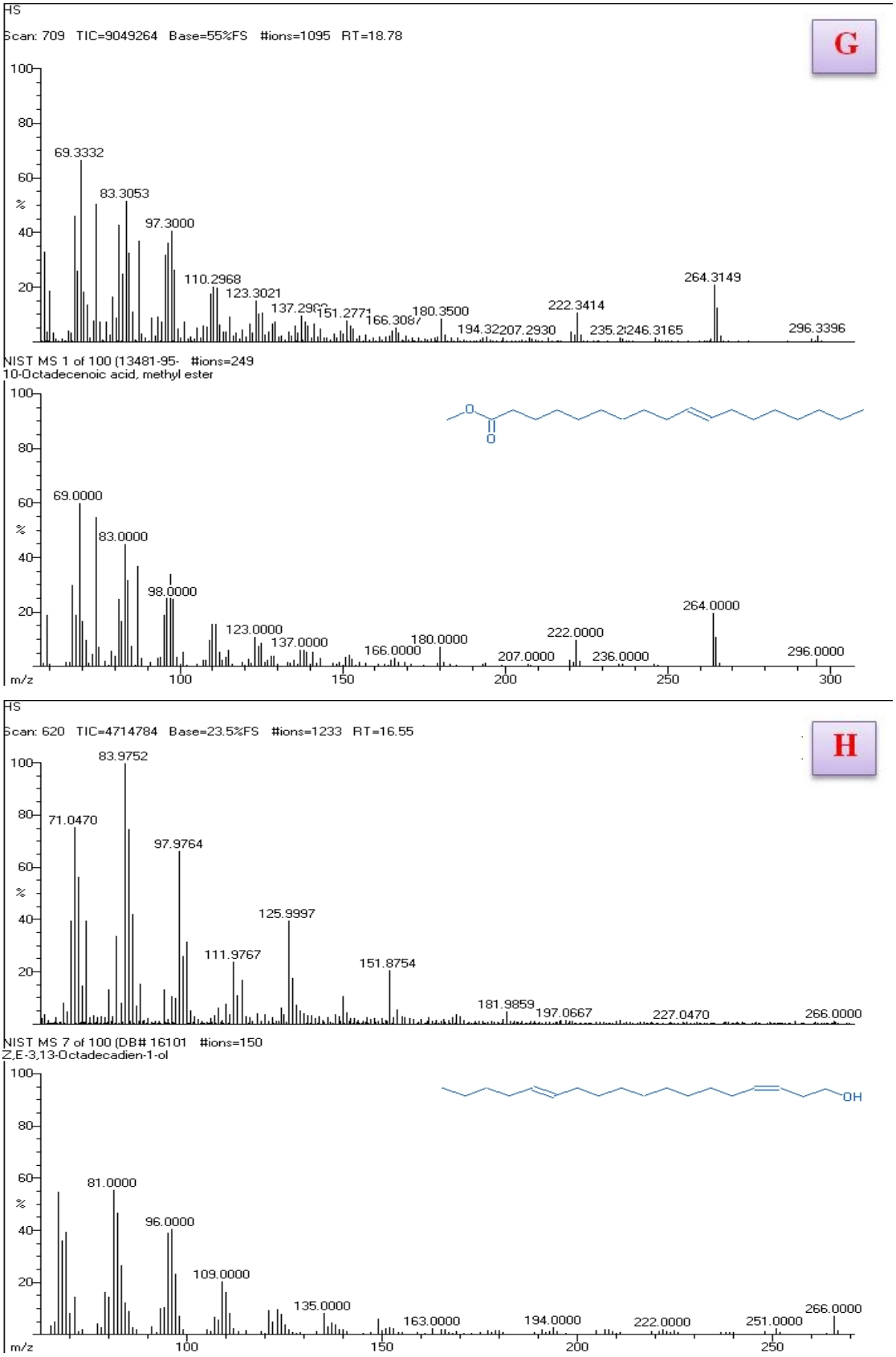

Figure 31.G-H. Compound identified in HS-MEOH extract: **G.** 10-Octadecanoic acid, methyl ester, **H.** Z,E-3,13-Octadecadien-1-ol

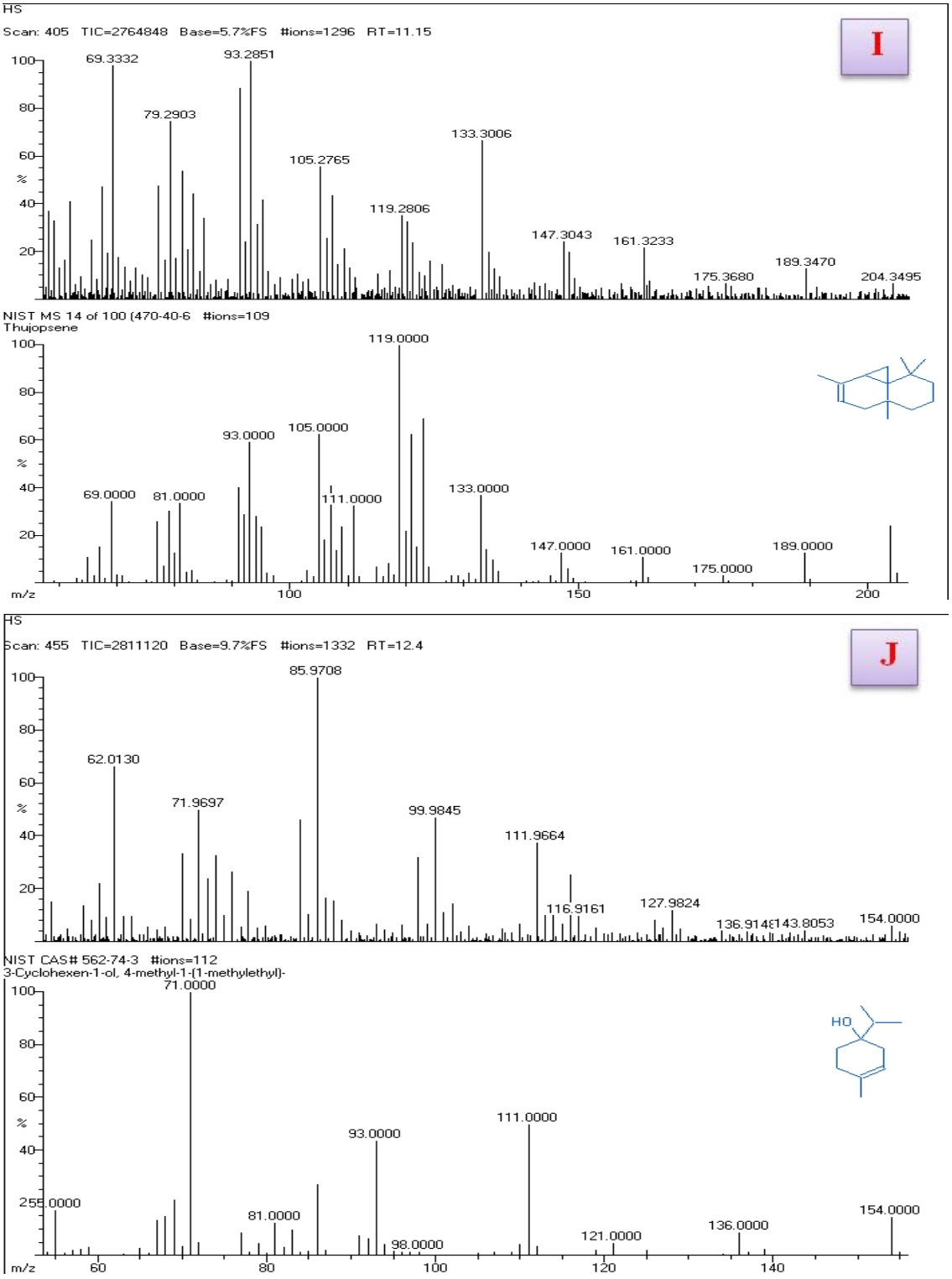

Figure 31. I-J. Compound identified in HS-MEOH extract: **I.** Thujopsene

, **J.** 3-Cyclohexen-1-ol, 4- methyl-1-1(1-methylethyl)

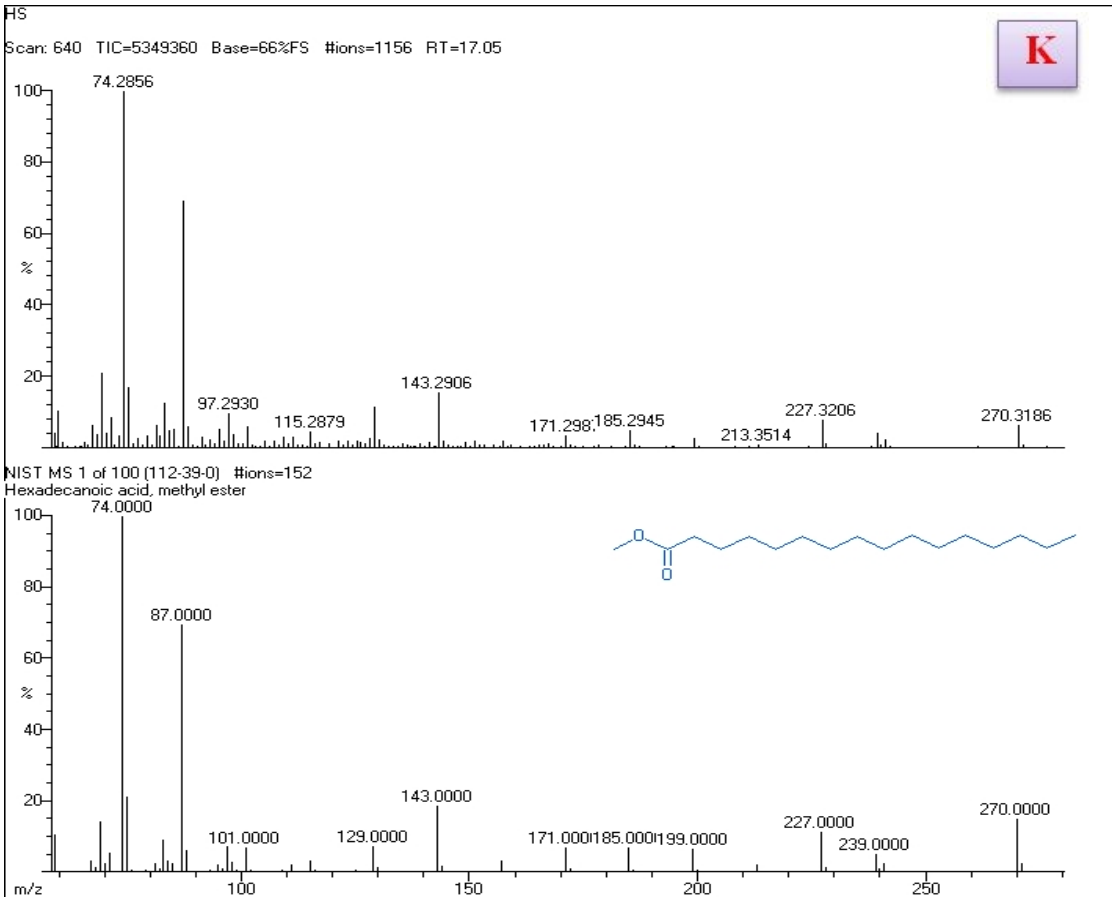

Figure 31. Compound identified in HS-MEOH extract: **K.** Hexadecanoic acid-methyl ester

4.12. Molecular docking study

The binding energies of different bioactive compounds obtained from *C. retrospiralis* and *Hydrocotyle sibthorpioides* are represented in Table 37 & 38**.**

The docking studies showed that identified compounds were able to bind the catalytic site of Peroxisome proliferators activated receptor gamma (3DZY). Docked conformation of different compounds of *C. retrospiralis* along with important amino acid residues of 3DZY are represented in Figures (Figure 33-41). The molecular docking results showed that the compound testosterone enanthate obtainined from methanol extract of *C. retrospiralis*, possess good binding affinity (-11.16 kcal/mol) with 3DZY showing interaction with protein residues ILE 268, ALA 271, ALA 272, GLN 275, TRP 305, ASN 306,LEU 309,ILE 310, PHE 313,ARG 316, LEU326, ALA 327, ILE 345, PHE 346, VAL 349, CYS 432, HIS 435, LEU 436 as compared to commercial drug metformin (Figure 32) (LYS431, GLU434, THR737, GLN741, GLN744, TYR777).

Again docked conformation of different compounds of *H. sibthorpioides* along with important amino acid residues of 3DZY are represented in figures (Figure: 41-51). The compound Corynan-17- ol,18,19-didehydro-10-methoxy acetate (ester) which was obtained from *H. sibthorpioides* possessed good binding affinity (-9.95 kcal/mol) by binding with amino acid residues ILE268,ALA 271, GLN 275,TRP 305, ASN 306, LEU 309,PHE 313, ARG 316, LEU 326, ALA 327, VAL 342, ILE 345,LEU 426,HIS 435(Figure: 45).

Table 37: Binding energy (Kcal/mol) at different poses of bioactive compounds of *C. retrospiralis*

Doking pose	**Metformin**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
I	-6.50	-4.53	-5.78	-5.58	-6.96	-7.16	-5.39	-6.57	-11.16	-6.17
II	-6.46	-4.51	-5.54	-5.23	-6.19	-6.75	-5.38	-6.39	-11.15	-5.67
III	-6.43	-4.36	-5.46	-5.19	-6.1	-6.74	-5.13	-6.13	-11.08	-5.6
IV	-6.16	-4.18	-5.26	-5.13	-6.5	-6.45	-5.13	-5.82	-10.66	-5.58
V	6.16	-3.93	-5.13	-5.02	-5.99	-6.38	-5.1	-5.68	-10.35	-5.53
VI	6.15	-3.93	-5.09	-5.01	-5.86	-6.19	-5.05	-5.67	-10.31	-5.28
VII	-6.15	-3.85	-4.87	-4.73	-5.59	-5.56	-4.93	-5.57	-10.1	-4.96
VIII	-6.10	-3.83	-4.84	-4.51	-5.54	-4.93	-4.87	-5.5	-10.08	-4.75
IX	-6.07	-3.82	-4.75	-4.42	-5.13	-4.1	-4.81	-5.32	-5.71	-4.62
X	-6.07	-3.37	-4.53	-4.06	-5.00	-4.1	-4.44	-4.79	-5.48	-4.34

Table 38. Binding energy (Kcal/mol) at different poses of bioactive compounds of *H. sibthorpioides*.

Doking pose	**Metformin**	1	2	3	4	5	6	7	8	9	10
I	-6.5	-5.67	-7.65	-6.25	-9.95	-6.64	-5.92	-5.08	-7.69	-5.7	-5.8
II	-6.46	-5.58	-7.63	-5.91	-8.24	-6.41	-5.84	-5	-7.67	-5.4	-5.2
III	-6.43	-5.24	-7.25	-5.18	-6.81	-6.4	-5.29	-4.98	-7.65	-5.4	-5.2
IV	-6.16	-5.17	-7.24	-5.13	-5.86	-6.31	-5.4	-4.91	-7.41	-5.4	-5.1
V	6.16	-5.17	-7.23	-4.86	-5.81	-5.99	-5.09	-4.87	-7.41	-5.3	-4.8
VI	6.15	-5.11	-7.22	-4.84	-5.38	-5.56	-5.05	-4.81	-6.88	-5.1	-4.5
VII	-6.15	-4.97	-6.44	-4.16	-5.35	-5.43	-4.99	-4.72	-6.88	-5	-4.5
VIII	-6.1	-4.95	-6.18	-4.03	-5.24	-5.11	-4.79	-4.45	-6.68	-5	-4.3
IX	-6.07	-4.75	-5.4	-3.71	-5.22	-4.71	-4.53	-4.44	-6.65	-5	-4.2
X	-6.07	-4.61	-5.05	-3.38	-5.06	-4.67	-4.52	-4.26	-6.46	-4.9	-4

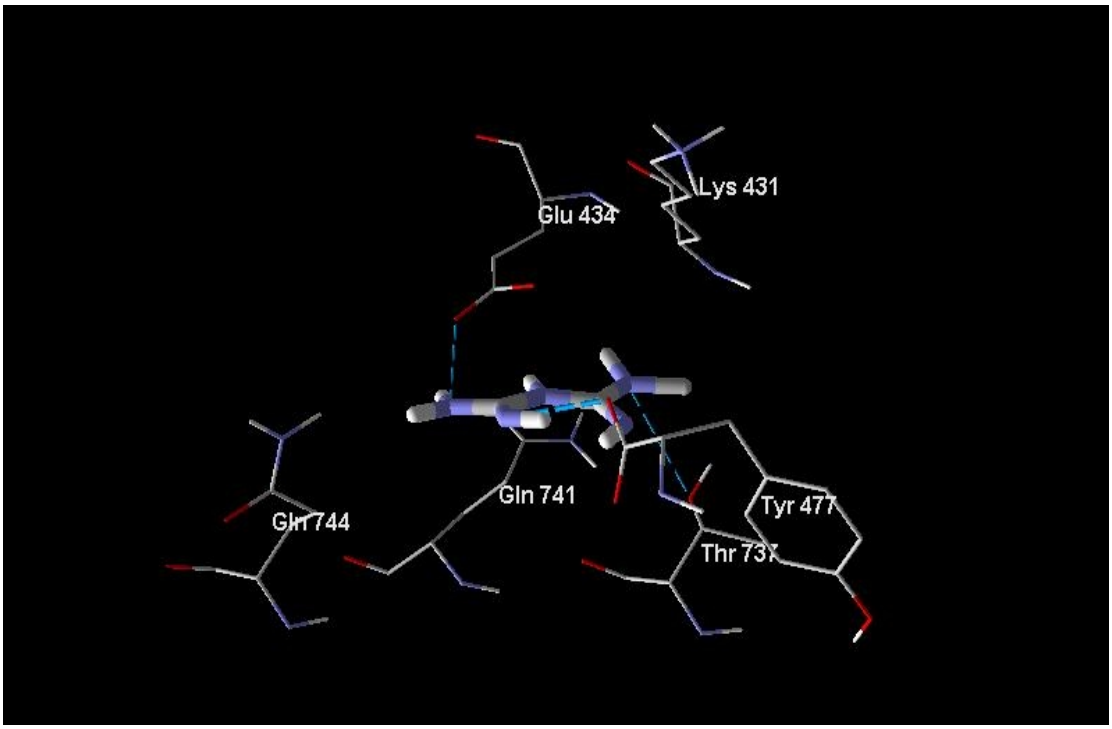

Figure 32. Docked conformation of Metformin along with the important amino acid residues of 3DZY

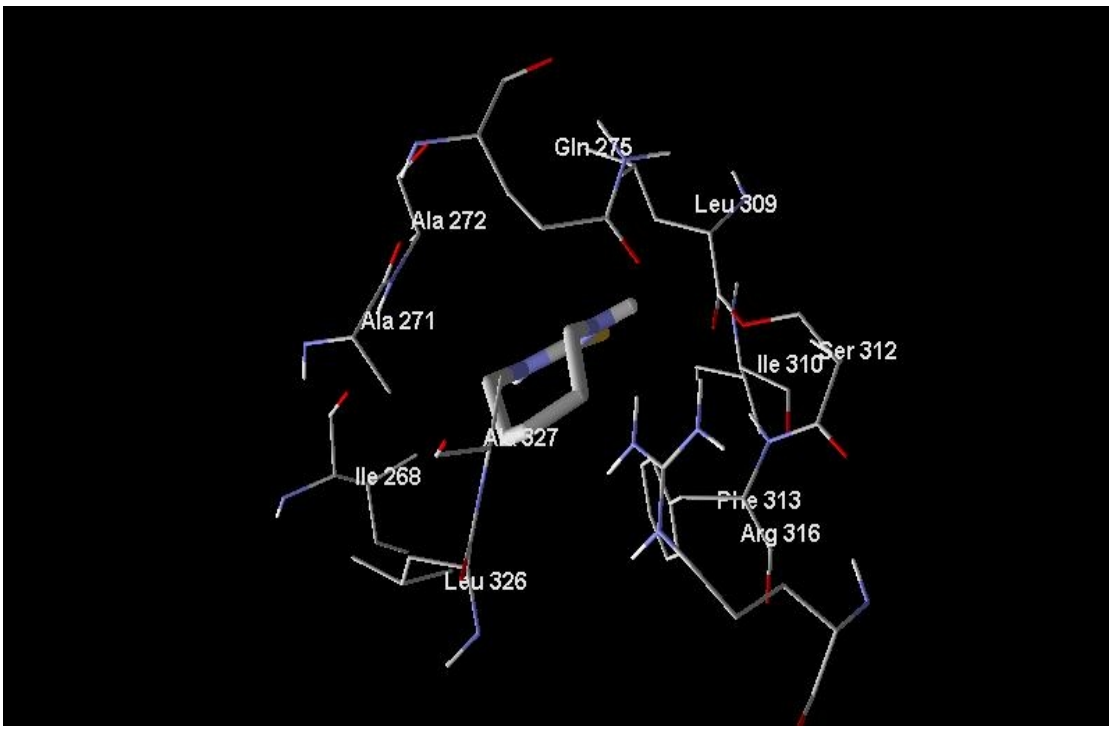

Figure 33. Docked conformation of **1,3- Diazocane -2 thione (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

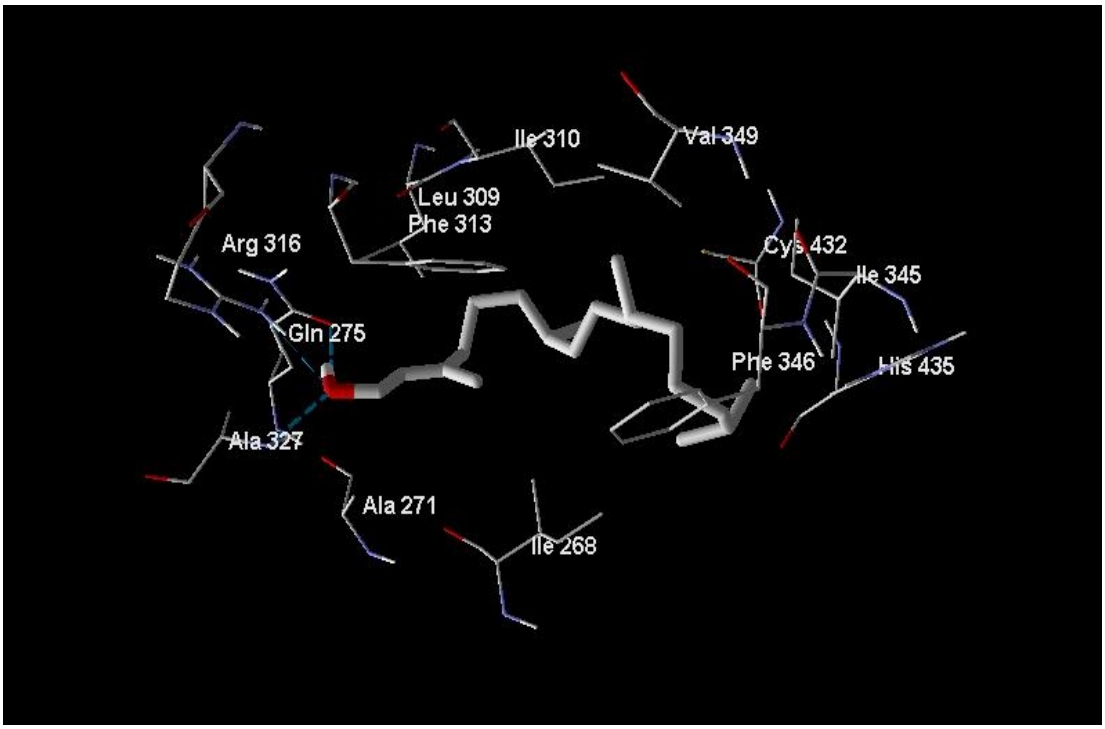

Figure 34. Docked conformation of **E 11 - Methyl-12-tetradecan-1-ol acetate (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

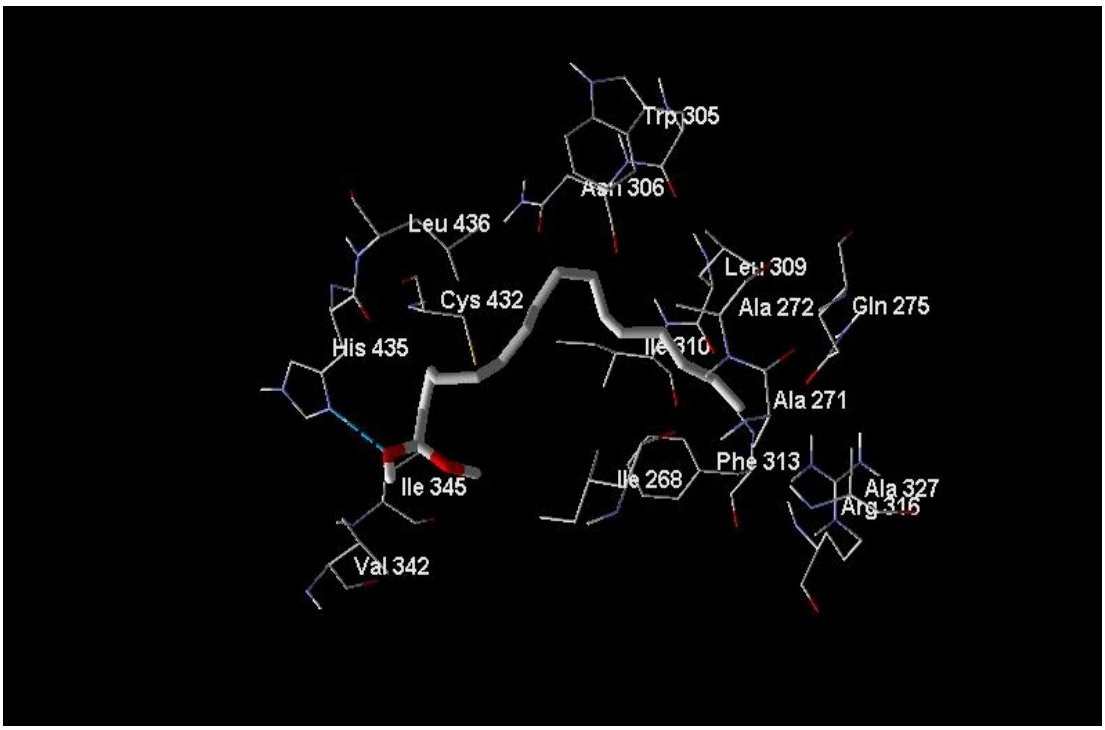

Figure 35. Docked conformation of **Hexadecanoic acid- methyl ester (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

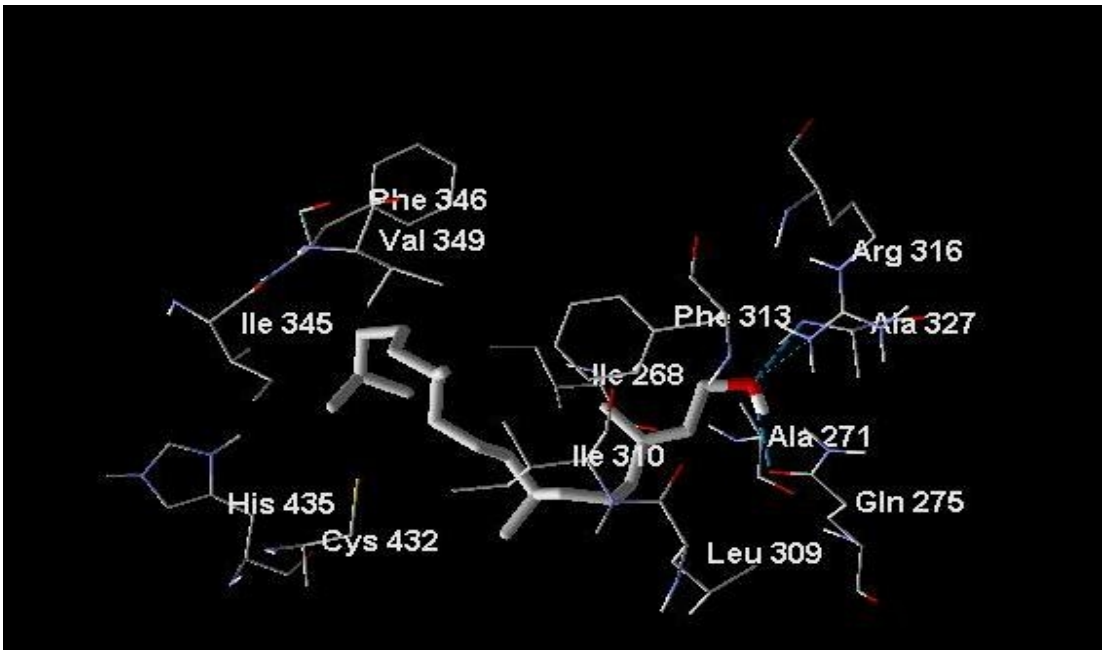

Figure 36. Docked conformation of **Phytol (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

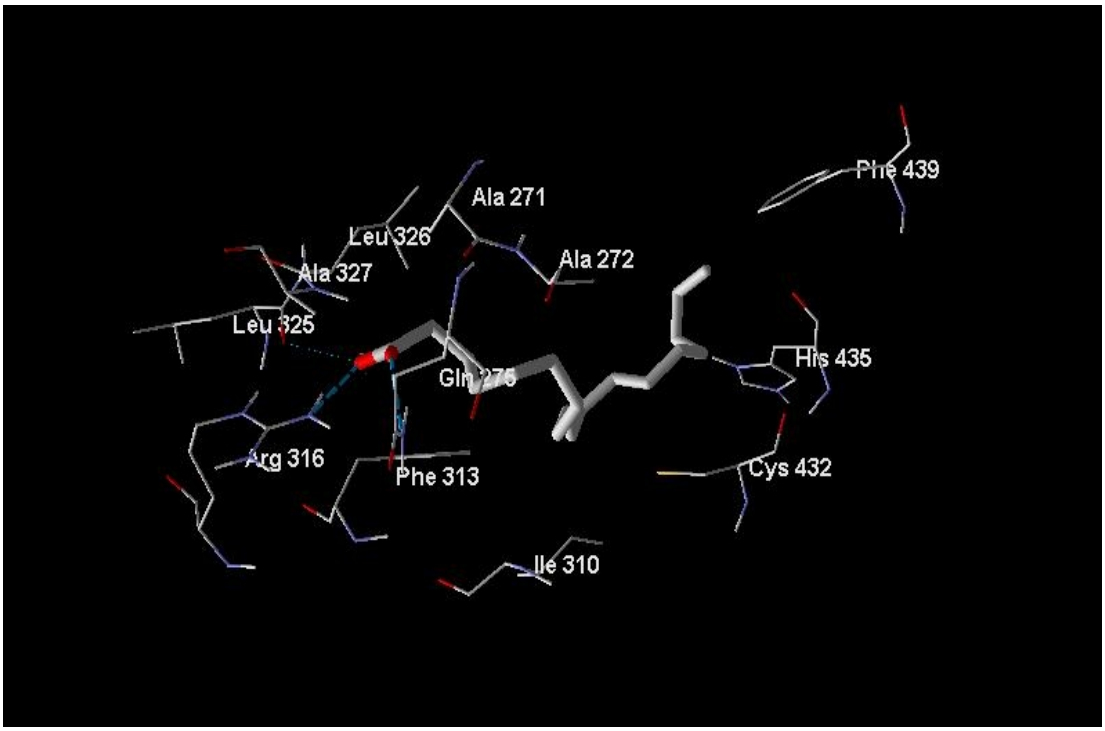

Figure 37. Docked conformation of **9, 12- Octadecadienoic acid (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

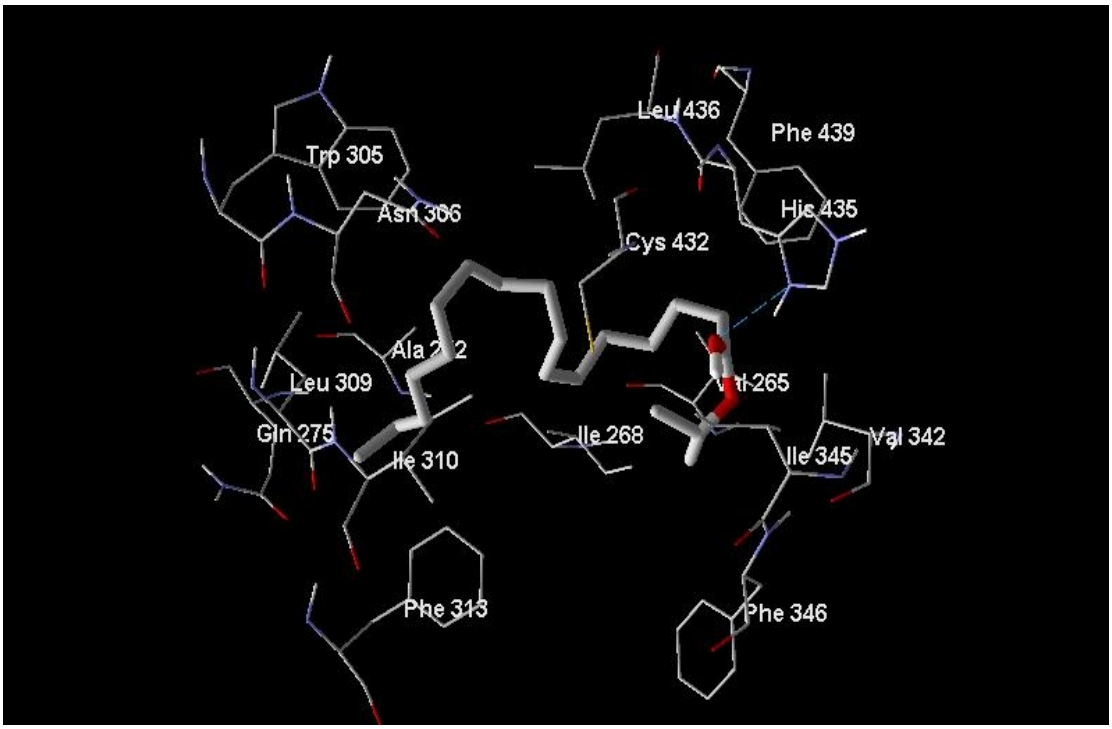

Figure 38. Docked conformation of **Isopropyl Stearate (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

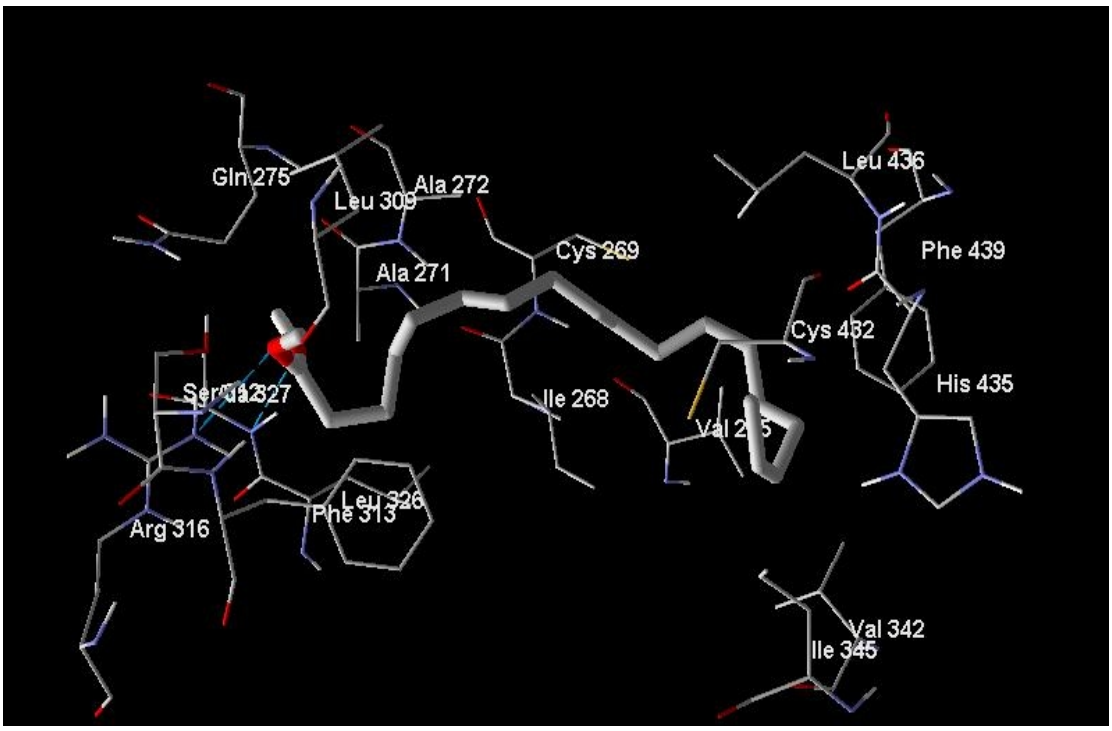

Figure 39. Docked conformation of **6,9,12-Octadecatrienoic acid, Phenyl methyl ester (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

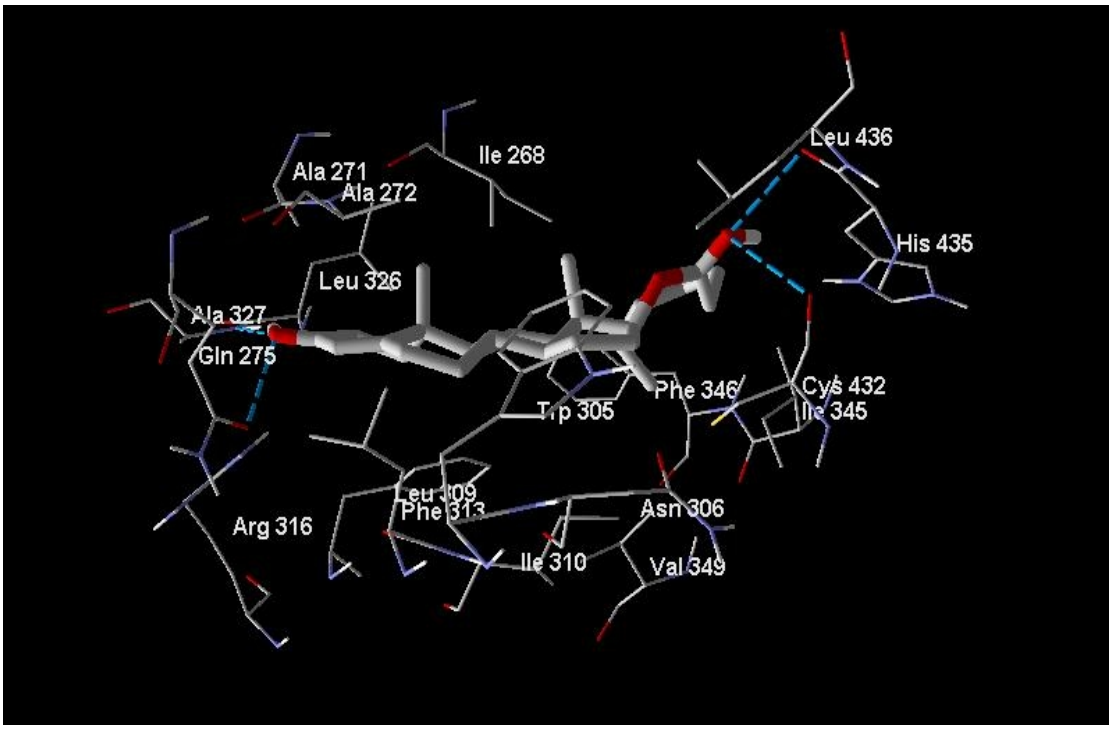

Figure 40. Docked conformation of **Testosterone Enanthate (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

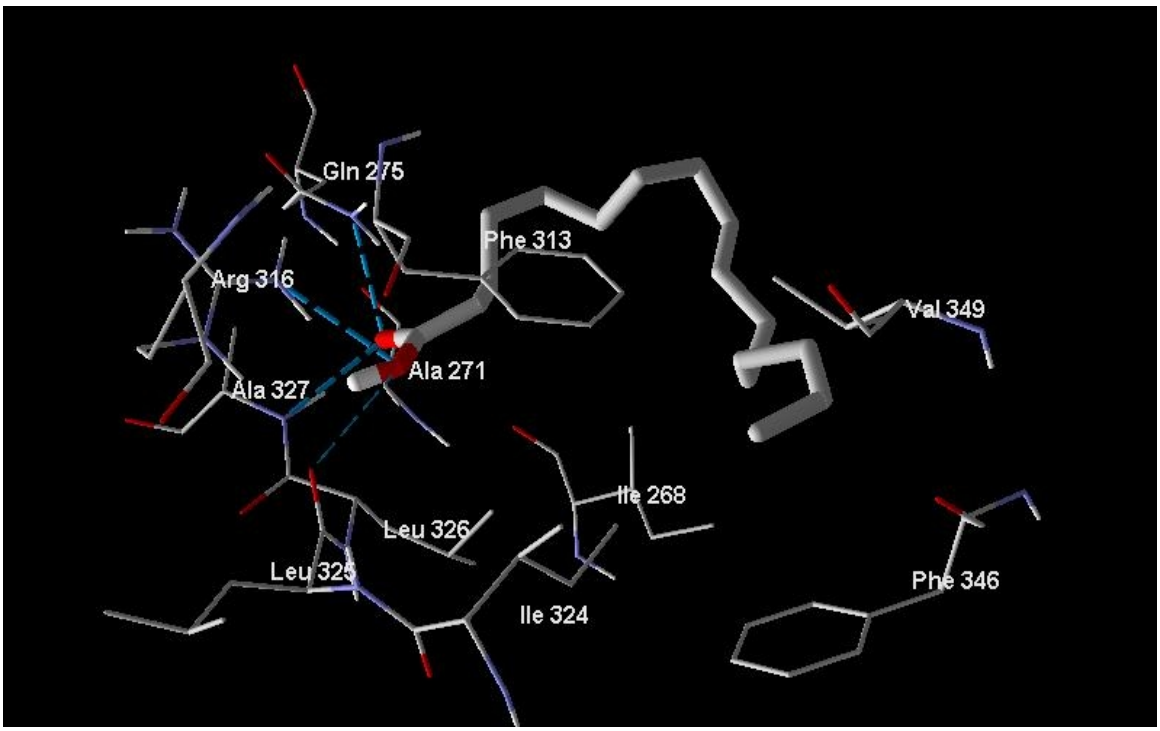

Figure 41. Docked conformation of **n-Hexadecanoic acid (***C. retrospiralis***)** along with the important amino acid residues of 3DZY

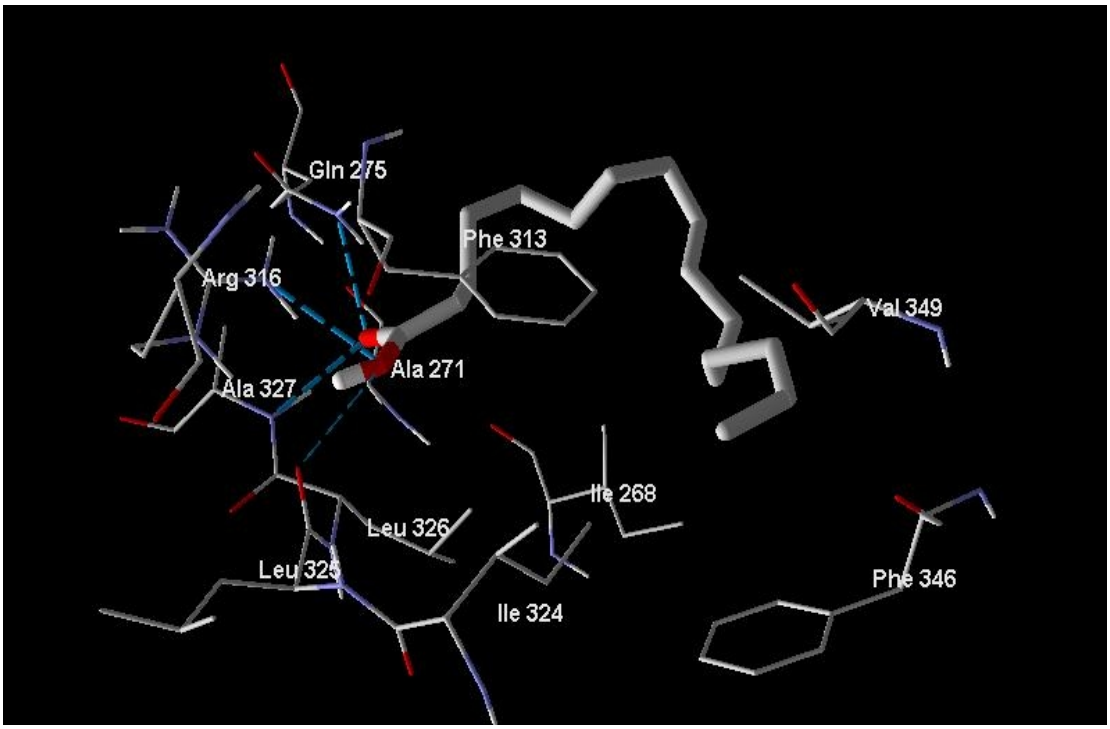

Figure 42. Docked conformation of **3 Eicosyne (***H. sibthorpioides* **1)** along with the important amino acid residues of 3DZY

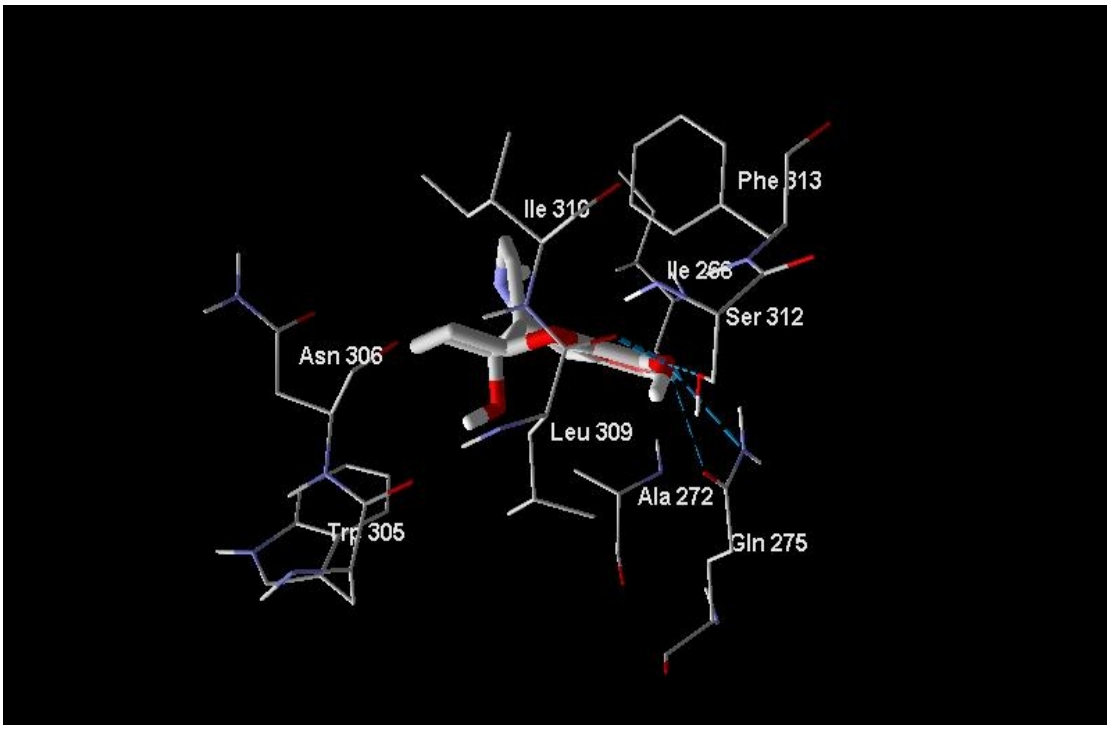

Figure 43. Docked conformation of **Morphinan- 3,14-diol, 4,5-epoxy(5 á) (***H. sibthorpioides* **2)** along with the important amino acid residues of 3DZY

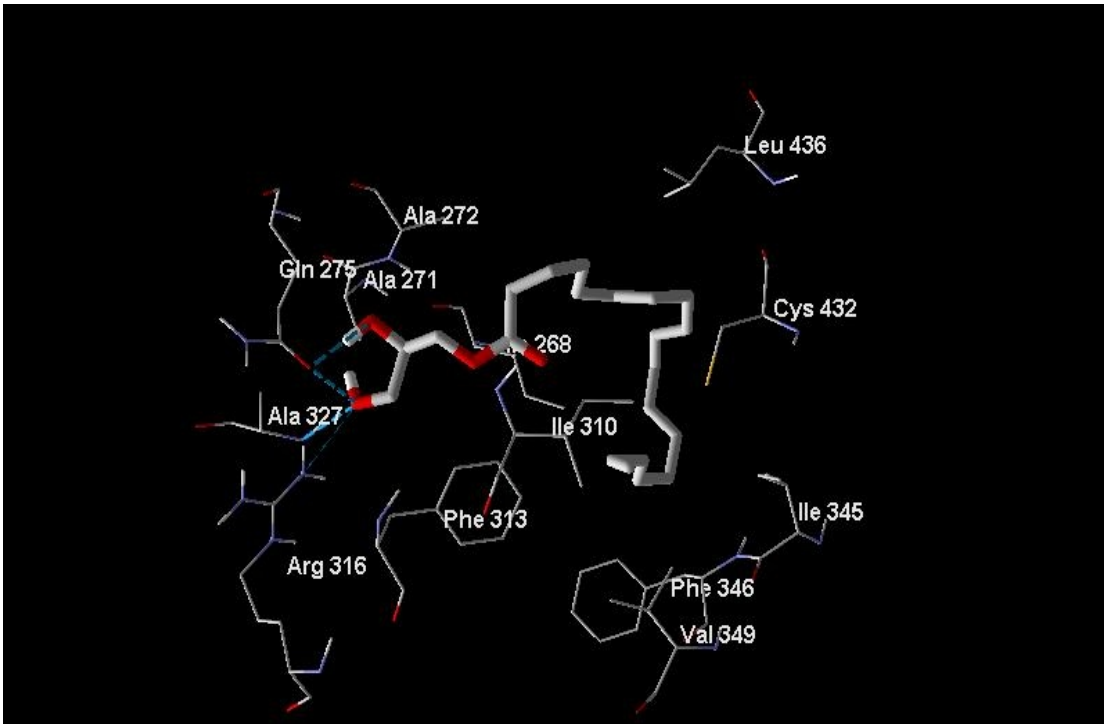

Figure 44. Docked conformation of **9,12,15-Octadecatrienoic acid, 2,3-dihydroxy propyl ester (***H. sibthorpioides* **3)** along with the important amino acid residues of 3DZY

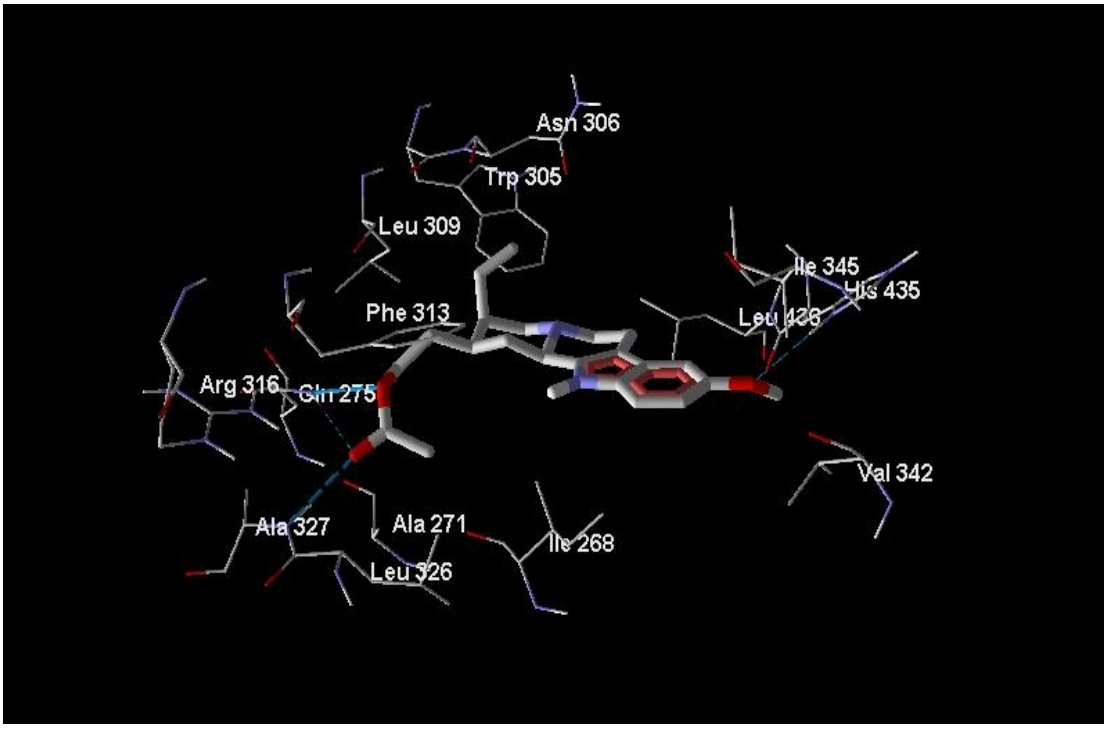

Figure 45. Docked conformation of **Corynan-17- ol, 18,19-didehydro-10-methoxy acetate (***H. sibthorpioides* **4)** along with the important amino acid residues of 3DZY

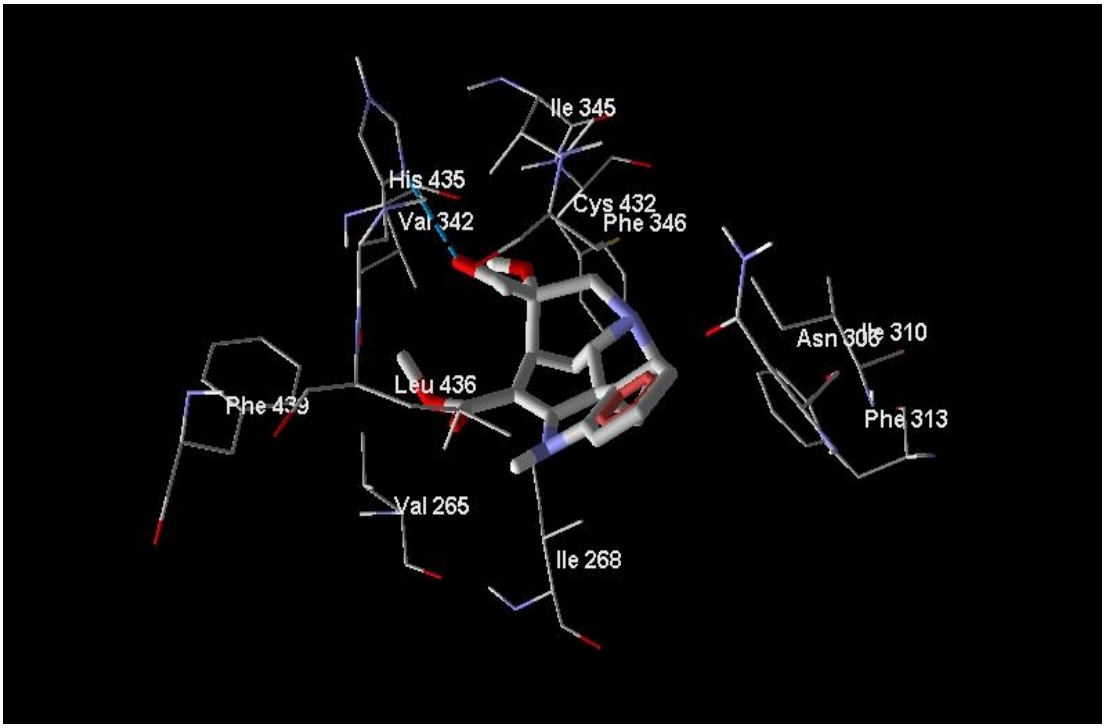

Figure 46. Docked conformation of **Curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo, methyl ester (***H. sibthorpioides* **5)** along with the important amino acid residues of 3DZY

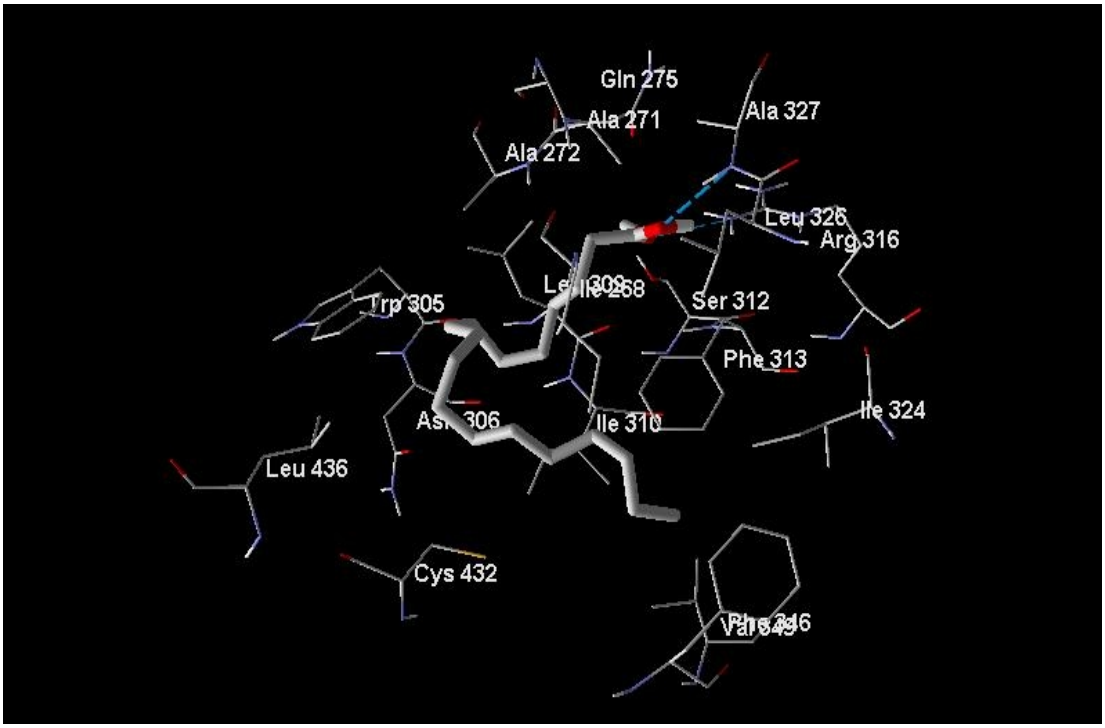

Figure 47. Docked conformation of **10-Octadecanoic acid, methyl ester (***H. sibthorpioides* **6)** along with the important amino acid residues of 3DZY

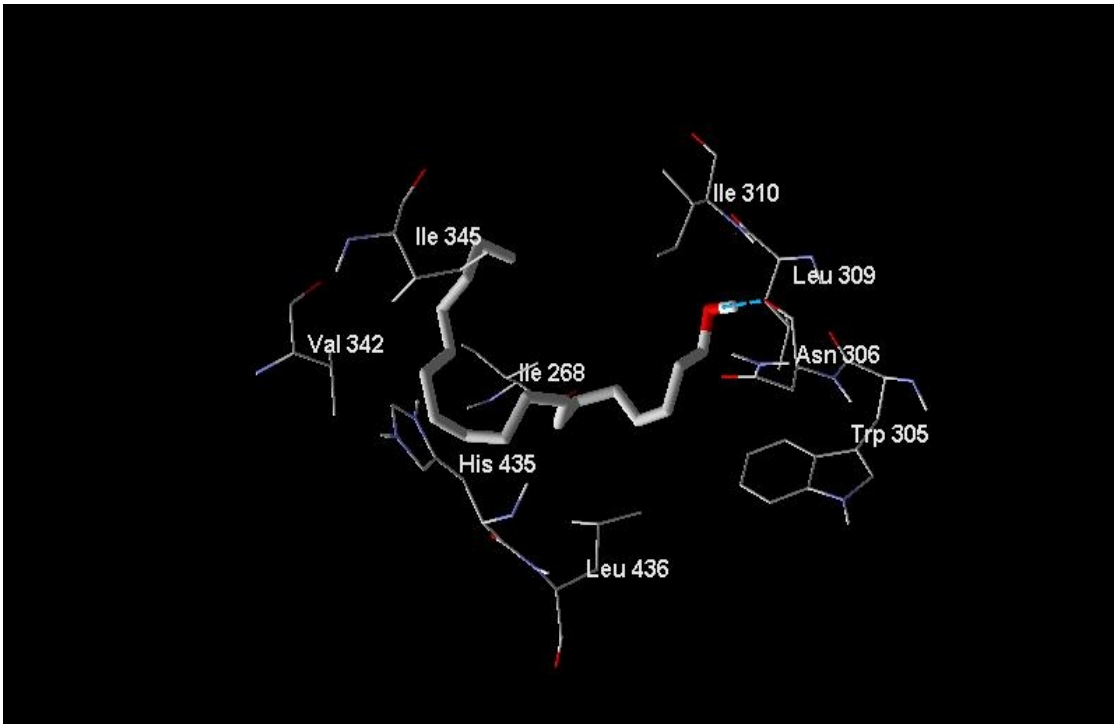

Figure 48. Docked conformation of **Z, E-3,13-Octadecadien -1 ol (***H. sibthorpioides* **7)** along with the important amino acid residues of 3DZY

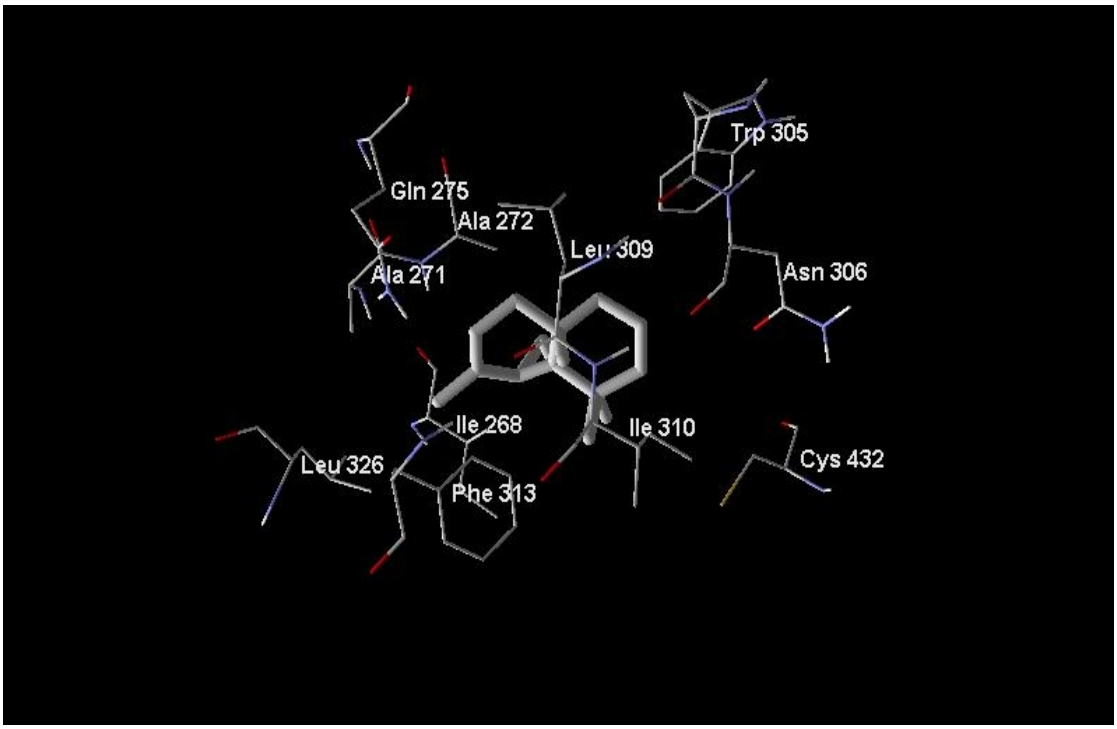

Figure 49. Docked conformation of **Thujopsene (***H. sibthorpioides* **8)** along with the important amino acid residues of 3DZY

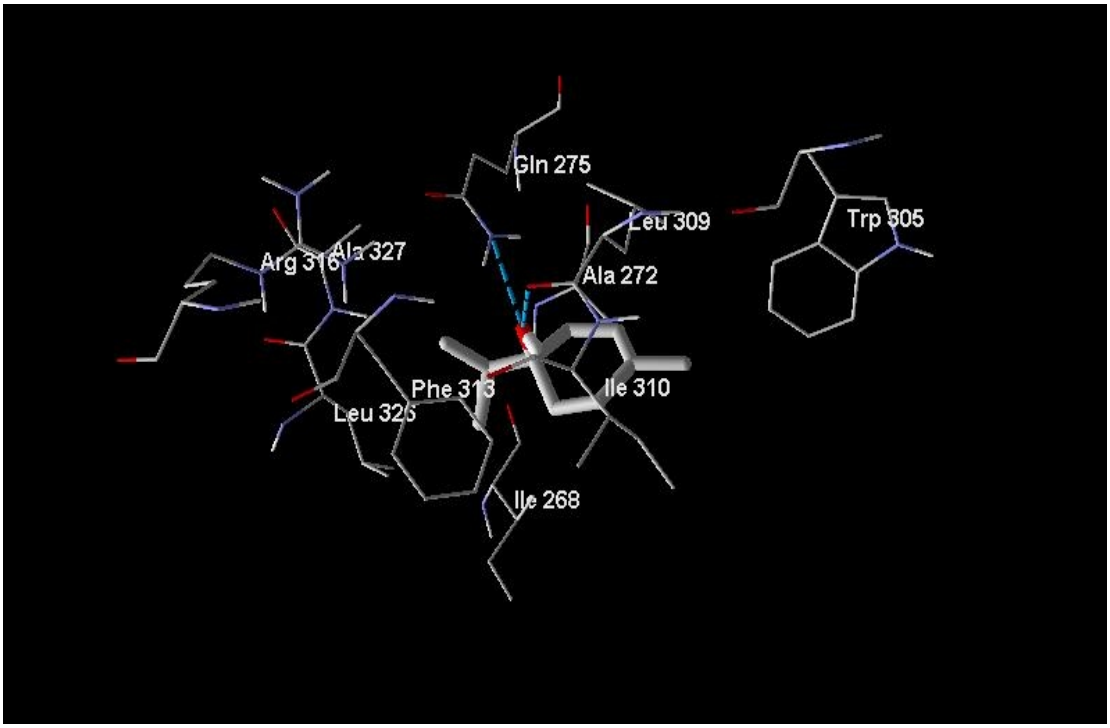

Figure 50. Docked conformation of **3-Cyclohexen-1-ol, 4- methyl-1-1(1-ethylethyl) (***H. sibthorpioides* **9)** along with the important amino acid residues of 3DZY

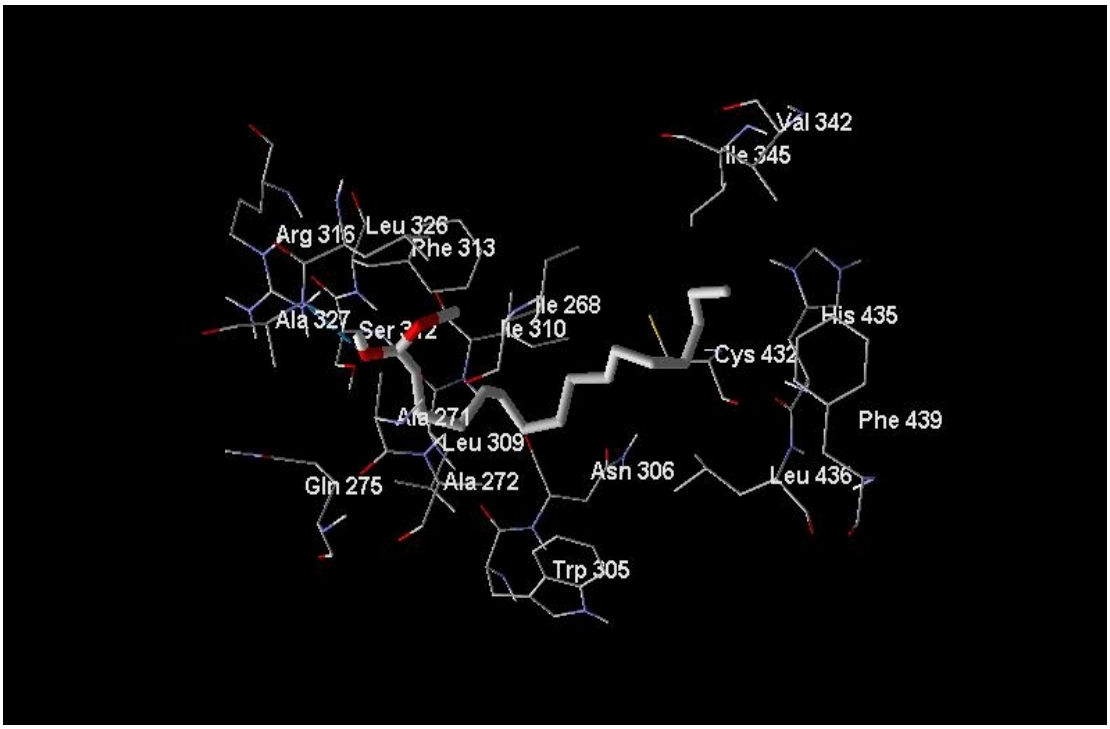

Figure 51. Docked conformation of **Hexadecanoic acid- methyl ester (***H. sibthorpioides* **10)** along with the important amino acid residues of 3DZY

4.13. Cytotoxic effect

The methanolic extract of *C. retrospiralis and H. sibthorpioides* showed significant cytotoxicity against the human hepatocarcinoma cell line ($HepG_2$) (Figure 52 A-B). Nuclei of the extract treated cells appeared smaller, indicating nuclear shrinkage. With an increase in concentration, a notable degree of nuclear fragmentation was observed. At a concentration 100 µg/ml these extract showed 17.38% and 30.41% cell death respectively for *C.retrospiralis* and *H. sibthorpioides* extracts, which accelerated with increasing concentration of the extract. At maximum concentration of the plant extracts tested i.e 500 µg/ml, the cell death increased to 57.12 and 68.32 respectively. The LD50 value of the *C. retrospiralis* and *H. sibthorpioides* extracts was determined to be 366.70 µg/ml and 375.5 µg/ml subsequently. The cells were visualized under Floid Cell Imaging Station (Life Technologies, USA). (Figue 53-54).

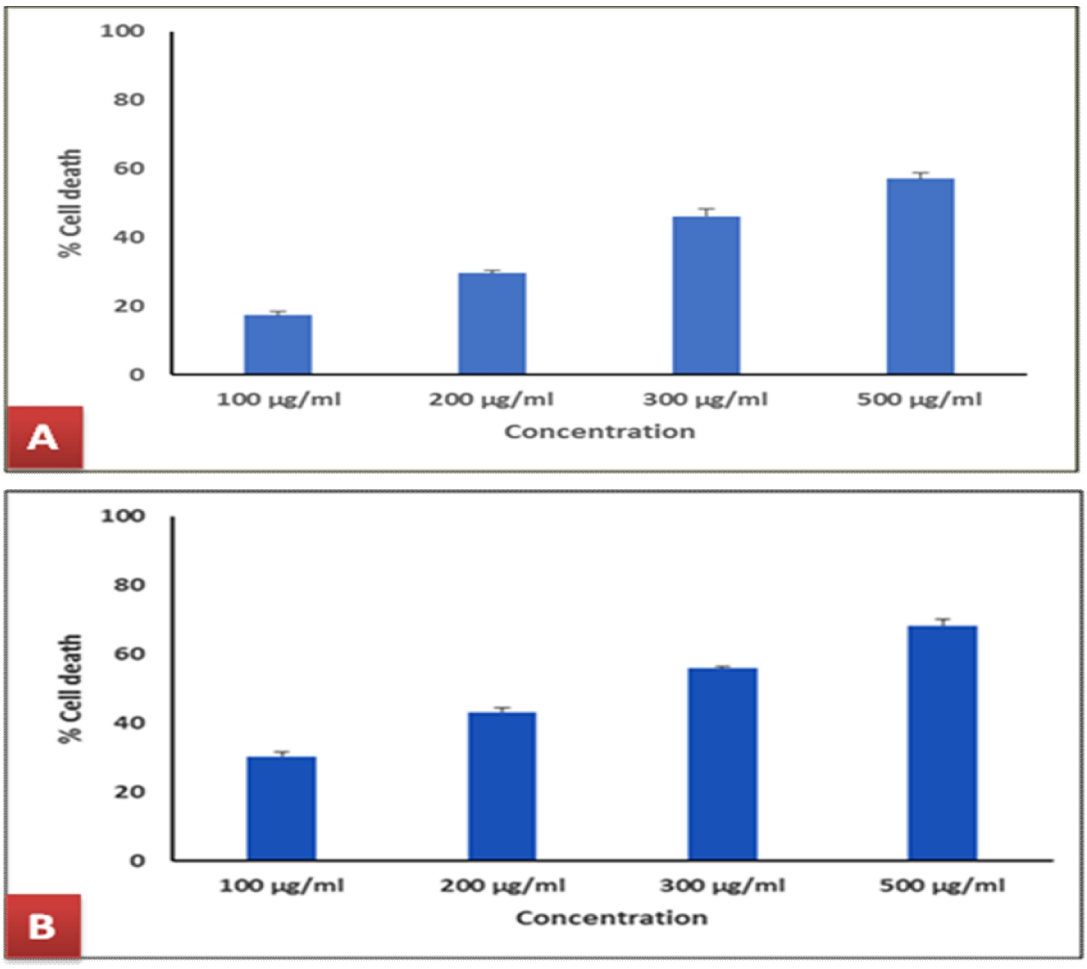

Figure 52. Cytotoxicity assay of the plant extracts on $HepG_2$ cell line: **A.** *C. retrospiralis*, **B.** *H. sibthorpioides*

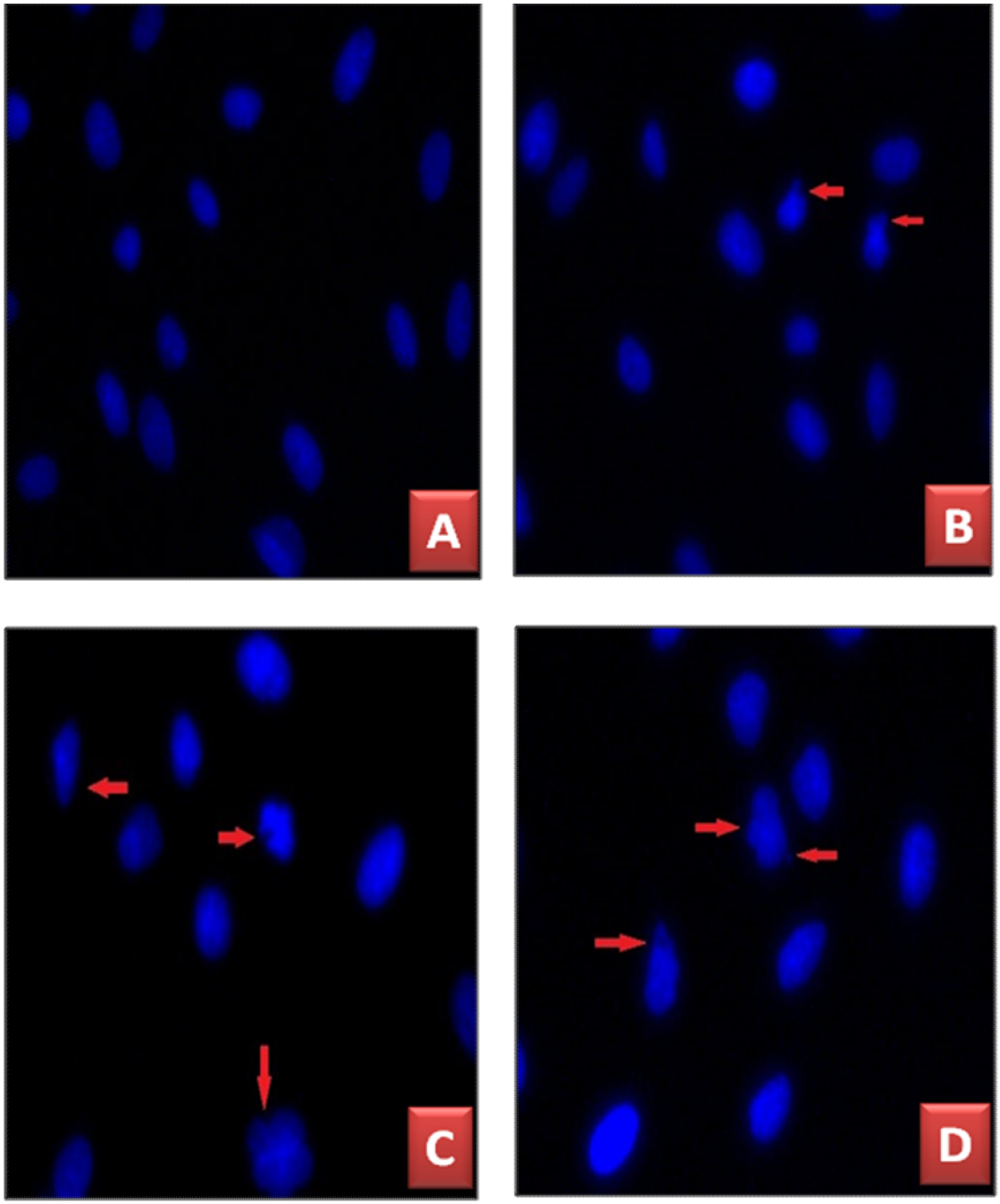

Figure 53. Changes in nuclear morphology by DAPI staining**.** Effect on $HepG_2$ cell lines treated with different concentrations of *C. retrospiralis* leaf extract: **A.** Control (0 µg/ml), **B.** 100 µg/ml, **C.** 300 µg/ml, **D.** 500 µg/ml. The red arrows point towards the effected cells.

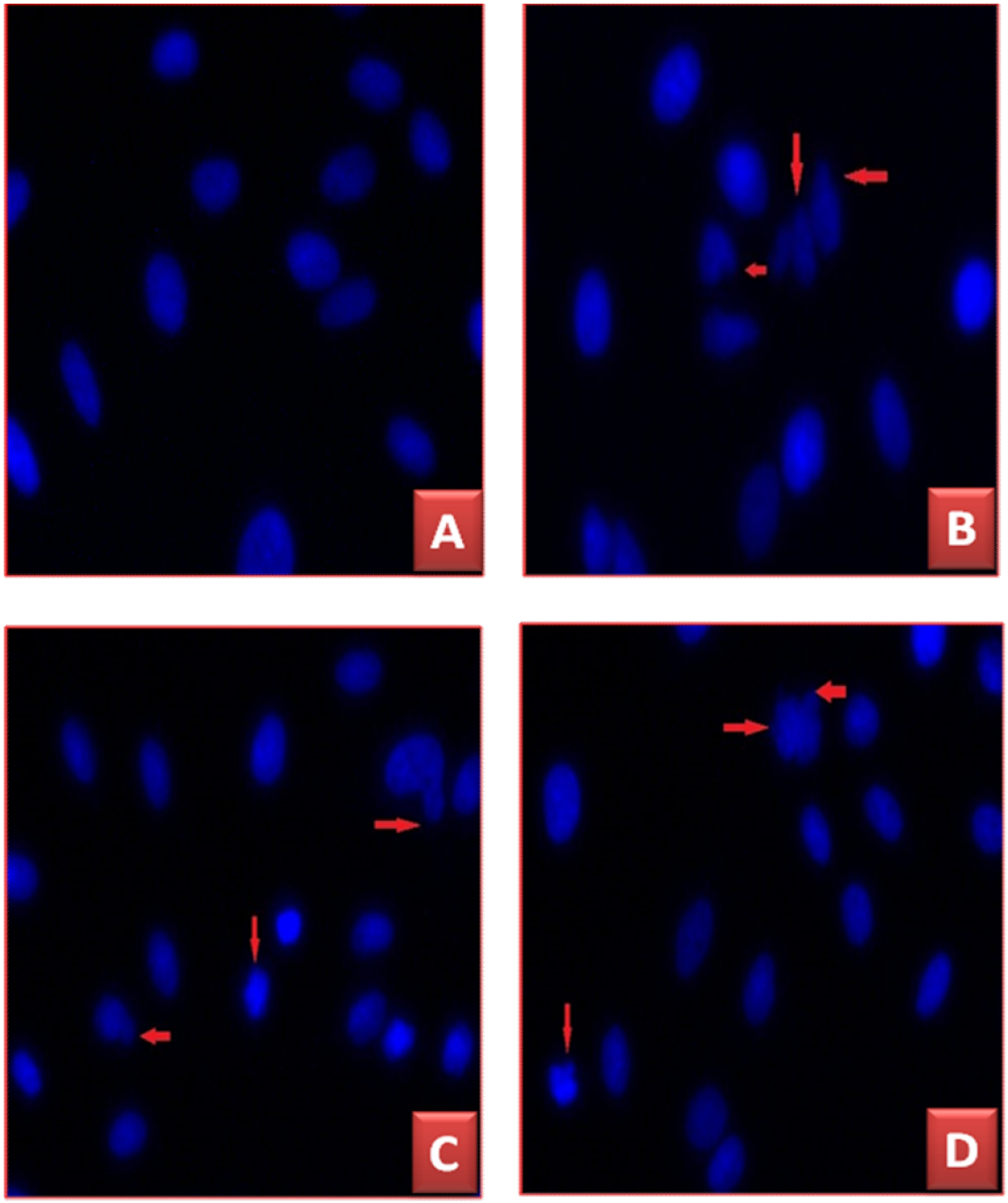

Figure 54: Effect on $HepG_2$ cell lines treated with different concentrations of *H. sibthorpioides* leaf extract: **A.** Control (0 µg/ml), **B.** 100 µg/ml, **C.** 300 µg/ml, **D.** 500 µg/ml. The red arrows point towards the effected cells.

5. DISCUSSION

For fulfilling most of the primary needs such as food, fodder, water etc. for times immemorial; human civilization have always depended up on wetlands from the beginning of their journey towards modern society (Swapna *et al*. 2011). Wetland ecosystem is also significant in providing livelihood to the thousands of people residing in the close proximity of these areas. Nowadays many researchers are trying to gather as much knowledge related to the use of wetland plants from the local people living in the vicinity of these wetlands. In the present study, many new aspects regarding the use of wetland plants from the different regions of northern part of West Bengal has been assessed. Briefly, an extended survey was conducted which revealed the usage of wetland plants as food and medicine by the local peoples. A total of fifteen plants were eventually selected on which the detail study was further conducted. Earlier workers have also reported the medicinal properties of wetland plants apart from their economic importance. Panda and Misra (2011) reported 48 ethnobotanically important wetland plants from the wetlands of Eastern Ghat region of Orissa. They reported some similar plants as described in Table 1-4 which included the plants like *Alternanthera sessilis, Bacopa monnieri, Eclipta prostrata, Enydra fluctuans, Glinas oppositifolius, Heliotropium indicum, Hygrophila auriculata, Nelumbo nucifera, Ipomoea aquatica, Ludwigia adscendens, Nymphaea nouchali, Nymphaea pubescens, Marsilea sp., Monochoria vaginalis, Nymphoides indica, Oldenlandia corymbosa, Pistia stratoes, Scoparia dulcis* that were found to be ethnobotanically important in the present study. Chowdhury and Mukherjee (2012) reported 84 wild edible plant species used by the local inhabitants of Malda, West Bengal. Among the 84 plants recorded, some were wetland plants and also found ethnobotanically very important in this current study. Some common wetland plants that appeared in the previous study include *Nelumbo nucifera, Alternanthera sessilis, Colocasia esculenta ,Bacopa monnieri, Enhydra fluctuans, Amaranthus spinosus, A. viridis, Argemone mexicana, Lasia spinosa, Chenopodium album, Diplezium esculentum, Euryale ferox, Hygrophila auriculata, Glinus oppositifolius, Ipomoea aquatica, Oxalis corniculata, Marsilea minuta, Nymphaea nauchali, Nymphaea pubescens, Ottelia alismoides, Portulaca oleracea, Ricinus communis, Xanthium indicum* etc. Similarly, Saha *et al*. (2014) documented ethnoveterinarily useful 60

different plants from Malda district of West Bengal. Among these 60 plants *Ricinus communis, Heliotropium indicum, Achyranthes aspera, Eclipta prostrata, Amaranthus spinosus* were also found ethnobotanically important in the present study.

Thus, in this study effort has been made to enlist the different locally available wetland plants and finally screen for the reported medicinal properties of the 15 selected plant samples collected from various wetlands of North Bengal region, for the presence of different phytochemicals along with the *in vitro* and *in vivo* estimation of antioxidant, antimicrobial, antidiabetic and anticancer activities.

Phytochemical screening of the powdered plant samples of *Cryptocoryne retrospiralis*, *Barringtonia acutangula*, *Hydrocotyle sibthorpioides, Eclipta prostrata*, *Enhydra fluctuans*, *Amaranthus spinosus*, *Marsilea minuta, Amaranthus viridis, Glinus oppositifolius, Portulaca oleracea, Hygrophila auriculata, Phyla nodiflora, Pilea microphylla, Diplazium esculentum and Ipomoea aquatica* revealed the presence and absence of glycosides, steroids, anthraquinones, amino acids, alkaloids, saponins, reducing sugar, resin, cardiac glycosides, terpenoid, tannin, carbohydrates, protein, phenol and flavonoids in these plants. Wadkar *et al*. (2017) reported the presence of saponins, glycosides, tannins, alkaloids, steroids and flavonoids in *C. retrospiralis*. Kathirvel *et al.* (2012) reported presence of alkaloids, phenols, flavonoids, protein, amino acid, carbohydrate, steroids, saponins, anthroquinones in *B. acutangula* which resembled the result of the present study. However in this study phytochemical screening of *H. sibthorpioides* reveals the presence of saponins, glycosides, tannins, amino acids, carbohydrates, protein, phenol and flavonoids not getting any other supporting works done by any worker. Lunavath *et al.* (2013) reported the presence of alkaloid, tannin, flavonoids, amino acid, saponins, glycosides and carbohydrate in *Eclipta prostrata* as seen in present study. Amino acids, alkaloids, saponins, reducing sugar, terpenoides, tannins, phenols, and flavonoids were also detected in *Enydra fluctuans*. Presence of saponins, flavonoids, protein, reducing sugar and phenols was reported in *E. fluctuans* previously by Kuri *et al.* (2014). Screening of phytochemicals in dried plant samples in two species of *Amaranthus* revealed that both *A. spinosus* and *A. viridis* contained glycosides, amino acids, reducing sugar, cardiac glycosides, protein, phenols, flavonoids and triterpenoids. However, saponins were detected in *A. spinosus* but it was found absent in *A. viridis*. Presence of cardiac glycosides, saponins and flavonoids were also

reported in *A. spinosus* previously (Amabye, 2015). Present study revealed that, amino acid, saponins, reducing sugar, tannins, carbohydrates, protein, phenols and flavonoids were present in detectable amount in dried powdered sample of *M. minuta*. Presence of similar type of active phytochemicals has been reported in *M. quadrifolia* (Meenatchi and Jenitha, 2015). In this study, in *Glinus oppositifolius* anthroquinones, resin and terpenoid were not detected and detection of alkaloid, carbohydrate, phenol, protein, steroid and tannin showed resemblances with the study of Ramaseshan *et al*. 2016. In *Portulaca oleracea* steroid were not detected along with anthraquinones, resins etc and the presence of glycosides, amino acids, saponins, cardiac glycosides, terpenoids, tannins, proteins and triterpenoids was confirmed by the qualitative tests. Similar works by Okafor and Ezejindu (2014) also supporting this result up to certain level. Phytochemical profile of *Hygrophila auriculata* showed the presence of amino acids, alkaloids, saponins, reducing sugar, terpenoids, tannins, carbohydrate, protein, phenols and flavonoids. This result is in accordance of the findings of Hussain *et al.* (2011). Also the presence of amino acid, alkaloids, flavonoids, terpenoids and saponins were detected in *Phyla nodiflora* which correlated with the similar investigations of by Priya and Ravindhran 2015. In *Pilea microphylla* amino acid, alkaloids, saponins, tannins, phenols and flavonoids were detected which reciprocated with the findings of Bansal *et al.* (2011). The phytochemical profiling of *Diplazium esculentum* revealed the presence of anthraquinones, alkaloid, saponin, reducing sugar, terpenoid, carbohydrate, protein, phenol, flavonoid and triterpenoids that resembles the works of Zannah *et. al.* (2017). Also, in *Ipomoea aquatica* presence of amino acid, reducing sugar, resin, protein, phenol, flavonoid and triterpenoid were detected, that resonated with the phytochemical screening of Sivaraman *et al.* (2010).

Among the naturally occurring aromatic phytocompounds, anthraquinones possess antioxidant, antimutagenicity and antitumor activities (Demirezer *et al*. 2001; Lee *et al.*, 2005; Kimura *et al.*, 2008). In our study only *B. acutangula* confirms the presence of anthraquinones. Tannin shows antiulcer, antiviral, antibacterial, anti-inflammatory and antioxidant activity in various experiments. Tannins are also effective in the treatment of burns due to its unique property to precipitate proteins from the exposed tissues and formation of a protective layer. Tannins are also used to treat gonorrhoea, leucorrhoea, piles, inflammation etc. Some studies also pointed towards the inhibitory effect of tannin on the HIV replication. In many astringent and

diuretic drugs mainly tannins are the principal compound (Lu *et al.*, 2004; Akiyama *et al.,* 2001; Kolodziej *et al.,* 2005). Since long, terpenoids have been used in pharmaceutical industries as antiseptic, antibiotics, anthelminthic and insecticidal (Duke, 1992; Parveen *et al*., 2010). These are multi-cyclic compounds and are derived from 5-hydrocarbon isoprene units (CH2=C(CH3)-CH=CH2) (Elbein *et al.*1999; Langenheim, 1994). Almost in all living organisms these natural lipids (terpenoids) are present and are commercially used in cosmetics, foods and agricultural products and also as flavouring agent and in fragrances (Elbein *et al.*, 1999; Harborne *et al.,* 1991).

Plant phenolics are the group of secondary metabolites bearing a common aromatic ring with one or more hydroxyl groups (Chirinos *et al.,* 2009). In the recent years, significant attention has been paid towards the research on plant phenolics (Manach *et al.,* 2004). As a result of intensive research, within the past decade, more than 8000 plant phenolic structures have been elucidated. Phenolic compounds are present at varying concentrations in different plants parts and thus contribute immensely towards the medicinal properties of the plants. Plant phenolics help in reducing inflammation and are also effective as vasodilators. Apart from this,phenolic compounds have anticancerous, antioxidant, antidiabetic and antimutagenic that has been proven scientifically by many workers alongwith their protective role in various neurodegenerative diseases (Padilla *et al.,* 2005; Mohanlal *et al*., 2013; Zhang *et al*., 2011; Jin *et al*., 2006; Kusirisin *et al*., 2009; Scalbert *et al*., 2005; Luo *et al*., 2002; Parekh and Chanda, 2007). In the present study, quantification of phenolics reveals that *Hydrocotyle sibthorpioides* contains highest amount of phenols and *Portulaca oleracea* contained the least.

Among other polyphenols flavonoids are the chief compounds and having antifungal, antiviral, anti-allergic, antibacterial, anticancer and anti-inflammatory activities (Di Carlo *et al.,* 1999; Montro *et al.,* 2005). It has 15 carbon atoms with a flavan nucleus. Flavonoids have the ability to scavenge most of the harmful oxidizing molecules or reactive oxygen/nitrogen molecules involved in various diseases (Bravo, 1998). Flavonoids are reported to exhibit protective role against cancers and cardiovascular diseases. The development of cancer is inhibited by flavonoids as it suppresses the production of enzymes (estrogen synthetase) involved in the synthesis of estrogen (Okwu and Omadamiro, 2005). Among the 15 studied plants, *A.viridis*

contains maximum amount of flavonoids and least amount of flavonoids was recorded in *P. oleracea.*

One of the most important and abundantly available biomolecule is carbohydrate. Glycosylated natural products are used as anticancerous and antimicrobial drugs, eg. nojirimycin, streptomycin etc (Dewick, 2001; Asano, 2003). Effective immunomodulatory activity of many polysaccharides isolated from medicinal plants from China has been reported (Jayabalan *et al.*, 1994). In the present dtudy, the highest amount of soluble sugar was present in *E. prostrata* and least present in *I. aquatica.* Chlorophylls are also known to possess several effective role in maintaining good health (Negishi, 1997; Dashwood *et al,* 1998). Numerous reports have suggested strong antioxidant activity of chlorophyllin and its usage to treat number of human diseases without evident harmful effects (Sato *et al*., 1984; Sato *et al*., 1985; Kamat *et al*., 2000; Kumar *et al.,*2001). In this study, highest amount of total chlorophyll was recorded in *E. prostrata* and least in *D. esculentum*. Chlorophyll a and Chlorophyll b was also recorded in these 15 plant materials studied. Proteins are identified as a potent antioxidant by some workers. Acording to our findings *A. spinosus* contains highest amount of protein and *P. microphylla* contains lowest. Alkaloids are the natural compounds and are known to have antimicrobial, antiprotozoal and antimalarial activities (Singh and Kapoor, 1980; Quetin- Lacqlercq *et al.,* 1995; Frederich *et al.,* 2002). Cardiac glycosides have antifungal activity along with cardioprotective ability (Abbassy *et al.,* 2007). Cardiac glycosides inhibit Na^+/K^+ pumps and increases the Na^+ and Ca^{2+} levels in the myocytes reducing cardiac arrest. Cardiac glycosides also increase the proficiency of heart under a controlled dosage as both toxic and therapeutic doses are very close to each other (Denwick, 2002).

Saponins possess a property of foaming in an aqueous solution; thereby saponins are efficient foaming and surface active agent. Industrial applications of saponins are not only important, but also the use of this phytochemical in medical science is highly prized. Saponins are compounds useful in precipitation and coagulation of RBCs (Okwu, 2004; Sodipo *et al.,* 2000) and exhibit antifungal (Aboaba *et al.,* 2001), antihyperglycemic (Sauvaire *et al.*, 1996; Vats *et al.,* 2003), antimicrobial (Mandal *et al.,* 2005), antioxidant (Gulcin *et al.,* 2004) and anti-inflammatory activities (Gepdireman *et al.,* 2005). In the present study, saponin was

detected in all plant samples except *C. retrospiralis* and *A. viridis* only. Plant extracts rich in saponins have been reported by Falodun *et al.* (2006) to be medicinally important. Steroid was detected only in *B. acutangula*, *E. prostrata* and *G. oppositifolius* amongst the 15 plant studied. There are several studies that suggest that plant steroids are able to reduce cholesterol, regulate immune responses and are pharmacologically very important for their ability to associate with various sex hormones (Shah *et al.,* 2009; Santhi *et al.,* 2011). Cardiovascular, antibacterial, anti-inflammatory, hepatoprotective and antitumor activity of some steroid was also reported by several workers (Bermejo *et al.,* 2000; Guisalberti, 1998; Emam *et al.,* 1997). Glycoside is a molecule in which sugar is bound to a non-carbohydrate moiety, usually a small organic molecule. Glycosides play numerous important roles in living organisms like fungi, bacteria and moots. Stored inactive glycosides can be activated by enzymes' hydrolysis, which causes the sugar parts to be broken off making the chemical available for use. Many of such glycosides are used for medications. (Imohiosen *et al*. 2014). In the present study, however, glycosides were not detected in *B. acutangula*, *Eclipta prostrata*, *E. fluctuans* and *M. minuta*.

In the present study, phytochemical screening was followed by quantification of some bioactive components. Highest amount of protein was observed in *A. spinosus* followed by *A.viridis* and lowest in *P. microphylla*. Andini *et al.* 2013 also reported similar types of result. Similarly the free amino acid content was also measured and *G. oppositifolius* contains highest level and the *C. retrospiralis* with lowest free amino acid levels. Likewise, very high phenol content was recorded in *H. sibthorpioides* and the lowest amount recorded in *P. oleracea*. Environmental stresses are known to be responsible for the production of phenolic compounds in plants; for example light intensity, duration of exposure has been shown to have great influence on the synthesis of phenols and flavonoids. Increased levels of flavonoids (antioxidants) have been reported in the plants available in higher altitudes as many prevailing stresses such as decreased pressure, low atmospheric temperature, exposure to higher UV rays etc augments its synthesis (Chanishvili *et al.*, 2007).

Carbohydrates are abundantly available biomolecules that find its application in various natural products that needs to be glycosylated, many of which have been used as anticancerous and antimicrobial drugs. For example, iminosugars like nojirimycin, aminoglycosides like streptomycin etc are some commonly used

glycosylated natural products (Dewick, 2001; Asano, 2003). Further, carbohydrates have been reported to exhibit antioxidative activity. Basu *et al.* (2012) reported Pusa Basmati polished seeds containing higher sucrose and starch content revealed better superoxide and hydroxyl scavenging capacity. Photosynthetic pigments like chlorophylls have been known to possess several beneficial properties for example chlorophyllin (water soluble analogue of chlorophyll) was proven to be more efficient than the parent compound (Negishi, 1997; Dashwood *et al.*, 1998). Vitamins are organic compounds which cannot be produced *in vivo* and are required in a very small quantity for performing several biochemical functions. Thus, it is essential to obtain from the diet or consume as a supplement (Peter, 1990). Vitamin C is known for its antioxidative activity and for its potential to prevent atherosclerosis (Addo, 2004).

In the present study, both qualitative and quantitative analysis revealed the presence of varied bioactive components in a varying amount in all the wetland plants studied. Though, in many cases differences in the finding with that of other workers was evident which may be attributed to numerous factors like age of the plant, time and percentage of humidity of the harvested plant samples, geographical differences, varying distribution of biological compounds in the plant organs and method used for determination. Hence, taking into the consideration of beneficial role of these phytochemicals (both primary and secondary metabolites) in human health, these wetland plants may be regarded as the potential source of complementary alternative medicines.

In recent times, natural products of plant origin such as flavonoids, phenols, terpenoids, steroids etc. have gained substantial attention due to their diverse application in therapeutics / nutraceuticals mainly because of their antioxidant activity (Takeoka and Dao, 2003; DeFeudis *et al.,* 2003) Antioxidants are the substances that are involved in inhibiting and scavenging free radicals thereby protecting humans against various infection and dreadful diseases. These antioxidant compounds may be extracted using different solvents from the plant samples. In the present study, for determining the antioxidative potential of the selected wetland plants, the samples were extracted in methanol. Here the highest extractive yield was observed in methanolic extract of *M. minuta* and lowest in *A. Spinosus*. The methanolic extracts have been shown to possess better antioxidant activities in comparison to the extraction in other solvents as propounded by various researchers (Zeliński and

Kozlowska, 2000; Hasmida *et al.* 2014). Plant derived natural antioxidants has recently received considerable attention because of its multifaceted activity in improving or correcting numerous human disorders / diseases. Moreover, in comparison to the synthetic drugs, ingestion of natural antioxidants are generally accepted to be safer. Presence of several antioxidant compounds makes it rather difficult to claim the role of a single compound which can be attributed towards the antioxidant potential of the plants. Thus numerous methods varying in the chemistry i.e in terms of target molecules or difference in the generation of free radicals have been developed in the measurement of total antioxidant activity. Many reports have been published periodically for revealing the antioxidative potential of plants (Bora *et al*., 2005; Gayathri *et al.*, 2005; Wang *et al.,* 2007; Mimica-Dukic*et al.*, 2008; Hort *et al.*, 2008; Lai *et al*., 2010; Paulsamy *et al.*, 2013; Valizadeh *et al.,*2015).

In the present study, methods such as ABTS scavenging, DPPH radical scavenging, FRAP assay, OH^- scavenging, nitric oxide scavenging, superoxide scavenging and metal chelating activity have been employed to evaluate the antioxidant potential of the methanolic extracts of plant samples. DPPH radical scavenging activity is a very common and an easy *in vitro* method to evaluate the antioxidant capacity of different plant extracts. In this method scavenging of the free stable DPPH radical (2,2'-diphenyl-1-picrylhydrazyl radical) by some antioxidant substances present in plant extract can be adjudged. The colour of DPPH solution is deep purple (absorption at 517 nm) which turns into yellow after being exposed with the proton donating compounds *ie.* antioxidants present in the extract solution (Sharma Bhat 2009). Generally, a decrease in the absorbance values of the reaction mixture indicates the antioxidative efficiency of samples under study. The DPPH scavenging activity highest observed in *H. sibthorpioides* followed by *B. acutangula, H. auriculata, C. retrospiralis*, *I. aquatica*, *P. oleracea*, *D. esculentum*, *A.viridis*, *E. prostrate, P.nodiflora, A. spinosus, G. oppositifolius*, *P. microphylla, M. minuta, E. fluctuans*.

The ABTS (2,2-azinobis-3-ethylbenzothiazoline-6- sulphonate) method in comparison to the DPPH assay offers flexibility in a way that this assay can be done at different pH levels and therefore useful while studying the antioxidant activity of various compounds in a wide range of pH of the reaction mixture. In this assay interaction between antioxidant and ABTS radical cation ($ABTS^{\bullet+}$) is studied which

has a typical color showing maxima at 645, 734 and 815 nm (Rice-Evans 1996, Miller and Rice-Evans 1996). The ABTS assay determines the relative ability of natural antioxidants in scavenging the $ABTS^{\bullet+}$ generated in aqueous phase and compared to the activity of trolox standard (Boligon *et al*. 2014). This assay is also very useful for the measurement of antioxidant activity of plant samples extracted in acidic solvents. Also as ABTS is soluble in both aqueous and organic solvents it is thus very useful in assessing antioxidant activity of samples in different solvent systems. In this present study, the studied plant extracts showed variable results, *H. sibthorpioides* showed maximum antioxidant activity and *E. fluctuans* showed the least activity. Other studied plant samples shows intermediate activity.

The ferric reducing antioxidant potential (FRAP) was determined using the method of Benzie & Strain (1996). The fresh FRAP reagent was prepared by mixing acetate buffer (300 mM; pH 3.6), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) (10 mM), and $FeCl_3 \cdot 6H_2O$ (50 mM) and the spectrophotometric measurement was performed at 593 nm. Vitamin C was used as a standard antioxidant to compare the antioxidant potential of the studied plant samples. In the present study, the highest FRAP activity was recorded for *B. acutangula* and least activity for *P. microphylla*. Similarly, hydroxyl radical scavenging activity was also used to evaluate the antioxidant potential of the 15 plant samples. Hydroxyl radicals are able to reduce disulfide bonds specifically in fibrinogen, resulting in abnormal spatial configurations and this reaction is found to be responsible for the occurrence of many diseases such as cancer, atherosclerosis and neurological disorders. This type of adverse effects of the reaction of hydroxyl radicals can be prevented by non-reducing substances obtained from natural sources (Lipinski 2011). In the present study, the scavenging potential of the plant extracts on hydroxyl radicals was measured at 560 nm in a spectrophotometer against a blank according to the method of Yu. *et al*. (2004). Among the 15 plant studied *C. retrospiralis* shows maximum activity followed by *P. oleracea, I. aquatica. D. esculentum, A. spinosus, H. sibthorpioides, G. oppositifolius, A.viridis, M. minuta.* NO scavenging activity of the plant extracts was also performed and for this study the NO radicals were generated from sodium nitroprusside at physiological pH. The nitric oxide thus generated interacts with free oxygen to form nitrite ions which is then estimated with the reaction with Griess reagent at 540 nm. Nitric oxide (NO) is also a very reactive free radical associated with many diseases

(Chen *et al.*, 2008; Ebrahimzadeh *et al.,* 2009). Scavenging of the harmful NO radicals by the plant extracts may lead to the screening and development of beneficial plant products which can be useful to combat the ill effects of excessive NO generated in human body. In the present study, sharp decrease was observed in absorbance with the increase in the concentration of plant extracts. It is evident from the study that plant samples having three to nine folds lesser effectiveness in NO scavenging activity than ascorbic acid. Among the plant extracts, the lowest IC50 value was recorded for *C. retrospiralis* and the highest IC50 value was recorded for *E. fluctuans*. Lower IC50 value indicates higher NO scavenging capacity of plant extract.

In living cells when oxygen is taken up by the cell superoxide anion radicals are immediately produced inside cells. Superoxide anion radicals are highly reactive free radicals produced endogenously by xanthine oxidase which converts hypoxanthine to uric acid. Harmful effects of these radicals on various cellular components leading to numerous diseases and are also involved in lipid peroxidation (Attarde *et al*., 2011). With the help enzyme superoxide dismutase biological systems naturally eliminates its toxic effect. (Chung *et al.* 2005). In this study *H. sibthorpioides* and *C. retrospiralis* showed very good scavenging activity. Ferrozine can chelate with Fe^{2+} and form a reddish complex (Dinis *et al,* 1994). In presence of other chelating agents this reaction results in a sharp decrease of the reddish colour of the ferrozine-Fe^{2+} complex. Measurement of the color reduction is measured spectrophotometrically to estimate the chelating activity of the extract. (Soler-Rivas *et al*. 2000). The antioxidant of plant extracts forms a complex with the metal ions and inhibit the electron transfer. In this way oxidation reaction is suppressed and no free radicals are produced. Among 15 plant samples studied *Portulaca oleracea* showed highest metal chelating activity and *B. acutangula* least.

On the basis of *in vitro* antioxidant activity two plants, *Cryptocoryne retrospiralis* and *Hydrocotyle sibthorpioides* were selected for further study. The methanolic extracts were tested against bacterial strains such as *B. subtilis, E. coli* (MTCC 452) and *Mycobactrium avium* (MTCC 1723). Both the plant extracts showed antimicrobial activity against *B. subtilis* and *E. coli*, but the extracts could not show any antibacterial activity against *M. avium*. Moreover, inhibition of spore germination of different plant pathogenic fungus like *Alternaria alternata*, *Curvularia lunuta* and *Fusarium oxysporum* was also exhibited by the plant extracts. Antibacterial activity

by the ethanolic extract of of *C. retrospiralis* was demonstrated by Wadkar *et al.* (2017). Antimicrobial efficacy of *Hydrocotyle javanica* against human pathogenic bacteria was also reported by Mandal *et al*. (2016). In case of antifungal activity, better result showed by *C. retrospiralis* in comparison to *H. sibthorpioides*. Presence of different bioactive compounds attributed for antibacterial as well as antifungal activity.

The in vitro hypoglycaemic activity of all the plant extracts were also evaluated by α-amylase inhibitory activity. α-amylase present in the small intestine and responsible for carbohydrate metabolism. Rising of postprandial blood glucose level is due to the breaking down of polysaccharides to monosaccharide in presence of this enzyme. This enzyme hydrolyses alpha bonds of large polysaccharides, such as starch and glycogen, and producing glucose and maltose. Thus any plant material or any compound able to inhibit α-amylase activity can be very useful in the treatment of diabetis (Bressler and Johnson, 1992). The risk of cardio-vascular diseases is related to the alteration in lipid profile has been encountered in diabetes mellitus (Maghrani *et al.*, 2005). Elevated levels of cholesterol, triglycerides and LDL with the decreased HDL-cholesterol are commonly encountered with hyperglycaemic condition (Uttra *et al.*, 2011).

The hypoglycaemic effect of both the plant extracts were also evaluated on streptozotocin induced diabetic rats. After 25 days of induction a significant decrease in blood glucose level was recorded in both plant extract feed rat groups. In this study, two concentrations 200 and 400 mg/kg of body weight were administrated and it was evident that crude extract helps to lower blood glucose level in dose dependent manner. Higher percentage (39.37) of lowering of blood glucose was recorded *H. sibthorpioides* with a concentration of 400 mg/kg of body weight. Comparatively, lower degree of blood glucose decrease was recorded when rats were administrated *C. retrospiralis* (28.34%). However, decrease in blood glucose concentration is quite higher (71.65%) in metformin applied group. Several authors reported promising activity of plant extracts against type II diabetes (Ahmad *et al*. 2008; Hafizur *et al*. 2012; Gaber *et al*. 2013). Kumar *et al.* (2011) demonstrated the antidiabetic activity of methanolic bark extract of *Albizia odoratissima* in alloxan induced diabetic mice.

Decreased body weight is one of the major phenomenons of diabetic patients. During diabetes, body cells unable to utilize glucose as energy source and seem to utilize proteins as an alternative energy source leading to metabolic imbalance in protein metabolism with consequent loss of body weight or continuous excretion of glucose from the body (Kirana *et al*. 2011; Rajasekar *et al.* 2014). In present study, a sharp decrease (28%) was observed in body weight of diabetic control rats. Whereas, increase in body weight was observed in *H. sibthorpioides* and *C. retrospiralis* administrated rats. It was observed that, gain in body weight is relatively higher in *H. sibthorpioides* (11.8 %) administrated rats when applied at a dose of 400 mg/kg body weight. However, the gain in body weight is higher (13.92 %) in metformin administrated rats. It was evident that both plant extracts reversed the detrimental effect of diabetes there by increasing the body weight of treated rats. Alamin et al. (2015) demonstrated significant increase in body weight in diabetes induced rats when administrated with the aqueous extract of *Tinospora bakis* at a concentration of 400 mg/kg of body weight.

Hepatoxicity is also very common in diabetic condition. Hepatoxicity is related with the elevation of liver marker enzymes (SGOT and SGPT) (Ghosh and Suryawanshi, 2001). Nephropathy in diabetic rats can be identified with the increased levels of serum urea and creatinine (chief marker) (Idonije *et al.*, 2011). Thus, the reduction in urea and creatinine levels, SGOT and SGPT of diabetic rats after treatment with the *C. retrospiralis* and *H. sibthorpioides* indicated that these extracts may have prohibited further kidney and liver damages upto certain level in the diabetic treated rats. Understanding the chemical nature of the extracts following the isolation and characterization of lead compounds responsible for biological activities is important for pharmaceutically exploiting these compounds, thereby leading to the discovery and development of newer drugs (Mariswamy *et al.,*2011).Thus, we have made an attempt to characterize the active compounds underlying the various activities shown by *C. retrospiralis* and *H. sibthorpioides* extract.

For partial characterization, a array of experiments were conducted. FTIR was performed to identify the functional groups present in the methanolic extract of *H. sibthorpioides* and C. retrospiralis. The main stretching vibrations of *C. retrospiralis* appear at 3394 cm^{-1} (broad), 2924 cm^{-1}2852 cm^{-1}, 2265 cm^{-1}2065cm^{-1}, 1640 cm^{-1}, 1407 cm^{-1} and 1237 cm^{-1}. Similarly we get same type of FTIR spectra of *H.*

sibthorpioides (Figure 14). The main stretching vibrations of *H. sibthorpioides* appear at 3397cm-1 (broad), 2926 cm^{-1}2855 cm^{-1}, 2265 cm-1, 2065 cm^{-1}, 1648 cm^{-1} and 1411 cm^{-1}. Spectral differences are considered as the objective reflection of componential differences. Therefore, fingerprint characters of FT-IR spectrum can be used to judge the origin of different extracts accurately and effectively, trace the constituents in the extracts, identify the medicinal materials true or false and even evaluate the qualities of medicinal materials (Mriswamy, *et al*. 2012).

HPLC profiling of all the preliminary selected fifteen plants were done to determine the presence of phenolic compounds in methanolic plant extracts. Earlier works with different plant extracts detected the presence of phenols and polyphenols, which contribute to the antioxidant potentiality (Siger *et al*. 2012). Highest numbers of peaks were detected in *M. minuta*. Pteridophytes are rich in phenols which are responsible for its several bioactivities. Finally selected two plants, viz. *C. retrospiralis* and *H. sibthorpioides* showed comparatively less numbers of peaks in their methanolic extracts. HPLC analysis of *H. sibthorpioides* revealed fifteen putative phenolic compounds. However, only twelve peaks could be detected in C retrospiralis. Kumari *et al*. (2016) detected catechin, epicatechin, quercetin and chlorogenic acid in *H. sibthorpioides* extract by ultra-high pressure liquid chromatography-mass spectrometry (UPLC-MS/MS).

The compounds identified in these samples may act as potential ligands or lead compounds against antidiabetic target proteins/enzymes. Moreover, both the extracts had revealed appreciable results in an *in vivo* antidiabetic study; thus, we have made an in silico molecular approach to understand some of the possible mechanisms of action behind the antidiabetic activity of *C. retrospiralis* and *H. sibthorpioides* to select the potential antidiabetic components by docking some of the compounds against target proteins/enzymes involved in glucose metabolism such as peroxisome proliferators activated receptor gamma (3DZY). Natural products have been described as a promising pool of structures for drug discovery, and a significant research effort has recently been undertaken to explore the PPARγ-activating potential of a wide range of natural products originating from traditionally used medicinal plants or dietary sources (Wang *et al*. 2014). Presence of diversified types of bioactive components may have attributed to the antioxidant, antimicrobial and antidiabetic activities exhibited by these wetland plants. There is no previous report of

such work from these plants of this region. These plants could be considered as the potential source for exploiting their applications in pharmaceuticals. Molecular docking approach to elucidate the probable mechanism of function by the plant extracts may be exploited to decrease time and labour as well as lowering the use of costly chemicals. In their study, Natarajan *et al*. (2016) applied molecular docking approach to elucidate (4Z, 12Z)-cyclopentadeca-4, 12-dienone from *Grewia hirsuta* with some targets related to type 2 diabetes. It was found that the compound (4Z, 12Z)-cyclopentadeca-4, 12-dienone is a promising candidate which docks well with various targets related to diabetes mellitus.

GCMS reveals presence of 9 compounds from *C.retrospiralis* and 10 compounds from *H. sibthorpioides* methanolic extract. Among these compounds some are known to have some role and others role is not yet known to us. Phytol is a compound found in *C. retrospiralis* is an acyclic diterpene alcohol and can be used as a precursor for the production of synthetic forms of vitamin E and vitamin K (Netscher and Thomas 2007). It is also an important compound reported with antimicrobial, cytotoxic, and antioxidant properties (Wei *et al.* 2011). Octadecadienoic acid is a monounsaturated fat and according to some workers its consumption reduces low-density lipoprotein (LDL) cholesterol, and possibly increases high-density lipoprotein (HDL) cholesterol. According to *Martin-Moreno* (1994) consumption of oleate in olive oil has been linked with a *decreased* breast cancer risk. Isopropyl Stearate used as a skin care product and in some medicine as inactive ingredients. Testosterone enanthate is a well known steroid which significantly increases sports person performance by increasing muscular strength within 6-12 weeks of administration (Rogerson 2007). n-hexadecanoic acid inhibits phospholipase A2, hence it is an anti-inflammatory compound. This study validate the use of oils rich in n-hexadecanoic acid for treating of rheumatic symptoms in ethno medical systemas well as Ayurveda of India (Vasudevan *et al*. 2012). Hexadecanoic acid is one of the major phytoconstituent detected by GC-MS analysis that is present in both the plant extracts which is known to possess strong antimicrobial activity (Preethi *et al.* 2010). Anti-inflammatory and cytotoxic potential of hexadecanoic acid, methyl ester was reported by Othman *et al*. (2015). It was reported by Hussein *et al.* (2016) that Curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo, methyl ester shows anti yeast activity.

The cytotoxic effect of the plant extracts were evaluated against human hepatocarcinoma cell line (HepG 2) was purchased and maintained in Ham's F-12 medium, with 10% Foetal Bovine Serum in a humidified CO_2 -Incubator at 5% CO_2 level and 37 °C. The methanolic extract of *C. retrospiralis* and *H. sibthorpioides* at different concentration showed significant cytotoxicity against the human hepatocarcinoma cell line (HepG 2). Nuclei of the extract treated cells appeared smaller, indicating nuclear shrinkage. With an increase in concentration, a notable degree of nuclear fragmentation was also evident when observed under Floid Cell Imaging Station (Life Technologies, USA). At a concentration 100 µg/ml these extract showed 17.38% and 30.41% cell death respectively for *C.retrospiralis* and *H. sibthorpioides* extracts, which accelerated with increasing concentration of the extract. At maximum concentration of the plant extracts tested i.e 500 µg/ml, the cell death increased to 57.12 and 68.32 respectively. For recent years it has been a new trend to search for alternative anticancerous drug from plant sources with less adverse effects. Quintanilla-Licea *et al.* (2016) reported cytotoxic effect of methanol extracts of *Pachycereus marginatus* (DC.) Britton & Rose and *Ibervillea sonorae* (S. Watson) against the murine lymphoma L5178Y-R cell line. Presence of bioactive compounds like alkaloids is the probable candidate for cytotoxicity against different cancer cell like. Methanol is good solvent and known to be efficient to dissolve active constituents from different dried plant materials. Orange *et al*. (2016) investigated the cytotoxicity effects of *Scrophularia oxysepala* methanolic subfractions and the underlying mechanism responsible for cell death in human breast carcinoma (MCF-7 cells) and mouse fibrosarcoma (WEHI-164 cells). Results clearly indicated that *S. oxysepala* methanolic subfractions induce apoptosis in MCF-7 and WEHI-164 cells.

6. CONCLUSION

- ✓ A review of literature pertaining to the different aspects of the present line of study has been presented.
- ✓ Ethnobotanical survey and selection of plant materials were done accordingly.
- ✓ Detailed description of all the procedure and protocols used in this investigation has been presented.
- ✓ Wetland plants collected from different areas of North Bengal were identified and processed for further experimentation. Identified samples were *Cryptocoryne retrospiralis* (Roxb.) Kunth., *Barringtonia acutangula* (L). Gaertn., *Hydrocotyle sibthorpioides* Lam., *Eclipta prostrata* (L.) L., *Enhydra fluctuans* DC., *Amaranthus spinosus* L., *Marsilea minuta* L. *Amaranthus viridis* L., *Glinus oppositifolius* (L)Aug.DC, *Portulaca oleracea* L., *Hygrophila auriculata* (Schumach) Heine., *Phyla nodiflora* (L) Greene., *Pilea microphylla* (L) Liebm., *Diplazium esculentum* (Retz) Sw., *Ipomoea aquatica* Frossk.
- ✓ Plant samples were dried , powdered and extracted using methanol and subjected to phytochemical analysis and determination of various biological activities
- ✓ Different phytochemicals including primary and secondary metabolites were screened in the powdered plant samples. In all the plant samples except *C. retrospiralis*, *A. viridis* and *I. aquatica* saponins were detected. Presence of alkaloids was detected in 9 plant samples except *H. sibthorpioides*, *A. spinosus*, *M. minuta*, *A. viridis*, *P. oleracea* and *I. aquatica*. The phenolics and flavonoids were detected in all the plant samples except *P. oleracea*. In *B.acutangula*, *E. prostrata* and *G. oppositifolius*, the phytochemical test for steroids showed positive result. In *C. retrospiralis*, *A. viridis*, *P. oleracea*, *P. nodiflora*, *P. microphylla* and *I. aquatica*, carbohydrates were not detected. The phytochemical screening showed positive results for the presence of anthroquinones only in case of *B.acutangula*, *P. nodiflora*, *D.esculentum*; but in other samples anthroquinones were not detected. Only *C. retrospiralis* and *D.esculentum* showed negative results for amino acid. Reducing sugars were not traced in *C. retrospiralis*, *P. oleracea* and *P. microphylla*. Also,

phytochemical screening of resins revealed their presence only in case of *B.acutangula*, *E. prostrata* and *I. aquatica*. On the other hand, cardiac glycosides were present in *A.spinosus*, *A. viridis*, *G. oppositifolius, P. oleracea* and tannins were detected in all the samples except *A.spinosus*, *D.esculentum* and *I. aquatica*. Likewise, phytochemical screening of glycosides revealed their presence in *C. retrospiralis*, *H. sibthorpioides*, *A.spinosus*, *A. viridis*, *G.oppositifolius*, *P. oleracea* and *P. nodiflora*. However, protein was not detected in *P. nodiflora* and *P. microphylla*; and terpenoids were detected in *C. retrospiralis*, *B.acutangula*, *E. prostrata*, *E. fluctuans*, *P. oleracea*, *H. auriculata.*

- ✓ Quantitativley, amount of total phenol, total flavonoid, protein, free amino acids, total sugar, reducing suger, chlorophyll, carotenoid and ascorbic acid content were estimated in these plants. The protein content observed maximum in *A. spinosus* and the amino acid content was recorded highest in *G. oppositifolius*. In *H.sibthorpioides* highest phenol content was recorded and in *A.viridis* highest flavonoid content observed. The total suger content of *E.prostrata* ranked highest and highest reducing suger content was recorded in *P. oleracea.*Highest ascorbic acid content observed in *H.sibthorpioides* and the carotenoid content estimated maximum in *A.viridis.*Total chlorophyll and Chl-a was recorded highest in *E.prostrata* and highest Chl-b content recorded in *A.spinosus* .
- ✓ Extractive values for these fifteen plants with methanol differ from 12.52% to 8.32%. Highest yield was achieved in *M. minuta* and lowest in *A.spinosus* .
- ✓ In the present study, methods such as DPPH radical scavenging, ABTS scavenging activity FRAP assay, OH^- scavenging activity, nitric oxide scavenging, superoxide scavenging and Metal chelating activity was employed.The DPPH scavenging activity mostly renged from 2.96 – 73.19 %. Among the plant extracts, lowest IC50 value for DPPH was recorded for *H.sibthorpioides* (1.539±0.065) and the highest IC50 value was recorded for *E.fluctuans* (9.672±0.082). ABTS scavenging activity mostly ranged from 04.96-76.09%. In case of FRAP assay highest activity was recorded in *B. acutangula* whereas lowest activity was exhibited by *P. microphylla.* Similarly all the extracts showed variable OH^- scavenging activity ranged from 8.34-81.23%. The NO

scavenging activity ranged from 04.65-60.29%. Almosr all the extracts showed variable superoxide scavenging activity. Among the plant extracts the lowest IC50 value was recorded for *H.sibthorpioides* and highest IC50 value recorded for *G. oppositifolius*. The metal chelating activity was found to increase in a dose dependent manner for the range of concentrations tested (0.1,0.5,1,2,4,6,8,10 mg/ml).All the extracts showed variable metal chelating activity that mostly ranged from 12.23-84.67%.

- ✓ *In vitro* α-amylase activity assay was also performed to determine wheather any antidiabetic property is present in the plant samples under study. All extracts showed variable α-amylase inhibitory activity that mostly ranged from 8.14-86.77%Among the plant extracts, the lowest IC50 value was recorded for *C. retrospiralis* and the highest IC50 value was recorded for *H.auriculata*
- ✓ Based on the results of all previous experiments, finally two plant samples showing overall best performance were selected for further experiments *eg*. antimicrobial properties, *in vitro* antidiabetic activity, Cytotoxic effect etc.
- ✓ Antifungal studies performed against *Alternaria alternata*, *Curvularia lunata* and *Fusarium oxysporum* by spore germination test revealed that the extracts of *C. retrospiralis and H. sibthorpioides* were efficient to inhibit the growth of the fungal spore upto certain level. Among, the three concentration used for the study, 500mg/mL was found to be effective against these fungi while no inhibition was observed by 250 and 100mg/mL concentration.
- ✓ Furthermore, an *in vivo* antidiabetic test in streptozotocin-induced diabetic rats was performed using *C. retrospiralis* and *H. sibthorpioides* as they were found to be more efficient in inhibiting α-amylase activity than *other* extracts.
- ✓ Before performing an *in vivo* test, acute toxicity test done to analyse the lethal doses of extracts revealed 500 and 250 mg/mL doses to be safer.
- ✓ Streptozotocin-induced diabetic rats treated orally with both the samples and metformin was able to reverse the diabetic induced changes to upto certain level. Various parameters such as fasting blood sugar level, triglycerides, LDL-cholesterol,total cholesterol, liver enzymes (SGPT and SGOT), serum urea and creatinine was reduced while significant increase in the body weight and HDL-cholesterol was also observed.

- ✓ The main stretching vibrations of *C. retrospiralis* appear at 3394cm^{-1} (broad), 2924 cm^{-1}2852 cm^{-1}, 2265 cm^{-1}2065cm^{-1}, 1640cm^{-1}, 1407cm^{-1} and1237 cm^{-1}. Absorption bands in the 3600-3200 cm^{-1} regions are from O-H stretching vibrations. Similar type of FTIR spectra of *H. sibthorpioides*. The main stretching vibrations of *H. sibthorpioides* appear at 3397cm^{-}1 (broad), 2926 cm^{-} ^{1}2855 cm^{-1}, 2265 cm^{-1}, 2065 cm^{-1}, 1648cm^{-1} and 1411cm^{-1}. From the FTIR spectra it confirmed that *C. retrospiralis* and *H. sibthorpioides* contain similar types of functional groups.
- ✓ The methanolic extract of *C. retrospiralis* and *H. sibthorpioides* were further subjected to GC-MS for partial characterization of the compounds present in the fractions. *C. retrospiralis* methanolic extract were identified to contain nine different compounds 1,3- Diazocane -2 thione, E 11 - Methyl-12-tetradecan-1-ol acetate, Hexadecanoic acid- methyl ester, Phytol, 9, 12- Octadecadienoic acid, Isopropyl Stearate, 6,9,12-Octadecatrienoic acid, Phenylmethyl ester, Testosterone Enanthate, n-Hexadecanoic acid and *H. sibthorpioides* methanolic extracts were identified to contain ten different compounds 3 Eicosyne, Morphinan- 3,14-diol, 4,5-epoxy(5 á), 9,12,15-Octadecatrienoic acid, 2,3-dihydroxy propyl ester, Corynan-17- ol,18,19-didehydro-10-methoxy acetate, Curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo, methyl ester, 10-Octadecanoic acid, methyl ester, Z,E-3,13-Octadecadien -1 ol, Thujopsene, 3-Cyclohexen-1-ol, 4- methyl-1-1(1-methylethyl), Hexadecanoic acid- methyl ester.
- ✓ To further understand the mechanism of action that may have been involved in antidiabetic activity revealed *C. retrospiralis* and *H. sibthorpioides* an *in silico* molecular docking studies was performed. The molecular docking results showed that the compound testosterone enanthate obtainined from methanol extract of *C. retrospiralis*, possess good binding affinity (-11.16 kcal/mol) with 3DZY showing interaction with protein residues ILE 268, ALA 271, ALA 272, GLN 275, TRP 305, ASN 306,LEU 309,ILE 310, PHE 313,ARG 316, LEU326, ALA 327, ILE 345, PHE 346, VAL 349, CYS 432, HIS 435, LEU 436 as compared to commercial drug metformin (LYS431, GLU434, THR737, GLN741, GLN744, TYR777).

- ✓ The compound Corynan-17- ol,18,19-didehydro-10-methoxy acetate (ester) which was obtained from *H. sibthorpioides* possessed good binding affinity (-9.95 kcal/mol) by binding with amino acid residues ILE268,ALA 271, GLN 275,TRP 305, ASN 306, LEU 309,PHE 313, ARG 316, LEU 326, ALA 327, VAL 342, ILE 345,LEU 426,HIS 435.
- ✓ The methanolic extract of *C. retrospiralis and H. sibthorpioides* showed significant cytotoxicity against the human hepatocarcinoma cell line ($HepG_2$). At maximum concentration of the plant extracts tested i.e 500 µg/ml, the cell death increased to 57.12 and 68.32 respectively. The LD50 value of the *C. retrospiralis* and *H. sibthorpioides* extracts was determined to be 366.70 µg/ml and 375.5 µg/ml subsequently.
- ✓ Presence of various bioactive constituents in these plants makes them the potential source for exploiting their applications in pharmaceuticals. However, further isolation and characterization of each compound responsible for the activity in addition to elucidation of the mechanism involved remains to be explored.

APPENDICES

Appendix A: List of thesis related publication

1. Choudhury J and Chakraborty U. (2017) Antioxidant activity and phytochemical screening of two important wetland plants *Enydra fluctuans* DC. and *Hygrophila auriculata* (Schumach.) Heine. *World Journal of Pharmacy and Pharmceutical Sciences*, **6:** 1424-1436.
2. Choudhury J, Majumdar S, Roy S and Chakraborty U. (2017) Antioxidant activity and phytochemical screening of two edible wetland pteridophytes *Diplazium esculentum* (Retz.) Sw. and *Marsilea minuta* L. a comparative study. *World Journal of Pharmceutical and Medical Research*, **3(9):** 195-203.

Appendix B: List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
$AlCl_3$	Aluminium chloride
ALT	Alanine aminotransferase
AS	*Amaranthus spinosus*
AV	*Amaranthus viridis*
BA	*Barringtonia acutangula*
BHT	Butylated hydroxy toluene
BSAE	Bovine serum albumin equivalent
CH_3COOH	Acetic acid
CR	*Cryptocoryne retrospiralis*
DE	*Diplazium esculentum*
DMRT	Duncan's multiple range test
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EF	*Enhydra fluctuans*
EP	*Eclipta prostrata*
$FeCl_3$	Ferric chloride
FRAP	Ferric Reducing Antioxidant Power
FT-IR	Fourier Transform Infrared Spectroscopy
GAE	Gallic acid equivalents
GC-MS	Gas Chromatography–Mass Spectrometry
GLDH	Glutamate ehydrogenase
GO	*Glinus oppositifolius*
H_2SO_4	Sulfuric acid
HA	*Hygrophila auriculata*
HCL	Hydrochloric acid
HDL	High-density lipoprotein
HPLC	High Performance Liquid Chromatograph
HS	*Hydrocotyle sibthorpioides*
IA	*Ipomoea aquatica*
IBM	International Business Machines Corporation
IC50	50% Inhibition concentration
LDL	Low-density lipoprotein
LGA	Lamarckian genetic algorithm
ME	Methanolic extract
MM	*Marsilea minuta*
NA	Nutrient Agar
Na_2CO_3	Sodium carbonate

Na_2SO_4	Sodium sulfate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NCCS	National Centre for Cell Science
NIST	National Institute of Standards and Technology
NO	Nitric oxide
PM	*Pilea microphylla*
PMS-NADH	Phenazine methosulfate- Nicotinamide adenine dinucleotide
PN	*Phyla nodiflora*
PO	*Portulaca oleracea*
RCSB	Research Collaboratory for Structural Bioinformatics
SD	Standard Deviation
SGOT	Serum Glutamic-Oxaloacetic Transaminase
SGPT	Serum Glutamic-Pyruvic Transaminase
SPSS	Statistical Package for the Social Sciences
TPTZ	2,4,6-Tris(2-pyridyl)-*s*-triazine

Appendix C: List of chemicals

1. α-Amylase enzyme
2. α-Glucosidase
3. 2,2′ Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ABTS
4. Acetic acid
5. Acetic anhydride
6. Acetone
7. Aluminum chloride
8. Ammonia
9. Butanol
10. Chloroform
11. Citrate buffer (pH 4.5)
12. Conc.H_2SO_4
13. Cupper acetate
14. 2-deoxyribose solution
15. Dichloromethane
16. Dichromatic acetic acid
17. 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-(3,3′-
18. dimethoxy-4,4′-diphenylene)di-tetrazolium chloride
19. DNS (3,5-dinitrosalicylic acid) reagent
20. DPPH (2,2-diphenyl-1- picrylhydrazyl)
21. Dragendroff's reagent
22. Ethylenediaminetetraacetic acid
23. Ethanol
24. Ethyl Acetate
25. Ethylenediamine tetraacetic acid
26. $FeCl_2$
27. $FeCl_3$
28. Fehling's solution I (A)
29. Fehling's solution II (B)
30. Ferric chloride
31. Ferrozine
32. $FeSO_4$, $7H_2O$
33. Folin-Ciocalteu reagent
34. Formic Acid
35. Gallic acid
36. Glacial acetic acid
37. Glibenclamide
38. Glucose
39. Hexane
40. Hydrated ferrous sulphate
41. Hydrochloric acid
42. Hydrogen peroxide
43. Hydroxylamine hydrochloride
44. Methanol

45. Na_2CO_3
46. Na_2SO_4
47. NaOH
48. Napthylethylenediamine dihydrochloride
49. n-Butanol
50. Nicotinamide-adanine dinucleotide phosphate (NADPH)
51. Ninhydrin reagent
52. Nitro-blue tetrazolium (NBT)
53. Petroleum ether
54. Phenazine methosulphate (PMS)
55. *p*-nitrophenol-a-D-glucopyranoside
56. Potassium dichromate
57. Potassium ferricyanide
58. Potassium hydroxide
59. Potassium persulfate
60. Pyridine
61. Sodium carbonate
62. Sodium hydroxide
63. Sodium nitroprusside
64. Starch
65. Streptozotocin
66. Trichloroacetic acid (TCA)

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ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL SCREENING OF TWO IMPORTANT WETLAND PLANTS *ENYDRA FLUCTUANS* DC AND *HYGROPHILA AURICULATA* (SCHUMACH.) HEINE

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ABSTRACT

Enydra fluctuans and *Hygrophila auriculata* have been consumed traditionally as a vegetable by the communities of North Bengal. Leafy vegetables are rich sources of antioxidants and essential nutrients. The antioxidant activities of the two plants were evaluated using methanolic (MeOH) extracts by DPPH free radical scavenging activity, ABTS, and FRAP assay. Qualitative analysis of phytochemicals was also done using standard methods. The presence of compounds like total phenols, flavanoid, protein, total sugar, reducing sugar, free amino acid and chlorophyll content were also evaluated in these two plants. However, *Enydra fluctuans* and *Hygrophila auriculata* exhibited significant differences in their antioxidant values.

KEYWORDS: phytochemicals, phenol, flavonoid, antioxidants, DPPH, ABTS, FRAP.

INTRODUCTION

Enydra fluctuans and *Hygrophila auriculata* have been consumed frequently as leafy vegetables by different communities of North Bengal, which encompasses the Northern region of West Bengal, India. The leafy vegetables are a rich source of minerals and vitamins and have been believed to be a good source of antioxidants which scavenge the reactive oxygen species (ROS) produced inside the cells and tissues. ROS are highly reactive and are able to induce oxidative damage to human body specially brain, kidney, liver, pancreas etc.[1,2] The compounds which reduces the attack of ROS and hence that of ROS related

health hazards are the antioxdants. ROS are formed in the body mainly by exogenous chemicals or endogenous metabolic processes in the human body. These ROS are capable of oxidizing bio-molecules eg. proteins, nucleic acids, lipids and can induce different diseases like cancer, neurological disorders, cirrhosis, atherosclerosis, arthritis etc.[3,4] Antioxidants are the compounds which can reduce or terminate the action of free radicals and thus reduce the high risk of these disorders.[5] All organisms are protected up to some extent against free radical damage with the help of antioxidant compounds eg. ascorbic acid, tocopherol, phenolic acids, polyphenols, flavonoids etc. Antioxidant supplements or dietary antioxidants are naturally present in daily food and can protect us against the degenerating effects of free radicals.[6] Currently, much attention of scientists has been focused on the use of natural antioxidants which can be incorporated in daily diet to protect the human body especially brain tissues from the oxidative damage caused by free radicals.

Wetlands or water bodies are a rich source of diverse groups of plants many of which are edible-being consumed by locals. Many of these are also components of ethnic medicines. In the present study, two such plants have been selected for scientific validation of their phytochemical properties and antioxidative actions.

E. fluctuans belonging to the family Asteraceae is a trailing herb, also floating on water; stem 27-54 cm long, rooting from the nodes. Sessile leaves are 2.3-7.4 cm long, linear-oblong, acute or obtuse, entire or subcrenate. Axillary and terminal sessile flowers are white. The vernacular name of this plant is Helencha, Hinchashak, Harhach and the English name Water Cress, Marsh Herb. This plant is recommended by Ayurvedic practitioners for the improvement of general health as well as gastrointestinal disorders.[7] Anticancer activity against Ehrlich's ascites carcinoma bearing Swiss albino mice were shown by flavonoids obtained from *E. fluctuans*. Significant decrease in the tumour cell volume and increase of life span with ethyl acetate fraction were also investigated earlier.[8,9] The flavonoid rich *E. fluctuans* has significant hepatoprotective effects.[10]

H. auriculata is a herbaceous, medicinal plant of the Acanthaceae family that grows in marshy places and is native to tropical Asia and Africa. In North Bengal it is commonly known as kulekhara. The parts of the plant are widely used in traditional medicine of almost all community of North Bengal for the treatment of many disorders, which include diseases of the urinogenital tract, dropsy from chronic Bright's disease, hyperdipsia, flatulence, diarrhea, dysentery, leukorrhea, gonorrhea, asthma, blood diseases, gastric diseases, inflammation,

cancer, rheumatism, painful micturition, menorrhagia.[11,12] Chloroform and alcoholic extracts have shown anti inflammatory effect of *Hygrophyla* leaves.[13,14]

Therefore, in the present study we have attempted to evaluate the comparative antioxidant activity of *E. fluctuans* and *H. auriculata* which are well known for their nutritive as well as medicinal values.

MATERIAL AND METHODS

Phytochemical screening

Phytochemical screening for the compounds like alkaloids, carbohydrates, glycosides, saponins, phytosteroid, phenols, tannins, flavonoids, proteins, amino acids, fats, gum and mucilages were done following standard protocols.

Collection of plant materials

Leaves of *Hygrophila auriculata* and *Enydra fluctuans* were collected from the wetlands of Dakshin Dinajpur (Latitude: 26°35'15" N to 25°10'55" N; Longitude: 89°00'30" E - 87 deg 48' 37" E) and Malda (Latitude: 24°40’20" N to 25°32’08" N; Longitude: 87°45’50" E to 88°28’10" E) districts of West Bengal, India. A flowering twig each of both plants was dried and submitted to Herbarium Facility at North Bengal University, Siliguri, West Bengal with the Accession Nos. 09822 and 09823 respectively.

Preparation of Extracts

The plants were collected from wetlands and washed thoroughly initially with tap water then with double distilled water and dried using blotting paper. The shade dried plant materials were then ground to obtain fine powder that was stored at -20°C for further use. The powdered sample was then extracted using methanol (MeOH) using the method described by Okwori *et al.* (2006)[15] with slight modification. Briefly, 10 g powdered samples were soaked in 100 ml of methanol for 72 hr at room temperature in a orbital shaker and filtered 3-4 times using Whatman No.1 filter paper. The filtrates were finally concentrated using rotary evaporator at 10°C.

DPPH free radical scavenging activity

DPPH (2,2-diphenylpicrylhydrazyl) free radical scavenging activity was done following standard protocol[16] with slight modifications. Different concentrations of plant extracts (0.1 mL) were put in the test tube and 2.9 mL of a methanolic solution of DPPH (0.1 mM)

was added. The mixture was kept in the dark at room temperature for 30 min and absorbance was measured at 517 nm against a blank and scavenging activity was calculated using the following formula.

DPPH Scavenging (%) = 100 × (A_o - A_s)/A_o

where A_o is the absorbance of the blank and A_s the absorbance of the sample.

ABTS scavenging activity

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay was based on a previously described method of with slight modifications.[17] ABTS radical ($ABTS^+$) was produced by the reaction of a 7 mM ABTS solution with potassium persulphate (2.45 mM). The $ABTS^+$ solution was diluted with ethanol to an absorbance of 1.5 ± 0.05 at 734 nm prior to the assay and stored in the dark at room temperature for 12 h. For assay, 100 µL of extract sample or ascorbic acid used as standard to 2 mL of diluted $ABTS^+$ solution, then absorbance was measured at 734 nm after exactly 6 min. The decrease in absorption was used for calculating scavenging effect using the following equation.

Scavenging effect (%) = 100 × (A_o - A_s)/A_o

where A_o is the absorbance of the blank; A_s is the absorbance of the sample.

FRAP assay

The FRAP (Ferric Reducing Antioxidant Power) was determined using a previously described method with slight modifications.[18] The freshly prepared FRAP reagent contained 500 mL of acetate buffer (0.3M; pH 3.6), 50 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (0.01M), and 50 mL of $FeCl_3 \cdot 6H_2O$ (0.05M). 100 µL of extracts were added to 2 ml of FRAP reagent and the absorbance was measured at 593 nm after 12 min. The results were calculated from the standard curve of $FeSO_4$ and expressed as mM Fe^{2+} equivalent.

Total phenols

The total phenol content was determined by slightly modified Folin-Ciocalteu method.[19] The reaction mixture contained 200 µL of extract, 800 µL of freshly prepared diluted Folin Ciocalteu (1:1) reagent and 2 mL of sodium carbonate (7.5%), kept in the dark at ambient conditions for 2 h to complete the reaction. The absorbance was measured at 765 nm. Gallic acid was used as standard and the results were expressed as mg gallic acid/g dried extract.

Total flavonoids

Total flavonoid content was determined according to the standard method using quercetin as a standard.[20] A volume of 500 µL of extract was added to 500 µL of $NaNO_2$ (5%). After 5 min at 25°C, 500 µL of $AlCl_3$ (10%) was added. After a further 5 min, the reaction mixture was mixed with 1 mL of 1 mM NaOH. Finally, the absorbance was measured at 510 nm. Quercetin was used as standard and the results were expressed as mg quercetin (QE)/g of dried extract.

Total chlorophyll and carotenoids estimation

0.5 g leaf tissue was extracterd in 80% (V/V) acetone for the estimation of chlorophyll and carotenoids and the calculation was done according to the following formula.[21, 22]

Total Chlorophyll = $17.32A_{645} + 7.18A_{663}$

Carotenoids = $(1000\ A_{470} – 1.63$ Chl a – 104.96 Chl b)/221

Protein content

1 g fresh plant tissue was homogenized in a prechilled mortar and pestle using 5 ml of 50 mM sodium phosphate buffer (pH 7.2) and PVP (polyvinylpyrrolidone) under ice cold condition and centrifuged at 10,000 rpm at -4ºC for 15 mins.[23] The supernatant obtained was used as the crude extract for estimation. For estimation, 1ml of extract and 5ml alkaline reagent was mixed thoroughly and allowed to stand for 15mins.[24] Then, Folin Ciocalteau's phenol reagent was added and incubated for 20 minutes and the absorbance was read at 690 nm.

Free amino acid

Free amino acids were estimated with standardized protocol with slight modifications.[25] 0.5 g leaf tissue was extracted in ethanol and filtered using Whatman No. 1 filter paper. To 1 ml of the filtrate, 1 ml of ninhydrin reagent was added and boiled in a water bath for 20 min. The absorbance was read at 570 nm and the free amino acid content was estimated using a standard curve of L-proline.

Total sugars and reducing sugars

Total and reducing sugar was extracted following the method of Harborne (1973).[26] 1 g fresh leaf tissue was extracted in 10 ml of 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath. Then the residue was dissolved in dH_2O and the final volume was made up to 5 ml which was then centrifuged at 5000 rpm for 10 min.

Estimation of total sugar was done by Anthrone reagent following the method of Plummer (1978).[27] To 1 ml of test solution, 4 ml of Anthrone reagent (0.2% Anthrone in conc. H_2SO_4) was added. The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10 mins. Then the reaction mixture was cooled under running tap water and absorbance was measured at 620 nm and sugar content was quantified using a standard curve of D-glucose.

Reducing sugar was estimated by Nelson-Somogyi method as described.[27] 1 ml of the test solution was mixed with 1 ml of alkaline copper tartarate solution and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water and 1 ml Nelson's Arsenomolybdate reagent was added along with 2 ml of dH_2O and mixed vigorously. A blue colour was developed, the absorbance of which was the measured in a colorimeter at 515 nm and reducing sugar content was quantified using a standard curve of D-glucose.

RESULTS

Phytochemical screening

The methanolic extract of the plants were screened for the presence of different phyto chemicals. The result revealed that both plants contain carbohydrate, saponins, phenols, tannins, flavonoids, proteins, triterpenes and alkaloids. Phytosteroid, fat and fixed oil, glycosides and anthroquinone were found to be absent in methanolic extract of both the plant samples (Table 1).

Table 1: Phytochemical compounds detected in the two plant extracts.

Sl No.	Phytochemicals	*E. fluctuans*	*H. auriculata*
1	Alkaloids	+	+
2	Carbohydrates	+	+
3	Glycosides	-	-
4	Saponins	+	+
5	PhytoSteroid	-	-
6	Phenols	+	+
7	Tannins	+	+
8	Flavonoids	+	+
9	Proteins & Amino acids	+	+
10	Amino acids	+	+
11	Fats & fixed oils	-	-
12	Gum and mucilages	+	-
13	Triterpenes	+	+
14	Cholesterol	-	+

15	Anthraquinone	-	-
16	Cardiac glycosides	-	+

+= Present; - = Absent.

Nutritional factors

The amount of different nutritional factors in both the dried plant samples was quantified. Results revealed that the protein and amino acid content is higher in *E. fluctuans*, where as total and reducing sugar content is much higher in *H. auriculata* (Table 2).

Table 2: Nutritional analyses in terms of total proteins, free amino acid, total sugars and reducing sugars in the extracts.

Extracts	Protein (mg/g dwt)	Free amino acids (mg/g dwt)	Total Sugars (mg/g dwt)	Reducing sugars (mg/g dwt)
EF	37.5 ± 0.06	0.33 ± 0.04	14 ± 0.67	0.11 ± 0.007
HA	14 ± 0.09	0.29 ± 0.05	154 ± 1.15	0.29 ± 0.004

EF= *E. fluctuans;* HA= *H. auriculata*; dwt= Dry Wt.

***In vitro* Antioxidant analysis**

Determination of antioxidant potential of the extract was carried out next. DPPH is a stable free radical containing an odd electron having a characteristic absorption at 517nm. The deep purple colour usually gets decolorized when exposed to antioxidant in the solution. Results obtained from DPPH radical scavenging activity showed dose-dependent inhibition in case of both the plants but HA showed much more DPPH scavenging activity in comparison to EF. The IC_{50} value for HA was found to be 2.7 mg/ml and 10 mg/ml for EF (Fig. 1).

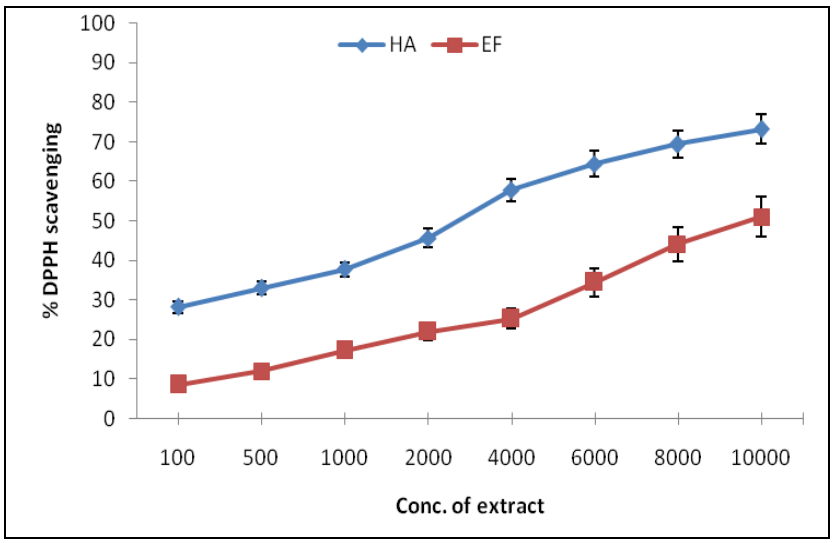

Figure 1. DPPH scavenging activity of different concentrations of plant extracts (µg/ml).

HA - *Hygrophila auriculata*, EF - *Enydra fluctuans*.

The study of ABTS scavenging activity also revealed that HA showed more efficacy in scavenging ABTS free radical as compare to EF. The IC_{50} value of the HA and EF was found to be 5.8mg/ml and 9.9 mg/ml respectively (Fig 2).

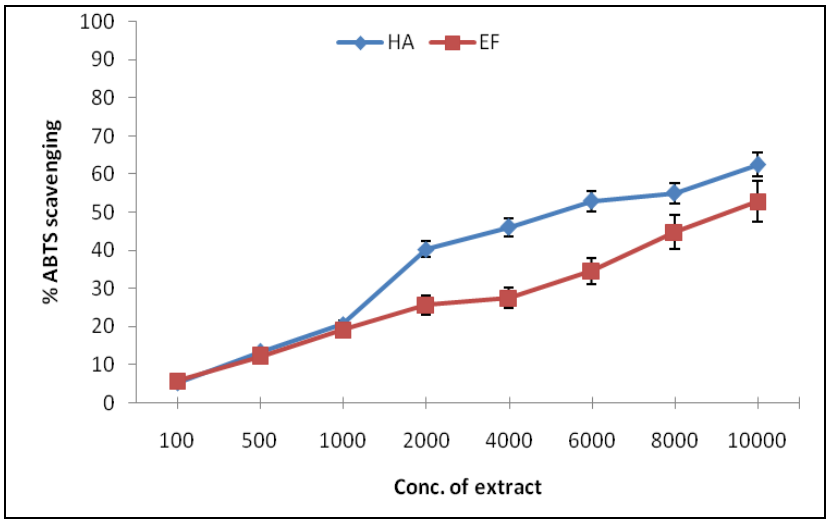

Figure 2: ABTS radical scavenging activity of different concentrations of plant extracts (µg/ml): HA - *Hygrophila auriculata*, EF - *Enydra fluctuans*.

Quantification of bioactive compounds revealed that *E. fluctuans* and *H. auriculata* contain 17.73 and 34.77 mg/g dwt of total phenol, 135 and 293 mg/g dwt of flavonoid, 1.634 and 1.623 mg/g fwt of chlorophyll, 0.47 and 0.46 mg/ g fwt of carotenoids respectively (Table 3).

Table 3. *In vitro* antioxidant potential of the extracts quantified in terms of FRAP, total phenols, total flavanoids, total chlorophyll and carotenoids.

Extracts	FRAP assay (mM Fe^{2+} eq.)	Total Phenols (mg/g dwt)	Total Flavanoids (mg/g dwt)	Total chlorophyll (mg/g dwt)	Carotenoids (mg/g dwt)
EF	0.161 ± 0.09	17.73 ± 0.24	135 ± 1.15	1.634 ± 0.06	0.40 ± 0.003
HA	0.223 ± 0.13	34.77 ± 0.19	293 ± 1.5	1.623 ± 0.03	0.41 ± 0.002

EF= *E. fluctuans;* HA= *H. auriculata*; dwt= Dry Wt.

DISCUSSION

Plants are the sources of different bioactive compounds. Ethnic groups posses the knowledge of beneficial role of different plants for healing of diseases. Wet lands are one of the neglected and unexplored depositories of plant species. In present study we screened two wet land plants *H. auriculata* and *E. fluctuans* for their antioxidant activities. These two plants regularly consumed by the peoples throughout the years.

Both the plants were subjected to the preliminary phytochemical screening to determine the nature of compounds present in their methanolic extract. The result revealed the presence of different compounds such as carbohydrate, saponins, phenols, tannins, flavonoids, proteins, triterpenes and alkaloids. Presence of alkaloids was also reported by previous workers in different species of *Hygrophila*.[28] According to Misra et al.[29] the presence of different bioactive compounds is the indication of its usefulness of these plants.

Phenolic compounds, secondary metabolites, the derivatives of the shikimate, pentose phosphate and phenyl propanoid pathways in plants, are reported to be distributed throughout the plant kingdom.[30] Phenols are one of the most commonly occurring groups of phytochemicals, with significant morphological and physiological important in plants. Phenolic compounds have been reported as major group of compounds that contribute to the antioxidant activity of plant extracts that has been correlated with DPPH scavenging assay. Presence of flavonoids was observed in case of both the plants in nearly equal quantity. Recently different activities of the flavonoids have been demonstrated by several authors. Protective effect of flavonoids to animal liver injury and liver fibrosis has been proved by some authors.[31, 32, 33] Carotenoids are one of the key non-enzymatic anti-oxidants that have a role in defense against water stress by scavenging of singlet oxygen and suppressing lipid peroxidation in all photosynthetic organisms.[34] In both the plant samples presence of carotenoids was observed in our study. The observation is in accordance with the earlier study. Presence of carotenoids in *E. fluctuans* was reported earlier by.[35]

DPPH scavenging activity was also exhibited by the methanolic extracts of the plants. HA showed much more DPPH scavenging activity in comparison to EF, which may be due to the presence of much more phenolic compounds in HA in comparison to EF. The phenolics serve as oxidation terminators by scavenging radicals to form resonance stabilized radicals and are generally correlated with DPPH scavenging assay.[36]

The ABTS scavenging activity is one of the most important parameters for determination of antioxidant activity. The ABTS radical cation decolorization assay can measure the relative antioxidant ability to scavenge the radical ABTS as compared with BHT. The blue and green ABTS radical cation was generated prior to adding antioxidant containing samples prevents interference, which stable absorbance was achieved, by adding the ethanolic extract of Desmodium gangeticum and the scavenging ability measured in terms of discolorization at

734 nm (Mathew et al., 2004).[37] Actually, the ABTS radical cation scavenging activity also reflects hydrogen-donating ability.[38]

Quantification of bioactive compounds revealed that *H. auriculata*, *E. fluctuans* and contain significant amount of phenols, flavonoids, chlorophyll and carotenoids. Phenolic compounds have been reported as major group of compounds that contribute to the antioxidant activity of plant extracts and they serve as oxidation terminators by scavenging radicals to form resonance stabilized radicals.[5] Flavonoids, including flavones, flavanols and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Plant flavonoids have antioxidant activity *in vitro* and also act as antioxidants *in vivo*.[39, 40]

CONCLUSION

From the present study it can be concluded that the presence of various phytochemicals, mainly the flavonoids and phenols may be responsible for the free radical scavenging activity of the two selected plants pointing to their use as potential source of natural antioxidant for the treatment of free-radical and age-related diseases. However, this can only be treated as a pointer since much more studies need to done before establishing them as biological antioxidants.

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ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL SCREENING OF TWO EDIBLE WETLAND PTERIDOPHYTES *DIPLAZIUM ESCULENTUM* (RETZ) SW AND *MARSILEA MINUTA* L.–A COMPARATIVE STUDY

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ABSTRACT

Pteridophytes are recognized for their biological and medicinal properties for long time but very less work has been done on wetland pteridophytes. In our present study we evaluated the antioxidant activity of two wetland pteriodphytes *Diplazium esculentum* (Retz.) Sw. and *Marsilea minuta* L. Presence of bioactive compounds like phenols, flavonoids and saponins was detected in both the plants. However, alkaloids, triterpenes and anthraquinones were exclusively detected in *Diplazium esculentum*. Methanolic extract of both the plants showed potential antioxidant activities. The IC_{50} values of *Diplazium esculentum* for DPPH, ABTS, metal chelating and superoxide ion scavenging activity were found to be 3.8, 4.6, 1.09 and 2.24 mg/ml. Dose dependent inhibition of free radicals was also observed in case of *M. minuta*. The IC_{50} values of *M. minuta* for DPPH, ABTS, metal chelating and superoxide ion scavenging activity were recorded to be 7.7, 6.4, 0.59 and 4.69 mg/ml respectively. The presence of compounds like total phenols, flavonoids, protein, total sugar, reducing sugar, free amino acid and chlorophyll content were also evaluated in these two plants. However, total phenol and flavonoids content was recorded higher in case of *D. esculentum*.

KEYWORDS: phytochemicals, phenol, flavonoid, antioxidants, DPPH, ABTS, FRAP.

INTRODUCTION

All living organisms require oxygen for its existence, which is a highly reactive molecule that damages living organisms by producing reactive oxygen species (ROS).[1,2] Free radicals or ROS, generated from the cellular reduction and oxidation processes, are considered responsible for several diseases such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases. The commonly formed reactive oxygen species are superoxide anions, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite radicals. Free radicals are extremely unstable due presence of unpaired electrons and can donate or receive single electron, which in turn accounts for their high reactivity with other biomolecules. Although, the existence of extremely developed antioxidative defence mechanism in human body balances the production of free radicals, but sometimes as a consequence of deficit in antioxidant levels, free radicals are generated which leads to oxidative stresses.[3-5] In recent years, much attention has been devoted to natural antioxidant and their associations with health benefits.[6] Plants are potential sources of natural antioxidants.[7]

In India, from ancient past we have the rich tradition of using plants with medicinal properties as food as well as in therapeutic diseases. Even in advanced countries use of natural resources, use of herbal medicines is climbing nowadays for their fewer side effects.[8]

Due to wide range of geographic expansion India is rich in biodiversity thereby providing a rich source of plants with different therapeutic activities waiting to be explored.

Wet lands are areas with immense biodiversity and are yet not properly evaluated for therapeutic plant resources. Similarly, pteridophytes growing in wet land attained very fewer attention of scientific community. However, in recent years, many workers have explored the biological and medicinal properties of pteridophytes.[5,9-12]

Diplazium esculentum (Retz.) Sw. is a pantropical species belonging to the family Athyriaceae occurring commonly throughout India, China, Cambodia, Laos, Vietnam and Malaysia with no threats, hence categorized as Least Concern. This plant grows in gregarious colonies in open marshy areas, stream banks and canals from sea level to 2,300 m.[13,14] Young frond and rhizomes are used as green vegetables. The vernacular name of this plant is *dhekia* in Santali *Dhekir Shaak* in Bengali, and the English name is Vegetable fern. Moreover, consuming as vegetable, *D. esculentum* has been reported to have profound use as traditional medicine in diseases like fever, dermatitis, measles dysentery, glandular dwellings, indigestion, diarrhea and various skin infections.[9,15]

Marsilea minuta L. is a wetland Pteridophyte of the Marsileaceae family that grows in marshy places as well as on the margin of ponds ditches, paddy fields etc and is native to tropical Asia and Africa. English name of this plant is water clover and four-leaf clover. In North Bengal it is commonly known as sushni, sunsuni or sunsunia sak. It has been used in the ayurvedic system of medicine for curing several ailments. *M. quadrifolia* showed antibacterial, cytotoxic and antioxidant activities.[16]

D. esculentum (Retz.) Sw. and *M. minuta* L. both have been consumed frequently as leafy vegetables by different communities of North Bengal, which encompasses the Northern region of West Bengal, India. Mostly they grow in wet lands of North Bengal and constitute a remarkable part of vegetables of poor and under privileged people of this region.

Therefore, in the present study we have attempted to evaluate the comparative antioxidant activity of two wet land pteridophytes *Diplazium esculentum* (Retz.) Sw. and *Marsilea minuta* L.

MATERIAL AND METHODS

Collection of plant materials

Fronds of *Diplazium esculentum* (Retz.) Sw. and *Marsilea minuta* L. were collected from the wetlands of Dakshin Dinajpur (Latitude: 26°35'15" N to 25°10'55" N; Longitude: 89°00'30" E - 87 deg 48' 37" E) and Malda (Latitude: 24°40’20" N to 25°32’08" N; Longitude: 87°45’50" E to 88°28’10" E) districts of West Bengal, India. Plants were dried and submitted to Herbarium Facility at North Bengal University, Siliguri, West Bengal with the Accession Nos. 09820 and 09821 respectively.

Phytochemical screening

Both the dried plant samples were screened for presence of compounds like alkaloids, carbohydrates, glycosides, saponins, phenols, tannins, flavonoids, proteins, amino acids and triterpenoids by following standard protocols.

Test for phenol

The powdered plant samples were mixed with 10 ml double distilled water and stirred in magnetic stirrer for 10 min. The mixture was then filtered using Whatman filter paper No.1. To the filtrate (1ml), equal volume (1ml) of 1% $FeCl_3$ was added and observed for the appearance of blue or green color indicating the presence of phenols.[17]

Test for flavonoid

Briefly, 2g of the sample was mixed thoroughly with 10ml of acetone which was evaporated by keeping the flask in a hot water bath for 5 min. Further, the sample was extracted using 10ml of warm double distilled water. The solution was thoroughly mixed, filtered while hot and allowed to cool at room temperature. To the filtrate (5ml), equal volume of 20% NaOH was added and change in appearance/color of the solution to yellow indicated the presence of flavonoid.[18]

Test for tannin

The crude plant powder (200mg) was mixed with 10ml of double distilled water and allowed to stand for 10 min before filtration. Then, 1ml of 5% $FeCl_3$ was added to 2ml of the filtrate. The formation of yellow brown precipitate indicates the presence of tannin.[19]

Test for alkaloid

The powdered sample (200mg) was mixed vigorously with 10ml of methanol for 1h at room temperature. The mixture was then filtered and to 2ml of methanolic filtrate, 2ml of 1% HCl was added. The solution was kept in boiling water bath for 5min. Then, Mayer’s - Wagner’s reagent (6-7 drops) was added to the filtrate. Formation of creamish/brown/red/orange precipitate indicates the presence of alkaloid.[20]

Test for cardiac glycosides

The method described by Trease and Evans (1989) with minor modification was followed for the qualitative screening of cardiac glycosides in the samples.[20] The methanolic filtrate (2ml) was mixed with 1ml glacial acetic acid, to which further 3-4 drops of 5% $FeCl_3$ was added. Then, 1 ml of concentrated H_2SO_4 was added carefully to the solution. Development of brown ring at the interface indicates the presence of cardiac glycosides. A violet colour may also appear below the brown ring.

Test for carbohydrates

The plant samples (500mg) were boiled in 30ml of double distilled water and filtered. Then, 2ml of aqueous extract was mixed with 2ml of Molish’s reagent (5%-α-naphthol in absolute ethanol) and shaken vigorously. To it, 2ml of concentrated H_2SO_4 was added carefully along the wall of the test tube. The presence of carbohydrate was inferred by the formation of reddish-ring at the junction of two liquids.[18]

Test for reducing sugars
The sample (500mg) was boiled in 30ml of double distilled water and filtered to obtain an aqueous extract/filtrate. The aqueous filtrate (1ml) was then mixed with 2ml of Fehling's solution (A: 7%CuSO4 in dH_2O containing 2 drops of H_2SO_4 (dil.), B: 12% KOH and 35% Sodium potassium tartarate in dH_2O. Mix A and B in equal amount) and boiled for 5mins. Formation of a brick red precipitate indicates the presence of reducing sugars.[18]

Test for protein
The method described by Pullaiah was followed for the detection of protein in the plant samples.[21] About 1g of the plant sample was mixed thoroughly with 10ml of double distilled water by a magnetic stirrer for 10h and filtered. 2ml of the filtrate was then mixed with 1ml of 40% NaOH in a test tube. Then, 1-2 drops of $CuSO_4$ was gently added to the solution. Change in the color of solution to violet indicates the presence of peptide linkages in a solution which in turn is an indication of the presence of proteins.

Test for saponin
The aqueous filtrate (0.5ml) prepared as above was mixed with 5 ml of double distilled water and shaken vigorously for about 30 seconds. The presence of saponins was indicated by the formation and persistence of the froth.[20]

Test for terpenoid
The methanolic filtrate (2ml) was mixed with 5ml of chloroform and 2ml of acetic anhydride. Then, to the mixture 1ml of concentrated H_2SO_4 was added carefully along the wall of the test tube. The formation of reddish brown ring at the interface indicates the presence of terpenoid.[22]

Test for steroid
The methanolic filtrate (5ml) was treated with 0.5ml of anhydrous CH_3COOH and cooled on an ice bath for 15mins. Then, 0.5ml of chloroform and 1ml of concentrated H_2SO_4 was added to the cold solution. Presence of the steroid may be inferred by the formation of reddish-brown ring at the junction of two liquids.[23]

Test for anthraquinone
About 0.5g of powdered sample was mixed with 5ml of chloroform, shaken for 5 min and filtered using Whatman No.1 filter paper. The filtrate (3ml) was mixed with 3ml of 10% ammonia solution and shaken properly. Development of pink/red/violet color in the aqueous layer after shaking indicates the presence of free anthraquinone.[18]

Test for amino acid
0.5 ml methanolic plant extracts were treated with few drops of ninhydrin reagent, heated inwater bath, a purple colour indicated the presence of amino acids.[24]

Test for glycosides
0.5 ml methanolic extracts of plant were added with 2ml of 50% hydrochloric acid. The mixtureswere hydrolyzed for 2 hrs on a water bath. After that 1 ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% sodium hydroxide solution were added. Pink to red colour designated the presence of glycosides.[24]

Test for triterpenoids
0.5 ml of methanolic plant extracts were evaporated and dissolved in 1ml chloroform. 1ml aceticanhydride was then added and chilled. After cooling, conc.H_2SO_4 was added. If reddish violate colour appeared, the existence of triterpenoids was confirmed.[24]

Preparation of Extracts
The plants were collected from different wetlands and washed thoroughly initially with tap water then with double distilled water to get rid of dart and dried using blotting paper. The shade dried plant materials were then ground to obtain fine powder that was stored at -20°C for further use. The powdered sample was then extracted using methanol (MeOH) using the method described by Okwori et al. with slight modification.[25] Briefly, 10 g powdered samples were soaked in 100 ml of methanol for 72 hr at room temperature in a orbital shaker and filtered 3-4 times using Whatman No.1 filter paper. The filtrates were finally concentrated using rotary evaporator at 10°C.

DPPH free radical scavenging activity
DPPH (2,2-diphenylpicrylhydrazyl) free radical scavenging activity was done following standard protocol with slight modifications.[26] Different concentrations of plant extracts (0.1 ml) were put in the test tube and then 2.9 ml of a methanolic solution of DPPH (0.1 mM) was added. The mixture was kept in the dark at room temperature for 30 min and absorbance was measured at 517 nm against a blank and scavenging activity was calculated using the following formula.

$$DPPH\ Scavenging\ (\%) = 100 \times (A_o - A_s)/A_o$$

Where, A_o is the absorbance of the blank and A_s the absorbance of the sample.

ABTS scavenging activity
The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay was based on a previously described method with slight modifications.[27] ABTS radical ($ABTS^+$) was produced by the reaction of a 7 mM ABTS solution with potassium persulphate (2.45 mM). The $ABTS^+$ solution was diluted with ethanol to an absorbance of 1.5 ± 0.05 at 734 nm prior to the assay and stored in the dark at room temperature for 12 h. For assay, 100 µl of extract sample or ascorbic acid used as standard to 2 ml of diluted $ABTS^+$ solution, then absorbance was measured at 734 nm after exactly 6 min. The decrease in absorption was used for calculating scavenging effect using the following equation.

$$Scavenging\ effect\ (\%) = 100 \times (A_o - A_s)/A_o$$

Where, A_o is the absorbance of the blank and A_s the absorbance of the sample.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined according to the Griess Illosvoy reaction.[28] The reaction mixture contained 2 ml of sodium nitroprusside (10 mM) in 0.5 ml phosphate buffer (0.5 M; pH 7.4). Various concentrations of the extracts (0.5 ml) were added in a final volume of 3 ml. After incubation for 60 min at 37°C, Griess reagent [N-(1-Naphthyl) ethylenediamine (0.1%) and sulphanilic acid (1%) in H_3PO_4 (5%)] was added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-(1-Naphthyl) ethylenediamine was measured spectrophotometrically at 540 nm. Ascorbic acid was used as a positive control. The scavenging ability (%) of the nitric oxide was calculated using the formula:

$$Scavenging\ effect\ (\%) = 100 \times (A_o - A_s)/A_o$$

Where, A_o is the absorbance of the blank and A_s the absorbance of the sample.

Metal chelating activity

Metal chelating activity of plat extract was measured by adding 0.1 mM $FeSO_4$ (0.2 ml) and 0.25 mM ferrozine (0.4 ml) subsequently into 0.2 ml of plant extract. After incubating at room temperature for 10 min, absorbance of the mixture was recorded at 562 nm. [29] Chelating activity was calculated using the following formula:

$$\text{Metal chelating activity} = 100 \times (A_o - A_s)/A_o$$

Where, A_o is the absorbance of the control and A_s the absorbance of the sample.

Superoxide scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of NADH and phenazine methosulfate under aerobic condition.[30] The 3 ml reaction mixture contained 50 ml of 1 M NBT, 150 ml of l M nicotinamide adenine dinucleotide with or without sample and Tris buffer (0.02 M, pH 8.0). The reaction was started by adding 15 ml of 1 M phenazine methosulfate to the mixture and the absorbance change was recorded at 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract.

FRAP assay

The FRAP (Ferric Reducing Antioxidant Power) of the methanolic extracts were determined by using a previously described method with slight modifications.[31] The freshly prepared FRAP reagent contained 500 ml of acetate buffer (0.3M; pH 3.6), 50 ml of 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) (0.01M), and 50 ml of $FeCl_3 \cdot 6H_2O$ (0.05M). 100 µl of extracts were added to 2 ml of FRAP reagent and the absorbance was measured at 593 nm after 12 min. The results were calculated from the standard curve of $FeSO_4$ and expressed as mM Fe^{2+} equivalent.

Total phenols

The total phenol content was determined by slightly modified Folin-Ciocalteu method.[32,33] The reaction mixture contained 200 µl of extract, 800 µl of freshly prepared diluted Folin Ciocalteu (1:1) reagent and 2 ml of sodium carbonate (7.5%), kept in the dark at ambient conditions for 2 h to complete the reaction. The absorbance was measured at 765 nm. Gallic acid was used as standard and the results were expressed as mg gallic acid/g dried extract.

Total flavonoids

Total flavonoid content was determined according to the standard method using quercetin as a standard.[34]A volume of 500 µlof extract was added to 500 µl of $NaNO_2$ (5%). After 5 min at 25°C, 500 µl of $AlCl_3$ (10%) was added. After a further 5 min, the reaction mixture was mixed with 1 ml of 1 mM NaOH. Finally, the absorbance was measured at 510 nm. Quercetin was used as standard and the results were expressed as mg quercetin (QE)/g of dried extract.

Chlorophylls

From the samples Chlorophyll was extracted following the method of Harborne with minor changes.[35] 1g of the dried powder was crushed with 80% acetone in a mortar pestle and filtered through Whatman No.1 filter paper in a dark room. The residue was re-extracted with 80% acetone until it became completely colorless attaining final volume upto 10 ml. OD was measured at 663nm and 645nm in a UV-VIS spectrophotometer against a blank of 80% acetone. Using the following formula the chlorophyll content was calculated.

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} dry weight.

Chlorophyll a = $(12.7\ A_{663} - A_{645})$ mg g^{-1} dry weight

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} dry weight.

Carotenoid content

Carotenoid content was estimated by the procedure of Lichtenthaler.[36] The desired amount of crude or diluted filtrate was taken directly into the cuvate and the absorbance was taken at 480nm, 645nm and 663nm wavelength against a blank in UV-VIS spectrophotometer 118 systronics. The amount of carotenoid was calculated using the standard formula as follows:

$A_{480} - (0.114 \times A_{663}) - 0.638\ (A_{645})$ µg g^{-1} dry weight

Protein content

1 g of fresh plant tissue was homogenized in a prechilled mortar and pestle using 5 ml of 50 mM sodium phosphate buffer (pH 7.2) and PVP (polyvinylpyrrolidone) under ice cold condition and centrifuged at 10,000 rpm at -4ºC for 15 mins.[37] The supernatant obtained was used as the crude extract for estimation. For estimation, 1ml of extract and 5ml alkaline reagent was mixed thoroughly and allowed to

stand for 15mins.[38] Then, FolinCiocalteau's phenol reagent was added and incubated for 20 minutes and the absorbance was read at 690 nm.

Free amino acid

Free amino acids were estimated with standardized protocol with slight modifications.[39] 0.5 g leaf tissue was extracted in ethanol and filtered using Whatman No. 1 filter paper. To 1 ml of the filtrate, 1 ml of ninhydrin reagent was added and boiled in a water bath for 20 min. The absorbance was read at 570 nm and the free amino acid content was estimated using a standard curve of L-proline.

Total sugars and reducing sugars

Total and reducing sugar was extracted following the method of Harborne.[22] 1 g fresh leaf tissue was extracted in 10 ml of 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath. Then the residue was dissolved in dH_2O and the final volume was made up to 5 ml which was then centrifuged at 5000 rpm for 10 min.

Estimation of total sugar was done by Anthrone reagent following the method of Plummer.[40] To 1 ml of test solution, 4 ml of Anthrone reagent (0.2% Anthrone in conc. H_2SO_4) was added. The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10 mins. Then the reaction mixture was cooled under running tap water and absorbance was measured at 620 nm and sugar content was quantified using a standard curve of D-glucose.

The amount of reducing sugar present in the plant samples was determined by Nelson-Somogyi method as described by Plummer.[40] In brief, 1 ml of the test solution was mixed with 1 ml of alkaline copper tartarate solution and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water and 1 ml Nelson's Arsenomolybdate reagent was added along with 2 ml of dH_2O and mixed vigorously. A blue colour was developed, the absorbance of which was the measured in a colorimeter at 515 nm and reducing sugar content was quantified using a standard curve of D-glucose.

RESULTS

Phytochemical screening

The dried plant samples were screened for the presence of different phytochemicals. The result revealed that both plants contain carbohydrate, saponins, phenols, tannins, flavonoids, proteins, triterpenes and alkaloids. Phytosteroid, fat and fixed oil, glycosides and anthroquinone were found to be absent in both the plant samples (Table 1).

Nutritional factors

The amount of different nutritional factors such as protein, carbohydrate and free amino acid in both the dried plant samples was quantified. Results revealed that the amount of total sugar is much higher in *D. esculentum* in comparison to *M. minuta* but in contrary the reducing sugar content is much higher in *M. minuta*. The protein content of both plant samples were quite similar but free amino acid content was found to be higher (0.42 mg/gm tissue) in MM (Table 2).

Table 1: Phytochemical compounds detected in the two plant extracts.

Phytochemicals	*D. esculentum*	*M. minuta*
Alakaloid	+	_
Carbohydrate	+	+
Glycosides	_	_
Saponins	+	+
Phytosteroid	_	_
Phenols	+	+
Tannins	_	+
Flavonoids	+	+
Protein	+	+
Amino acid	_	+
Triterpens	+	_
Anthroquinones	+	_
Cardiac glycosides	_	_

Table 2: Nutritional analyses in terms of total proteins, free amino acid, total sugars and reducing sugars in the extracts.

Sample	Protein (mg/g dwt)	Free amino acids (mg/g dwt)	Total Sugars (mg/g dwt)	Reducing sugars (mg/g dwt)
DE	26.50±01.41	0.15±0.010	35.00±0.32	0.130±0.01
MM	25.50±02.04	0.42±0.210	11.00±0.13	0.435±0.03

DE =*Diplazium esculentum*; MM=*Marsilea minuta*; dwt= Dry Wt.

***In vitro* Antioxidant activity**

DPPH scavenging activity

DPPH is a stable free radical having characteristic purple colour having absorption maxima at 517 nm. It loses its colour when react with antioxidant. In case of both the plants dose-dependent scavenging activity was observed. Methanolic extract of *D. esculentum* showed much more scavenging activity than *M. minuta*. The IC_{50} value for DE was found to be 3.8 mg/ml and 7.7 mg/ml for MM (Fig. 1).

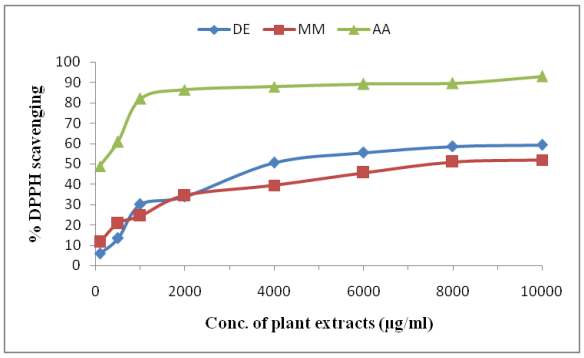

Figure 1: DPPH scavenging activity of different concentrations of plant extracts (µg/ml); DE-*Diplaziumesculentum*, MM-*Marsileaminuta*, AA-Ascorbic acid (standard).

ABTS scavenging activity

Proton radical scavenging is an important feature of antioxidants. ABTS is a protonated radical that has characteristic absorption maxima at 734 nm, which decreases in presence of antioxidants. In both the plant samples, scavenging effect of ABTS radical increased with concentration. The scavenging activity of DE was relatively higher than MM. The IC_{50} values were recorded 4.6 mg/ml and 6.4 mg/ml for DE and MM respectively (Fig. 2).

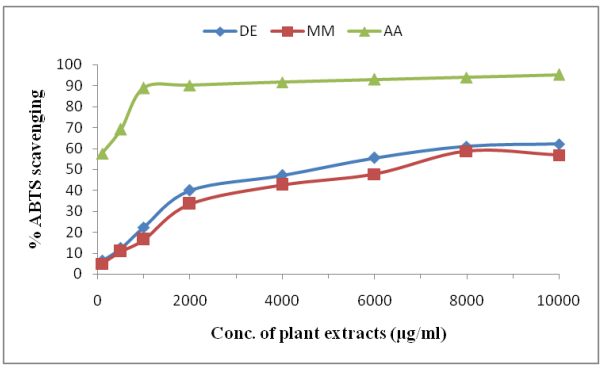

Figure 2: ABTS radical scavenging activity of different concentrations of plant extracts (µg/ml), DE-*Diplazium esculentum*, MM-*Marsilea minuta*, AA-Ascorbic acid (standard).

Nitric oxide scavenging activity

Nitric oxide scavenging activity of DE and MM was determined with varied concentration ranging from100 to 10000 µg/ml. At low concentration the scavenging activity of DE was found to be greater than MM, however with increase in concentration both the plant samples showed almost similar type of scavenging efficiency (Fig. 3).

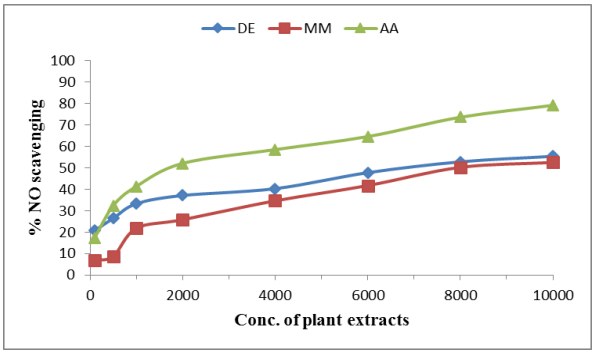

Figure 3: NO scavenging activity of different concentrations of plant extracts (µg/ml).

Metal chelating activity

The methanolic extract of both the samples evaluated for their chelating capacity. With increasing concentration of plant extract decrease in absorbance of Fe^{+2}-ferrozine complex was observed in both the cases. Efficacy of metal chelating was found to be greater in MM in comparison to DE. The IC_{50} values for metal chelating activity were recorded 0.59 and 1.09 mg/ml for MM and DE respectively (Fig.4).

Superoxide scavenging activity

Dose dependent superoxide radical scavenging activity was demonstrated by methanol extract of DE and MM. Though at higher concentration both the samples showed almost similar degree of scavenging, but IC_{50} value for superoxide anion scavenging activity were recorded 2.24 and 4.69 mg/ml for DE and MM (Fig. 5).

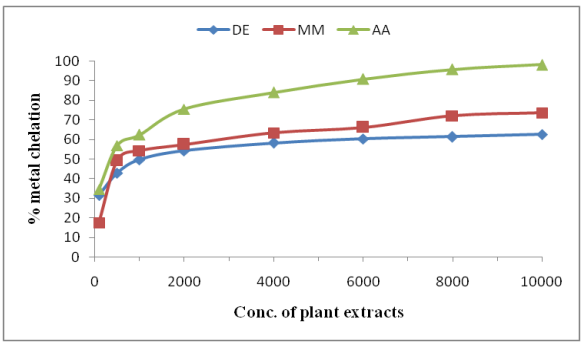

Figure 4: Metal chelating activity of plant extracts at different concentrations (µg/ml).

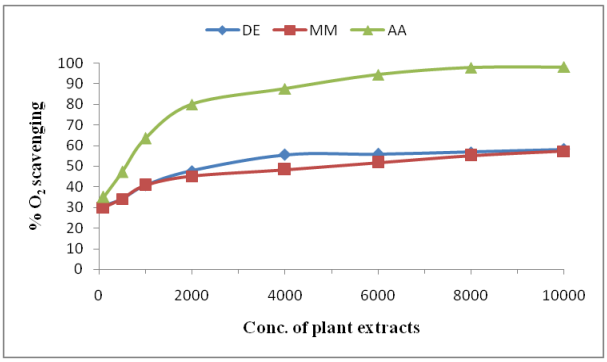

Figure 5: Superoxide anion scavenging activity of different concentrations of plant extracts (µg/ml).

Hydroxyl ion scavenging activity

The potential of methanolic extracts of DE and MM to inhibit hydroxyl-radical-mediated deoxyribose damage was assessed at a concentration of 100 to10000 µg/ml. A concentration dependent inhibition of hydroxyl ion was observed in both plant extracts (Fig. 6).

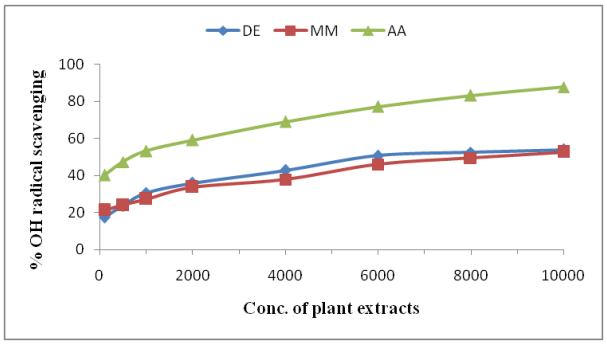

Figure 6: Hydroxyl ion scavenging activity of plant extracts at different concentrations (µg/ml).

FRAP Assay

Ferric Reducing Antioxidant Power capacity of the plant extracts was also determined. Results revealed that FRAP of DE is higher than that of MM. Total phenol and flavonoids content also showed similar type of observation. The phenolic and flavonoids content of DE was found to be higher in comparison to MM. However, chlorophyll, carotenoids and ascorbic acid content was recorded higher in *M. minuta* methanol extract (Table 3).

Table 3: *In vitro* antioxidant potential of the extracts quantified in terms of FRAP, total phenols, total flavanoids, ascorbic acid, carotenoids and total chlorophyll.

Sample	FRAP assay (mM Fe2+ eq.)	Total Phenols (mg/g dwt)	Total Flavonoids (mg/g dwt)	Ascorbic acid (mg/g dwt)	Carotenoids (mg/g dwt)	Total chlorophyll (mg/g dwt)
DE	0.095±0.002	0.054±0.04	0.238±0.021	0.459±0.11	0.279±0.005	0.648±0.004
MM	0.121±0.005	0.018±0.01	0.127±0.090	0.478±0.09	0.517± 0.003	1.424±0.100

DE =*Diplazium esculentum;* MM=*Marsilea minuta*; dwt= Dry Wt.

DISCUSSION

Pteridophytes are known to have different bioactive compounds which have therapeutic activity. In present study we have evaluated the antioxidant activity of two wet land pteridophytes *D. esculentum* and *M. minuta*, which are generally consumed by poor and underprivileged peoples. The phytochemical screening revealed that both the plant samples contain carbohydrate, saponins, phenols, tannins, flavonoids, proteins, triterpenes. These compounds are known to contribute for antioxidant activities of plants.[41] Presence of hydroxyl functional group in flavonoids empower them to act as antioxidant.[42]

DPPH scavenging activity was exhibited by methanolic extract of both plant samples. DPPH scavenging activity of DE was much greater than that of MM. This may be due to the presence of much more phenolic compounds in DE in comparison to MM. Similar type of observation was also recorded previously.[43] The ABTS radical cation decolorization assay can measure the relative antioxidant ability to scavenge the radical ABTS.[44] The ABTS scavenging activity is one of the most important parameters for determination of antioxidant activity. Methanolic extract of both plant samples showed considerable amount of scavenging activity. Nitric oxide is a free radical produced in mammalian cells involved in regulation of various physiological processes. However, excess production of nitric oxide is associated with several inflammatory diseases.[45,46] So, development of antioxidant mechanism to combat nitric oxide has become a recent area of research. In present study, the methanolic extract of *Diplazium esculentum* and *Marsilea minuta* was evaluated for its inhibitory effect on nitric oxide production. It was found that both the isolates could inhibit the production of nitric acid from sodium nitroprusside at physiological pH.

In present study, methanolic extracts of *Diplazium esculentum* and *Marsilea minuta* inhibited the formation of chelating agent complex, which reduces the red color of the complex. This measurement of color reduction therefore allows estimation of the chelating ability of the coexisting chelator.[44] Superoxides are produced from molecular oxygen by both enzymatic and noenzymatic pathway.[47] These superoxide anions are responsible for the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, protein and DNA.[48,49] A dose dependent superoxide radical scavenging activity was demonstrated by methanol extract of both the plants.

Hydroxyl ion scavenging activity was also demonstrated by methanolic extract *Diplazium esculentum* and *Marsilea minuta*. Hydroxyl ion, which can react with biomolecules in living cells cause severe damage, scavenging activity is considered as prime criterion to be an antioxidant.[50] In presence of antioxidant, ferric-ferric cyanide complex is reduced to the ferrous form, thus providing the basis of FRAP assay. Higher absorbance

indicates a higher ferric reducing power.[51,52] In our study we showed that in presence of both the plant extracts ferric-ferric cyanide complex was reduced to the ferrous form.

Quantification of bioactive compounds revealed that methanolic extract of *Diplazium esculentum* and *Marsilea minuta* contain significant amount of phenols, flavonoids, chlorophyll and carotenoids. Phenolics are important plant secondary metabolites and are known to be distributed throughout plant kingdom. Phenols are one of the most commonly occurring groups of phytochemicals, with significant morphological and physiological importance in plants. Phenolic compounds have been reported as major group of compounds that contribute to the antioxidant activity of plant extracts and has been correlated with DPPH scavenging assay.[53] Along with chlorophyll presence of carotenoids was observed in both the plant samples in present study. Carotenoids are one of the key non-enzymatic anti-oxidants that have capacity to suppress lipid peroxidation in all photosynthetic organisms.[54]

CONCLUSION

In present study we have evaluated the antioxidant activity of two wet land pteridophytes *Diplazium esculentum* and *Marsilea minuta*. Both the plants are used as vegetables by local peoples. In our study we found that both the plants have potential antioxidant activity as well as nutritional values. However, the overall result indicated that, *Diplazium esculentum* had more nutritional and antioxidative properties in comparison to *Marsilea minuta*. Finally, it could be concluded that the consumption of these underutilized vegetables should be popularised as they could be natural sources of antioxidative phytochemicals and nutrients.

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