Studies on *Grewia nervosa* for Bioprospecting

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Biotechnology

By

Surya Nandan Meena

Goa University,

Taleigao Goa

January, 2017

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CERTIFICATE

This is to certify that the thesis entitled " ***Studies on Grewia nervosa for Bioprospecting****" submitted by Mr.* ***Surya Nandan Meena*** *for the Award of the Degree of Doctor of Philosophy in Biotechnology is based on original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any university or institution.*

Place: Goa University

January, 2017

Dr. Sanjeev Ghadi

(Research Guide)

Professor, Department of Biotechnology

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STATEMENT

I, hereby, state that the present thesis entitled "***STUDIES ON Grewia nervosa FOR BIOPROSPECTING****"* is my original contribution and that the same has not been submitted on any previous occasion for any degree. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Place: Goa, India

January, 2017

Surya Nandan Meena

Dedicated to

Lord Bajrang Bali,
Soul of my wife
(late.Poonam)
&
My family

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I need a garden of flowers to present a flower each to all those who have rendered invaluable help in my research work and in the presentation of the results in this book.

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Surya Nandan Meena

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CHAPTER 1: INTRODUCTION

Since thousands years, medicinal plants have been use worldwide for health care. Furthermore, 75% of the world population depend directly or indirectly on medicinal plants and their products. The secondary metabolites present in medicinal plants is correlated with therapeutic properties. On the other hand, therapeutic potential of some plants predominantly depends on composition of nutritional and cosmetic metabolites.

Indian traditional system of medicine comprises of Ayurveda, Yoga, Unani, siddha and homeopathy (AYUSH) and are widely used for treatment of various diseases. In India, Ayurveda developed between 2500 and 500 BC and currently is the most accepted system of medicine (Subhose et al. 2004). Drugs of plant origin have always been assumed of a first line of defense to cure most human diseases (John, 1984; Veale et al. 1992). Ayurvedic system is not only practiced in India and its subcontinents but it is well flourished in many European countries and United States. The Ayurveda treatment based on medicinal plants includes crude extracts from various parts of plant *viz*. flower, fruits, leaves, bark, and roots. Most of the ayurvedic companies depend on the plant derived products for manufacturing ayurvedic drugs. Based on traditional use of medicinal plants, 121 Ayurvedic drugs have been discovered in last century (Anesini and Perez, 1993). Currently, Ayurvedic system of treatment has been socially and scientifically strengthened in India. Crude herbal preparations are now being scientifically evaluated and properly disseminated. Medicinal chemistry techniques have also helped in intensifying the ayurvedic system of medicine. Scientists can now predict the compound/s present in the medicinal plants and can access the same compound from many other related plants of same or different family or alternatively chemically synthesize the lead compound in the laboratory.

India is a rich repository of herbal plants and is categorized among the twelve mega herbal diversities of the world. In India, around 20,000 medicinal plants have been identified, however only 7000-7500 plants are being use by ayurvedic practitioners. The Konkan region of India is endowed with rich herbal biodiversity. The Konkan region is marked by Sahyadri mountain range (Western Ghats) on the eastern side, Arabian sea from the western side, Gangavali river of Karnataka and region of Mayura River constituting the northern boundary. Medicinal plants from konkan region commonly exploited in traditional system of health care are *Aloe barbadense, Ceropegia hirsute, Cicer arietinum*, and *Anisochilus carnosus* for digestive or gastro-intestine problems. *Zingiber officinale* for acidity and ulcer problems and *Citrus aurantifolium* for dysentery problems (Kamble et al. 2008). *Mangifera indica* L. and *Spondias mangifera* L. for Diarrhea; *Annona reticulata* L., *Anacardium occidentale* L., *Acorus calamus* L., *Andrographis paniculata* (Burm.f.) wall., *Alstonia scholaris, Rauvolfi serpentine, Amorphophallus paeoniifolius* (Denns) Nicolson, *Artemisia parviflra* L., *Chrysanthemum indicum* L., *Benincasa hispida* Cogn, *Cucurbita maxima* L and *Tamarindus indica* L. for curing stomach ache; *Calotropis gigantean, Agave americana* L., *Carissa spinarum* L, *Colocasia esculenta* (L.) Schott, *Coccinia grandis, Cucumis sativus* L, *Terminalia chebula* Retz, *Terminalia arjuna* (Roxb.) Wight & Arn., *Terminalia tomentosa* Willd and *Terminalia paniculata* Roth. for wound healing; *Polyalthia longifolia* Sonn., *Buchanania lanzan* Spreng is being used for fever; *Aerva lanata* (L.) Juss. used for kidney stone problems; *Achyranthes aspera* L., *Justicia adhatoda* L., *Asparagus racemosus* Willd., *Hemidesmus indicus (*L.), *Chromolaena odorata* L. *Ananas comosus* (L.) Merr and *Kalanchoe pinnata* (Lam.) Pers. is used for the treatment of cold. Many other important medicinal plants found in the konkan region that have great medicinal potential are

Achyranthes aspera L., *Andrographis echioides* L., *Anisochilus carnosus* L., *Aloe barbadensis* Mill., *Bauhinia variegata* L., *Terminalia chebula*, *Cymbopogon martini* (Roxb.), *Chlorophytum tuberosum*, *Gymnema sylvestre*, *Chlorophytum borivilianum* L., *Cissus quadrangularis* L., and *Ocimum tenuiflorum* L.

Bioprospecting of medicinal plants is being continuously explored and with the advent of new techniques, the potential of medicinal plants for search of novel compound is becoming a reality. Approximately 25% of the commercial drugs marketed worldwide are of plant origin. The important plant originated drugs such as digoxin from *Digitalis* sp., vincristrine and vinblastine from *Catharanthus roseus*, quinine and quinidine from *Cinchona* sp., morphine and codeine from *Papaver somniferum* and atropine from *Atropa belladona* (Yue-Zhong, 1998). Additionally, other plant originated compounds such as muscarine, cannabinoids, physostigmine, yohimbine, colchicine, phorbol esters and forskolin have also been demonstrated with pharmaceutical, physiological and biochemical properties (Williamson et al. 1996).

The pharmaceutically important compounds are synthesized in the plant via a long complex biochemical pathway and is difficult to be reproduced in laboratory conditions. For example vinblastine and taxol are complex plant metabolites potentially used as anticancer drugs and cannot be synthesized *in vitro.* Thus, fulfilling the requirement of such compounds worldwide is achieved by use of state of art facility to increase production of secondary metabolites.

Medicinal plants have been and will remain important sources of pharmaceutical compounds. Phytotherapy involving use of plants or plant extracts for medicinal purpose and

is not part of human diet. The principle of phytotherapy correlates the secondary metabolites to therapeutic properties in the plants. Plant secondary metabolites are organic in nature and do not participate in plants growth and development. The major plant secondary metabolites are terpenoids, non-protein amino acids, amines, glycosides, flavonoids, phenolics, tannins, alkaloids, saponins etc. The type and production of these metabolites vary from plant to plant and are mainly dependent on genus, family, species, type of explant, tissue specific and time specific factors.

Drugs from medicinal plants have high structural diversity, high specificity to their target and other properties that make them ideal or better candidate for drug as compared to the lead compounds that are synthetic in origin (Frank & Carter, 2005). Although India is rich repository of medicinal plants, several problems create hurdles in the promotion of the medicinal plants worldwide. Out of all identified medicinal plants, very few plants are exploited for scientific research and explored for commercial potential. Similarly, lack of molecular markers to identify medicinal plants lead to wrong identification of plants. Additionally, medicinal plant have several synonyms, while some of them having same synonyms in different parts of India. The quality of herbal preparations differ from batch to batch as there is no standard protocol for making herbal preparations to treat particular disease. Further, the quality of herbal medicines depend on soil texture, nutritional composition, climate conditions and periodic variations. These factors affect both quantity and quality of active compounds of pharmacological importance in the plants. Lack of proper conservation and preservation of medicinal plants that are continuously being exported as a raw material has resulted in many of these plant species being listed as endangered species. Further several plants that are traditionally known for their medicinal potential are still

awaiting scientific evaluation and validation. Due to lack of specific markers for identification of medicinal plants, adulteration of raw herbal drugs with other related plants is rampant. Indian herbal pharmacopoeia includes only 32 herbal plants whereas, current manufacturers employs about 500 plants. Thus, faulty identification of herbal plants, extinctions of important medicinal plants due to overexploitation, adulteration and lack of scientific justification for medicinal potential of many traditional medicinal plants are some major hurdles associated with the Indian herbal repository. Thus, validation of the therapeutic potential of Indian medicinal plants, their sustainable use and promotion is an urgent requirement for bioprospecting of unexplored native plants.

Plant tissue culture involving callus culture, single cell suspension culture and subsequent organogenesis enables the preservation of rare and endangered species of plants. Further, tissue culture techniques can be employed for the production of pharmacological important secondary metabolites. Taxol a diterpenes alkaloid used as an anticancer drug worldwide is obtained from the bark of Taxus tree traditionally. The scarcity of taxol has been overcome by production of this alkaloid from a different species of Taxus through tissue culture techniques (Jennewein et al. 2005). Likewise tissue culture techniques for the production of analgesic drugs such as Morphine and Codeine have been achieved by callus and cell suspension culture of *Papver sominiferum* (Yu et al. 2002). Barberine, a well-known alkaloid used as an antibacterial is being produced from the cell culture of *Coptis japonica*, *Thalictrum* sp. and *Berberis* sp. (Dubey et al. 2004). Tal et al. (1983), reported *in vitro* production of diosgenin using single cell suspension culture of *Dioscorea deltoidea.* Vincristine and Vinblastine, the well known anticancer drugs used in chemotherapy were originally obtained from *Catharanthus roseus* at a very low yield i.e. 0.0005%.

Subsequently, cell culture was employed to produce more quantity of these compounds from the *C. roseus* (Taha et al. 2009). Further, clone of *Artemisia annua* producing high yield of antimalarial drug i.e. Artemisinin has also been obtained (Baldi & Dixit, 2008).

With the increasing demand of herbal drugs and remarkable developments in the purification technology, spectroscopic techniques and rapid *in vitro* assays, scientific community have regained their interest to screen potential pharmaceutical compounds from medicinal plants. I*n vitro* assays are employed for the high throughput screening of medicinal plants for their therapeutic potential. IC_{5o} value of the crude extract determined by various *in vitro* assays help in evaluation of crude extract as a possible drug lead, especially if the IC_{50} value of crude extract is below 100µg/ml (Cos et al. 2006). Although most of the *in vitro* assays are microplate based, *in vitro* enzyme inhibition assays based on colorimetric or spectrometry are also employed to assess the medical medicinal plants (Ellman et al. 1961; Pohanka et al. 2011; Beretta and Facino, 2010). After evaluation of important pharmaceutical property of crude extract of medicinal plant using *in vitro* assays, isolation, separation and identification of the lead compound present in the herbal mixture is required. Bioassay guided purification and identification of lead compound has played a crucial role in the process of drug discovery. This process comprises the repetitive preparation of various fractionations in different solvents and assessment of their pharmaceutical potential. By use of various techniques such as GC-MS, LC-MS/MS, LC-FTIR, H-NMR, C-NMR-MS and CE-MS, it is now possible to isolate and detect compound of our interest from the crude herbal preparations.

The main focus of the present study is bioprospecting of a selected medicinal plant from the Konkan region of Western Ghats, India. The selection of the medicinal plant for the present study was solely based on the criteria that plant is known for its traditional medicinal properties but awaiting scientific validation. Based on the literature search on medicinal plants belong to Konkan region around Goa, it was observed that that tissue culture, pharmacognosy and phytochemical work on *G. nervosa* L. was lacking. Hence, the *G. nervosa* L. was selected for the present investigation with the following objectives:

1.1. The objectives of the Ph.D. research work

1 Tissue culture of *G. nervosa.*

2 Assessment of the medicinal properties associated with the *G. nervosa.*

3 Partial purification & identification of bioactive compound for a selected pharmacological activity.

CHAPTER 2

REVIEW OF LITERATURE

This chapter comprises a detailed review of traditional as well scientifically reported medicinal properties for *G. nervosa.* Information on molecular identification and tissue culture of *G. nervosa* reported in literature will also be presented. Further, various assay systems to detect antioxidantive, antiinflammatory, antilithiatic, antityrosinase, anticancer, antidiabetic properties as well as methods generally adopted to determine the cytotoxicity, mutagenicity of plant crude extracts would also be discussed. The chapter also describes the various isolation, purification and identification techniques adopted for characterization of pharmacological important compound/s from various medicinal plants.

2.1 *Grewia nervosa* L. Panigrahi

Grewia nervosa L. is a shrub with palmately nerved leaves, whitish flower, buds suburbanite edible fruits and belongs to the family Malvaceae. *G. nervosa* (Lour.) Panigrahi (English Name: Microcos). The plant also known by other synonyms: *Microcos nervosa* (Lour.), *Microcos paniculata* L., *Microcos glabra* Jack, *Microcos mala* Buch-Ham., *Arsis rugosa* Lour., *Fallopia nervosa* Lour., *Grewia affinis* L., *Grewia microcos* L., *Grewia muenterii* Walp. and *Grewia ulmifolia* Roxb. Although, *G. nervosa* is commonly known by several synonyms, due to different languages in India, the plant has several local names. The plant is called as Pisoli and Thengprenkeorong in Assamese; Asar, Garo, Borsubret, Bolchibins in Bengali; Shiral in Hindi; Abhranga, Khasi, Dieng sohdkhar, Dieng Sohlienghadem in Kannada; Kottakka in Marathi; Hasoli and shirala in Malayalam; Tatam and Visalam in Tamil and Asali and Chivra in Konkani. Although, the recent name of the plant is *G. nervosa,* researchers still prefer using the older synonyms i.e. *Microcos paniculata*. *G. nervosa* L. is a member of genus that includes 60 species distributed more or less throughout mainland India, Andaman and Nicobar islands, Sri Lanka, China, Cambodia,

Myanmar, Thailand, Vietnam, Indonesia and Malaysia. (Ghani et al. 1998; Kirtikar et al. 1987).

2.2 Traditional use of *Grewia nervosa* as therapeutic agent

Traditionally, *G. nervosa* is known for curing several ailments. Water extract from boiled leaves is used to treat jaundice and kidney stone. Kalita & Deb, (2004) reported that leaves boiled along with turmeric and snail shell is being used for the treatment of jaundice, cold, heat stroke and dyspepsia. Additionaly, aqueous extract of bark of *G. nervosa* is used to treat Hepatitis B infection (Biswas et al. 2010). Leaves, bark, root and fruit of *G. nervosa* have been reportedly used to treat fever, dirrhoea as well as sprayed as insecticidal agent (Luo, 2009; Bandara et al. 2000; Feng et al. 2008). Leaves paste of the *G. nervosa* is also reported to cure digestion problems (Rahman et al. 2012). Since, ancient times the leaves of *G. nervosa* have been added in the Chinese herbal tea. Additionally, warm paste of fresh leaves of *G. nervosa* is also known to be applied on fractured body parts (Debnath et al. 2014).

2.3 Scientific studies on *Greiwa nervosa*

Although the plant is traditionally claimed to have several medicinal properties, they have not been scientifically validated. However, since the last decade *G. nervosa* is attracting the attention of researchers. Bandara et al. (2000), purified a novel alkaloid from the stem bark of *M. paniculata* i.e. n-methyl- 6 beta-(deca-1', 3', 5'-trienyl) -3 beta-methoxy-2 beta-methylpiperidine, that reportedly demonstrated insecticidal activity against *Aedes aegypti* second instar larvae, a carrier of Dengue virus. Feng et al. (2008), isolated two novel piperidine alkaloids, microcosamines A (1) and B (2) from the leaves of *M. paniculata,* that depicted larvicidal activity against *Culex quinquefasciatus*.

The antioxidantive potential of ethyl acetate extract (EAE), butanol extract (BE), aqeuous extract (AE) and petroleum ether extract (PEE) of *M. paniculata* has been evaluated using three different *in vitro* model assay systems viz. 1,1-diphenyl-2- picrylhydrazyl (DPPH) method, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) method and Co (II) EDTA-induced luminol chemi-luminescence by flow injection method. Unlike other extracts, only EAE depicted higher antioxidant potential. Further, five compounds viz. methyl 3β-O-p-hydroxy-E-cinnamoyloxy-2α, 23- dihydroxyolean-12-en-28-oate, epicatechin, 3-trans-feruloyl maslinic acid, maslinic acid and sucrose were isolated from EAE of *M. paniculata*. Amongst the five, epicatechin depicted antioxidant activity and was further identified as a natural antioxidant compound present in the EAE of *M. paniculata* (Fan et al. 2010).

Rahman et al. (2011), revealed the cytotoxic and analgesic activity of ethanol leaves extract of *M. paniculata* on mice. In comparison to Diclofenac sodium, a standard analgesic drug, ethanol leaves extract depicted significant analgesic effect on mice at 250 and 500 mg/kg oral doses. However, ethanol leaves extract depicted significant toxic effect on brine shrimp with LC_{50} as 60 µg/ml.

Aziz et al. (2014) extracted fruit of *M. paniculata* using various organic solvents viz. Methanol fruit extract (MFE), Chloroform fruit extract (CFE) and aqueous fruit extract (AFE). Antibacterial activity by agar disc diffusion test, toxicity study by brine shrimp lethality assay and larvicidal activity on mosquito larvae were studied using standard protocols. MFE depicted significant broad range of antibacterial activity compared to CFE & AFE. Moreover, MFE exhibited cytotoxic effect on brine shrimps (LC_{50} of 52.7 µg/ml). Out

of the three extracts exposed to *Culex quinquefasciatus* (4th instar larvae), CFE and MFE demonstrated significant larvicidal activity with LC_{50} of 441.7 and 342.1 µg/ml respectively. Thus the organic fraction of *M. paniculata* extracts depicted insecticidal activity as well as toxicity effect.

Aziz et al. (2015), explored anti-helminthic, anti-inflammatory and antidiabetic activity of aqueous extract from the fruits of *M. paniculata*. Aqueous fruit extract (AFE) of *M. paniculata* depicted significant anti-helminthic activity at 50 mg/mL leading to killing of *Pheretima posthuma* at 55.25, 34.24 min respectively. AFE demonstrated significant anti-inflammatory activity at 41.05%, whereas, proteinase was inhibited at 250 µg/mL with IC_{50} value of 285.47µg/mL.

Aziz et al. (2015), analyzed the major phytochemicals of *M. paniculata* and depicted the presence of alkaloids, saponins, tannins, triterpenoids, flavonoids and carbohydrates. Although all the extracts showed significant proteinase inhibitory activity, highest inhibition was exhibited by the methanol bark extract i.e. 75.94% with a IC_{50} of 61.31 µg/mL. Methanol and aqueous extracts from fruits at the doses of 200 and 400 mg/kg body weight showed significant inhibition of ear edema and granuloma formation. These extracts also significantly reduced the paw licking and abdominal writhing of mice. In addition, BME (400 mg/kg), FME (200 and 400 mg/kg) depicted significant analgesic activities at 60 min in the tail immersion test. Additionally, significant post-treatment antipyretic activities were observed with BME at 200 and 400 mg/kg as well as with FME at 400 mg/kg.

2.4 Pharmaceutical scope of *G. nervosa*

Most of the researchers have primarily focused on the fruits of the *G. nervosa* for exploring medicinal properties. Although, leaves and stem bark have also been used, roots have not been explored for pharmaceutical potential. Further, most of the studies revealed the antioxidant activities of *G. nervosa* with few reports on antiinflammatory and antidiabetic activity from the fruits.

2.5 Molecular identification of the *G. nervosa* species

The information provided by herbal specialists about medicinal plants, herbal products, medicinal uses, origin and families may be incorrect or insufficient (Bellakhdar, 1997). Difficulty in identification arises when the same medicinal plant has different synonyms, and in some case the same name is used for multiple plant species (Bellakhdar et al. 1991). Increased demand for medicinal plant products in the market has led to over harvesting of even non-endangered species whereas faulty identification has resulted in incorporation of toxic or unsuitable species in the market, causing health risk to consumers (Barthelson et al. 2006; Ize-Ludlow et al. 2004).

Generally, identification of medicinal plants is mainly based on morphological, phytochemical, and anatomical characters. Identification of medicinal plants based on chemical variability impede its true identity as chemical composition of a plant species varies and is dependent on growth, storage condition and harvesting processes. Identification based on microscopic analysis is laborious, inefficient and time consuming.

Biotechnology and plant genetics have provided various techniques for identification of medicinal plants up to species level (Kumar et al. 2009). Identification using DNA

barcoding ensures accurate, rapid automated species identification. Species identification using DNA sequences are routinely used e.g. Fungi (Gardes and Bruns, 1993), animals (Arnason et al. 1991; Tang et al. 1995; Caldeira et al. 1998; Milinkovitch et al. 2004), plants (Garnock-Jones et al. 1996). Progress in the barcoding research is monitored by Consortium for the Barcode of Life (CBOL) and are focused on retrieving the barcode sequence from all plants species (Hollingsworth et al. 2009).

The fundamental problems with the DNA based primers is their inability to discriminate interspecies and intra-species variation in the sequence (Chen et al., 2010; Lahaye et al., 2008; Chase and Fay, 2009).

CBOL, recommends the combination of matK and ribulose-biphosphate carboxylase (*rbcL*) universal primers with the option of supplementing these with two or more other primer for the discrimination of plants species.

Currently, *matK, rbcL psbA-trnH, ITS2*, and *rpoC1* are popularly used for barcoding and identification of plants. Some researchers used *psbA-trnH* barcode to identify the medicinal pteridophytes species as well as plants belonging to *Dendrobium* genus (Yao et al. 2009; Ma et al. 2010). ITS2 is a potential universal DNA barcode for the identification of plants (Chen et al. 2010). Approximately, 92.7% cases (6600 plants belonging to different phyla i.e. Gymnosperms, Mosses, Angiosperms, Ferns, Algae, Liverworts, and Fungi) have been correctly identified, using ITS2. The ITS2 universal primer has been used to identify plants belonging to many families i.e. Rosaceae, Rutaceae, Asteraceae etc. (Yao et al. 2010; Gao et al. 2010; Luo et al. 2010; Pang et al. 2011; Liu et al. 2012).

The other widely acceptable reliable DNA barcode marker is *rbcL* (Kress et al. 2009). The *rbcL* sequence has been used to discriminate several medicinal plants (Sheng-Guo et al. 2008; Doyle et al. 1997; Hamdam et al. 2013) and is capable of identifying genetic differences in plant populations. This primer detects rapid and small amount of mutations in plant species, enabling discrimination of closely related species (Hamdam et al. 2013).

2.6 Tissue culture studies on *G. nervosa*

Increasing use of natural products as medicines in the worldwide healthcare sector has created demand for medicinal plants and their raw products. Uncontrolled harvesting of medicinal plants by herbal based pharmaceutical companies has caused extinction of many red listed medicinal plants. Recently World Conservation Union reported that 70% plants of medicinal importance are under threat (Paunescu, 2009). Over harvesting, inappropriate agricultural based practices in forest areas, colonization, global warming are some the main causes attributed to the loss of natural biodiversity (Pitman & Jorgensen, 2002).

There is an urgent need to ensure conservation and sustainable use of medicinal plants. In order to protect & preserve plant biodiversity alternative methods to conserve the genetic resources of medicinal plants need to be explored. Although, *G. nervosa* is not an endangered species, *in vitro* micropropagation of *G. nervosa* has been carried out by researchers especially by those involved in medical bioprospecting of this plant. Further, secondary metabolites of pharmacological importance are available in different parts of plant (flowers, fruits, leaves, bark and roots) and it is possible to obtain the same via callus culture or single cell suspension culture. So, using callus or single cell suspension culture, economical mass production of pharmacological important compounds would be possible.

In vitro culture techniques include shoot/root tip culture, somatic embryogenesis, callus culture, single cell suspension culture etc. These techniques not only aid in conservation of genetic resources of endangered medicinal plants but would also promote mass production of pharmaceutically important secondary metabolites.

In vitro culture techniques have been employed to conserve several threatened, endangered, rare plant species such as *Picorrhiza kurroa, Gymnema sylvestre, Salaca oblonga, Oroxylum indicum, Celastrus paniculata, Saussaurea lappa, Ginkgo biloba, Swertia chirata* and *Tinospora cordifolia, Holostemma annularis* (Sharma et al. 2010).

In several studies, callus has been commonly used for studying the pharmacological activity of a medicinal plant. The callus can be used instead of plant extract, to treat a particular disease in a geographically isolated region where the intact plant is not available. Further implementation of the cell culture techniques for medicinal plants would help in conservation of biodiversity.

Sokmen et al. (2004), prepared methanol extract from the callus of *Origanum acutidens* and evaluated it for antioxidant, antimicrobial and antiviral activity. Results indicated that methanol callus extract (MCE) did not inhibit reproduction of the influenza Aichi virus in Madin-darby Canine Kidney Epithelial Cells line (MDCK), however, methanol extract from callus depicted toxicity against the virus, indicating presence of antiviral compounds in the callus extract. Although, methanol callus extract did not depict antimicrobial activity, methanol callus extract depicted free radical scavenging activity with IC_{50} value of 71.5± 5.3 µg/ml. Similarly, Gulluce et al. (2003), evaluated the anti-bacterial and antioxidant property of methanol callus extract (MCE) of *Satureja hortensis* L. Although

MCE did not depict any antibacterial activity, free radical scavenging activity with IC_{50} value of 23.76± 0.80 µg/ml was detected.

The ultimate objective of all plant cell culture studies is to obtain bioactive compounds from derived callus or single cell suspension culture. Several secondary metabolites have been obtained through callus culture of medicinal plants viz. Alliin (Malpathak & David, 1986); Altamisine (Goleniowski & Trippi,1999); Saikosaponins (Wang & Huang,1982.); L-Canavanine (Ramirez et al. 1992); Pyrethrins (Rajasekaran et al. 1991); Naringin & Limonin (Barthe et al. 1987); Caffeine (Waller et al. 1983); Secoiridoid glucosides (Skrzypczak et al. 1993); L-DOPA (Brain, 1976); Camptothecin (Thengane et al. 2003); Thebaine (Day et al. 1986); Betacyanin (Schroder & Bohm, 1984) and Lithospermic acid B and Rosmarinic acid (Morimoto et al. 1994).

Apart from callus culture, single cell suspension culture of medicinal plants is an alternative option for rapid mass production of bioactive compounds of pharmaceutical importance. Bioactive compounds that have been successfully produced on a large scale using single cell suspension culture are Canthinone alkaloids (Anderson et al. 1986); Rosmarinic acid (De-Eknamkul & Ellis, 1985); Theamine, γ-glutamyl derivatives (Orihara & Furuya, 1990); Capsaicin (Johnson et al. 1990); Diosgenin (Heble & Staba, 1980); L-Ephedrine D-pseudoephedrine (O'Dowd et al. 1993); Ginkgolide A (Carrier et al. 1991); Cerebroside (Jang et al. 1998); Nicotine (Mantell et al. 1983); Ginsenosides (Zhong & Zhu, 1995); Morphine, Codeine (Siah & Doran, 1991); Reserpine (Yamamoto & Yamada, 1986); Taxol (Wu et al. 2001) and Berberin (Kobayashi et al. 1987).

2.7 Antioxidant activity of *G. nervosa*

In living organism's reactive oxygen species (ROS) like hydroxyl ($OH^{\cdot}$-), superoxide ($O2^{\cdot}$-) and peroxyl ($ROO^{\cdot}$, $^{\cdot}OOH$) are produced during stress. Proteins, RNA, DNA, lipids and enzymes are the major targets of reactive oxygen species resulting in dysfunction or mutations leading to major degenerative diseases (Ames et al. 1993). Free radicals of ROS have been observed in pathogenesis of various human diseases such as Alzheimer's disease, heart disease, cancer (Diaz et al. 1997); ageing (Burns et al. 2001); inflammation, neuron disorders, diabetic problems and atherosclerosis (Chen et al. 2006).

Although, human possess innate defence system to combat excess production of ROS, excess air and water pollution as well as unhealthy food habits have decreased the capacity to overcome the effects of free radicals. Therefore, antioxidants that can neutralize free radicals are of great importance to sustain the redox balance of human body which ultimately would regulate the progression of several diseases (Willet, 1994).

For several years, researchers have been exploring the antioxidant potential of thousands of medicinal plants worldwide (Yen et al. 1995; Masaki et al. 1995). The free radical scavenging or antioxidant property of medicinal plants is attributed to plant secondary metabolites such as flavonoids, phenolic acids and diterpenes (Pietta, 1998; Shahidi et al. 1992).

All protocols used for evaluating the antioxidant potential of plant extract are based on electron paramagnetic resonance (Rosen et al. 1984), spectrophotometry/colorimetric (Nishikimi et al. 1972) and chemi-luminescence principle (Robinson et al. 1997). The major assays to evaluate the antioxidant potential are 1,1-diphenyl-2-picrylhydrazine (DPPH)

method (Blois, 1958); nitro blue tetrazolium (NBT) reduction method or superoxide anion scavenging assay (Beauchamp and Fridovich, 1971); reducing power method (Oyaizu, 1986); hydroxyl radical scavenging assay (Halliwell et al. 1987); hydrogen peroxide scavenging assay (Ruch et al. 1989); total radical trapping antioxidant potential (TRAP) method (Lissi et al. 1992); Trolox equivalent antioxidant capacity (TEAC) assay (Salah et al. 1995); ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996); thiobarbituric acid (TBA) method, ferric thiocyanate (FTC) assay, (Mackeen et al. 2000); oxygen radical absorbance capacity (ORAC) assay (Ou et al. 2001) and 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay (Rice-Evans and Miller, 1994; Re et al. 1999; Baltrusaityte et al. 2007).

Amongst all antioxidant assays described above, FRAP assay is quick and reproducible and can be linearly related to the molar concentrations of pure or mixture of antioxidants present in the plant crude extract. FRAP assay is easy to perform as chemicals and equipments used in this assay are inexpensive and are easily available (Benzie & Strain, 1996).

Till now several medicinal plants have been screened for antioxidant activities using FRAP assay (Velioglu et al. 1998; Lee & Shibamoto, 2000; Kitts et al. 2000; Liu & Ng, 2000; Wang and Jiao, 2000; Tiwari, 2001; Lee et al. 2003).

The antioxidant property of some secondary metabolites like polyphenols and flavonoids from medicinal plants have been studied (Sies, 1991; Friedman & Kimball, 1986; Dreosti, 1991; Hertog et al. 1993; Hertog et al. 1995; Dalton & Ahmad, 1995; Langley-Evans, 2000; Pietta, 2000; Tiwari, 2001; Lee et al. 2003; Modun et al. 2003). Both black and

green tea, the most consumed beverages worldwide have been reported to contail antioxidants (Hertog et al. 1993; Langley-Evans, 2000; Lie and Xie, 2000).

2.8 Anti-inflammatory activity

Inflammation in human body is a natural indirect defence process initiated in response to any external toxic or harmful stimuli. Unchecked inflammation results in chronic disorders. Arachidonic acid metabolic pathway plays an important role in the inflammation process. Lipoxygenase (LOX) is the key enzyme, responsible for leukotrienes biosynthesis from Arachidonic acid. The leukotrienes are the mediators of allergic and inflammatory reactions. Lipoxygenase oxidizes linoleic and Arachidonic acid at different sites. Depending on the oxidation site, four types of iso-enzymes such as 15-LOX, 12-LOX, 8-LOX and 5-LOX have been identified (Porta et al. 2002). However due to difficulty in obtaining human LOX, most of the *in vitro* studies are carried using 5-LOX from soybean (Skrzypczak-Jankun et al. 2003).

Standard drugs that are used for the treatment of inflammation, inhibit the activity of two key enzymes i.e. lipoxygenase and cyclooxygenase, as these two enzymes are crucial in the metabolism of arachidonic acid. However, drugs for the treatment of various inflammatory induced diseases such as arthritis, Alzheimer's and osteoporosis result in side effects such as ulcers, cardiac aberrations, hypertension etc. (Hougee, 2008). Thus the, focus has shifted towards natural drugs (Conforti et al. 2008; Buhrmann et al. 2011). In recent years, natural antiinflammatory agents of plant origin, have gained special attention for the treatment of inflammation. Nowadays, patients with chronic inflammatory disease like

arthritis worldwide, are being advice to use anti-inflammatory drugs of herbal origin on priority basis.

Medicinal plants are a premium source for obtaining novel inhibitors for 5-LOX. Screening of medicinal plants using *in vitro* assays for 5-LOX inhibition have led to safe, cheap and easily available anti-inflammatory drugs of natural origin with minimal adverse side effects (Wagner, 1993; Bauer, 1999).

Sreejayan and Rao (1996), reported the role of polyphenols in the treatment of inflammatory disorders. Polyphenols such as curcumins, tannins and flavonoids (isoquercitrin, quercetin, apigenin, sideritoflavon, luteolin, silibinin, gnaphalin, baicalein, rhamnetin, centaureidin) from the medicinal plants are reported to control the inflammatory reactions by free radical scavenging potential or by inhibiting lipoxygenases (LOX) and cyclo-oxygenases (COX) enzymes (Sadik et al. 2003; Lee et al. 2003).

Schneider and Bucar (2005), reviewed 150 species of medicinal plants belonging to various families. Thirty natural compounds were identified as potent inhibitors of 5-lipoxygenase. Additionally, medicinal plants and the bioactive compounds with potential of 5-lipoxygenase inhibition activity belonged to a broad range of families and different classes of chemical compounds has been reported.

Over production of nitric oxide (NO), a free radical reactive oxygen species, also induces many chronic inflammatory diseases (Stuehr & Nathan, 1989; Nathan & Hibbs, 1991; MacMicking et al. 1997; Guzik et al. 2003). Crude extracts from many medicinal

plants have been demonstrated to control these inflammatory diseases by scavenging nitric oxide free radicals (Kanno et al. 2006; Lee et al. 2007; Lee et al. 2010).

2.9 Anti-lithiatic activity

2.91 Anti-urolithiasis potential of medicinal plants

Recurrent nucleation and aggregation of renal stones in the kidney has become a worldwide problem. Calcium oxalate (CaOx) is found in 80% of kidney stone patients. The kidneys of other 20% patients contain stone made of uric acid, struvite, cysteine etc. (Park & Pearle, 2007). Nucleation and aggregation of kidney stone is a very complex process (Miller et al. 2007).

2.92 Medicinal plants in kidney stone treatment

From ancient times, people have used decoctions or extracts of various medicinal plants for dissolving kidney stones. Although, the rationale behind these phytoremedies are still unclear, many of these are able to limit the recurrent urolithiasis.

Herniaria hirsuta L, a medicinal plant is traditionally known for its anti-lithiatic activity. Although extract of *H. hirsuta* L. enhances the nucleation i.e. number of crystals, it simultaneously decreases the size of crystals (Atmani & Khan, 2000). Further, *H. hirsuta* L. extract successfully inhibited the binding of crystals to renal cells (Atmani et al. 2004).

Phyllantus niruri L., a famous medicinal plant of Brazil, has also been traditionally used to treat kidney stone. Barros et al. (2003), justified the crystal nucleation inhibition property using water extract of *P. niruri* L. Similarly, *P. niruri,* extract was demonstrated to inhibit the formation of calcium oxalate crystals (Campos and Schor, 1999). Garimella et al.

(2001), confirmed the *in vitro* inhibition of calcium and phosphate precipitation by *Vigna unguiculata* L. seed extract. Additionally, various other medicinal plants from China have also been proved scientifically to inhibit calcium oxalate crystallization (Chen et al. 2007; Koide et al. 1995). Other medicinal plants and juice from citrus plants have been found to decrease the rate of calcium oxalate crystallization (Oussama et al. 2005; Kulaksizoglu et al. 2008).

In India, although several medicinal plants have been traditionally known for their anti-lithiatic property, very few of them are actually marketed as herbal drugs. Some herbal drugs in Indian market are Calcuri (Charak Pharmaceuticals, Bombay, India), Cystone (Himalaya Drug Company, India), and Chandraprabha bati (Baidyanath, India). Though, *G. nervosa* is known for its potential to cure urinary problems, no scientific report is available to justify its traditional anti-lithiatic property.

2.10 Tyrosinase inhibition

In the human, the major defence against UV light and darkening of the skin is induced by production of excess melanin pigments (Freidman, 1996). Kojic acid, Hydroquinone, Azelaic acid, Arbutin, and Catechins are commercially avalaible products for skin depigmentation (Maeda et al. 1991). Tyrosinase plays an important role in the biosynthetic pathway of melanin production. Most of the available treatments for skin whitening, inhibit tyrosinase. Due to excessive use of face whitening creams, skin of the face become thin and a black area may develop around the eyes called 'bleach panda effect'. These common irreversible adverse effects of synthetic products are ochronosis, cutaneous damage etc. Thus people prefer natural or homemade remedies for skin whitening such as honey, turmeric,

cow' s milk, gram flour, tender, coconut water, citrus fruits, cucumber, papaya, almond, tomato, potato, milk thristle, strawberry, blueberry, etc.

Tretinoin, hydroquinone, α-hydroxyl acids (AHAs), kojic acid, L-ascorbic acid, monobenzone, azelaic acid and magnesium ascorbyl phosphate are some of the synthetic tyrosinase inhibitors commonly used in skin whitening creams.

Most of the secondary metabolites such as phenols, flavonoids, terpenes, alkaloids, steroids, fatty acids that are present in the medicinal plants are reported to have anti-tyrosinase activity (Chang, 2009; Curto et al. 1999; Matsuda et al. 1996; Kubo & Kinst-Hori, 1999; Kim et al. 2005).

Extracts from *Prosopis Africana*, *Portulaca pilosa, Stryphnodendron barbatimao*, *Entada Africana* and *Cariniana brasiliensis* have been reported to inhibit tyrosinase activity (Baurin et al. 2002). Further, ten different varieties of Korean tea were screened for their skin whitening potential and a green tea variety was found strongly inhibit tyrosinase activity (No et al. 1998). Further, five common Asian tea varieties (Horsetail, Inulae Flos, Chinese Leucas, Indian Wikstroemia and Broomweed) were also tested for tyrosinase inhibition activity and Inulae Flos variety was found to be potent source of tyrosinase inhibition (Lu et al. 2010). Elsewhere, out of 100 medicinal plant extracts tested, *Dryopteris crassirhizoma, Glycyrrhiza glabra, Morus alba, Rheum palmatum, Myristica fragrans, Sophora japonica, Chaenomeles speciosa,* and *Gastrodia ellata* demonstrated tyrosinase inhibition (Lee et al. 1997).

Hibiscetin heptamethyl ether (Chang, 2009), phydroxybenzoic acid (Briganti et al. 2003), lupeol (Seiberg et al. 2000), 2,6-dimethoxy-4-hydroxy acetophenone, 3,4-dihydroxybenzoic acid, ferulic acid (Maeda et al. 1991) and 2'-O-ethylmurrangatin (Nerya et al. 2003) isolated from medicinal plants were screened for anti-tyrosinase activity, but only dihydroxybenzoic acid and lupol inhibited tyrosinase.

2.11 Anti-cancer assays

According to the World Cancer Report, 12.7 million new cancer cases and 7.6 million deaths (around 13% of all deaths) occurred in 2008. Among all types of cancers, lung, female breast, colorectal and stomach cancers accounted for 40%. Breast cancer was the most common type of cancer diagnosed in women i.e. 23% of all new cases in women (Ferlay, 2012).

Breast cancer is prevalent in human as well as other mammals. Although most of the human cases of breast cancer are reported in women, male breast cancer is also known to occur (US, NIH). Globally, breast cancer accounts for 22.9% of total cancers (except non-melanoma skin cancers) in women. In 2008, breast cancer caused 458,503 deaths globally, 13.7% of total cancer deaths in women. It is 100 times more common in women than in men even though men tend to have lesser outcomes due to delays in diagnosis (World Cancer Report, 2008). Further, every year one in four new cancers diagnosed worldwide is a breast cancer in females (Bray et al. 2013).

MCF-7 (Michigan Cancer Foundation-7) is a well-known breast cancer cell line isolated from a 69-year-old Caucasian woman and is widely employed for *in vitro* breast cancer studies.

Various plants are recognized in *Ayurveda* for treatment of cancer. Recently, many plants have also been screened for their anticancer activities. *Vinca* alkaloids was the first natural anticancer agent discovered from plant (Noble, 1990). Currently, 60% of the anticancer drugs available in the market are derived from natural sources. Thus, the conventional herbs lead the trail for the discovering biologically active novel molecules (Sakpakdeejaroen & Itharat, 2009). Hence, in spite of the artificial and combinatorial chemistry as well as molecular modelling approaches, medicinal plants are an important source of new drugs (Abu-Dahab & Afifi, 2007).

Many medicinal plants have adaptable antioxidant properties and have shown defensive effect against breast cancer. As already reported, antioxidant properties are correlated with the occurrence of active phytoconstituents such as vitamins, carotenoids, terpenoids, flavonoids, polyphenols, enzymes, minerals, alkaloids, polysaccharides, saponin, lignins, xanthones and pigments (Gupta & Sharma, 2006; Pandey & Rizvi, 2009; Saxena et al. 2013).

2.12 Gas chromatography-mass spectroscopy (GC-MS) analysis

Chromatography techniques have been used for the detection of primary and secondary metabolites present in the crude or pure biological samples. Since, last three decades, chromatography techniques have been efficient in terms of accuracy and up-to date database of the compounds in the libraries. Gas Chromatography (GC) coupled with Mass spectroscopy (MS) provides robust identification of the compounds based on commercially available libraries like NIST & Wiley online library. GC-MS technique mainly used to detect and characterize targeted or non-targeted compounds from the mixture of metabolites.

Selection of the solvent system for the extraction of sample is mainly dependent on metabolite target, sample matrix, biological problem etc. Researchers generally use a wide range of solvent system ranging from polar to non-polar solvents.

GC-MS studies have been used routinely for identification of several secondary metabolites such as phenols and phenolics acids (Robbins, 2003; Saitta et al. 2009; Zhang & Zuo, 2004; Zuo, et al. 2002), flavonoids (Zhang & Zuo, 2004; Fuzfai & Molnár-Perl, 2007; Gao et al. 2010), alkylresorcinols (Zarnowski et al. 2002), phytoestrogens (Lee et al. 2004), secoiridoids & ligstrosides (Saitta et al. 2009; Rios et al. 2005), diterpenes and diterpenic acids (Popova et al. 2010), phenolic diterpenes, pentacyclic triterpenes (Razborsek et al. 2007), sterols, stanols, and esters (Esche et al. 2012; Shin et al. 2010), lignans (Meagher et al. 1999), stilbenes (Lamikanra et al. 1996; Rimando et al. 2004) and alkaloids (Torras-Claveria et al. 2010). Using GC-MS technique the chemical ingredients of crude extracts from *Datura metel*, a famous medicinal plant were determined (Hossain et al. 2013). Likewise, using GC-MS, Islam et al. (2015), revealed sixteen bioactive compounds from the essential oil of *Cuscuta reflexa.* Panda et al. (2015), reported many important secondary metabolites from the crude leaves extract of *Clerodendrum viscosum* and based on the important metabolites of pharmaceutical importance, this plant was claimed for treatment of various diseases. Similarly, Nanadagopalan et al. (2015), have revealed the presence of 27 important bioactive compounds in *Sterculia urens* leaves extract.

2.13 Anti-Diabetic assays

Diabetes is one of the major disease worldwide, affecting around 194 million people. As per claim by World health organization (WHO), 300 million people will be suffering from

diabetes by 2025 (World Health Organization, 2010). Between type-1 and type-2, the most prevalent is type 2 diabetes mellitus (Gershell, 2005) and accounts for 9 % of deaths. The most common therapeutic approaches to control diabetes is to evolve strategies to control hyperglycaemia (Rhabaso-Lhoret & Chiasson, 2004).

Post-prandial hyperglycaemia can be decreased by reducing the digestion and absorption of glucose through the inhibition of carbohydrate hydrolysing enzymes such as α-amylase and α- glucosidase. Inhibition of these enzymes results in delay of the digestion of the carbohydrates, causing a reduction in the rate of glucose absorption (Rhabaso-Lhoret & Chiasson, 2004).

2.13.1 α- amylase enzyme

α- amylase (EC 3.2.1.1) is a starch hydrolysing enzyme that cleaves the α-bond associated with starch to form glucose and maltose (Maureen, 2000). Commercially, α-amylase is isolated from animals and humans (Voet and Voet, 2005). In plants, the α-amylase is synthesized near the starch deposit sites and is consequently present along the digestive tract of phytophagous animal's i.e insects to mammals (Bowles, 1990). Pancreatic α-amylase is the key enzyme in the digestive system. It catalyses the initial step in the hydrolysis of starch to a mixture of small oligosaccharides consisting of maltose, maltotriose and a number of α-(1-6) and α-(1-4) oligoglucans. These small oligosaccharides are then hydrolysed by α-glucosidase and further degraded to glucose, which on absorption enters the bloodstream.

Inhibition of α-amylase, results in decline in the carbohydrate digestion and absorption by the small intestine. Therefore, glucose level in the blood can be minimized (Laar et al. 2008;

Cheng & Fantus, 2005). Acarbose and voglibose are currently used as α-amylase and α-glucosidase inhibitors respectively, but are known to induce side effects such as bloating, flatulence and diarrhoea (Chakrabarti & Rajagopalan, 2002). The adverse side effects are attributed to excessive inhibition of pancreatic α–amylase leading to the abnormal bacterial fermentation of undigested carbohydrates in the colon (Bischcoff, 1994).

2.13.2 Inhibitors of α-amylase by crude extracts of medicinal plants

Many researchers have investigated the role of medicinal plants in the inhibition of α-amyalse. Approximately, 800 plant species are reported to demonstrate anti-diabetic activity. In Indian traditional system of medicine, water extracts of *Psidium guajava* and *Syzygium cumini* L. have been proved to depict α-amylase inhibition (Karthic et al. 2008). Anti-diabetic activity of seed extracts of *S. cumini* has also been confirmed by *in vitro* studies using rat (Mentreddy, 2007; Kumar et al. 2008). Conforti & Cols (2005), reported the α-amylase inhibition (80%) activity of *Amaranthus caudatus* L. with respect to control. Aqueous extracts of plants such as *Vaccinium myrtillus, Melissa officinalis, Khaya senegalensis, Galega officinalis, Tamarindus indica, Camellia sinensis*, *Taraxacum officinale, Mitragyna inermis, Holarrhena floribunda, longepedunculata Fresen* etc. have also been reported to depict α-amylase inhibition (Funke & Melzing, 2006). Further, out of 41 plants belonging to Mangolian traditional medicine, only extracts of *Ribes pullchelum, Geranium pretense, Paeonia anomala*, *Vaccinium uliginosum*, *Leontopodium ochroleucum*, *Rhodiola rosea*, *Pentaphylloides fruticosa* demonstrated greater than 30% inhibition of α-amylase activity (Kobayashi et al. 2003). Nine medicinal plants from Labanon were also screened for

inhibition of α-amylase activity, wherein methanol extracts of *Marrubium radiatum* & *Salvia acetabulosa* were observed to inhibit α-amylase activity (Loizzo et al. 2008).

Since thousands of years, Ayurveda has been using Indian traditional medicines for many diseases. In Ayurveda, many medicinal plants are reported to have anti-diabetic activity with no side effects. (Bhat et al. 2011; Bhutani & Gohil, 2010). Six medicinal plants viz. *Azadirachta indica, Ocimum tenuflorum, S. cumini*, *Linum usitatissimum* and *Murraya koenigii*, have been routinely used in Ayurveda as medicines. Amongst these plants *Ocimum tenuflorum* demonstrated α-amylase inhibition (Bhat et al. 2011). Some proteins isolated from various medicinal plants have also been reported to promote α-amylase inhibition (Wang et al. 2011). *Phaseolus vulgaris* contain a protein that inhibits α-amylase (Yamada et al. 2001; Berre-Anton et al. 1997).

2.13.3 α- glucosidase enzyme

The α-glucosidase (EC 3.2.1.20) is an exo-type carbohydrate hydrolysing enzyme widely present in various microorganisms, plants, and animal tissues (Skeggs et al. 1956). The enzyme is known by various other names viz. glucoinvertase, Maltase, maltase-glucoamylase, α-D-glucosidase, α-glucopyranosidase, glucosidoinvertase, glucosidosucrase, α-glucoside hydrolase, α-1, 4-glucosidase, α-D-glucoside glucohydrolase. The enzyme is present in the brush borders of small intestine and cleaves the 1, 4-α bonds of starch (Larner, 1960; Bruni et al. 1970; Flanagan & Forstner, 1978; Sivikami & Radhakrishnan, 1973; Sorensen et al. 1982).

2.13.4 α- glucosidase inhibitors

α-glucosidase cleaves the 1,4- α-glucoside bond and releases the α-glucose units leading to increase in absorption rate of sugar in digestive tract followed by increase in the blood glucose level. Clinically, α-glucosidase inhibitors are used as anti-hyperglycaemic agent (Casirola et al. 2006). Currently, acarbose and voglibose are used as α-glucosidase inhibitors to control diabetes. However, side effects such as gastrointestinal diarrhoea and flatulence have been associated with these drugs (Van et al. 2005). Hence, anti-glucosidase drugs from natural sources such as medicinal plants are an attractive alternative approach to treat diabetes (Patricia et al. 2005; Kyung & Moo, 2000; Ye et al. 2002). Out of 900 Korean medicinal plants screened for α-glucosidase inhibition, 72 plants were observed to inhibit α-glucosidase activity (Watanabe et al. 1997). The IC_{50} values for α-glucosidase inhibition from *Dalbergia odorifera*, *Pueraria thunbergiana*, *Glycyrrhiza glabra* and *Sophora japonica* were reported as 19.5 mg/mL, 21.8 mg/mL, 28.1 mg/mL and 26.5 mg/mL respectively.

2.14 Enzyme kinetics

Enzymes act as a catalyst in all enzymatic reactions. Any molecule, whether organic or inorganic that interacts with the enzyme inhibiting the reaction is known as an inhibitor. The enzyme inhibitors are generally low molecular weight compounds and they partially or completely inhibit the enzyme. Loss in enzymatic activity may reversible or irreversible. The range of enzyme disruption by inhibitors may be due to interactions of inhibitor with single amino acid or with multiple side chains of various amino acids. Inhibitors interact with enzymes active sites either by covalent or non-covalent interactions. Like substrate, different inhibitors may have same/different active binding sites in the enzyme.

In the drug discovery research, many compounds and their derivatives are synthesized or obtained from the natural sources for evaluating enzyme inhibition. Therefore analysing enzyme-substrate relationship and enzyme inhibition pattern is a necessary component in all drug design programs (Sami et al. 2011). Enzyme kinetic study helps in understanding the relationship between inhibitors function/structure with the enzymes active sites leading to discovery of novel enzyme inhibitors. These approaches are still widely used in medicinal chemistry and in high throughput screening technology while working with thousands of inhibitory compounds (El-Metwally et al. 2010).

Rational drug design uses three-dimension structures of enzymes to predict all possible active sites and in accordance with 3D active site, find a suitable inhibitor molecule for a particular disease. The 3D modelling approach for drug discovery programs indeed helps in evaluating the ideal inhibitory compound from the long list of novel compounds available in the database.

Enzyme inhibition is completely dependent on the structure of the inhibitory compound and does not follow any trend with particular families groups of compounds. Different compounds isolated from same source may exhibit same or different type of enzyme inhibition. Terpenes isolated from *Luculia pinciana* (Rubiaceae) such as 24-trihydroxyursolic acid depicted competitive inhibition, whereas, 5-methoxy-8-hydroxycoumarin, oleanolic acid, scopletin, and ursolic acid depicted non-competitive inhibition (Kang et al. 2009).

Purified alkaloids from several medicinal plants were also analyzed for α-glucosidase inhibition. Vasicine and vasicinol alkaloids obtained from *Adhatoda vasica* Nees

(Acanthaceae) depicted competitive inhibition (Gao et al. 2008). Similarly, β-carboline glucoalkaloid isolated from *Buthus martensii* Karsch demonstrated uncompetitive inhibition (Kim, 2013). Out of the two novel phenylpropanoyl amides, viz. A & B obtained from the leaves extract of *Piper sarmentosum*, compound B alone inhibited the α-glucosidase in a non-competitive manner (Damsud et al. 2013).

Quinones are also reported to demonstrate α-glucosidase inhibition. Compound 1, 3-dihydroxy-2-methylanthraquinone and 1-hydroxy-2-methylanthraqunione from *Rubia cardifolia* L. inhibit the enzyme in competitive mode whereas 1,2-dihydroxyanthraquinone from same plant showed non-competitive inhibition (Kang et al. 2009).

Pure flavonoid compounds such as quercetin, morin, naringenin fisetin and quercitrin depicted mixed type inhibition of α-glucosidase activity (Iio et al. 1984). Further five flavonoid compounds viz. scopoletin, tanacetin, chrysoeriol, selagin and quercetagetin-3, 6-dimethylether isolated from the ethyl acetate and aqueous extracts of *Crossostephium chinense* L. exhibited competitive inhibition (Wu et al. 2009). Acacetin obtained from aerial part of *Brickellia cavanillesii* (Asteraceae) demonstrated a mixed-type inhibition of α-glucosidase activity (Escandon-Rivera et al. 2012).

The curcuminoid compounds bisdemethoxycurcumin from *Curcuma longa* has been reported to exhibit non-competitive inhibition of α-glucosidase (Du et al. 2006). 3-O-Caffeoylquinic acid and its isomer 5-O-caffeoylquinic acid isolated from the leaves of *Nerium indicum* (Apocynaceae) showed non-competitive type of α-glucosidase inhibition (Ishikawa et al. 2007). Two derivatives of coumarine isolated from the stem extract of *Luculia pinceana* (Rubiaceae) showed non-competitive inhibition of α-glucosidase (Kang et

al. 2009). Further, chebulagic acid & chebulinic acid compounds isolated from the fruit extract of *Terminalia chebula* Retz. (Combretaceae) has also been reported to belong to the non-competitive class of inhibitors of α-glucosidase activity (Gao et al. 2007).

Under the class of steroids, 3-(Z)-Butylidenephthalide isolated from *Ligusticum porter* and 6-Hydroxyacetyl-5-hydroxy-2, 2-dimethyl-2H-chromene isolated from the *B. cavanillesii* inhibited α-glucosidase in a non-competitive manner (Brindis et al. 2010; Escandon-Rivera et al. 2012).

2.15 Comet & micronuclease assay

The data on evaluation of genotoxic and mutagenic effects of medicinal plants on human or animals models is almost negligible (Hartmann et al. 2004; Suter, 2006; Smart et al. 2011). Since, people have been using medicinal plants for centuries, researchers have faith in medicinal plants and a general belief that they lack toxic effects (Luyckx & Naicker, 2008).

Because of modern scientific techniques such as high throughput screening, *in vitro* assays, bioinformatics, ADMETOX, *in silico* drug design tools, metabolomics etc., now it is easy to evaluate the mutagenicity and genotoxicity of natural plant extracts or bio-active compounds.

Both, the European Centre for the Validation of Alternative Methods (ECVAM) and Organization for Economic Co-operation and Development (OECD) have validated and approved several assays to check the genotoxic and mutagenic effects of medicinal plant extracts. These assays are categorized into three main categories i.e. (1) *in silico* methods such as DEREK (Greene, 2002); TOPKAT (Snyder & Smith, 2005) and 3D DNA docking

(Snyder et al. 2004; Snyder & Smith, 2005). (2) *In vitro* assays: Ames and vitotox assays to check toxic effects of test sample in prokaryotic organisms, while micronuclease, Unscheduled DNA synthesis (UDS), Sister chromatid exchanges (SCE), Mouse lymphoma (MLA), comet (single-cell gel electrophoresis assay) and H2AX assays are employed for eukaryotic organisms (yeast, human and animal). The main limitation associated with *in vitro* assays is that they do not reflect the actual environmental conditions of animal body such as temperature, pH, electrolyte concentrations, interactions of cells within tissues, extracellular matrix etc. Moreover the culture medium of assays is not homeostatic throughout the assay (Hartung, 2011). (3) *In vivo* methods: in order to address the above problems observed for *in vitro* assays, *in vivo* methods such as micronucleus assay, unscheduled DNA synthesis (UDS) assay, Mouse spot test, Transgenic rodent (TGR) mutation assay, Somatic mutation and recombination test (SMART) are used.

The micronucleus (MN) test and comet assay are sensitive, rapid and widely used methods in the detection of mutagenicity and genotoxicity of synthetic and natural chemicals (Kumar et al. 2010; Nwani et al. 2011).

Herbal medicines can be potentially toxic to human health. Scientific research has indicated that many of the plants used in traditional and folk medicine are potentially toxic, mutagenic, and carcinogenic (Mengs, 1988; Ferreira-Machado et al. 2004). Comet and micronuclease assays were used by Cavalcanti, et al. (2006) to reveal the genotoxic effects of oil obtained from *Copaifera langsdorffii* Desf. Similarly, using comet assay, Li et al. (2008), demonstrated the DNA breakage effect by artesunate, a compound derived from *Artemisia annua* L.

2.16 Isolation, purification and identification of compounds

So far, crude extracts/compounds from medicinal plants are important sources in drug discovery programme. Biodiversity of medicinal plants promotes the possibilities of discovering novel bioactive compounds against several challenging diseases. These bioactive compounds are secondary metabolites of plants and are classified into three main categories i.e. phenolic, alkaloids and terpenoids. The use of alkaloids (Andrade et al. 2012), phenolics (Zhang et al. 2011) and terpenoids (Brusotti et al. 2012) against several diseases, indicate the vital role of these natural source in several recent pharmaceutical applications. Since, compounds found in the medicinal plants are present at low concentration, efficient concentration, isolation, purification and identification is a big challenge. Many researchers have focussed their studies in standardization of extraction (Winjngard et al. 2012) and identification (Wu et al. 2013) of the natural compounds from the crude extracts.

Although, *in vitro* assays do not replicate the *in situ* environment of animal or human being, these assays are quick, reliable, globally accepted and the test compound to be assayed is required in minute amount. Most *in vitro* methods are photometric or colorimetric based methods. Pharmaceutical compounds from the medicinal plants are worth exploring especially if the IC_{50} value is less than 100 µg/ml (Cos et al. 2006). *In vitro* enzyme inhibition assays have played an important role in assessing the pharmaceutical potential of bioactive compounds present in the crude extract. After validation of the specific activity, isolation of inhibitor can be achieved by bioassay guided extraction.

2.16.1 Isolation of analytes from plant material

For efficient isolation of natural compound/s from medicinal plants, several solvent combinations are being used with increasing polarity (Jerzmanowska, 1970; Nyiredy, 2004). Techniques available for the extractions of compounds are soxhlet extraction, ultrasonic extraction (sonication), accelerated solvent extraction, microwave-assisted extraction, steam distillation, membrane processes, supercritical fluid extraction, solid-phase micro-extraction and sample disruption method.

Amongst extraction processes, soxhlet extraction is the most common method for the isolation of bioactive compounds from natural resources. Soxhlet technique is generally used for the isolation of low to medium volatile, thermo-stable compounds.

2.17 Separation and purification of compound

2.17.1 Thin layer chromatography and column chromatography

Thin layer chromatography (TLC) is the initial step for selection of an appropriate mobile solvent system for better separation of analytes during chromatography techniques. TLC is a method where, liquid phase moves up through stationary phase (silica gel). In contrast, in column chromatography (CC), mobile solvent system migrates down along with solid phase.

2.17.2 High performance liquid chromatography (HPLC)

HPLC is a well-known technique used worldwide for the separation and purification of compounds from crude preparations. Column chromatography can separate a wide range of compounds with lowest to highest polarity (McMaster, 2007). There are two types of HPLC i.e. normal and reverse phase HPLC.

2.17.2.1 Normal-phase HPLC

In this type of column chromatography, generally silica is used as a polar stationary phase the sample is loaded on the top of the column, moves down and is subsequently eluted with increasing polar mobile phase. Normal phase column chromatography has also proved very useful in purification of several compounds from medicinal plants. Stivioside and phenolic acids have been successfully isolated from *Stevia rebaudiana* and *Plumbago zeylanica* L. respectively (Gupta et al. 1993; Tateo et al. 1999).

2.17.2.2 Reversed-phase HPLC

Reversed phase column chromatography involves a hydrophobic stationary phase which has strong affinity towards hydrophobic compounds. Octadecyl silane (ODS), a non-polar hydrophobic stationary phase is usually used in reversed phase column chromatography. Reverse phase is generally used for the separation and purification of analytes such as Phenylpropanoids from *Allium sativum* L. (Ichikawa et al. 2003), Ginkgolide A and B from *Ginkgo biloba* (Mesbah et al. 2005), Triterpenes from *Chenopodium quinoa* (Kuljanabhagavad et al. 2008), mahanimbine and mahanine from *Murraya koenigii* (Pandit et al. 2011).

2.17.3 Mass spectroscopy

Mass spectroscopy (MS) techniques require very small quantity of sample and impurities of sample can be easily differentiated from spectrum of main compound. Mass spectroscopy is quick and robust and can be applied for large molecular weight compounds.

2.17.4 Nuclear Magnetic Resonance

Nowadays, NMR has became a basic technique to reveal the structure of a natural/synthetic compound. NMR technique is non-destructive and require high amount of pure sample (Smith et al. 1997). Further, this technique is not applicable for exploring the structure of high molecular weight compounds i.e. 30KDa (Chalmers et al. 2006).

2.17.5 Infra-red spectroscopy

Near infrared spectroscopy is a high throughput method allowing a large number of samples. Near infrared spectroscopy uses electromagnetic rays between 700-2500 nm. It can detect many bonds in the compounds *viz*. CH, -OH, -NH etc. Near infrared spectroscopy has been used to reveal the carotenoids content in *Zea mays* L. (Brenna & Berardo, 2004; Berardo et al. 2009); phytochemical profile of *Mentha haplocalyx* (Dong et al. 2014); flavin composition of black tea (Hall et al. 1988); as well as phenolic and alkaloid content in leaves of green tea (Hall et al. 1988).

CHAPTER 3

MOLECULAR APPROACHES FOR IDENTIFICATION OF *G. nervosa* AND TISSUE CULTURE STUDIES

In order to confirm the morphology based taxonomic identification of *G. nervosa*, molecular studies using universal markers were undertaken. Tissue culture studies on *G. nervosa* was also undertaken to produce callus and single cell suspension culture that could be potentially used as a source material for isolation of the compounds of pharmacological significance.

3.1. Methodology for molecular identification of *G. nervosa*

3.1.1 Sample collection and DNA Extraction

Identification of *G. nervosa* was achieved with the help of a botany expert and a herbarium of *G. nervosa* (Sample no. 5312) deposited in the Department of Botany, Goa University. Fresh young leaves of *G. nervosa* (Fig. 3-a) collected from the Goa university campus (5°27'35.05" N, 73°49'58.46" E, 52 m) were used as a source for isolation of genomic DNA. Genomic DNA was isolated using a standard protocol described by Deshmukh et al. 2007. 300 mg of fresh leaves were homogenized in centrifuge tube followed by addition of 1 ml TE buffer (Appendix 1). The homogenate was vortexed for 5 min and later centrifuged at 12,000 g for 3 min at 4°C. The supernatant was discarded and the pellet was repeatedly washed 4-5 times with 70% ethanol. 1000 µl of extraction buffer (Appendix 2) was added to the pellet and again centrifuged at 8,400 g for 5 min. The pellet was resuspended in 450 µl of Resuspension buffer (Appendix 3) followed by addition of 80 µl of 10% SDS and incubated at 70ºC for 15 min. The tube was cooled to room temperature (30±2°C) followed by addition of 300 µl of 7.5 M ammonium acetate and kept on ice for 30 min. The suspension was centrifuged at 12,000 g for 15 min. The upper clear aqueous layer was transferred to microfuge tube. Equal amount of ice-cold isopropanol was added and centrifuged at 12,000 g for 15 min. The supernatant was decanted and the DNA pellet was washed twice with 70% ethanol. The pellet was resuspended in 100 µl TE buffer. 10 µl of RNaseA (10µg/ml) was

added and incubated at 37ºC for 1 h. Equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 g for 10 min. The aqueous layer was transferred to a fresh 1.5 ml microfuge tube. Two volumes of ice-cold ethanol were added followed by centrifugation at 12,000 g for 5 min at room temperature. The DNA pellet was washed with 70% ethanol and re-suspended in 100 µL of TE buffer and stored at -20°C until further use.

Fig. 3- a: *Grewia nervosa* L. Panigrahi

3.1.2. Primers for DNA amplification

Two pairs of universal primers (*rbcL* and *ITS2*) used for amplifying *rbcL* and *ITS2* genes from *G. nervosa* are shown in Table 3-I.

Table 3- I: Primers (*rbcL* and *ITS2*) used in the present study.

rbrbcL	FP	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	26 bp	Asahina et al. (2010)
	RP	5'-GTAAAATCAAGTCCACCRCG-3'	20 bp	
ITS2	FP	5'-ATGCGATACTTGGTGTGAAT-3'	20 bp	Gu et al. (2013)
	RP	5'-GACGCTTCTCCAGACTACAAT-3'	21bp	

3.1.3. Polymerase chain reaction (PCR) and Sequencing

Polymerase chain reaction was conducted to amplify the *rbcL* & *ITS2* genes from *G. nervosa*. PCR reaction mixture was prepared as shown in Table 3-II. The parameters for amplification are shown in Table 3-III. The amplified products were electrophoresed on 1% agarose gel. After confirming the size of amplified DNA, the DNA (approximately 500-600 bp) was excised from the gel and recovered using the Genie gel extraction kit as per the manufacturing instructions. The quality and quantity of the recovered DNA were checked by Nanodrop analysis. The pure recovered DNA was sequenced at Banglore Genei pvt. Ltd. (India).

Table 3- II: Composition of PCR reaction mixture used for the amplification of *rbcL* & *ITS2* genes from *G. nervosa*.

Components	Volume (µl)	Concentration
Sterile Milli Q water	13.6	--
10X polymerase buffer	2.0	1X
dNTPs mix	2.0	1 Mm each
Template DNA	1.0	100 ng
Forward primer	0.7	0.5 µM
Reverse primer	0.7	0.5 µM
Taq polymerase	0.5	1.5U
Total	20.0	

Table 3- III: Polymerase chain reaction parameters used for the amplification of *rbcL* & *ITS2* genes from *G. nervosa*.

Parameters	Temperature; Time	No. of Cycles
Initial denaturation	94 ºC; 5 min	1
Denaturation Annealing Extension	94 ºC; 30 sec 56 ºC; 30 sec 72 ºC; 45 sec	40
Final extension	72 ºC; 10 min	1

3.2. Tissue culture of *G. nervosa*

3.2.1. Surface sterilization of leaves explant from *G. nervosa*

Immature leaves of *G. nervosa*, growing in the Goa university campus (15°27'35.05" N, 73°49'58.46" E, 52 m) were used as explant for tissue culture. The leaves were washed thoroughly in running tap water for 5 minutes followed by rinsing 2-3 times with sterile distilled water. The explant was dipped in 70% ethanol for 30 seconds. Additionally, leaves were further surface sterilized with freshly prepared 0.1% w/v mercuric chloride for 8 minutes. Finally, the explant was thoroughly washed 3-4 times with sterile distilled water to remove traces of mercuric chloride.

3.2.2. Culture medium for induction of callus

MS medium (Murashige & Skoog, 1962) containing 3% sucrose (Analytical grade, Himedia, India) and 0.8% agar (Bacteriological grade, Himedia, India), was used for the culturing leaf explant. 2, 4-Dichlorophenoxy acetic acid (2, 4-D) and Kinetin (Kn) were added at desired concentration to MS medium either singularly or in various combinations.

3.2.3. Inoculation of surface sterilized explant

Surface sterilized leaves (2-3 cm) were incubated on MS medium (Appendix 4) containing auxin and kinetin in various ratios. The tubes were incubated at 25˚C for 30 days with a photoperiod of 16 h light & 8 h dark.

3.3. Results & Discussion

3.3.1. Molecular identification of *G. nervosa*

The universal DNA markers employed for identification of plant species should contain significant differences in the sequences so as to differentiate at species level and simultaneously possess conserved regions to demonstrate minimum variability among same species (Kress & Erickson, 2008; Taberlet et al. 2007).

Using universal *rbcL* primer, a single amplified DNA band of 553bp was obtained (Fig. 3-b) as demonstrated by several other workers (Kress & Erickson, 2007; Newmaster et al. 2006). Partial DNA sequence of 220 bp was obtained from DNA sequencing. The sequence of amplified fragment of *rbcL* is depicted in Fig. 3-c. The sequence was analyzed using BLAST (Basic Local Alignment Search Tool) program at NCBI (National Center for Biotechnology Information) using default parameters. The *rbcL* sequence comparison of *G. nervosa* obtained using present study demonstrated 94% identity with *rbcL* sequence of different isolates of *M. paniculata* (Fig. 3-d).

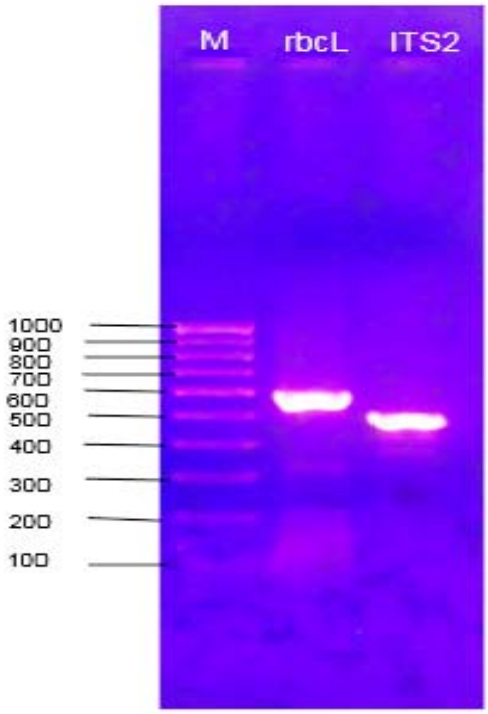
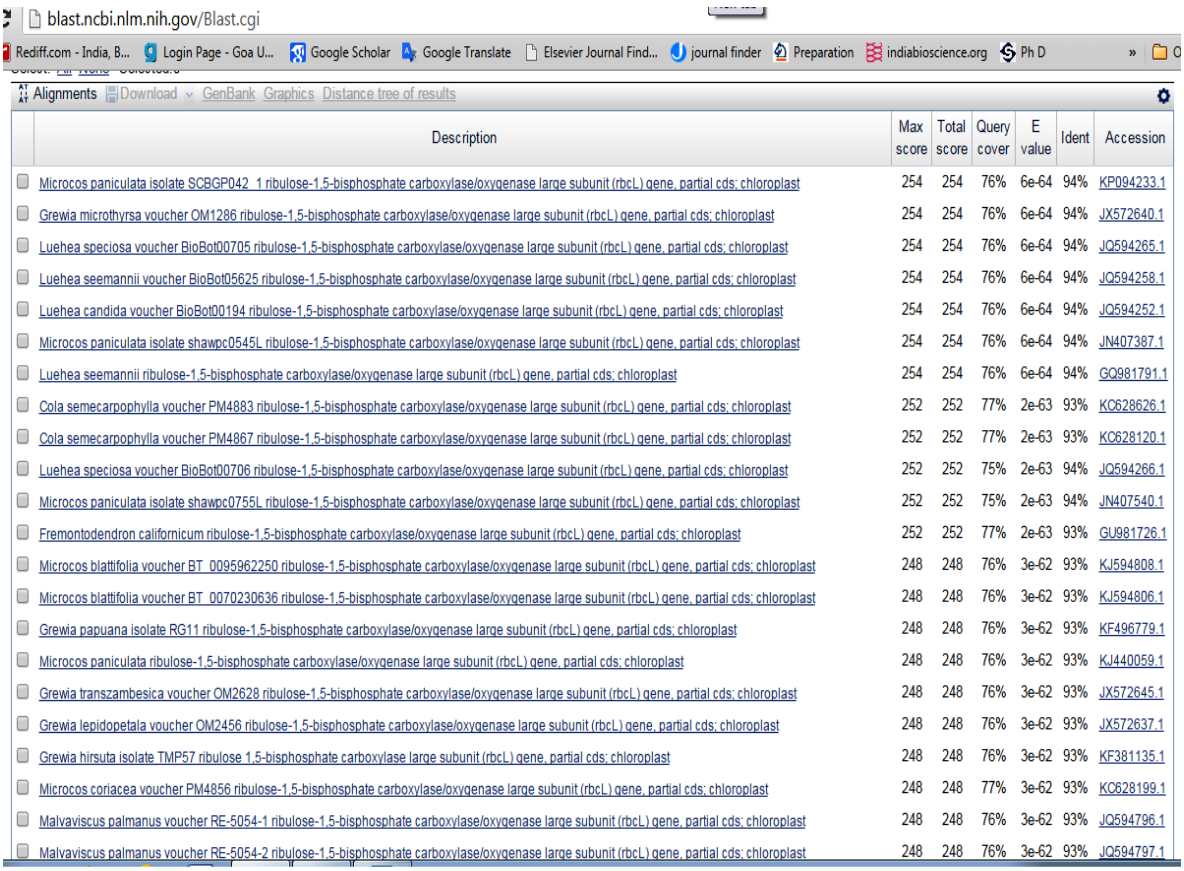


Fig. 3- b: PCR amplification of *rbcL* & *ITS2* of *G. nervosa*, M-100 bp ladder, *rbcL* -553bp and *ITS2*- 490 bp.

```
        10        20        30        40        50
ATTCTCTACGTGTATATGTCGTCAGCGGTGTTAGAGAGTATAAATGTGAC

        60        70        80        90       100
TTATTATACTCCTGATATGAAGTCAAAGATATGAATCATGGCAGCCTTCC

       110       120       130       140       150
GAGTAACTCCTCGACCCGGAGTGCTCTGAGGAAGCAGGAGCCGCGGTAGC

       160       170       180       190       200
TGCTGAATCTTCTACTGGTACATGGACAACCGTGTGGACCGATTCTTTCA

       210       220
CATATAGACAATAGATGCGT
```

Fig. 3- c: *rbcL* sequence of *G. nervosa*



blast.ncbi.nlm.nih.gov/Blast.cgi

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Microcos paniculata isolate SCBGP042_1 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	254	254	76%	6e-64	94%	KP094233.1
Grewia microthyrsa voucher OM1286 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	254	254	76%	6e-64	94%	JX572640.1
Luehea speciosa voucher BioBot00705 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	254	254	76%	6e-64	94%	JQ594265.1
Luehea seemannii voucher BioBot05625 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	254	254	76%	6e-64	94%	JQ594258.1
Luehea candida voucher BioBot00194 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	254	254	76%	6e-64	94%	JQ594252.1
Microcos paniculata isolate shawpc0545L ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	254	254	76%	6e-64	94%	JN407387.1
Luehea seemannii ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	254	254	76%	6e-64	94%	GQ981791.1
Cola semecarpophylla voucher PM4883 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	252	252	77%	2e-63	93%	KC628626.1
Cola semecarpophylla voucher PM4867 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	252	252	77%	2e-63	93%	KC628120.1
Luehea speciosa voucher BioBot00706 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	252	252	75%	2e-63	94%	JQ594266.1
Microcos paniculata isolate shawpc0755L ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	252	252	75%	2e-63	94%	JN407540.1
Fremontodendron californicum ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	252	252	77%	2e-63	93%	GU981726.1
Microcos blattifolia voucher BT_0095962250 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	KJ594808.1
Microcos blattifolia voucher BT_0070230636 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	KJ594806.1
Grewia papuana isolate RG11 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	KF496779.1
Microcos paniculata ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	KJ440059.1
Grewia transzambesica voucher OM2628 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	JX572645.1
Grewia lepidopetala voucher OM2456 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	JX572637.1
Grewia hirsuta isolate TMP57 ribulose 1,5-bisphosphate carboxylase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	KF381135.1
Microcos coriacea voucher PM4856 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	77%	3e-62	93%	KC628199.1
Malvaviscus palmanus voucher RE-5054-1 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	JQ594796.1
Malvaviscus palmanus voucher RE-5054-2 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	248	248	76%	3e-62	93%	JQ594797.1

Fig. 3- d: BLAST results of *rbcL* gene sequence of *G. nervosa* at NCBI.

To identify the variable sites within *rbcL* of *G. nervosa* in comparison to the *rbcL* sequences belonging to various isolates of *M. paniculata*, multiple sequence alignment was done using Clustal W programme of Bio-edit software. The results of Clustal W multiple sequence alignment is shown in Fig. 3-e.

G+C content in the *rbcL* is observed to be constant among different species of plants. Total G+C content of *rbcL* region of *G. nervosa* was 45.5% and is almost equal to the G+C content of *rbcL* reported from other *M. paniculata* isolates as well as %G+C of *rbcL* from plants belonging to other families (Wong et al., 2013). Totally 33 variable sites were

identified in the *rbcL* sequence of *G. nervosa* in comparison to *rbcL* sequences from other *M. paniculata* isolates (Fig. 3-e). Identification of variable sites in the *rbcL* of different isolates belonging to the same species indicates the potential of *rbcL* marker for intra-species discrimination.

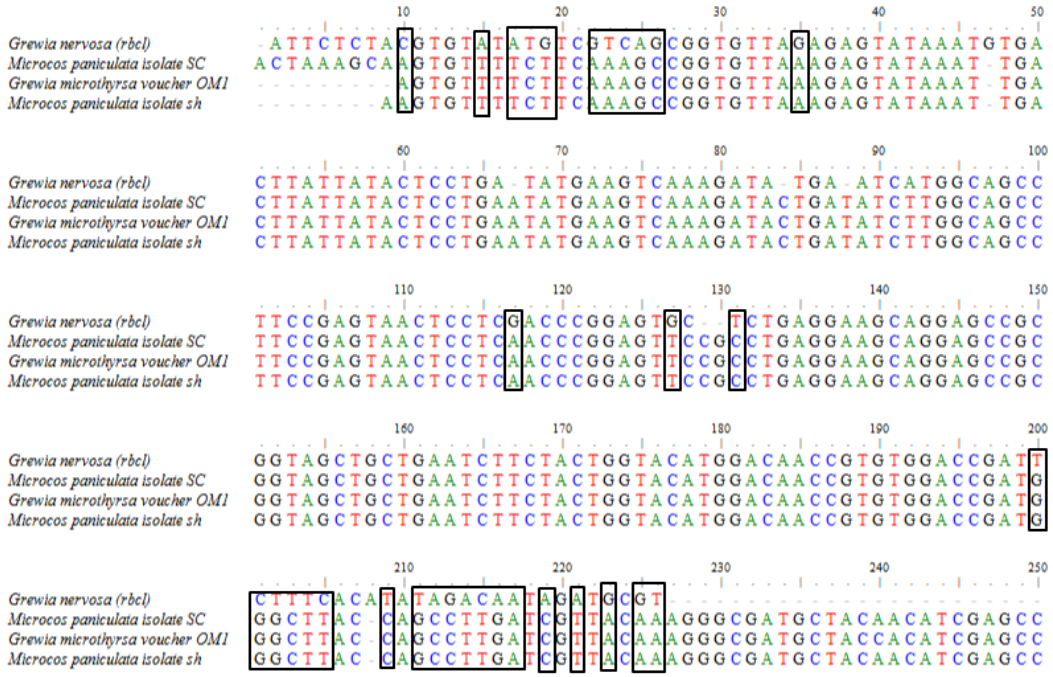


Fig. 3- e: Clustal W multiple alignment of *rbcL* sequence of *G. nervosa* with the *rbcL* sequences from other related plants.

Similarly, amplification of *ITS2* from *G. nervosa* using universal primers resulted in amplification of 490 bp DNA band (Fig. 3-b). The resultant amplified DNA was sequenced and a partial sequence of 316 bp was obtained (Fig. 3-f). *ITS2* sequence was subjected to BLAST at NCBI with default parameters. The partially sequenced *ITS2* of *G. nervosa*

demonstrated similarity to the *ITS2* sequences of *M. paniculata* with maximum identity of 85% (Fig. 3-g).

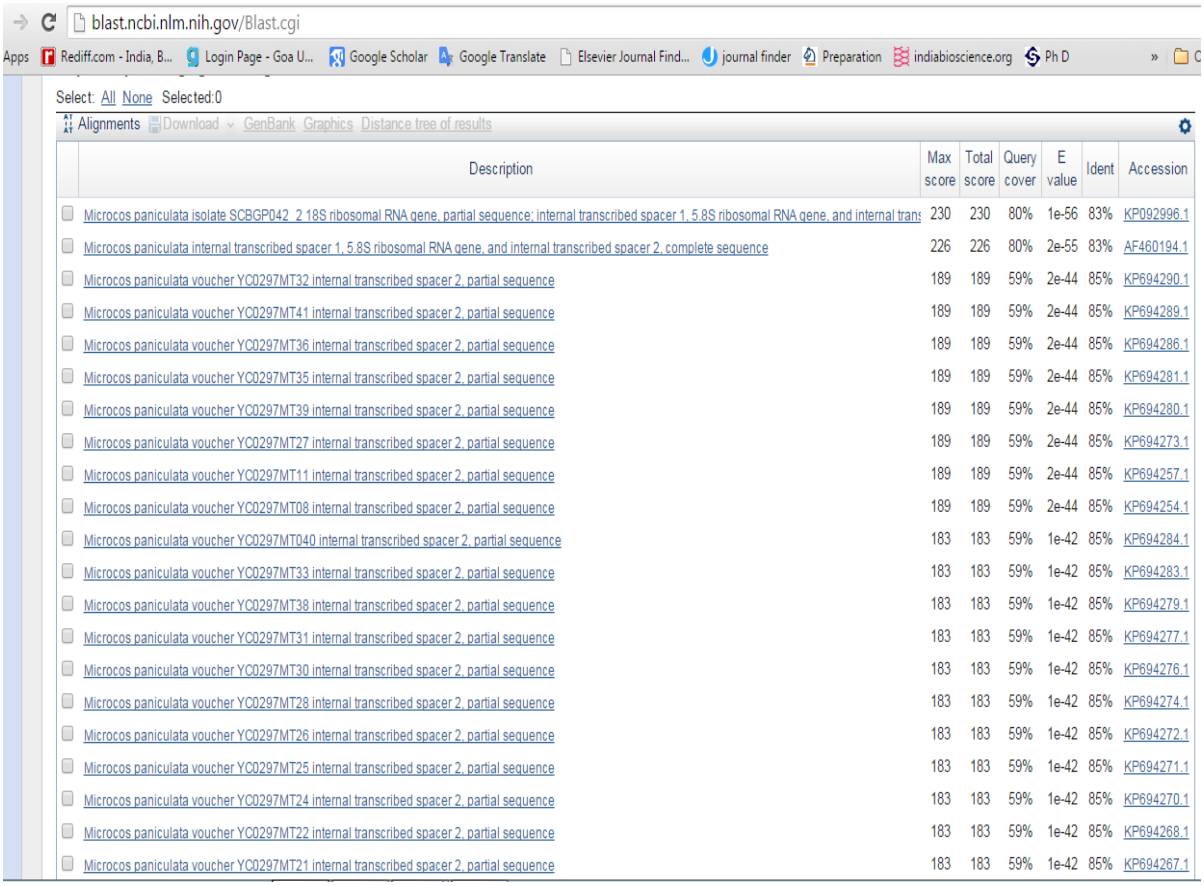
Yo et al. (2010) studied the length of *ITS2* in plants belonging to different groups and concluded that the average length of *ITS2* of di-cotyledon plants is 221 bp. Further, Yo et al. (2010), identified the potential of *ITS2* as marker to differentiate closely related species of dicotyledon plants. Amongst, all markers, *ITS2* has proved to be a standard DNA barcode tool for identification of closely related species belonging to various families and genera. Using *ITS2* marker, Gao et al. (2010), successfully identified several species of plants belonging to family Asteraceae & Fabaceae. Similarly, Pang et al. (2012), identified the *Ephedra* sp. from closely resembling species. Four primers viz. *rbcL*, *matK, psbA-trnH* and *ITS2* were also used to differentiate *Bupleuri radix* from a complex mixture of adulterants. Amongs all markers tested, *ITS2* was reliable and successfully differentiated the *Bupleuri radix* isolate from closely related plants (Chao et al. 201 4).

Multiple sequence alignment of *ITS2* of *G. nervosa* was carried out with *ITS2* sequences from various other isolates of *M. paniculata.* The results of clustal W multiple sequence alignment are shown in Fig. 3-h. Total G+C content of partially sequenced *ITS2* region is 59.1%. 48 variable sites were observed during multiple sequence alignment of the *ITS2* sequences of *G. nervosa* with *ITS2* sequences of the *M. paniculata* isolates.

Moreover, Ashfaq et al. (2013), used three markers viz. *rbcL, matK* and *ITS2* to discriminate between 20 diploid and five tetraploid cotton species (*Gossypium* sp.). The study successfully identified all isolates of *Gossypium* sp. Further, *ITS2* provided correct

identification with success rate of 98.93% followed by 97.7% with *rbcL* and 62.8% with *matK* primers.

Fig. 3- f: Sequence of internal transcribed spacer region 2 (*ITS2*) of *G. nervosa*.



Description	Max score	Total score	Query cover	E value	Ident	Accession
Microcos paniculata isolate SCBGP042_2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	230	230	80%	1e-56	83%	KP092996.1
Microcos paniculata internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	226	226	80%	2e-55	83%	AF460194.1
Microcos paniculata voucher YC0297MT32 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694290.1
Microcos paniculata voucher YC0297MT41 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694289.1
Microcos paniculata voucher YC0297MT36 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694286.1
Microcos paniculata voucher YC0297MT35 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694281.1
Microcos paniculata voucher YC0297MT39 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694280.1
Microcos paniculata voucher YC0297MT27 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694273.1
Microcos paniculata voucher YC0297MT11 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694257.1
Microcos paniculata voucher YC0297MT08 internal transcribed spacer 2, partial sequence	189	189	59%	2e-44	85%	KP694254.1
Microcos paniculata voucher YC0297MT040 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694284.1
Microcos paniculata voucher YC0297MT33 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694283.1
Microcos paniculata voucher YC0297MT38 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694279.1
Microcos paniculata voucher YC0297MT31 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694277.1
Microcos paniculata voucher YC0297MT30 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694276.1
Microcos paniculata voucher YC0297MT28 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694274.1
Microcos paniculata voucher YC0297MT26 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694272.1
Microcos paniculata voucher YC0297MT25 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694271.1
Microcos paniculata voucher YC0297MT24 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694270.1
Microcos paniculata voucher YC0297MT22 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694268.1
Microcos paniculata voucher YC0297MT21 internal transcribed spacer 2, partial sequence	183	183	59%	1e-42	85%	KP694267.1

Fig. 3- g: BLAST of *ITS2* sequence of *G. nervosa* at NCBI.

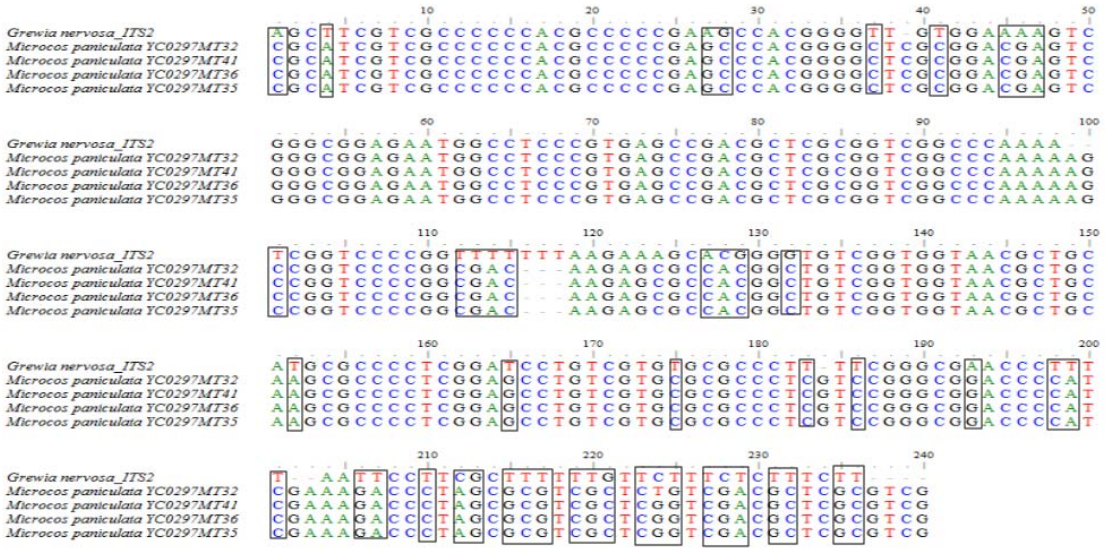


Fig. 3- h: Clustal W multiple alignment of internal transcribed spacer region 2 (*ITS2*) sequence of *G. nervosa* with the *ITS2* sequences of *Microcos paniculata* available at NCBI database.

Data obtained from molecular identification of *G. nervosa* using two different methods such as *ITS2 & rbcL* are conclusive enough for the confirmation of *G. nervosa* and supports the morphology based identification of the *G. nervosa* from the Goa University campus.

3.3.2. Induction of callus from the leaves of *G. nervosa*

Callus induction in *G. nervosa* was carried out by culturing the leaf explant on MS medium supplemented with different additives with various ratios of 2, 4-D & kinetin. The results of callus induction in presence of various ratios of 2, 4-D & kinetin are depicted in the Table 3-IV.

Table 3- IV: Callus induction in *G. nervosa* by using the different ratios of phyto-hormones.

2,4-D+Kinetin (mg/L)	Callus yield %
1.5+0.5	66.66
1.5+1.0	33.33
1.5+1.5	66.66
1.5+0.25	0
1.5+0.25	0
0.25+0.25	80
0.5+2.0	100
0.75+1.5	60
1.0+1.0	100
1.5+0.5	100
2.0+0.5	100
2.5+0.25	100

As soon as the leaf explants were inoculated, the explants started proliferation. New proliferated mass appeared from the axil of leaves that developed into large friable callus after 4 weeks of inoculation. After successful initiation of the callus culture (30 day culturing as shown in Fig. 3-i), the newly formed callus was further sub-cultured on the same medium.

Fig. 3- i: Callus induction from immature leaf explant of *G. nervosa*.

In the present study, *in vitro* induction of callus (Fig. 3-i) and single cell suspension culture (Fig. 3-j & 3-k) from *G. nervosa* was attempted and established successfully. Tissue culture studies have been reported on the other plants that belong to the Malvaceae viz. *Hibiscus sabdariffa* L. (Sylvere et al. 2010), *Gossypium hirsutum* (Ouma et al. 2004), *Theobroma cacao* (Minyaka et al. 2008). MS medium was chosen for callus induction as MS medium has been commonly reported for callus induction especially from medicinal plants (Murashige & Skoog, 1962).

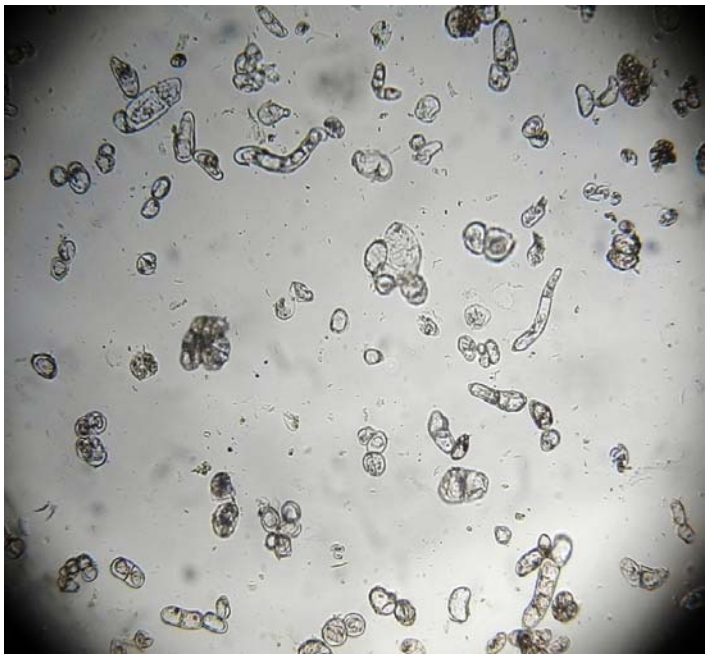


Fig. 3- j: Single cells suspension culture from callus of *G. nervosa*.

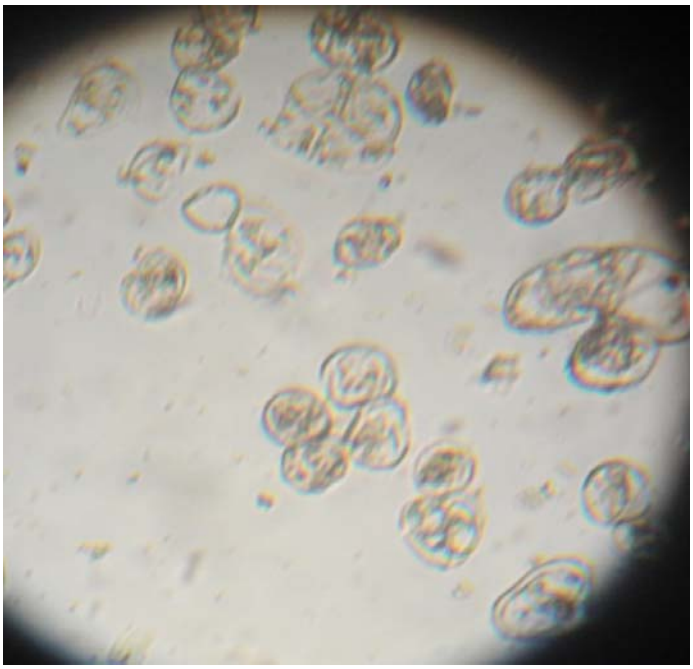


Fig. 3- k: Sub cultured single cell suspension of *G. nervosa*.

In the present study, initially matured leaves of *G. nervosa* were inoculated in to MS medium with 2, 4-D and Kinetin in various ratio (Table 3-IV). However, no callus induction was observed (Fig. 3-l). Infact, the use of matured leaf as an explant resulted in brown coloration of the medium. This is attributed to secretion of phenolic secondary metabolites that were being leached out from matured leaves. Callus induction and growth have been known to be inhibited by phenolic compounds that are leached from explants (Poudyal et al. 2008). The phenolic content reported in the leaves and fruit extracts of *G. nervosa* is 7.11 ±1.76 mgTAE/g (Meena et al. 2013) and 6.28 ± 0.19mg GAE/g (Fu et al. 2010) respectively. To delay or prevent the secretion of phenolics from matured leaves in the medium, activated charcoal was added to the MS medium. Although, Thomas, (2008), has recommended the uses of activated charcoal for adsorption of phenolics especially in plant tissue culture, activated charcoal did not affect the level of callus induction from leaves the of *G. nervosa* (Fig. 3-m). Finally, callus growth was observed from the immature young leaves of *G. nervosa* as less phenolics were produced. Chemical and physical parameters used for induction of callus were similar to those used with matured leaves.

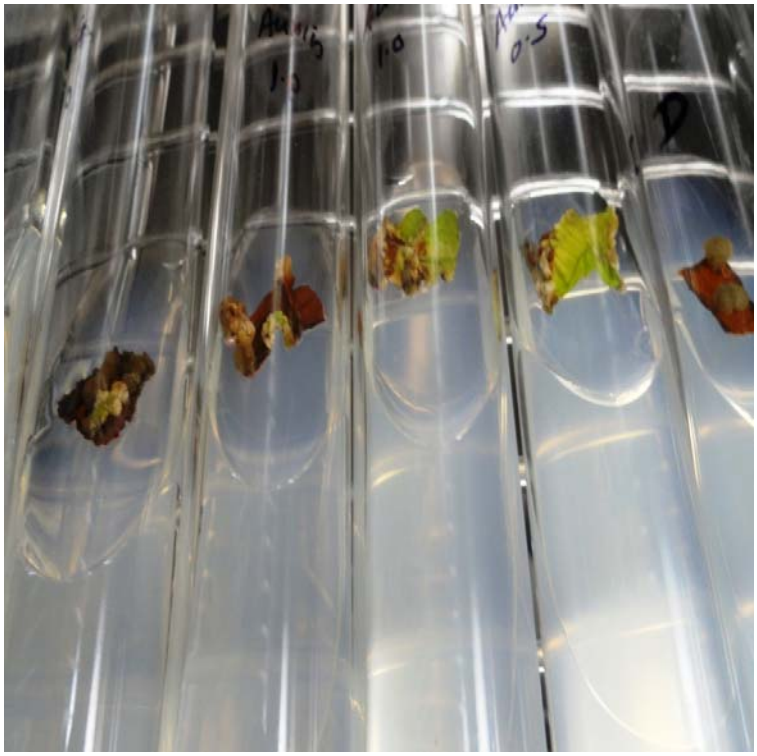


Fig. 3- l: Inoculation of mature leaf explant of *G. nervosa* in MS medium.

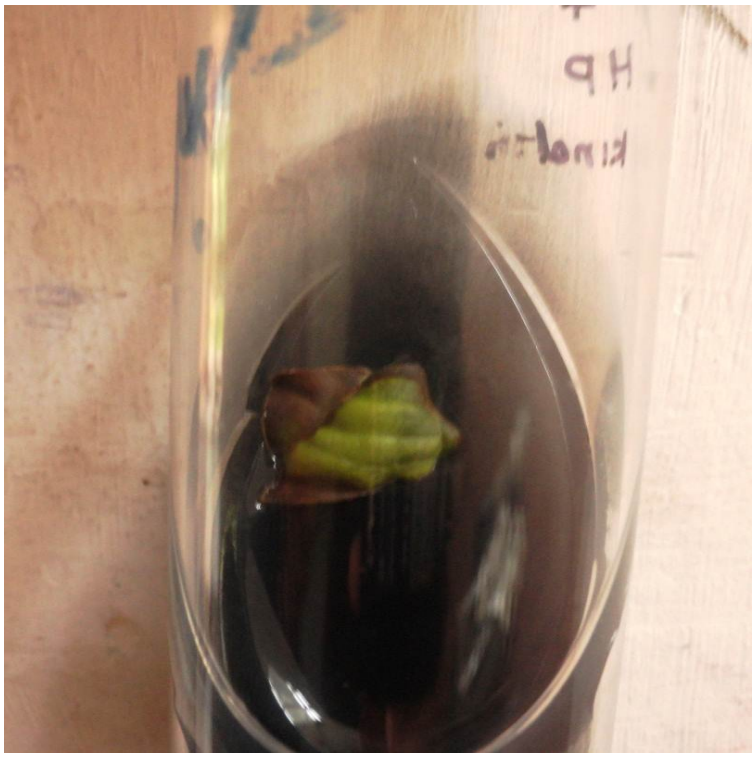


Fig. 3- m: Inoculation of mature leaf explant of *G. nervosa* in MS+ charcoal medium

3.3.2.1. Growth regulators

The use of growth regulators in tissue culture for callus induction and single cell suspension culture are important and can be used for production of secondary metabolites (DiCosmo and Towers, 1984). The leaves have always been an ideal explant for callus induction. The concentration of phyto-hormones in the tissue culture medium is also a crucial step for the standardization of callus induction protocol. Han et al. (2009), summarized the successful callus induction studies in several plants using combination of various phyto-hormones. Use of auxin alone or in combination with cytokine significantly modifies the accumulation or chemical composition of tissue culture product (Mantell & Smith, 1984). Ono & Tian,

(2011), have reviewed the effect of phyto-hormones on callus induction and growth of plants belonging to several families.

In the present study, various combinations of 2.4-D and kinetin were used for callus induction from fresh leaves of *G. nervosa*. Callus induction was observed at all combination of 2, 4-D & kinetin. However, combination of 2, 4-D & kinetin (0.5+2.0mg/L) is most efficient for induction of callus from all the inoculated explants.

CHAPTER 4

EVALUATION OF PHYTOTHERAPY POTENTIAL OF *G. nervosa*

4.1 Source of medicinal plant

G. nervosa plant located on the campus of Goa University (15°27'35.05" N; 73°49'58.46" E, 52 m) served as the source material for obtaining explants. The plant was identified with the help of Prof. M.K. Janarthanam, further deposited in the herbarium collection of Department of Botany, Goa University with the sample number 5312.

4.2 Collection and preparation of plant sample for pharmacological assays

The leaves, bark and roots of *G. nervosa* were obtained from the plant and used as source material for preparation of methanol and aqueous extract. The explants were washed under tap water and dried under shade for 48 h to eliminate moisture. The explants were extracted with methanol or water by soxhlet extractor for 24 h at 65ºC. The extracts were further concentrated using rota evaporator to produce a thick paste or powder from six different samples *viz*. methanol leaves extract (MLE), aqueous leaves extract (ALE), methanol bark extract (MBE), aqueous bark extract (ABE), methanol root extract (MRE) and aqueous root extract (ARE). Callus obtained by tissue culture of leaves, used as a source material and also extracted in methanol and water as methanol callus extract (MCE) & aqueous callus extract (ACE). All samples were stored in closed air tight bottles at 4 ºC and immediately used for assays.

4.3 Methodology

4.3.1 Anti-oxidant activity of *G. nervosa* extracts

At low pH ferric tripyridyl triazines (Fe III TPTZ) is reduced to its ferrous form (dark blue color), that can be estimated at 593nm. The change in the absorbance is directly related to the total antioxidant power of the extract.

4.3.1.1 Reagents

- 300mM acetate buffer, pH 3.6
- 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 ml of HCL
- 20mM $Fecl_3.6H_2O$
- FRAP reagent (The above three reagents mixed in the ratio of 10:1:1 respectively)
- Standard: 1mM ascorbic acid

4.3.1.2 Procedure

Total antioxidant activity in the plant extracts were measured by ferric reducing antioxidant power (FRAP) assay as mentioned by Benzie & Strain, (1999). All methanol & aqueous extracts of *G. nervosa* were resuspended in acetate buffer and the antioxidantive activity was determined at various concentrations of extracts. Ascorbic acid (100µM-1000µM) was used as standard. Various concentrations (25, 50, 75, 100 µg/ml) of plant extracts were mixed with 3 ml of FRAP solution. The absorption at 593nm was determined at "0" min followed by incubation at 37°C in water bath. Spectrophotometric absorption at 593nm was again determined at 4 min interval. The assays were conducted in triplicate and standard deviation was calculated.

FRAP value of sample (µM) = (Change in absorbance of sample from 0 to 4 minute / Change in absorbance of standard from 0 to 4 minute) X FRAP value of standard (1000 µM).

4.3.2 Determination of anti-inflammatory activity

In vitro inhibition of lipoxygenase activity is routinely used for testing the antiinflammatory activity of medicinal plant or their crude extracts using linoleic acid as substrate. 5-

lipoxygenase is mainly responsible for production of inflammatory leukotrienes in body. High production of these inflammatory agents leads to asthma and allergy that may also lead to death.

4.3.2.1 Reagents

- 0.2M Borate buffer (pH 9.0)
- Linoleic acid solution: 10 µl of linoleic acid was mixed with 30 µl ethanol and 20 ml of borate buffer
- Lipoxidase (Himedia Pvt. Ltd.): 400U/ml
- Ascorbic acid: 10 mM

4.3.2.2 Procedure

The anti-lipoxygenase assay was performed using linoleic acid (Himedia, India) as substrate, lipoxidase (Himedia, India) as an enzyme and 10 mM ascorbic acid as a standard. 2% MLE, MBE, MRE, ALE, ABE, ARE, MCE and ACE of *G. nervosa* re-suspended in 0.2 M borate buffer, (pH 9.0) were used as a test sample to determine the anti-lipoxygenase activity (Shinde et al. 1999).

4.3.3 Determination of anti-lithiatic activity

The *in vitro* anti-lithiatic activity assay was determined by nucleation assay and aggregation assay as mentioned by Patel et al. (2010).

4.3.3.1 Nucleation assay

This assay involves the study of crystallization in the presence and absence of inhibitor. Calcium chloride and sodium oxalate were prepared at a final concentrations of 11.25 mM/l and 7.5 mM/l respectively. Both these solutions were dissolved in Tris buffer (0.05mol/l, pH 6.5) containing 0.15mol/l, NaCl. Different concentrations of a MLE (100 to1000 µg/ml) of *G. nervosa* were resuspended in Tris buffer. 950µl of calcium chloride solution was mixed with 100 µl of plant extracts. Further, 950µl of sodium oxalate solution was added and incubated at 37°C for 30 min. The optical density of the solution was measured at 620 nm. The rate of the nucleation was estimated by comparing the induction time in the presence and absence of the MLE (Hennequin et al.1993; Atmani et al. 2004).

4.3.3.2 Aggregation assay

The method used in the present study is as described by Atmani and Khan, (2000) with some minor modifications. 5 ml of 50 mM sodium oxalate was incubated with various concentrations of MLE (100 to1000 µg/ml) followed by drop wise addition of 50mM calcium chloride to make up the final volume to 10 ml and further incubated at 60°C for 1h. Later, the mixture was incubated overnight on shaker at 37°C. The crystals were harvested by centrifugation and dried at 37°C. The crystals were dissolved in 0.05mol/l Tris and 0.15 M/l NaCl to a final concentration of 0.8mg/ml. The optical density was measured at 620 nm (Masao et al. 2000). The percentage aggregation inhibition rate (Ir) was then calculated by comparing the turbidity in the presence and absence of the plant extract using the formula.

Ir = 1 - (turbidity of sample/turbidity of control) ×100.

In vitro synthesis of calcium oxalate crystals was carried out by slow addition of 1 g of calcium carbonate ($CaCO_3$) to 0.25 g of oxalic acid ($H_2C_2O_3$) with constant stirring at 50°C. After mixing, the solution was incubated for 12 h at room temperature. The formation of calcium oxalate crystals were observed under compound microscopes at 100X magnification. Crystal size and aggregation inhibition effect of *G. nervosa* extract was analyzed by incubating different concentrations of MLE with calcium oxalate crystals at various time intervals (6, 12, and 24 h). The effect caused by MLE on size and aggregation of crystals was observed under compound microscope and compared with control sample (sample not treated with MLE).

4.3.4 Determination of anti-tyrosinase activity

Tyrosinase enzyme found in the melanocyte cells catalyzes the conversion of tyrosine to melanin via several intermediates. The formation of melanin is estimated at 475 nm.

4.3.4.1 Reagents

- 50mM potassium phosphate buffer (pH 5.8)
- 20mM L-tyrosine
- 125u/ml Tyrosinase (SRL Pvt. Ltd.)

4.3.4.2 Procedure

Plant extracts from various explants such as MLE, MBE, MRE, ALE, ABE, ARE, MCE & ACE were mixed at various concentrations (200, 400, 600, and 800µg/ml) with 40 µl tyrosinase and the volume was made to 200 µL using potassium buffer. The mixture was incubated for 37°C for 5 min. Tyrosine (80 µL) was added to mixture followed by incubation

for 30 min at 37°C. Optical density was estimated at 475 nm. The % inhibition of the tyrosinase was calculated using the formula:

$$\% \text{ inhibition} = (A-B)/A \times 100.$$

Where A is the absorbance at 492 nm without test sample, B is absorbance at 492 nm with test sample. All readings were taken in triplicate and standard deviation was determined.

4.3.5 Determination of antiproliferative activity

4.3.5.1 Cell culture of MCF-7 cell line

Breast cancer cell lines (MCF-7 maintained in 10% Di-methylsulfoxide (DMSO) at -80ºC was used for the MTT assay. The MCF-7 cell line was taken from the stock and re-suspended in 5 ml of RPMI medium (Roswell Park Memorial Institute medium) under aseptic condition. The cell suspension was centrifuged at 2000 rpm for 2 minutes. The supernatant was discarded and cell pellet was re-suspended in 10 ml of fresh RPMI medium. The cell suspension was added to the culture plate and rotated clockwise and anticlockwise for uniform suspension of the cells. The plate was incubated in CO_2 incubator calibrated to 37ºC, 5% CO_2 and 32% relative humidity (RH).

4.3.5.2 Sub culturing of MCF-7 cell line

The stock culture plate was observed under microscope (10X) to determine the colony formation. The spent medium from the culture plate was decanted and 1.5 ml of trypsin-EDTA (Himedia Pvt. Ltd.) was added and further incubated in CO_2 incubator for 5 min. The adhered cells in trypsin-EDTA were detached from the plate surface using pipette by slanting the plate. The cells were re-suspended into fresh RPMI medium. 5 ml of cell suspension was

taken on a haemocytometer slide for counting the cells under compound microscope. In the present experiment, $1x10^4$ cells/well re-suspended in RPMI medium was added to 24 cell culture well plate. The plates were incubated in CO_2 incubator for 24 h.

4.3.5.3 MTT assay (Cell proliferation assay)

Living cells oxidizes 2-(4, 4-dimethyl-2-tetrazoyl)-2, 5-diphenyl-2, 4-tetrazolium salt (MTT) to form colored insoluble formazon. The formation of formazon can be quantified spectrophotometrically at 596 nm and directly correlates to the number of viable cells in the medium.

4.3.5.3.1 Reagents

- MCF-7 breast cancer cell line
- RPMI-1660 medium (Genetic Biotech Asia Pvt ltd.)
- 10% Fetal bovine serum (FBS)
- 2-(4, 4-dimethyl-2-tetrazoyl)-2,5-diphenyl-2,4-tetrazolium (MTT) dye
- DMSO

4.3.5.3.2 Procedure

The effect of MLE, MBE, MRE, ALE, ABE and ARE on the cell proliferation and cell cytotoxic was determined using MCF-7 cell line. For MTT assay, MCF-7 cells ($1x\ 10^3$) were seeded in a 96-well plate (triplicate) and were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 in a humidified incubator incubated for 24 h, prior to the addition of respective plant extracts (0 day). After 24 h, plant extracts were added at 200 or 400 µg/ml and incubated further for 5 days. The effect of plant extract on cell

proliferation and cell cytotoxicity was determined on day 0, 3 and 5. The MTT dye was added to the wells and incubated at 37°C for 1 h. MTT solution was removed followed by addition of DMSO to each well. The plates were then shaken and optical density was determined using plate reader at 595 nm to determine cell proliferation. The cell viability was determined using the formula:

$$\text{Viability } \% = (\text{Optical density of sample/optical density of control}) \times 100.$$

The cytotoxicity was calculated by comparing the absorbance between the test sample and the control. IC_{50} values were calculated as the concentrations of plant extracts that demonstrated 50% inhibition of cell proliferation on tested cell line. The experiments were performed twice in triplicates and standard deviations were calculated for each data.

4.3.5.4 Trypan blue cell death assay

This is a simple colorimetric assay to check the cytotoxicity of compound or crude extracts on cancer or normal cell line. Trypan blue, a diazo dye is used to stain the cells of the medium. Dead cells are stained by trypan blue dye whereas viable cells remained unstained. Thus the number of dead stained cells can be quantified using a haemocytometer.

4.3.5.4.1 Reagents

- MCF-7 breast cancer cell line
- RPMI-1660 Medium
- Trypan blue dye

4.3.5.4.2 Procedure

The trypan blue assay was conducted as described by Blair, 1985. Typan blue assay was carried out with the MLE, MBE & MRE of *G. nervosa*. The number of non-viable cells were checked at 200µg/ml & 400 µg/ml of plant extracts. The MCF-7 cell suspension was prepared (approx10^4 cells/ml) and 100 µl was seeded into a 96 well micro titer plates and incubated at 37˚C. The spent media was removed and fresh RPMI medium was added to each well and further incubated for 24 and 48 h. Later, the spent medium from the well was decanted and 50 µl of trypan blue was added to each well. After 30 sec, trypan blue was completely removed from the each well and after which MCF-7 cells observed under microscope. Viable cells exclude trypan blue, whereas dead cells stain blue due to uptake of trypan blue. The total number of cells as well as stained cells were counted.

4.3.6 Anti-diabetic assays

Type-2 diabetes is the most common form of endocrine metabolic disorder characterized by chronic hyper-glyceamia (high blood glucose level) in which the human body fails to produce enough insulin (Patel et al. 2012). The α-amylase and α-glucosidase are carbohydrate digestive enzymes that hydrolyzes dietary starch and are responsible for augmentation of postprandial glucose. Therefore drugs that inhibit these key enzymes is one of the main approach to tackle diabetes (Mohamed et al. 2012). α-amylase hydrolyses alpha bonds of large, α-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose whereas α-glucosidase help in more absorption of carbohydrates in the gut, thereby increasing postprandial glucose levels.

4.3.6.1 α- amylase inhibition assay

Estimation of the α-amylase activity by DNSA method is based on the principle that di-nitro salicylic acid (DNSA) reacts only with reducing sugars (eg. glucose) instead of non-reducing sugars (eg. sucrose). The α-amylase act on long chain carbohydrate sugars substrate such as starch that beak down into simple reducing sugars such as glucose and maltose. Addition of DNS reagent reacts with reducing sugars producing a colored complex, 3-amino-5-nitrosalicylic acid that strongly demonstrate absorption at 540 nm.

4.3.6.1.1 Reagents

- Acarbose, (Fluka Pvt. Ltd.)
- 20 mM Phosphate buffer (pH 6.9)
- α- amylase (SRL Pvt. ltd. India)
- Maltose (Himedia Pvt. ltd.)
- 3, 5-dinitrosalicylic acid (Himedia Pvt. ltd.)
- Starch (Sigma-Aldrich Pvt. ltd.)

4.3.6.1.2 Procedure

The α-amylase inhibition activity was spectrophotometrically determined using 3, 5-dinitrosalicylic acid method as described by Miller, 1959. Various concentrations (50µg/ml to1000 µg/ml) of MLE, MBE, MRE, MCE, ALE, ABE, ARE and ACE were evaluated for inhibition of α-amylase activity. The extracts of *G. nervosa* were incubated with 15µl of α-amylase (1U/ml) and the volume was adjusted to 2 ml with addition of 20mM phosphate buffer (pH 6.9) followed by additional incubation at 37°C for 30 min. 1ml of starch solution

(1% w/v) was added and further incubated for 15 min at 37°C. The reaction was stopped by addition of 1 ml DNSA reagent, followed by heating in boiling water bath for 5 min. Two blanks were set, one is without addition of plant crude extracts and other is without addition of α-amylase. The absorbance was estimated at 540nm. 0.2% (w/v) maltose was used as standard. Acarbose (Fluka, Sigma-Aldrich Pvt. Ltd. & Glucobay®50, Bayer pharmaceuticals Pvt. Ltd.) was taken as a standard inhibitor of α-amylase. Results were expressed as % inhibition of α-amylase activity and is defined as the % decrease in the maltose production rate over the control and was determined by using the following formula.

%Inhibition = 100 - {[(maltose) test/ (maltose) control] × 100}.

4.3.6.2 α- glucosidase inhibition assay

α- glucosidase enzyme act on p-nitrophenyl-α -D-glucopyranoside (PNPG) substrate and produce p-nitrophenol (PNP) and D-glucose. The presence of yellow colored p-nitrophenol is estimated at 400 nm using a spectrophotometer.

4.3.6.2.1 Reagents

- α- glucosidase (Sisco Research laboratories Pvt. Ltd.)
- 3mM nitrophenol-α-D-glucopyranoside (PNPG)
- 0.1M Na_2CO_3
- Acarbose (Fluka)
- 20mM phosphate buffer (pH 6.9)

4.3.6.2.2 Procedure

The α-glucosidase inhibition was spectrophotometrically determined using nitrophenol-α-D-glucopyranoside (PNPG) (Kim et al. 2011). α- glucosidase (7.5µl of 0.5 U/ml) was incubated with variable concentrations of plant crude extracts (MLE, MBE, MRE, MCE, ALE, ABE, ARE, ACE) at 37°C in triplicates. After 15 min, 100 µl of PNPG (3mM, pNPG; Himedia Pvt. Ltd.) was added. The reaction mixture was further incubated for 10 min at 37°C. The reaction was terminated by adding 750 µl of Na_2CO_3 (0.1M) and the absorbance was determined at 405 nm. Standard anti-diabetic drug, acarbose was used as a positive control for α-glucosidase inhibition assay. The percentage inhibition of the enzyme activity was determined by using the following formula:

Inhibition rate (%) = {1-(absorbance of test control - absorbance of sample)/ absorbance of control} ×100.

4.3.7 Kinetic analysis of α-glucosidase inhibition

For kinetic studies, α- glucosidase enzyme was incubated for 15 min with various concentrations of MRE of *G. nervosa* (0, 1, 2, 10 µg/ml). After incubation, pNPG was added at different concentrations (0.023-1.9mM) to individual reactions, absorbance was determined at 405nm with the time interval of 3 sec for 180 sec. The Km and Vmax values were determined from Michaelis-Menten graph and type of α-glucosidase inhibition was determined by of Lineweaver–Burk plot.

4.3.8 Statistical analysis

Statistical analysis was performed using Excel from Microsoft Pvt. Ltd. Data was analyzed with student's t-test and ANOVA. Within the single experiment, the significant of each dose

against their respective control values was also evaluated by the student's t-test. Response of α-glucosidase to various doses of plant crude extracts was determined by one-way ANOVA with pairwise Tukey's test to identify differences between specific treatment groups.

4.4 Results & Discussion

4.4.1 Antioxidative activity of *G. nervosa* extracts

Amongst all extracts, only MLE, ALE & MCE demonstrated antioxidative activity as shown in Fig. 4-a.

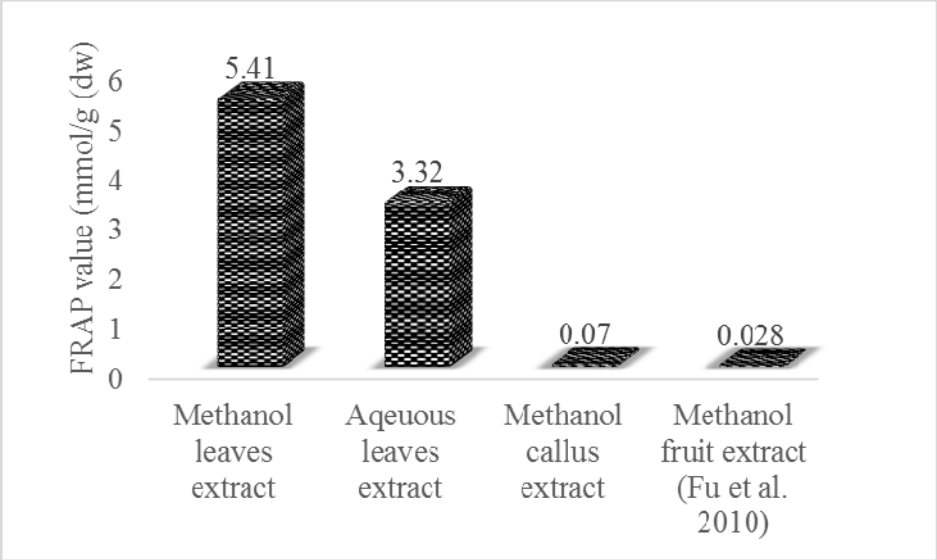


Fig. 4- a: Antioxidative activity of methanol leaves extract, aqueous leaves extract and methanol callus extract of *G. nervosa*.

MLE demonstrated higher antioxidative activity i.e. 5.41± 0.23 mmol /g (dw) compared to MCE & MCE i.e. 3.32±0.45 & 0.07±0.23mmol/g (dw) respectively (Fig. 4-a). The antioxidative activity from the fruit extract of *G. nervosa* has already been reported (Fu et al. 2010). However, the FRAP value (28.5 ± 0.43 µmol/g,dw) observed in the fruit extract

is much lower than the FRAP value of MLE reported in the present study (Fu et al. 2010). Similarly the FRAP values reported by Ksiksi & Hamza, (2012), in various plants belonging to Malvaceae family viz; *Sida acuta* Burm.f (3.70±0.15 mmol/g,dw), *Sida alba* L. (4.06±0.05 mmol/g,dw), *Sida cordifolia* L. (3.47±0.04 mmol/g,dw), *Sida rhombifolia* L.(3.69±0.38 mmol/g,dw), *Sida urens* L. (3.18±0.03 mmol/g,dw) and *Cienfuegosia digitata* Cav. (4.33±0.21 mmol/g,dw) are comparatively lower than the FRAP values reported in MLE of *G. nervosa* in the present study.

Although, ALE also depicted antioxidative activity, it was relatively lower than MLE. Likewise, Sharma & Joshi, (2011), demonstrated higher antioxidative activity in the MLE from *Andrographis paniculata* in comparison to that observed in ALE, Additionally, antioxidative activity from the MLE of *Limonia crenulata* (Roxb.) i.e. 77.89 ± 0.8 % was also higher than the antioxidative activity observed in the aqueous leaves extract i.e. 70.59 ± 2.7 % (Merinal & Viji, 2012).

4.4.2. Anti-inflammatory activity

The methanol and aqueous extracts of *G. nervosa* from leaves, bark, roots and callus were analyzed for the anti-inflammatory activity using the 5-LOX inhibition assay. As observed from Fig.4-b, the MBE demonstrated inhibition of lipoxygenase activity. The ABE as well as the aqueous/methanol extract of leaves, roots & callus did not inhibit lipoxygenase even at higher concentrations. In comparison to positive control i.e. ascorbic acid, anti-inflammatory activity demonstrated by the MBE appears to be promising. The compounds present in the crude extract of bark may demonstrate higher anti-inflammatory activity in its pure form than

ascorbic acid. Further, additional detailed work is required to isolate, purify, and characterize the anti-inflammatory compounds present in the MBE of *G. nervosa.*

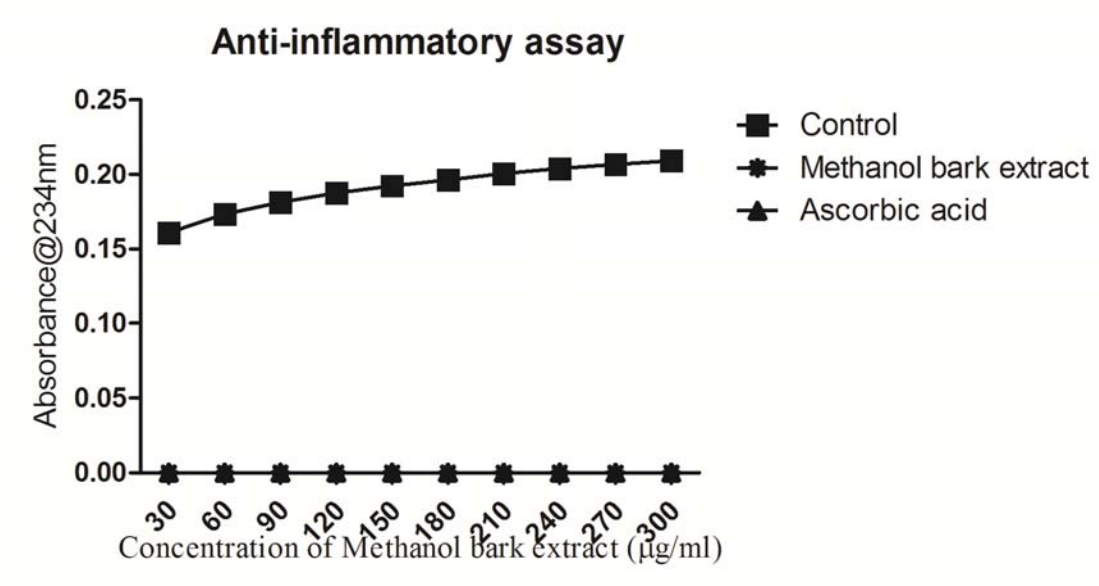


Fig. 4- b: Inhibition of lipoxygenase activities by the methanol bark extract from *G. nervosa.*

Lipoxygenase plays an important role in the pathophysiology of several inflammatory diseases (Wedi and Kapp, 2001). Various chemical constituents originating from plants such as flavonoids, coumarins, quinones, pentacyclic triterpenes, sesquiterpenes, alkaloids and polyacetylates have been reported to inhibit 5- lipoxygenase activity (Werz, 2007). The anti-inflammatory activity in medicinal plants belonging to Malvaceae family such as *Sida acuta* Burm. f, *Sida alba* L., *Sida cordifolia* L., *Sida rhombifolia* L., *Sida urens* L. and *Cienfuegosia digitata* Cav. have also been documented (Kiessoun et al. 2010). Similarly, Schneider et al. (2005), have extensively evaluated the lipoxygenase inhibition from 180 medicinal plants belonging to different species.

4.4.3 Anti-lithiatic activity

Fig. 4-c & 4-d depict the potential of MLE of *G. nervosa* to inhibit the crystal formation as well as aggreagation of CaOx (calcium oxalate). The CaOx is the most common component of kidney stones (Khan, 1992), Thus if nucleation or aggregation of CaOx can be prevented or minimized, kidney stone problem could be prevented. Nucleation rate of the CaOx crystals were observed to decreases in response to increasing MLE concentrations (Fig. 4-c.). Increase in inhibition rate of CaOx crystal aggregation (28.8% to 129%) was also observed with increasing concentration of the MLE (100 µg/ml-1000 µg/ml) (Fig. 4-d). Beghalia et al, (2008), have evaluated the extracts of ten different plant species from West and South of Algeria for inhibitory effect on calcium oxalate crystalization. Extracts of *Erica multiflora, Ammodaucus leucotrichus, Ajuga iva, Atriplex halimus, Erica arborea, Globularia alypum, Stipa tenacissima, Globularia alypum, Chamaerops humilis* and *Tetraclinis articulate* successfully inhibited into formation of calcium oxalate monohydrate crystals. Similarily, Patel et al. (2011), have evaluated the anti-urolithiatic activity of fruits extract of *Piper longum*.

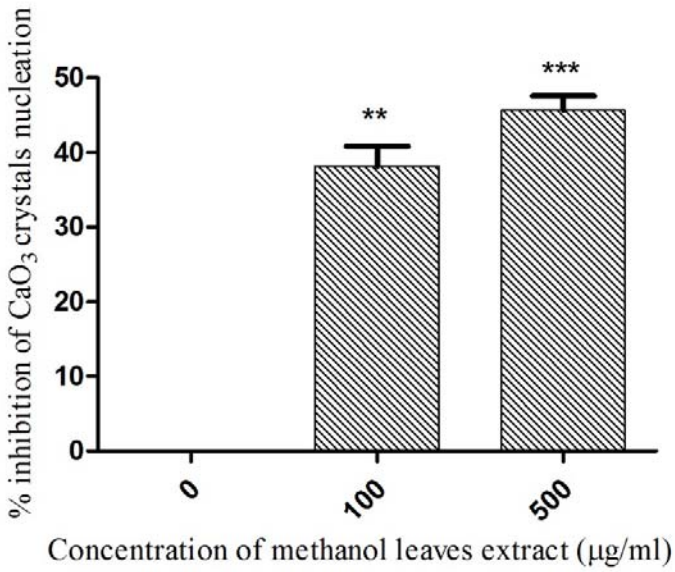


Fig. 4- c: Effect of various concentrations of methanol leaves extract of *G. nervosa* on nucleation of calcium oxalate crystals.

In vitro synthesis of calcium oxalate crystals was carried out by addition of 1 g calcium carbonate ($CaCO_3$) to 0.25 g Oxalic acid ($H_2C_2O_3$) with constant stirring at 50°C. After mixing the solution for 12 h at 30°C, the calcium oxalate crystals were observed under compound microscopes at 100X (Fig. 4-e). MLE did not alter the size and shape of the calcium oxalate crystals even after prolonged incubation for 24 h. Further study is required, to confirm the anti-lithiatic potential of *G. nervosa*.

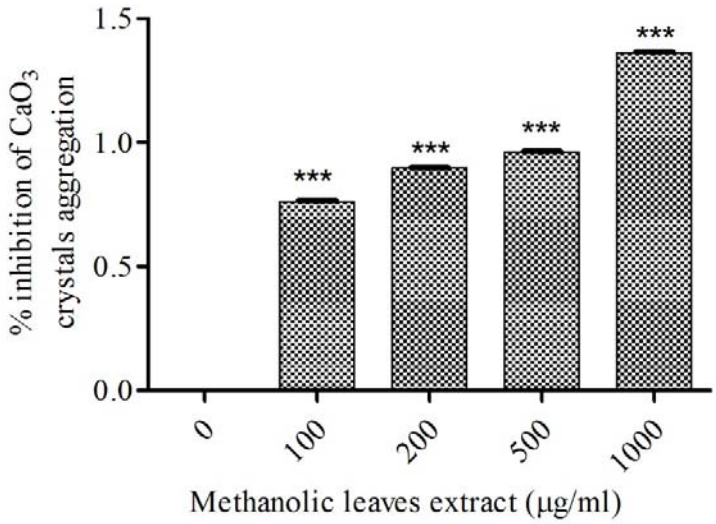


Fig. 4- d: Effect of various concentrations of methanol leaves extract of *G. nervosa* on calcium oxalate crystal aggregation.

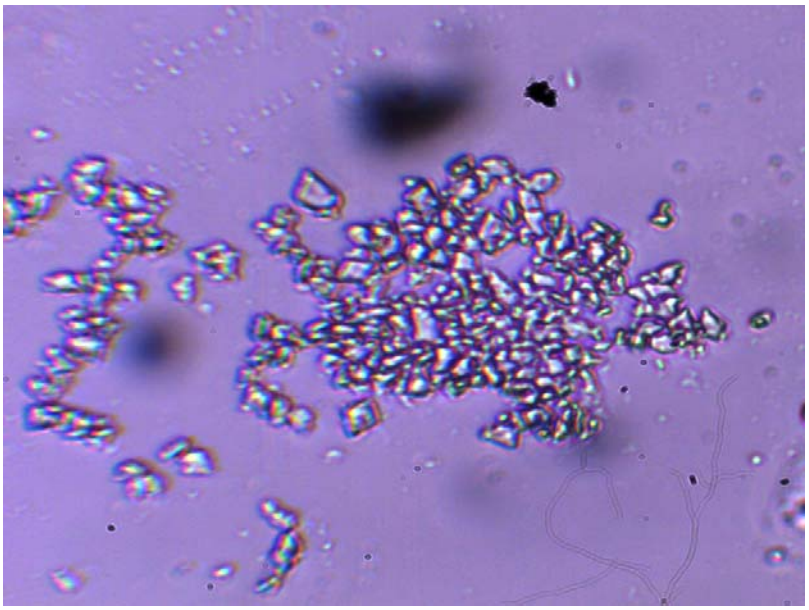


Fig. 4- e: Calcium oxalate crystals under compound microscopes (100X).

4.4.4. Anti-tyrosinase activity

Amongst all the extracts of *G. nervosa* that were tested, only ABE demonstrated dose dependent inhibition of tyrosinase activity (Fig. 4-f). The ABE (600µg/ml) demonstrated 42.24 % inhibition of tyrosinase activity. Thus, *G. nervosa* may be a novel source of a unique compound with anti-tyrosinase activity. Though, anti-tyrosinase inhibition depicted by ABE of *G. nervosa* is weak, it is comparable to the anti-tyrosinase activity reported from different medicinal plants commonly found in India. Vaibhav and Lakshaman, (2012), analyzed the crude extracts (1000 µg/ml) of various common Indian medicinal plants for inhibition of tyrosinase activity. The results indicated that the rhizome extracts of *Glycyrrhiza glabra,* bark extract of *Azadiracta indica*, leaves extract of *Camellia sinensis*, seed extract of *Nelumbo nucifera*, bark extract of *Acasia catechu* and leaves extract of *Mangifera indica* inhibited tyrosinase at 76.53, 43.59, 38.35, 32.76, and 44.4% respectively.

Further out of 263 plant species from Jeju Island, Korea that were evaluated for anti-tyrosinase activity, only 11 species *viz. Myrica rubra* Siebold, *Distylium recemosum*, *Veratrum patulum*, *Morus alba*, *Rhus javanica*, *Cornus walteri*, *Morus bombysis*, *Maackia furrier*, *Toxicodendron succedaneum*, *Limonium tetragonum* and *Sophora flavescence* demonstrated significant tyrosinase inhibition (in the range of 62-87%) at 500 µg/ml of extract (Moon et al. 2010). In a similar kind of study, ethanol bark extract from *Stryphnodendron adstringens* also demonstrated 95% inhibition of tyrosinase activity (Souza et al. 2012).

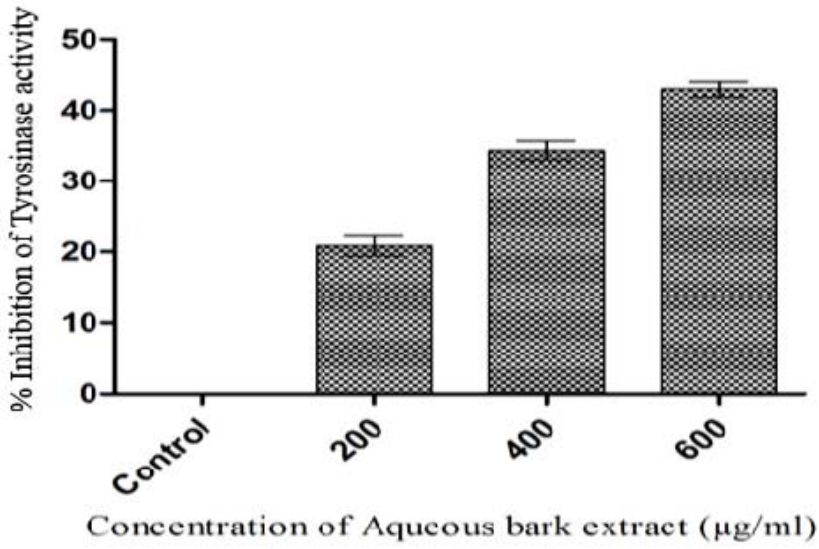


Fig. 4- f: Dose dependent inhibition of tyrosinase activity by aqueous bark extract of *G. nervosa*.

4.4.5 Anticancer activity

4.4.5.1 Anti-proliferation activity of *G. nervosa* extracts on MCF-7 cell line

As a part of ongoing effort to evaluate the potential of *G. nervosa* extracts for various bioactivity, methanol and aqueous extracts of root, leaves, and bark of *G. nervosa* were evaluated for anti-proliferation activity on MCF-7 cell line. Fig. 4-g, depicts the results of antiproliferation studies conducted with 200 and 400 µg/ml of the methanol extracts from various explants over a period of five days. The MRE, MLE, and MBE demonstrated concentration as well as time dependent inhibition of cell proliferation. On third day, in comparison to control (without plant extract), cell line incubated with 200 µg/ml of MRE and MLE depicted 55.8% and 60.12% cell proliferation respectively, whereas 73.66% cell

proliferation was observed in cell line incubated with 200 µg/ml MBE. However, by day five, in comparison to control (without plant extract), curtailed cell proliferation of 50.24% and 50.83% was observed with cell line incubated in MBE and MLE respectively, whereas cell proliferation of 53.63% was observed in presence of MRE. Thus by fifth day, 50% inhibition of proliferation was demonstrated by 200µg/ml, in all the three methanol extracts. The time dependent effect on inhibition of cell proliferation was more evident with MBE in comparision to MRE and MLE.

Further, incubation of MCF-7 cell line with 400µg/ml of MBE, on day three, decrease cell proliferation of 43.63% was observed in relation to normal cell proliferation rate observed in control cell line (without plant extracts) whereas reduced cell proliferation of 56.05% and 61.08% was observed in case of cell line treated with MLE and MRE. Further curtailing of cell proliferation was evident on fifth day, as cell proliferation of 21.01%, and 20.08% was observed in presence of MBE and MLE respectively whereas only 26.57% cell proliferation was observed in presence of MRE.

Inhibition of cell proliferations have also been reported in extracts of *Sida acuta*, *Sida Cordifolium*, *Sida rhombilifolia*, *Urena lobata* and *Viscum album* that belong to Malvaceae family. Addition of 250 µg/ml of methanol extract of *S. acuta* or *U. lobata* to HepG-2 cells line (Human liver cancer cells) resulted in curtailed cell proliferation of 32.98% and 36.85% respectively on third day in comparison to control cell line (Pieme et al. 2010). Additionally, extracts from *Sida acuta* demonstrated cell line specific inhibition of proliferation. The methanol extract (200µg/ml) demonstrated the inhibition of proliferation of cell lines such as

T-549 (95%), BT-20 (97%), and PC-3 (97%), but did not inhibit proliferation of MCF-7 cell lines (Fadeyi et al. 2013).

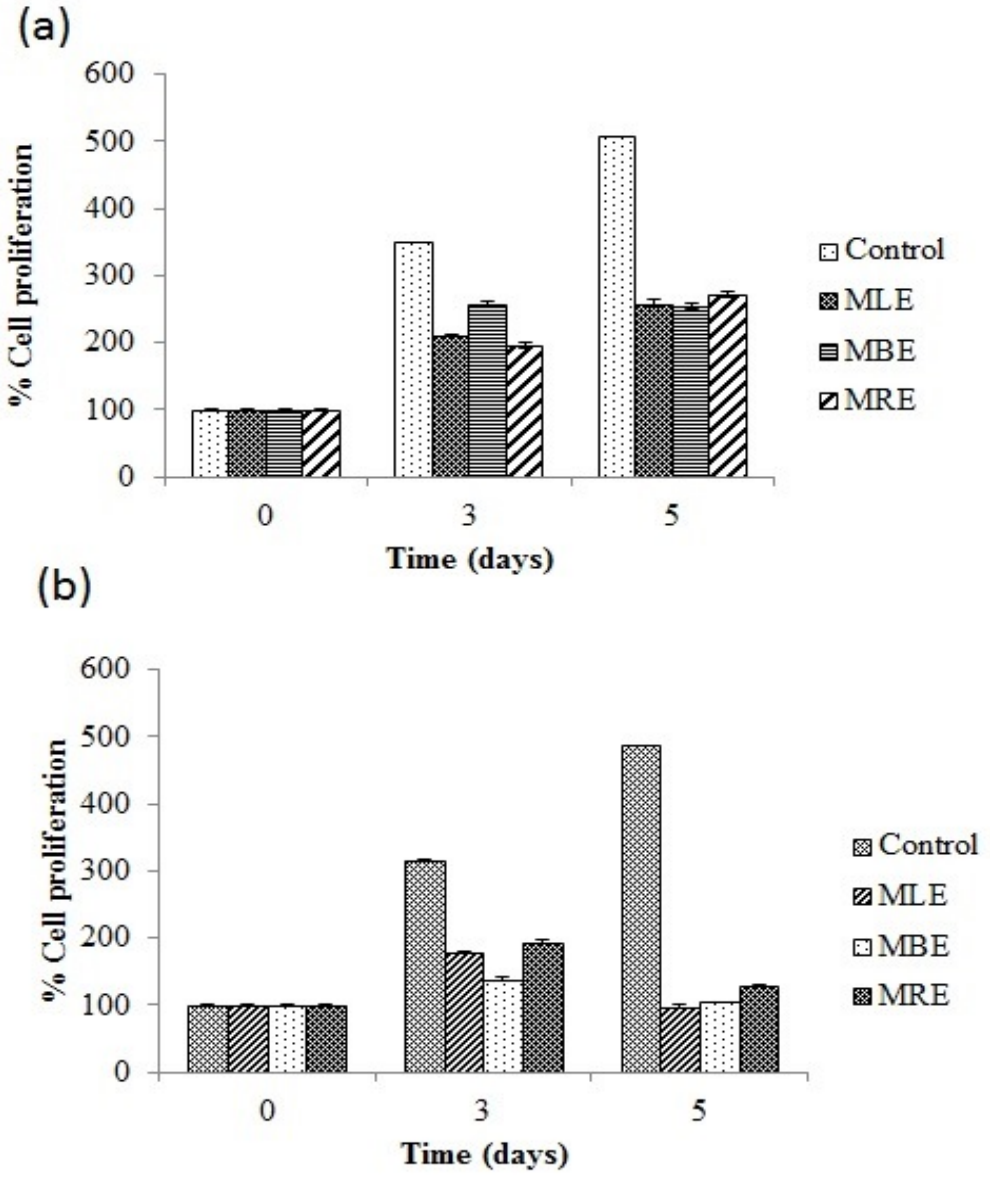


Fig. 4- g: Antiproliferative activity of methanol bark extract (MBE), methanol leaves extracts (MLE) and methanol root extract (MRE) of *G. nervosa* on MCF-7, breast cancer cell line, using MTT assay at (a) 200 µg/ml and (b) 400µg/ml.

The ARE, ABE, and ALE of *G. nervosa* did not inhibit the proliferation of MCF-7 cell line (Fig. 4-h). On the contrary, ABE & ALE marginal augmented MCF-7 cell line proliferation on fifth day. These observations are in concurrence with the results of Talib & Mahasneh (2010), in which, forty four aqueous extracts from sixteen plants did not inhibit MCF-7 cells in comparison to anti-proliferative effect observed with methanol extracts. Thus non-polar compounds are primarily responsible for the antiproliferative activity in these plants. Antiproliferative activity have been commonly associated with non-polar compounds and have been reported in *Schisandra sphenanthera, Scutellaria barbata, Achillea santolina*, and *Typhonium flagelliforme* (Abu-Dahab & Afifi, 2007; Huyke et al. 2007; Yu et al. 2007; Lai et al. 2008).

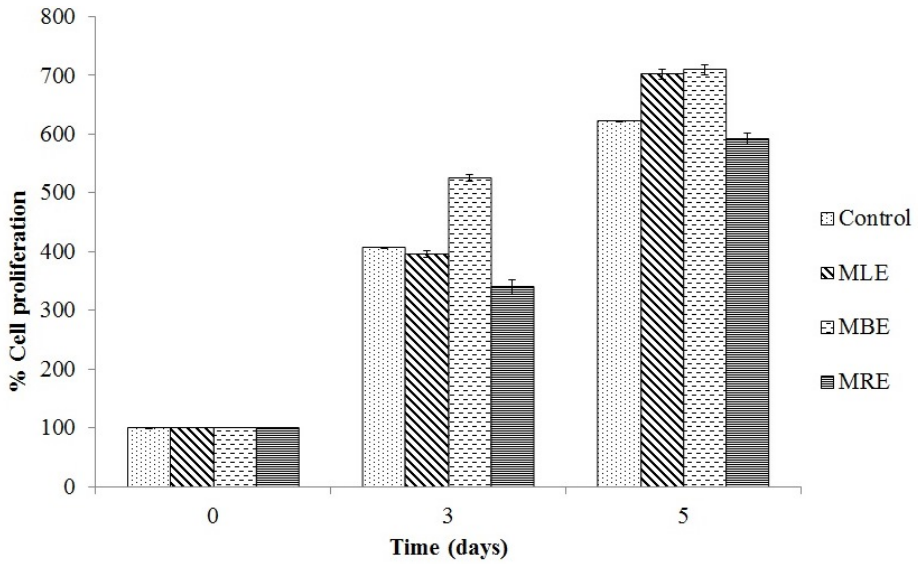


Fig. 4- h: Antiproliferation activity of aqueous bark extract (ABE), aqueous leaves extract (ALE) & aqueous root extract (ARE) of *G. nervosa* (200µg/ml), on MCF-7 breast cancer cell line, using MTT assay

The methanol extracts were further evaluated for cytotoxic activity at various concentrations to determine the IC_{50} value. All the methanol extracts (MBE, MLE and MRE) inhibited cell proliferation and inhibited MCF-7 cell line with IC_{50} value of 850µg/ml for MBE, 1100 µg/ml for MLE and 1200 µg/ml for MRE (Table 4-I). Although the cytotoxic activity depicted by methanol extracts is comparatively weak in comparison to some of the standard anticancer drugs, the cytotoxic activity can be further enhanced by purification of active compound from the *G. nervosa*. IC_{50} for extract of *Urena lobata* belonging to family Malvaceae that was reported for antiproliferative activity on breast cancer (MB-MDA 435) cells was determined as 181mg/ml (Pieme et al. 2012). Similarly, using MTT assay, extract of *G. asiatica* belonging to Malvaceae family also depicted antiproliferation activity against MCF-7 cell line and the IC_{50} value (mg/mL) was reported as 58.65% (Marya et al. 2011). Thus results of the present study on G. nervosa depict significant higher inhibition of cancer cells at lower concentrations than the above reported values.

Table 4- I: Determination of IC_{50} value of methanol bark extract, methanol leaves extract & methanol root extract of *G. nervosa* on MCF-7 cell line.

Concentration (µg/ml) of extracts	% Cell viability in presence of		
	Methanol bark extract (MBE)	Methanol leaves extract (MLE)	Methanol root extract (MRE)
0	100	100	100
200	55.36	61.66	70.26
400	53.05	54.33	68.38
600	52.48	49.49	66.71
800	48.91	49.09	54.33
1000	42.05	46.69	48.68
1200	44.73	56.67	48.37
1400	35.60	46.80	46.58
1600	31.63	38.80	45.11

4.4.5.2 Cell death activity of plant crude extracts

Fig. 4-i, depicts the effect of methanol extracts (MLE, MBE and MRE) on MCF-7 cell death. Compared to typical cell death observed in control cell line (no plant extracts), the presence of 200 or 400 µg/ml of methanol plant extracts resulted in higher cell death on day 3 in comparison to day 5. Addition of 200 µg/ml of methanol extract caused maximum cell death

(52-59%) on day 3 compared to cell death (35-40%) observed on day 5. Further addition of 400 μg/ml of methanol extracts demonstrated higher cell death (56-64%) on day 3 in comparison to cell death (27-36%) on day 5. The concentration dependent cell death was more prominent on day 3 in the presence of 200 or 400 μg/ml *G. nervosa* extracts.

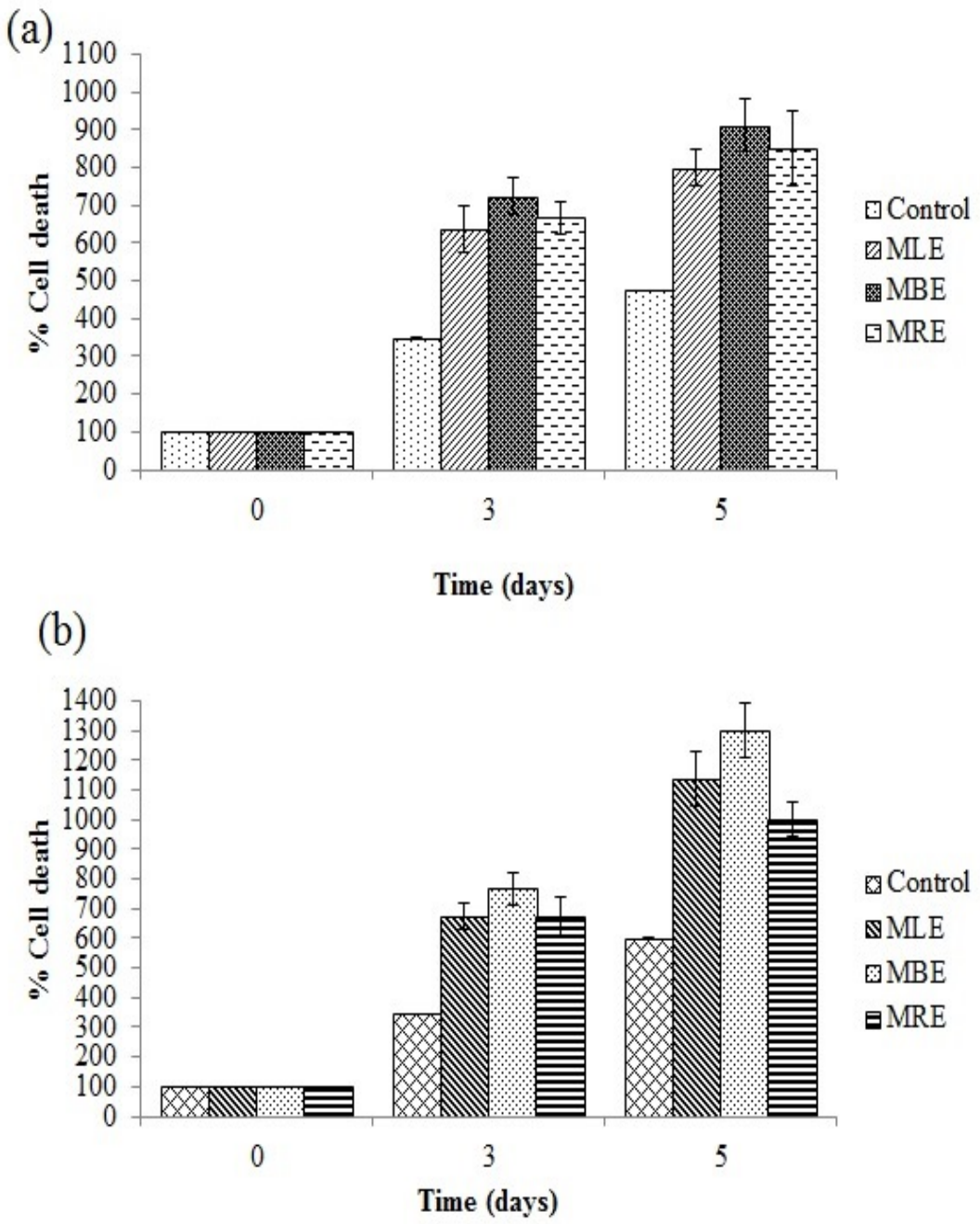


Fig. 4- i: Relative MCF-7 cell staining with trypan blue dye in presence of methanol bark, leaves & root extracts of *G. nervosa* (a) 200 µg/ml and (b) 400µg/ml.

4.4.6 Anti-diabetic activity of plant crude extracts

4.4.6.1 Inhibition of α- amylase activity

Various concentrations of methanol and aqueous extract of leaves, bark, and root (0.05–1mg/ml) of *G. nervosa* were evaluated for their potential to inhibit α-amylase activity. A standard maltose calibration curve was prepared using DNSA assay and was used for estimation of reducing sugars that were released by enzymatic action on starch (Fig. 4-j). Acarbose, a standard anti diabetic drug was used as a positive control and at 100 μg/ml, 91.3% inhibition of α-amylase activity was observed (Fig. 4-k). The MLE at concentration of 1 mg/ml demonstrated 97.5% inhibition of α-amylase activity (Fig. 4-l). Aqueous or methanol extract of bark and root as well as aqueous extract of leaves did not inhibit α-amylase activity. The α-amylase inhibition activity observed in the leaves extract of *G. nervosa* is attributed to the presence of phytochemicals observed in this plant. As reported in the literature, at least one of the phytochemicals such as alkaloids, glycosides, galactomannan gum, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides or terpenoids from medicinal plants have been linked to anti-diabetic activity (Mentreddy, 2007).

As observed from Fig. 4-l, a dose dependent inhibition of α-amylase activity was observed in the MLE of *G. nervosa*. Likewise, 97.5% inhibition of α-amylase activity was also reported from *Cinnamomum tamala* (Kumanan et al. 2010). Additionally, *Hibiscus sabdariffa* belonging to the Malvaceae family have also been reported to inhibit α-amylase activity by 100% at 10 ml/g, fresh wt (Hansawasdi et al. 2000). Recently, 46 medicinal plants depicting very high α-amylase inhibition activity have been extensively reviewed (Sales et al. 2012). The α-amylase inhibition activity observed in *Psidium guajava* var. Pomiferum,

Syzygium cumini Skeels and *Cajanus cajan* were 98% at 200mg/ml, 98% at 200 mg/ml and 100% at 2 mg protein respectively. However, the concentrations that demonstrate α- amylase inhibition activity in above plants are higher in comparison to the reported in present study. Thus MLE of *G. nervosa* demonstrates higher α-amylase inhibition activity at lower concentration.

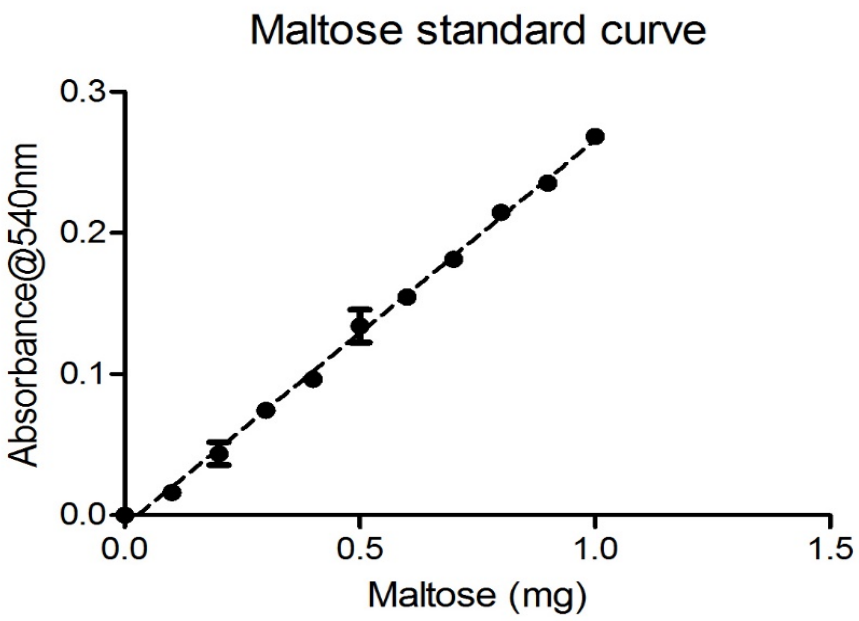


Fig. 4- j: Standard maltose calibration curve.

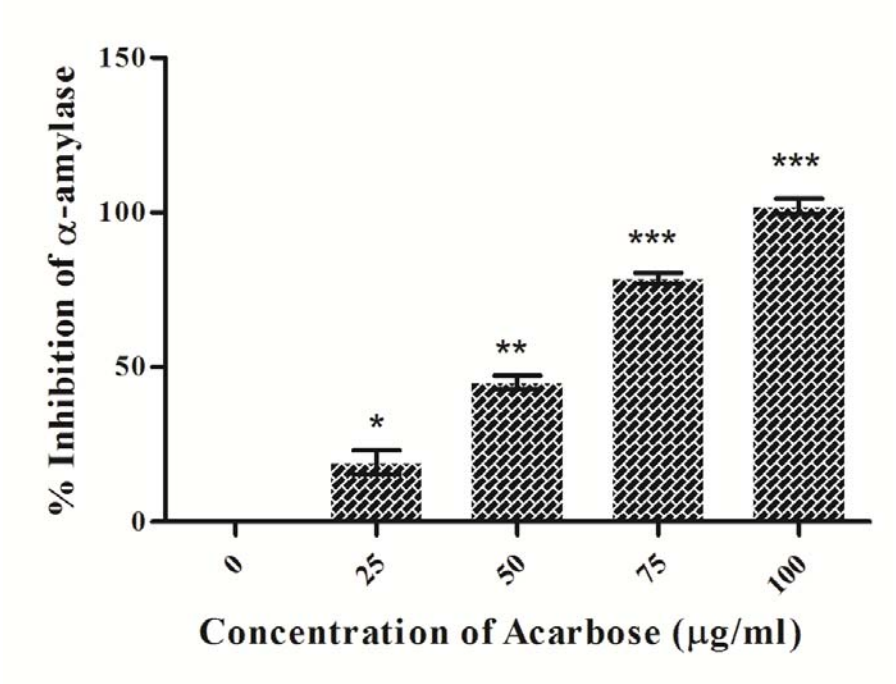


Fig. 4- k: Dose dependent inhibition of α-amylase by Acarbose

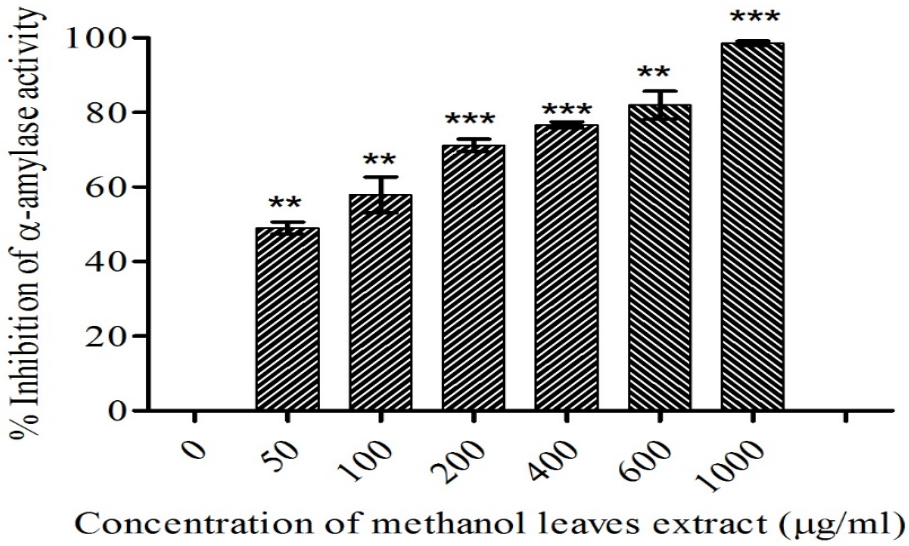


Fig. 4- l: Dose dependent inhibition of α-amylase by methanol leaves extract of *G. nervosa*.

4.4.6.2 α- glucosidase inhibition activity

MLE, MBE, MRE, and ARE of *G. nervosa* also demonstrated significant inhibition of α-glucosidase activity. The inhibition of α-glucosidase activity was observed at all the doses of MLE, MBE, MRE and ARE in comparison to their respective controls (t-test). Acarbose, a standard anti diabetic drug was used as positive control and demonstrated maximum 91.3% inhibition of α-glucosidase activity at 400 µg/ml (Fig. 4-m). Dose dependent inhibition of α-glucosidase activity by MLE ranged from 4.4± 0.96% at 7.5µg/ml to 90.87± 2.3% at 60µg/ml. Further one way ANOVA analysis of the % inhibition of α-glucosidase activity observed in the control and different doses of MLE showed significance at $P < 0.0001$ ($F = 323.4$) (Fig. 4-n). Inhibition depicted by MBE ranged from 18.27± 3.2% at 0.5µg/ml to 94.13± 0.11 % at 60µg/ml and significance was justified by one way ANOVA analysis at $P < 0.0001$ ($F = 1425$) (Fig. 4-o). Similarly, inhibition of α-glucosidase depicted by MRE ranged from 11.77± 2.5% at 0.05µg/ml to 95.63± 0.85 % at 0.9µg/ml and was statistically significant at $P < 0.0001$ ($F = 1566$) (Fig. 4-p). Inhibitions of α-glucosidase activity, depicted by ARE ranged from 29.23± 0.97% at 0.2µg/ml to 90.70± 2.17% at 1.6µg/ml. One way ANOVA of the percentage inhibition of α-glucosidase observed in the control and different doses of ARE demonstrated significance at $P < 0.0001$ ($F = 148$) (Fig. 4-q).

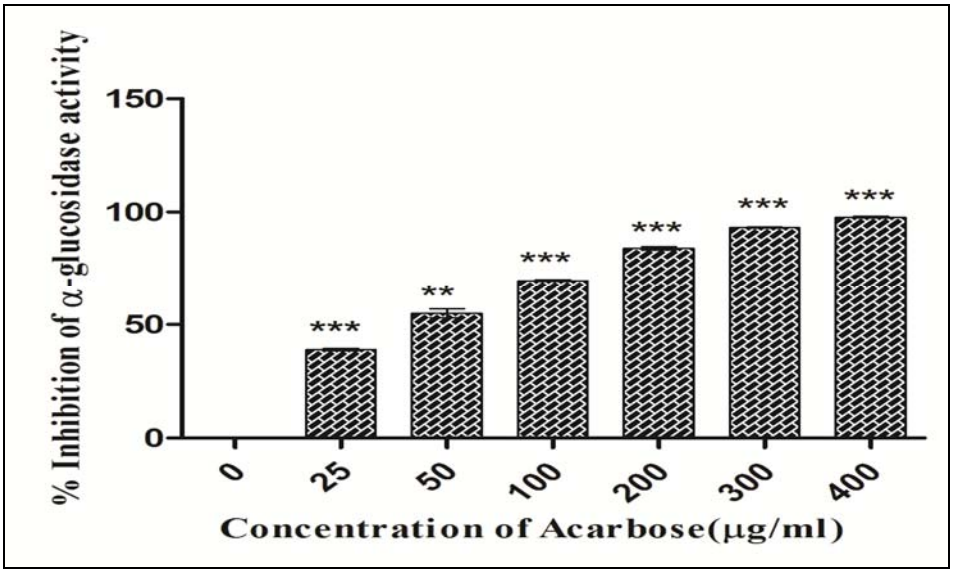


Fig. 4- m: % inhibition of α-glucosidase activity at various doses of acarbose.

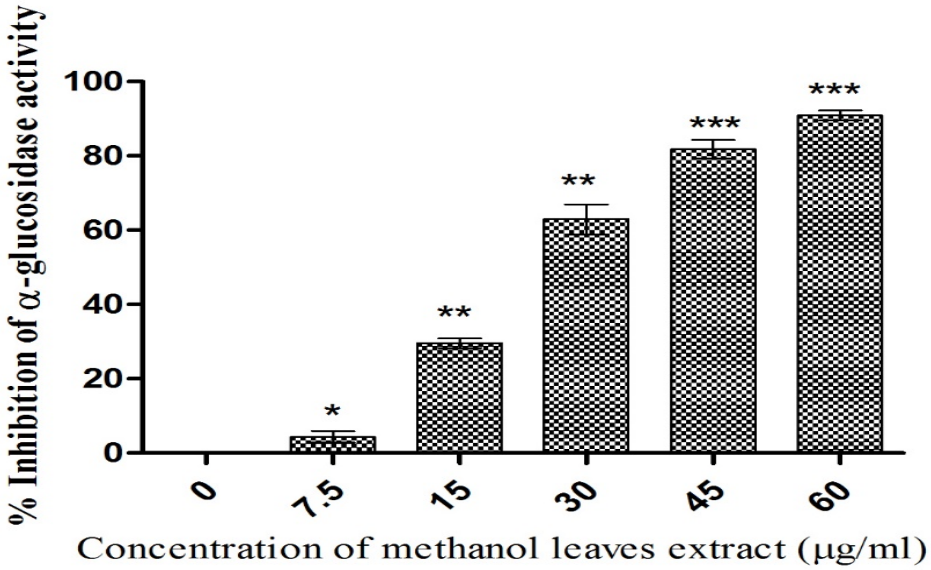


Fig. 4- n: % inhibition of α-glucosidase activity at various doses of methanol leaves extract of *G. nervosa.*

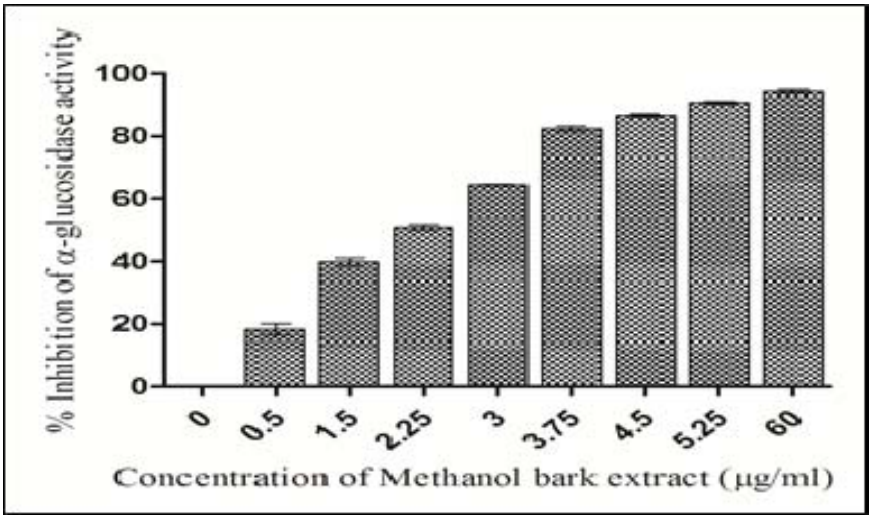


Fig. 4- o: inhibition of α-glucosidase activity at various doses of methanol bark extract (MBE) of *G. nervosa.*

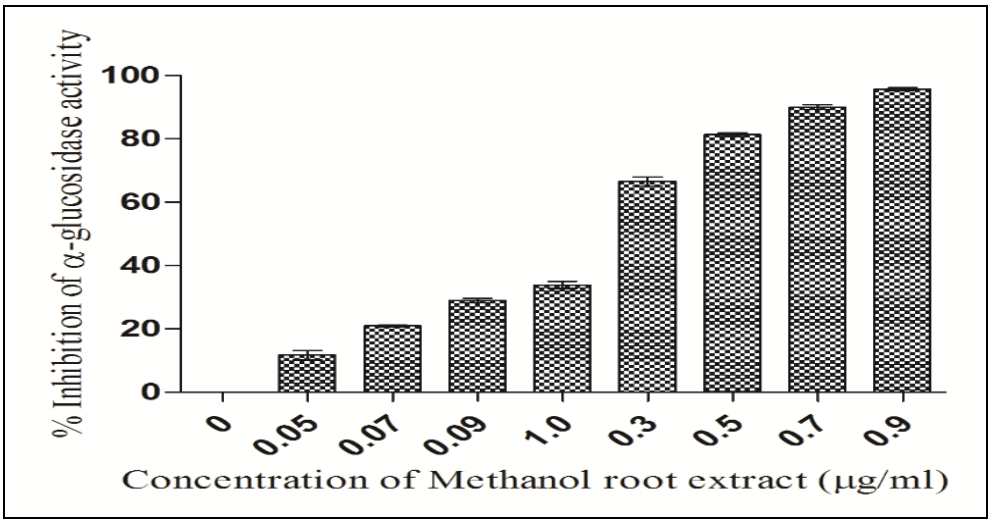


Fig. 4- p: % inhibition of α-glucosidase activity at various doses of methanol root extract (MRE) of *G. nervosa.*

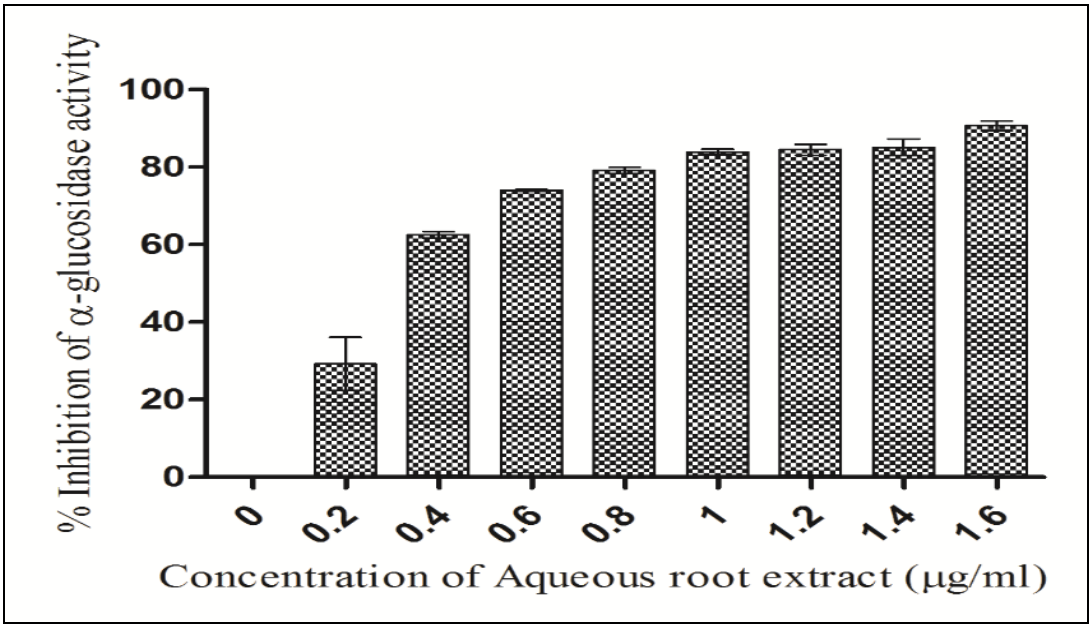


Fig. 4- q: % of inhibition of α-glucosidase activity at various doses of aqueous root extract (ARE) of *G. nervosa.*

In vitro studies using MLE, MBE, MRE, and ARE demonstrated significant inhibition of α-glucosidase, suggesting the presence of potential enzyme inhibiting compound (s) in the *G. nervosa* extracts . The α-glucosidase activity inhibition at various doses of MLE, MBE, MRE, and ARE were compared with each other. A linear increase in percentage inhibition of α-glucosidase activity was observed with the increase doses of MLE, MBE, MRE, ARE and acarbose. The percentage inhibition of α-glucosidase activity by *G. nervosa* plant extracts was highest in MRE (95.63 % at 0.9µg/ml) followed by MBE (94.13 % at 60µg/ml) and MLE (90.87± 2.3% at 60µg/ml). All four extracts of *G. nervosa* demonstrated more inhibition of α-glucosidase activity than the standard anti-diabetic drug, acarbose (97.72 % inhibition at 400µg/ml). The inhibition of α-glucosidase at nano-gram level by plant extracts would definitely help in delaying the absorption of carbohydrate in the

gut, thereby lowering postprandial glucose levels and thus is a potential candidate (s) for evaluation for anti-diabetic studies. *Abelmoschus esculentus, Abroma augusta, Abutilon indicum, Thespesia populnea*, *Hibiscus vitifolius* (Kunnumakkara et al. 2007), *Hibiscus rosa sinensis* belonging to family Malvaceae have also depicted hypoglycaemic effect leading to slowing down of carbohydrate metabolism, decrease glucose absorption released in, therefore less postprandial glucose (Rhabasa-Lhoret & Chiasson, 2004; Oboh et al. 2013).

Inhibition of α-glucosidase activity depicted by *G. nervosa* can be correlated to presence of phytoconstituent such as phenolics, tannins, alkaloids, saponin etc. in *G. nervosa* (Meena et al. 2013). Furthermore several reports, have revealed the major phytochemicals for inhibition of α-glucosidase activity (Gao et al. 2008; Matsui et al. 2001; Tabopda et al. 2008; Du et al. 2006; Mbaze et al. 2007; Adisakwattana et al. 2009).

4.4.7 Enzyme inhibition kinetic of methanol root extract

In order to check the mode of inhibition of α-glucosidase activity by MRE, the Km and Vmax values of control (not treated with plant extracts) were determined using Michaelis–Menten plot followed by comparison of Km & Vmax of α-glucosidase in presence of plant extracts (Fig. 4-r). The mode of inhibition of α-glucosidase by MRE is depicted in the Lineweaver-Burke plot. As observed in Fig. 4-s, the inhibition of α-glucosidase activity by MRE was non-competitive type or the Km value unchanged i.e. Km of control enzyme is 0.10 mM, whereas, Km of enzyme in presence of MRE is 0.09 mM. In the absence of extract Vmax was 0.16 $mM.s^{-1}$, whereas in presence of MRE the Vmax was reduced to 0.06 mM^{-1} (Table 4-II).

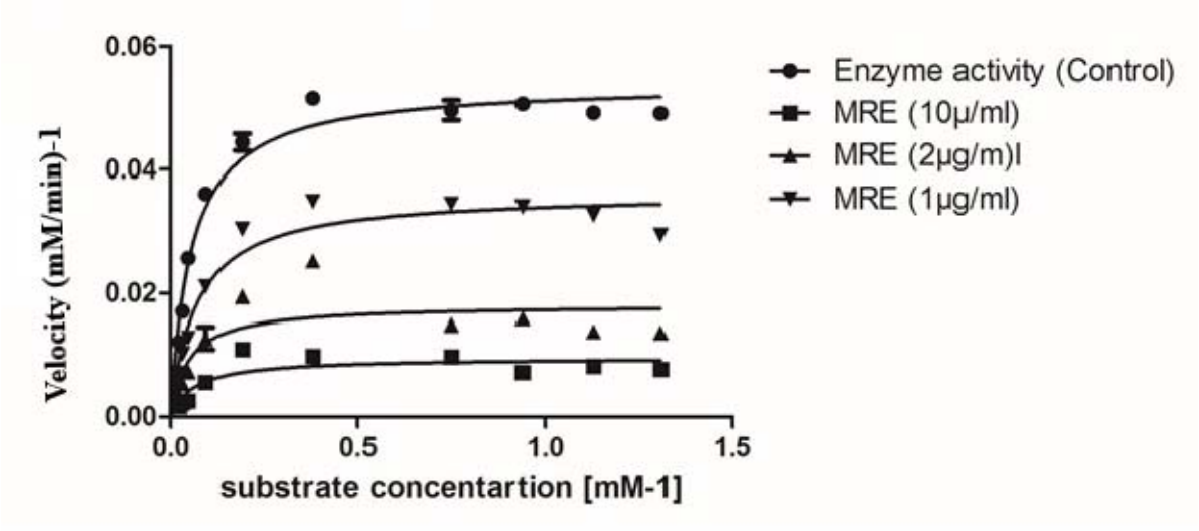


Fig. 4- r: Michaelis–Menten kinetics of α-glucosidase in absence and presence of methanol root extract of *G. nervosa.*

Thus, MRE of *G. nervosa* depicted non-competitive type inhibition of α-glucosidase activity. In non-competitive type reactions, Michaelli's-Menten constant (Km) remains same, whereas maximum velocity (Vmax) of the enzymatic reactions decreases. In this type of reaction, inhibitor reduces the activity of the enzyme irrespective of substrate binding.

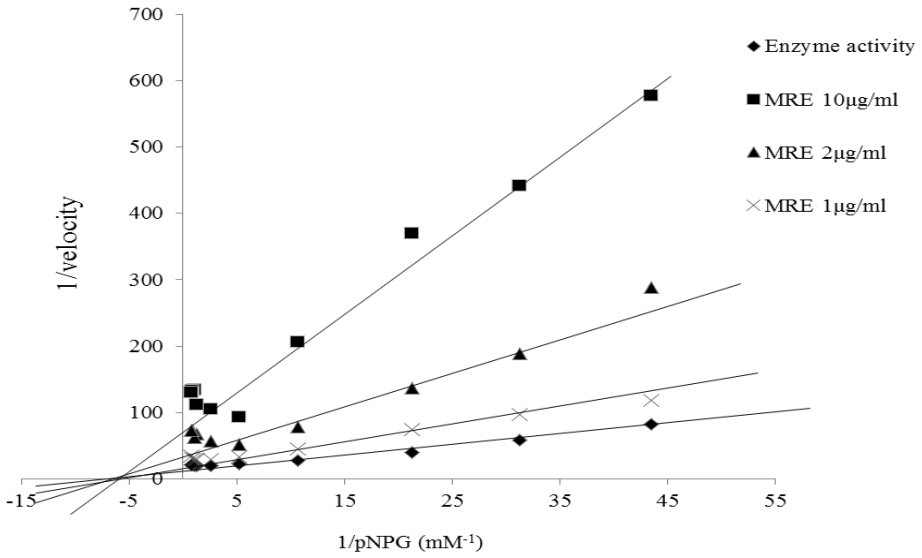


Fig. 4- s: Line-weaver Burk plot depicting the type of inhibition of α-glucosidase with various concentrations of methanol root extract of *G. nervosa.*

Table 4- II: Km & Vmax of the α-glucosidase activity in presence and absence of methanol root extract (MRE) of *G. nervosa.*

	Km (mM)	Vmax $(mM/min)^{-1}$
Control (Absence of MRE)	0.10	0.16
MRE	0.09	0.06

For the management of type 2 diabetes, use of drugs which shows non-competitive type inhibition of α-glucosidase enzyme should be given preference over the drugs that depict competitive type inhibition (eg. Acarbose). Competitive type inhibition depends on the substrate concentration and inhibition potential of these drugs can be overcome by increasing

the concentration of substrate, if a type 2 diabetes patience is given acarbose tablets and he is having excess food with more carbohydrates than the acarbose effect on enzyme would be overcome by higher concentration of carbohydrates, wherase, if a patient taking drug which shows non-competitive type inhibition and simultaneously having higher carbohydrate containing diet, the carbohydrates would not affect the enzyme inhibition potential of drug.

Thus, MRE of *G. nervosa* has better potential to inhibit α-glucosidase than acarbose. The purification and identification of compound in MRE as well as toxicity study would help in drug development for management of type 2 diabetes.

CHAPTER 5

GC-MS ANALYSIS OF METHANOL EXTRACTS OF *G. nervosa*

The GC-MS analysis have been routinely used to analyze and identify the volatile compounds by considering retention time with molecular masses. It is one of the easiest technique to explore the chemical profile of any plant crude extract. Novel compounds and compounds of higher molecular weight cannot be identified by GC-MS technique. This chapter deal with the determination of various secondary metabolites in the methanol extracts of leaves, bark and root (MLE, MBE, MRE) using GC-MS analysis. The compounds that are identified in the methanol extracts can in turn be correlated with potential pharmacological properties.

5.1 METHODOLOGY

Methanol extracts from leaves, bark & root of *G. nervosa* used in previous experiment were used for GC-MS analysis. The dried extracts obtained by soxhlet extracts were re-suspended in methanol at a final concentration of 10 mg/ml. The samples were filtered through whatmann filter paper no.2 to remove any impurities. GC-MS analysis was carried out to identify different phyto-constituents in MLE, MBE and MRE using GC SHIMADZU QP2010 system. The ion source temperature was 200°C and injector temperature was maintained at 250°C. The oven temperature was gradually increase from 40°C (isothermal for 3 min.) to 280°C in the next 10 min. The GC running time was 40 min. Mass spectra were taken at 69kvV with a scan interval of 0.5 seconds in the range of 35–500 m/z. The individual peaks and the retention time were compared with of NIST and Wiley library for the identification of the compounds.

5.2 RESULTS & DISCUSSION

5.2.1 Phytoconstituent detected in MLE

The chromatogram profile of phytochemicals present in the MLE is depicted in Fig. 5-a. α-tocopherol (20.48%), Dendroban-12-one (16.39%), Phytol (14.25%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (α-linolenic acid) (8.20%), n-hexadecanoic acid (4.61%), γ-sitosterol (4.48%), 5,7-dioxatetracyclo[7.4.0.0(3,10)0.(4,8)] tridecane, 2-methylene-11-(1-methylethyl)-1,6,6-trimethyl- (2.80%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (3.19%) and 9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)- (2.77%) were the major phytochemicals detected in MLE of *G. nervosa* (Table 5-I).

α- tocopherol is a well-known antioxidant, associated with anticancer activity (Patra et al. 2001). Similarily, antioxidants present in the extracts of *Rheum officinale* Baill. *Sanguisorba officinalis* Linn. & *Paris polyphylla*. Smith have been linked with cytotoxicity against MCF-7 and AS49, adenoma carcinoma cell line (Li et al. 2007).

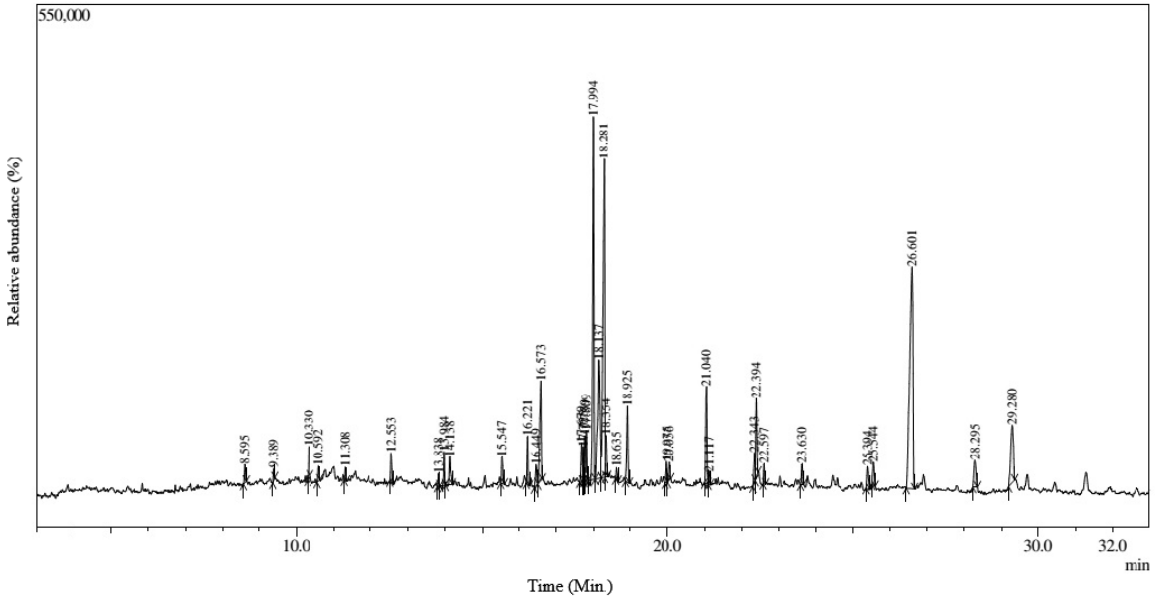


Fig. 5- a: GC-MS chromatogram of methanol leaves extract of *G. nervosa.*

Table 5- I: Compounds identified in the methanol leaves extract of *G. nervosa* by GC-MS.

R. time	Area	Area%	Height	Name	Formula	Mass
8.595	37090	0.55	19347	2-tetrazene, 1,1-diethyl-4,4-dimethyl	$C_6H_{16}N_4$	144
9.389	19970	0.30	12700	Benzofuran, 2,3-dihydro	C_8H8_O	120
10.330	54057	0.81	33168	4-hydroxy-3-methylacetophenone	$C_9H_{10}O_2$	150
10.592	29324	0.44	15593	2-hydroxy-1,3-dimethoxybenze	$C_8H_{10}O_3$	154
11.308	21495	0.32	13888	2-butanone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-	$C_{13}H_{22}O$	194
12.553	56597	0.84	30532	4-methyl-2,5-dimethoxybenzaldehyde	$C_{10}H_{12}O_3$	180
13.838	21038	0.31	13285	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	$C_{11}H_{14}O_3$	194
13.984	50900	0.76	35203	2-propenoic acid, pentadecyl ester	$C_{18}H_{34}O_2$	282
14.138	77941	1.16	28659	4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180
15.547	56800	0.85	27925	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296
16.221	94758	1.41	50261	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
16.449	54237	0.81	21735	Benzenepropanoic acid, 2,5-dimethoxy-	$C_{11}H_{14}O_4$	210
16.573	308967	4.61	104820	n-hexadecanoic acid	$C_{16}H_{32}O_2$	256

17.679	92628	1.38	40876	Oxalic acid, monoamide, N-(4-ethylphenyl)-, pentyl ester	$C_{15}H_{21}NO_3$	263
17.725	74585	1.11	36358	Pyrazol-5(4H)-one, 3-methyl-1-[4,6-bis(dimethylamino)-1,3,5-triazin-2-yl)-	$C_{11}H_{17}N_7O$	263
17.759	77949	1.16	47634	9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292
17.809	95170	1.42	52651	Bicyclo[7.7.0]hexadec-1(9)-ene	$C_{16}H_{28}$	220
17.994	954808	14.25	380754	Phytol	$C_{20}H_{40}O$	296
18.137	549157	8.20	124234	9,12,15-octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278
18.281	1098446	16.39	334521	Dendroban-12-one	$C_{16}H_{25}NO_2$	263
18.354	96939	1.45	43693	Octadecanoic acid	$C_{18}H_{36}O_2$	284
18.635	21237	0.32	12855	5,7-dioxatetracyclo[7.4.0.0(3,10)0.(4,8)]tridecane, 2-methylene-11-(1-methylethyl)-1,6,6-trimethyl-	$C_{18}H_{28}O_2$	276
18.925	187880	2.80	79842	5,7-dioxatetracyclo[7.4.0.0(3,10)0.(4,8)]tridecane, 2-methylene-11-(1-methylethyl)-1,6,6-trimethyl-	$C_{18}H_{28}O_2$	276
20.056	41076	0.61	18978	2-oxa-6-azatricyclo[3.3.1.1(3,7)]decane, 6-(phenylsulfonyl)-	$C_{14}H_{17}NO_3S$	279
21.040	213919	3.19	102123	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330
21.117	29052	0.43	12699	Hexadecanoic acid	$C_{19}H_{38}O_4$	330

22.343	64187	0.96	30750	8-[2-thienyl] theophylline	$C_{11}H_{10}N_4O_2S$	262
22.394	185538	2.77	82082	9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	$C_{21}H_{36}O_4$	352
22.597	39628	0.59	21039	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358
23.630	44839	0.67	21978	Squalene	$C_{30}H_{50}$	410
25.544	67050	1.00	24222	β-tocopherol	$C_{28}H_{48}O_2$	416
26.601	1372161	20.48	233277	α-tocopherol-β-D-mannoside	$C_{35}H_{60}O_7$	592
28.295	103166	1.54	27730	Stigmasterol	$C_{29}H_{48}O$	412
29.280	299893	4.48	60061	γ-sitosterol	$C_{29}H_{50}O$	414

Furthermore, phytol detected in MLE has already been scientifically validated to with antiproliferative activity on hepatocellular cancer cell lines such as Huh-7 and HepG2 (Kim et al. 2015). Furthermore, γ-sitosterol detected in MLE of *G. nervosa* has also scientifically linked as antiproliferative agent against MCF-7 (breast cancer cells) and A 549 (lung carcinoma cells) (Sundarraj et al. 2012). Thus the concomitant presence of these three phytochemicals in the MLE can be correlated to the antiproliferative activity with MCF-7 cell line as demonstrated in chapter 4.

The presence of significant amount of α-linolenic acid (8.20%), an essential ω-3 fatty acid in the MLE is a silent feature, as it has associated with several neuroprotective properties (Blondeau et al. 2015). α-linolenic acid has been observed in various ranging concentrations from 0.19-0.35% in the fruit extract of *Quercus brantis* to 14.79% in whole plant extract of *Peganum harmala* (Khodadoust et al. 2014, Moussa & Almaghrabi, 2015). Additionally, n-hexadecanoic acid detected in the MLE of *G. nervosa* has been reported to be a potent inhibitor of phospholipase A2 and thus MLE would inhibit inflammations (Aparna et al. 2012). The other minor compounds detected in MLE, linked with various pharmacological activities and have been summarized in Table 5-II.

Table 5- II: Bioactivities of minor compounds detected by GC-MS in methanol extract of *G. nervosa*.

Minor compounds detected in MRE, MBE and MLE with their bioactivities	
Benzofuran, 2,3-dihydro	Antiangiogenic activity (Dharmalingam & Nazni, 2013), Antimicrobial, Antiinflammatory (Rajeswari et al. 2013)
Hexadecanoic acid, methyl ester	Antioxidant, Flavor, Hypocholesterolemic Pesticide, 5-Alpha reductase inhibitor (Sermakkani & Thangapandian, 2012)
Benzenepropanoic acid, 2,5-dimethoxy-	Antimicrobial (Gunasekaran et al. 2013)
9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z) (Linolenic acid ester)	anti-inflammatory, hypocholesterolemic, anticancer, Hepatoprotective, nematicide insecticide, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor antiandrogenic, antiarthritic, anticoronary, insectifuge (Merrill et al. 2013)
Octadecanoic acid	Emulsifying agent, solubilizing agent, tablet and capsule lubricant. skin cleansers(http://health.howstuffworks.com/skin-care/cleansing/products/ stearic-acid-in-skin-cleansers.htm)
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	Hemolytic, pesticide, flavor, antioxidant (Gnanavel & Saral, 2013)
9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	Antipyretic, Analgesic, Antiseptic, Anticonvulsant (Gnanavel & Saral, 2013)
Squalene	Cholesterol lowering effect, anticancer (Kelly , 1999)
Stigmasterol	anticancer, antioxidant, hypoglycemic thyroid inhibition activity (Panda et al.2009)

5.2.2 Phytoconstituent detected in MRE

The chromatogram profile of separated constituents from MRE is shown in Fig. 5-b. 6-methylene-1,3-cyclooctadiene (49.18%), 5-trimethylsiloxy (trimethylsilyl) indole (11.12%), n-hexadecanoic acid (10.14%), para-fluorofentanyl (9.70%), β-sitosterol (4.21%) 6-methylenecycloocta-1,3-diene (3.59%) and 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (2.95%) were detected as major phytochemicals present in MRE of *G. nervosa* (Table 5-III).

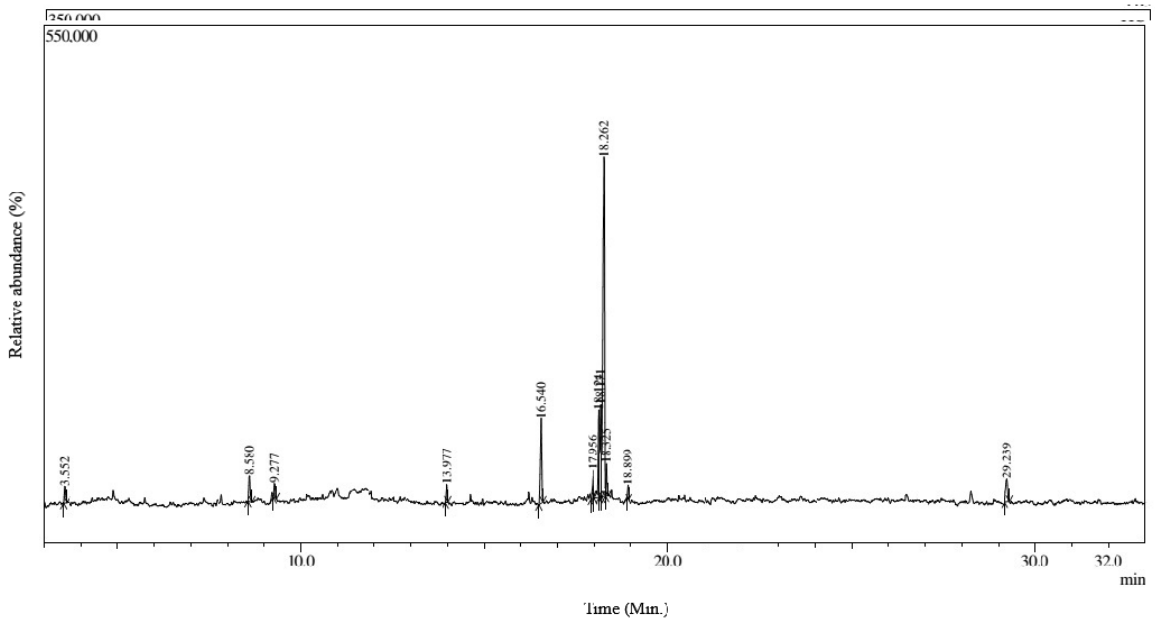


Fig. 5- b: GC-MS chromatogram of methanol root extract of *G. nervosa*.

Table 5- III: Compounds detected in the methanol root extract of *G. nervosa* by GC-MS.

R. Time	**Area**	**Area%**	**Height**	**Name**	**Formula**	**Mass**
8.580	66394	2.95	27488	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$	144
9.277	34376	1.53	16138	6-Methylenecycloocta-1,3-diene	C_9H_{12}	120
13.977	29990	1.33	19499	Ethane, 1,1,2-trichloro-	$C_2H_3Cl_3$	132
16.540	227942	10.14	89982	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
17.956	80819	3.59	30029	6-Methylenecycloocta-1,3-diene	C_9H_{12}	120
18.124	218240	9.70	93531	Para-fluorofentanyl	$C_{22}H_{27}FN_2O$	354
18.171	249995	11.12	98767	5-Trimethylsiloxy(trimethylsilyl)indole	$C_{14}H_{23}NOSi_2$	277
18.262	110605	49.18	36038	6-Methylene-1,3-cyclooctadiene	C_9H_{12}	120
18.325	64083	2.85	34367	Hexadecanoic acid	$C_{18}H_{36}O_2$	284
18.899	27513	1.22	15122	Dehydrocholic acid ((5β)-3,7,12-Trioxocholan-24-oic acid)	$C_{24}H_{34}O_5$	402
29.239	94764	4.21	24628	β-Sitosterol	$C_{29}H_{50}O$	414

Amongst the detected compounds from MRE, only few compounds could be associated with medicinal properties. The presence of β-sitosterol in MRE has been extensively reviewed and associated with several pharmacological activities. β- sitosterol (4.21%) detected in MRE is of great pharmacological importance has been reported to demonstrate anti-inflammatory activity (Prieto et al. 2006; Loizou et al. 2010), inhibition of MCF-7 cells (Breast cancer) proliferation (Chai et al. 2008) and *in vitro* cytotoxic effects on HT-29 cell line (colon carcinoma) (Manayi et al. 2013). β- sitosterol in diet has also been reported to reduce the growth of MCF-7 cell line, indirectly aids in treatment of breast cancer (Ju et al. 2004). β-sitosterol was detected as a major compound in the extract of *Aloe vera* and has been correlated with angiogenic (Moon et al. 1999), genotoxicity (Paniagua-Perez et al. 2005), analgesic, anthelminthic, anti-mutagenic, (Villasenor et al. 2002), immunomodulatory (Bouic et al. 1996), antioxidative (Baskar et al. 2012; Vivancos & Moreno, 2005), neuroprotective (Shi et al. 2013) and anti-diabetic activity (Radhika et al. 2013).

n- hexadecanoic acid (10.14%) is commonly known as palmitic acid. Aparna et al. (2012) have reported the link between anti-inflammatory activity and n-hexadecanoic acid. 3,5-Dihydroxy-6-methyl-2, 3-dihydro-4H-pyran-4-one (2.95%) has also been reported as antimicrobial, anti-inflammatory and anti-proliferative agent (Zab et al. 2012). Furthermore, Beppu et al. (2012) have demonstrated the nerve stimulatory activity of 3, 5-Dihydroxy-6-methyl-2, 3-dihydro-4H-pyran-4-one in rats.

5.2.3 Phytoconstituent detected in MBE

The chromatogram profile of constituents present in MBE is depicted in Fig. 5-c. Ethane-1, 1, 1-trichloro- (41.86%), 6-Methylenecycloocta-1, 3-diene (16.87%), Ethane, 1, 1, 2-

trichloro- (7.31%) and 7-methylenebicyclo[4.2.0]oct-2-ene (2.49%) were the major phytoconstituent in MBE of *G. nervosa* (Table 5-IV). Although MBE depicted anti-proliferation activity of MCF-7 cells using MTT and trypan blue assays, as reported in previous chapter, none of the compounds detected in MBE could be associated with anti-proliferation activity. All the major compounds detected in MBE are not associated with any known pharmacological activity except one minor compound i.e. Diazinon oxon (0.93%) that is reported to demonstrate acetyl cholinesterase inhibition activity (Kousba et al. 2007).

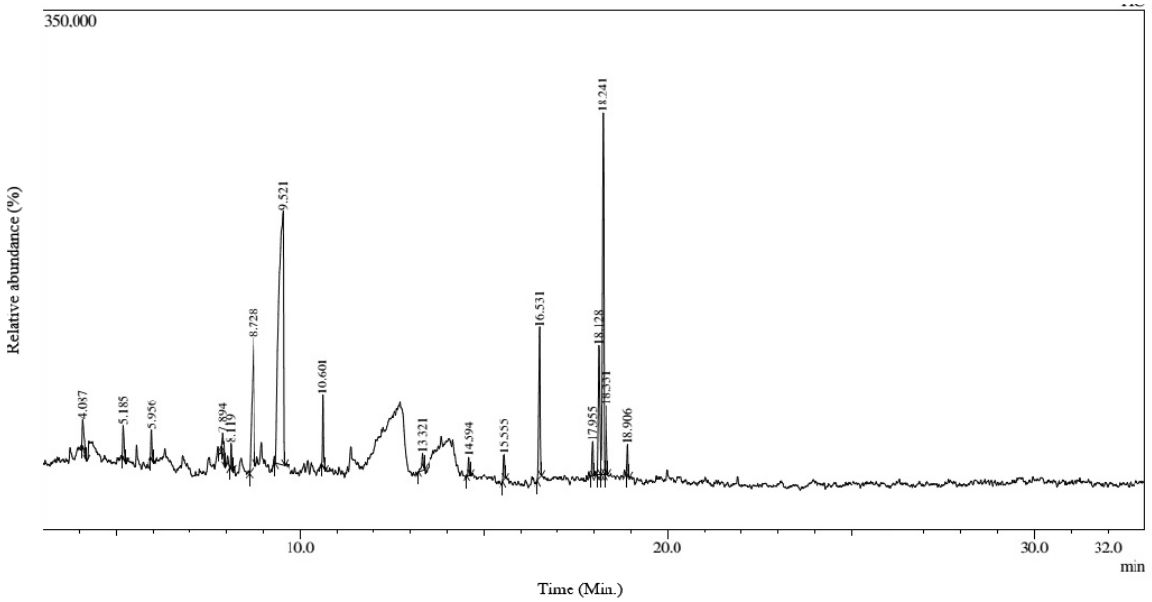


Fig. 5- c: GC-MS chromatogram of methanol bark extract of *G. nervosa.*

Table 5- IV: Compounds identified in the methanol bark extract of *G. nervosa* by GC-MS.

R.Time	**Area**	**Area%**	**Height**	**Name**	**Formula**	**Mass**
8.119	26611	0.74	16324	1-hepten-5-yne, 2-methyl-3-methylene	C_9H_{12}	120
9.521	1510008	41.86	171212	Ethane-1,1,1-trichloro-	$C_2H_3Cl_3$	132
10.601	89952	2.49	47989	7-methylenebicyclo[4.2.0]oct-2-ene	C_9H_{12}	120
13.321	33721	0.93	10888	Diazinon oxon	$C_{12}H_{21}N_2O_4P$	288
15.555	38936	1.08	17024	Cyclopropa [5,6]-A-nor-5α-androstane-3,7-dione	$C_{23}H_{32}O_4$	372
16.531	263843	7.31	101343	Ethane, 1,1,2-trichloro-	$C_2H_3Cl_3$	132
18.241	608436	16.87	243625	6-methylenecycloocta-1,3-diene	C_9H_{12}	120
18.906	41160	1.14	20790	2-methyl-3-methylene-1-hepten-5-yne	C_9H_{12}	120

Vast phytochemical diversity was observed in explants of *G. nervosa*. Amongst all the methanol extracts, MLE demonstrated maximum number (36) of phytochemicals belonging to variety of major classes of secondary metabolites in comparison to that of MBE that demonstrated 17 different phytochemicals whereas MRE depicted least number of phytochemicals (12 numbers). It is interesting to note that of all phytochemicals detected by GC-MS in methanol extracts, no single phytochemical were commonly detectedin all three different explants i.e. leaves, bark and roots. However, hexadecanoic acid, pentadecanoic acid, octadecanoic acid, linolenic acid, β-sitosterol, γ-sitosterol were identified in MLE and MRE, whereas, Cyclopropa[5,6]-A-nor-5.α-androstane-3,7-dione, 3',6.β-dihydro-17.β-, benzene, fluoro-, p-bromofluorobenzene, ethane, 1,1,2-trichloro-, 2-methyl-3-methylene-1-hepten-5-yne, Fenitrothion, 7-methylenebicyclo[4.2.0]oct-2-ene, 6-methylenecycloocta-1,3-diene, 2-methyl-3-methylene-1-hepten-5-yne were detected in MBE and MRE. No common compound was observed between MLE & MBE (Table 5-V). Earlier explanations of medicinal properties from *G. nervosa* primarily focused on leaves, flower and bark extracts. The GC-MS analysis of MRE of *G. nervosa* in the present studies have depicted several compounds of pharmacological importance.

Table 5- V: Common compounds present in different parts of methanol extracts of *G. nervosa*.

SL NO.	**COMPOUNDS**	**MLE**	**MBE**	**MRE**
1	Cyclopropa[5,6]-A-nor-5.alpha.-androstane-3,7-dione, 3',6.beta.-dihydro-17.beta.-	-	+	+
2	Benzene, fluoro-	-	+	+
3	p-bromofluorobenzene	-	+	+
4	Ethane, 1,1,2-trichloro-	-	+	+
5	2-methyl-3-methylene-1-hepten-5-yne	-	+	+
6	Fenitrothion	-	+	+
7	7-methylenebicyclo[4.2.0]oct-2-ene	-	+	+
8	6-methylenecycloocta-1,3-diene	-	+	+
9	Hexadecanoic acid	+	-	+
10	Pentadecanoic acid	+	-	+
11	Octadecanoic acid	+	-	+
12	2-methyl-3-methylene-1-hepten-5-yne	-	+	+
13	Linolenic acid	+	-	+
14	β-Sitosterol	+	-	+
15	γ-Sitosterol	+	-	+

Major classes of secondary metabolites present in the leaves, bark and roots of *G. nervosa* are shown in Table 5-VI. Fatty acids were identified in leaves and roots of *G. nervosa*. The presence of α-linolenic acid (8.20%), an essential ω-3 fatty acid in the MLE is of great significant. α-linolenic acid has been observed (0.19-0.35%) in the fruit extract of *Quercus brantis* and 14.79% in whole plant extract of *Peganum harmala* (khodadoust et al. 2014; Moussa & Almaghrabi, 2015).

Table 5- VI: Class of secondary metabolites detected in methanol extracts of leaves, root and bark of *G. nervosa*.

Compound category	**MLE (%)**	**MBE (%)**	**MRE (%)**
Fatty acid	18.77	-	10.14
Sterols	4.8	2.71	6.37
alkaloids	-	-	20.82
Alkanes	16.39	49.17	60.3
Vitamins	20.48	-	-
Diterpene	14.25	-	-
Others	`25.2	48.12	3.0

Phytosterols are distributed throughout the *G. nervosa* plant parts with almost same proportion. The phytosterols possess several bioactive properties that would have healthy implications on human (Normen et al. 2002). γ-sitosterol (4.48%) detected in MLE of *G. nervosa* has been already confirmed to depict antiproliferative activity against MCF-7 and lung carcinoma cell line, A 549 (Sundarraj et al. 2012).

The leaves of *G. nervosa* are a potent source for vitamins i.e. α-tocopherol (20.48%) whereas other explants of *G. nervosa* did not reveal the presence of any vitamin. Vitamin E is a well-known antioxidant promoting immunity against viruses and bacteria, as well as supports formation of red blood cells (Meydani et al. 2005). The predominant presence of α-tocopherol, an antioxidant reflects the antioxidant property of leaf extract (Patra et al. 2001).

Alkaloids have been observed in roots of *G. nervosa.* A survey indicated that alkaloids are associated with several pharmacological activities. Thus roots of the *G. nervosa* can be a potent source for novel pharmacological activities or compound/s. Plant alkaloids are reported to have various pharmacological activities such as antimyopia and anticholinergic (McBrien et al. 2013; Gu et al. 2011), antibacterial, antiviral, antiinflammatory, anticancer and antidiabetic activities (Kim et al. 2010; Zha et al. 2010; Zhang et al. 2010; Agyapong et al. 2013).

Amongst all the methanol extracts, only MRE depicts the presence of diterpenes i.e. phytol (14.25%). Diterpenes are chemical compounds composed of two terpene units and have been also detected in animals and fungi. As already discussed in above section, phytol is well known for its anti-proliferative effect on hepatocellular cancer cell lines such as Huh-

7 and HepG2 (Kim et al. 2015). Other recognized pharmacological uses of phytol are as a chemotherapy agent for the treatment of schistosomiasis (de Moraes et al. 2014). Phytol has also use to control the blood glucose level in type 2 diabetes (Elmazar et al. 2013).

CHAPTER 6

TOXICITY STUDIES OF METHANOL ROOT EXTRACT OF *G. nervosa*

During the process of drug discovery from natural source, it is imperative to evaluate the cytotoxic and genotoxic effects on animal model. Assessing the beneficial versus toxicological effects of plant extracts/drug is essential for justifying its relevance for phytotherapy (Rodeiro et al. 2006).

Traditionally, *G. nervosa* is well known medicinal plant used for treatment of various disease. However, potential risk of *G. nervosa* extract on human health has not been evaluated. Ames (1983), demonstrated cytotoxic and mutagenic activities of various medicinal plant extracts and consequently they were correlated with the occurrence of cancer. Thus, understanding health benefits as well as toxicity studies of *G. nervosa* extracts is significant.

The present chapter deals with cytotoxic, mutagenic and genotoxic effect of methanol root extract (MRE) on adult zebra fish *(Danio rerio)* blood cells and human leukocytes. MRE of *G. nervosa* was chosen for the present study, because MRE demonstrated excellent antidiabetic activity (as described in chapter 4) than standard drug acarbose. Further, phytotherapy potential of all the explants of *G. nervosa* such as flower, leaves, bark were have been extensively explored by several researchers, but none of the report is available on medicinal potential of root extract of *G. nervosa*.

6.1 METHODOLOGY

6.1.1 Experimental animal

Adult zebra fishes *(Danio rerio)* were obtained from a local aquaculture farm, Margao, Goa. The fishes were acclimatized up to one month in a 50 L glass aquarium tank (50 L). The tank was equipped with aeration, heating & light to maintain photoperiodism. The temperature was set at 28±1ºC, whereas photoperiodism was set at 14:10, light: dark and

was constantly maintained throughout the experiment. Once, every week, 50% of tank water was replaced with fresh water. Further reduction in water level due to evaporation was replenished to desired level by adding fresh water every day. *Danio rerio* fishes were fed with brine shrimp (*Artemia salina*) along with dry shrimp flakes and pellets food twice in a day (Brand et al. 2002). The physiochemical parameters such as hardness, pH and total dissolved oxygen of water were measured using standard techniques.

6.1.2 Determination of LC_{50} of MRE for zebra fish

Acute toxicity studies were conducted to determine the LC_{50} after 24h exposure of adult Zebra fishes (length 2.6-2.8 cm & weight 0.2-0.3 g) to MRE of *G. nervosa*. The experiments were carried out as per instructions of Organisation for Economic Cooperation and Development (OECD) for testing of chemicals (BRM, 1997). Prior to the definitive test, the range finding test was conducted to determine the concentrations of the plant extract. Ten zebra fishes of both sexes were exposed to a particular dose in 5L capacity rectangular glass tank containing different concentrations of the plant extract (2, 4, 6, 8 and 10 mg/L), obtained after range finding test. The experiment was set in triplicate to obtain the 24 h-LC_{50} value of the test chemicals for the species. Mortality was monitored continuously and the fishes were considered dead when operculum movement was not detected and the fishes failed to respond when touched with a glass rod. The dead fishes were immediately removed from the tank. The data was tabulated and the lethal dose that caused 50% mortality was calculated according to Spearman–Karber method (Dede & Kaglo, 2001).

6.1.3 *In vivo* exposure of zebra fish and sample collection

Zebra fish *(Danio rerio)* (10 animals per group) were exposed to with three different concentrations of MRE (0.1, 0.5 and 1mg/L) for 72 h with MRE. Temperature, pH, resistivity, conductivity, oxygen and salinity were maintained during the experiment (Delince, 2013). Cyclophosphamide was used as positive control. Blood samples were collected from the caudal vein of the control, positive control and MRE exposed fishes using a hypodermic syringe under dim light at intervals of 24, 48 and 72 h. the blood sample was transferred immediately to a microcentrifuge tube placed in an ice to prevent endogenous DNA damage and to inhibit DNA repair in the unfixed cells (Siu et al. 2004).

6.1.4 Human blood collection, isolation of leukocytes and *in vitro* exposure

Peripheral blood (2 ml) was collected in heparinized conical centrifuge tube from the vein of a healthy human donor and centrifuged at 2,000 rpm for 15 min. The whitish portion of blood formed just below the upper plasma layer was collected. Cells were washed twice with 0.5 ml of NH_4Cl (0.85 %) to remove RBC. Lymphocytes that appeared in the form of white pellet were subsequently re-suspended in PBS (phosphate buffer saline) and stored at 4 °C. Human lymphocytes were exposed to the three different concentrations of MRE (0.1, 0.5 and 1 mg/ml) for 0, 1 and 3 hrs respectively at 37°C. Distilled H_2O was used as control and H_2O_2 (4mM) was used as the positive control.

6.1.5 Trypan blue assay

- The cytotoxic effects of MRE on blood cells of zebra fishes and human blood cells were analysed using trypan blue dye exclusion method (Tennant, 1964).
- Cell suspension and 0.4% trypan blue solution were mixed in 1:1 ratio.

- The haemocytometer was filled with cell suspension and incubated for 1-2 minutes at room temperature (30±2°C).
- Cell in each square (1 x 1 mm) was enumerated under trinocular light compound research microscope (BX53, Olympus Japan) and average number of cells per square were determined. For an accurate determination, the total number of cells overlying one 1 mm 2 were kept between 20 – 50 cells/square. Square that contain cells more than 200 cells were counted after desired dilution.

Cell viability and cell number was calculated as follows:

Cell count

Cells per ml = Average cell count per square x dilution factor x 10^4

Total cells = cells per ml x original volume

$$\text{Percentage of viability} = \frac{\text{Total viable cells}}{\text{Total cells}} \times 100$$

6.1.6 Comet assay

The alkaline comet assay was performed using the protocol mentioned by Tice and Strauss, (1995), with modifications as mentioned by Singh et al. (1988).

6.1.6.1 Sample and slide preparation

- Using cardiac puncture method, 0.2 ml of blood sample was collected from adult zebra fishes followed by dilution in 1ml of phosphate buffer saline (pH 7.4) and stored at 4°C for further use.

- 250µl of 1% normal melting agarose (NMA) was placed on frosted microscope slide, covered with cover glass. After fixing on ice for 5min and the glass cover was removed.
- 25µl of cell suspension containing approximately10^5 blood cells were mixed with 75µl of 0.5%, low melting agarose (LMA) and the cell-agarose suspension was poured over the first layer and covered with cover glass (dim light) for the suspension gel.
- 100µl LMA was again poured over the second layer and covered with cover glass under dim light. Slides were kept on ice for 5 min and then the glass cover was removed.

6.1.6.2 Slide processing

6.1.6.2.1 Lysis

The slides were incubated in freshly prepared cold lysis solution at 4°C overnight, followed by washing in PBS to discard any excess salts.

6.1.6.2.2 Unwinding

The slides were placed in the electrophoretic unit containing freshly prepared electrophoresis buffer (300mM NaOH, 1mM EDTA, (PH>13) for 20 min

6.1.6.2.3 Electrophoresis

Gel was run for 20 min at 300 mA, 25 V under dim light.

6.1.6.2.4 Neutralization

The slides were neutralized three times by soaking in 400 mM Tris, pH 7.5 for 5 min.

6.1.6.2.5 Staining

After draining, the slides were stained with 30 µl ethidium bromide (20µl/ml), covered with cover slip and analyzed immediately

6.1.6.2.6 Screening

Two slides per sample and 25 comets per slide were scored using image analysis software along with CCD camera attached to fluorescent microscope with green and blue filters. Comet assay images were analyzed by using CASP software. 50 comets for each groups were analyzed and the %DNA in tail region were taken into consideration and compared with control and other groups.

6.1.7 Micronucleus test

- Clastogenic effects of MRE on zebra fish were analysed using micronuclease test (Russo et al. 2004).
- Human peripheral blood (0.05-0.1 ml) was obtained in a heparinized syringe from the vein of healthy human.
- A thin smear of human blood was made on pre cleaned slide with the help of a glass slide
- Fixing of the human blood cells on slides were achieved by dipping the slides in methanol 5-10 min followed by drying for at least 1 hour.
- The slides were stained using using 0.13% May Grunewald's stain for 2-3 minutes followed by washing with double distilled water (DDW) and later dried.
- The slides were later stained in 6-10% Giemsa stain in phosphate buffer for 30 minutes followed by washing with distilled water and dried.

- The slides were observed under using 40/100X objective lenses of compound microscope and micro nucleated cells were enumerated (Matsumoto & Colus, 2000).

6.1.8 Statistical analysis

The statistical analysis was performed by using PRISM, STASTICA 6.0 software packages. Data was analyzed with student's t-test and ANOVA. Within the single experiment, the significant of each dose against their respective control values was evaluated by the student's t-test. Dose-response as well as the time-response of peripheral blood cells of fishes and human leukocytes to MRE were determined by one-way ANOVA with pairwise Tukey test to identify differences between specific treatment groups.

6.2 RESULTS & DISCUSSION

6.2.1 Cytotoxicity effect

The LC_{50} for MRE of *G. nervosa* was observed to be 5.1 mg/ml at 24 h. The cytotoxicity studies conducted to assess the MRE toxicity indicated that more than 99% cells were viable in control as well as treated groups (n=3), (Fig. 6-a). Positive control (Cyclophosphamide) used in the present study demonstrated time dependent cytotoxicity on human leukocytes and zebra fish blood cells (Fig. 6-b).

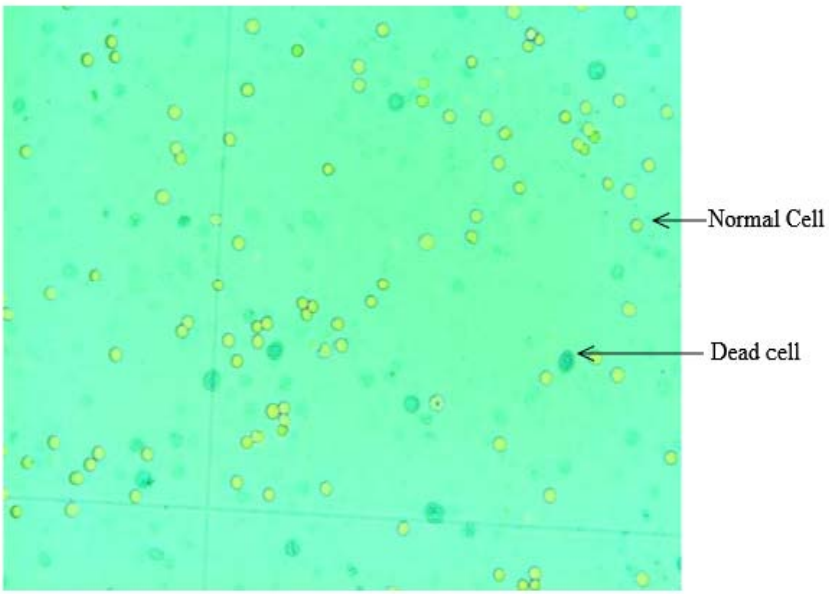


Fig. 6- a: Viable and dead cells observed during trypan cell viability assay on zebra fish lymphocyte

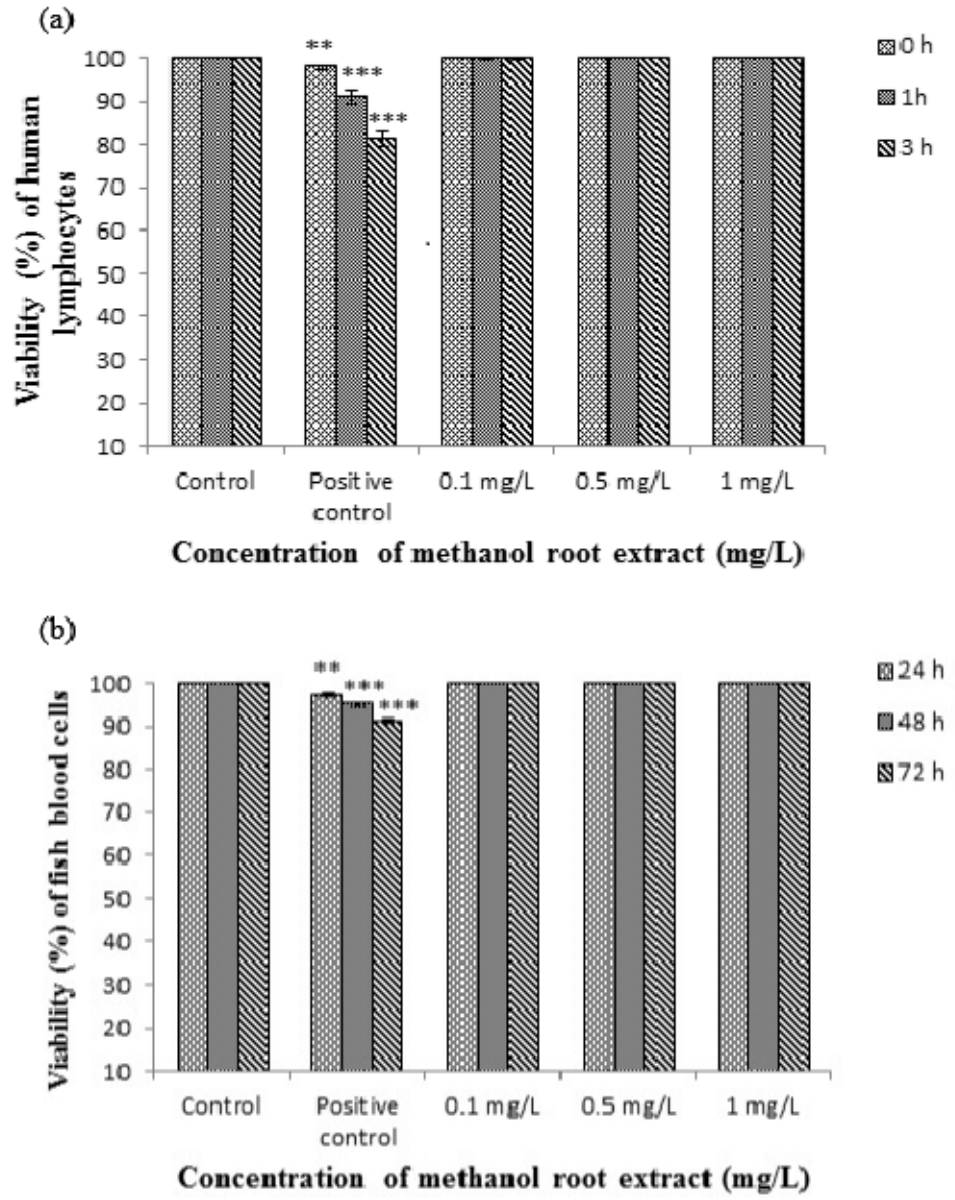


Fig. 6- b: Percentage of viable cells in human leukocytes (a) and Zebra fish blood cells (b) exposed to various concentrations of methanol root extracts of *G. nervosa* at different time intervals. Data are mean ± SD. Note: ** = P<0.01, *** = P<0.001

6.2.2 Mutagenic effects

The dose and the time dependent DNA damage by MRE on human leukocytes and their statistical significance are represented in Fig. 6-c. None of the tested dose of MRE demonstrated DNA damage in human leukocytes of study except the studies involving 1 mg/L of MRE that induced significant DNA damage at 3h.

The DNA damage induced by various concentrations of MRE extracts in the zebra fish blood cells at different intervals is shown in Fig. 6-d. A significant increase ($P > 0.05$) of DNA damage was observed for higher dose (1000µg/L) at 72h. However, lower doses (100 and 500 µg/L) did not depict DNA damage, regardless of duration of exposure.

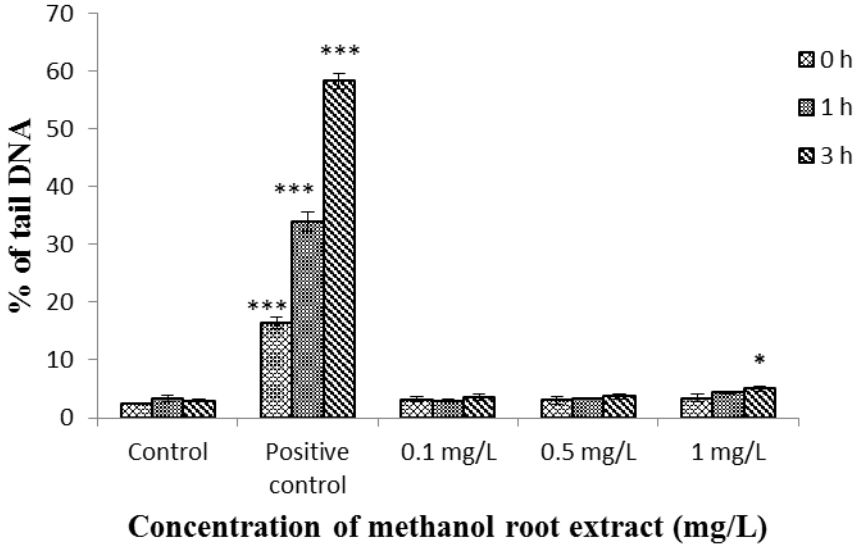


Fig. 6- c: Percentage of DNA damage in human leukocytes exposed to various concentration of methanol root extracts of *G. nervosa* at 0, 1 and 3 h time intervals. Data are mean ± SD [@ = P<0.05 denote statistically significant difference from the control (Dose response study) and damage at 24 h, (Time response study) student's t-test significance]. Note: *=p<0.05, ***=P<0.001.

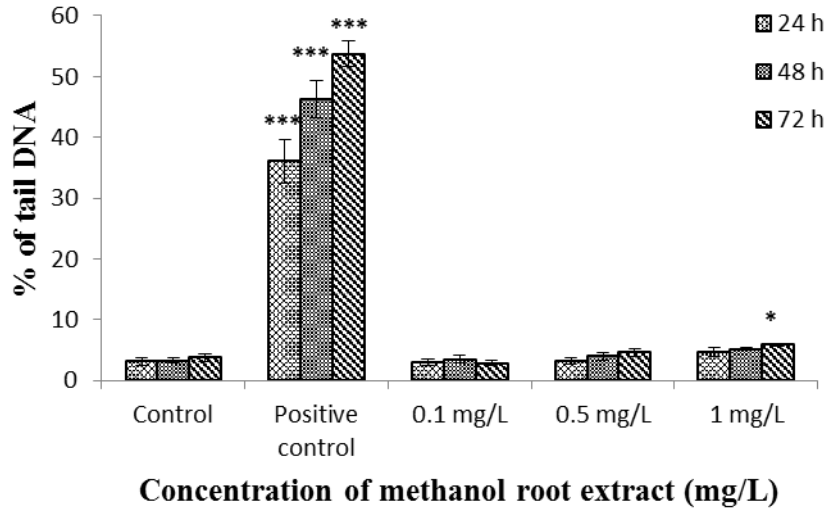


Fig. 6- d: Percentage of DNA damage in blood cells of zebra fish exposed to various concentration of methanol root extract of *G. nervosa* at 24, 48 and 72 h time intervals. Data are mean ± SD [@ = P<0.05 denote statistically significant difference from the control (Dose response study) and damage at 24 h, (Time response study) Student's t-test significance].

6.2.3 Genotoxic effects

The dose and the time dependent frequency of occurrence of micronucleated zebra fish blood cells in response to various concentrations of MRE (0.1, 0.5 and 1 mg/L) were observed after 24, 48 & 72 h. The microscopic observation of micronucleated peripheral blood cells of zebra fish is shown in Fig. 6-e. In the present study, no significant increase (P>0.05) of micronuclei were observed at all tested doses of MRE as well as at different time intervals. All experimental groups demonstrated equal frequency of micronucleated

cells in comparison to that obtained in the negative control. Whereas, the positive control H_2O_2 used in the present study showed significant dose & time dependent DNA damage ($P<0.001$) in leukocytes (Fig. 6-f). These results indicate that MRE of *G. nervosa* is not mutagenic at tested doses and time intervals.

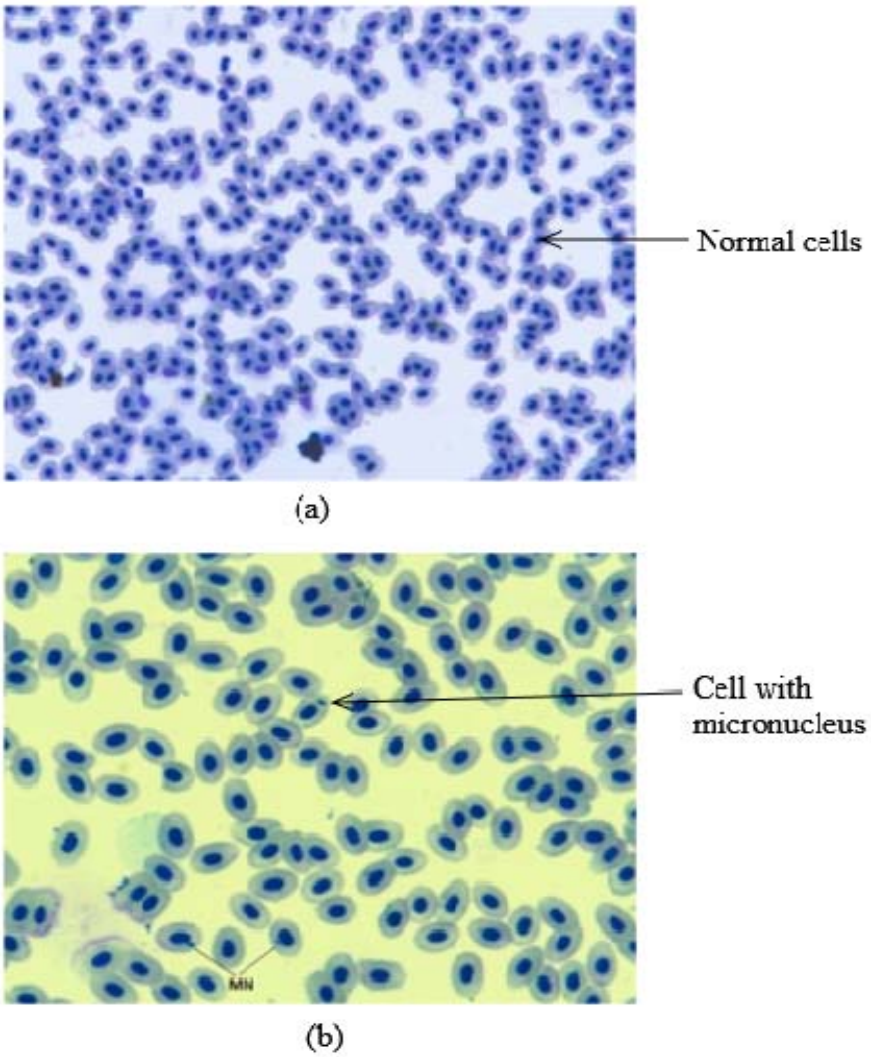


Fig. 6- e: Fish peripheral blood cells with Micronuclei (a) 200X (b) 400X

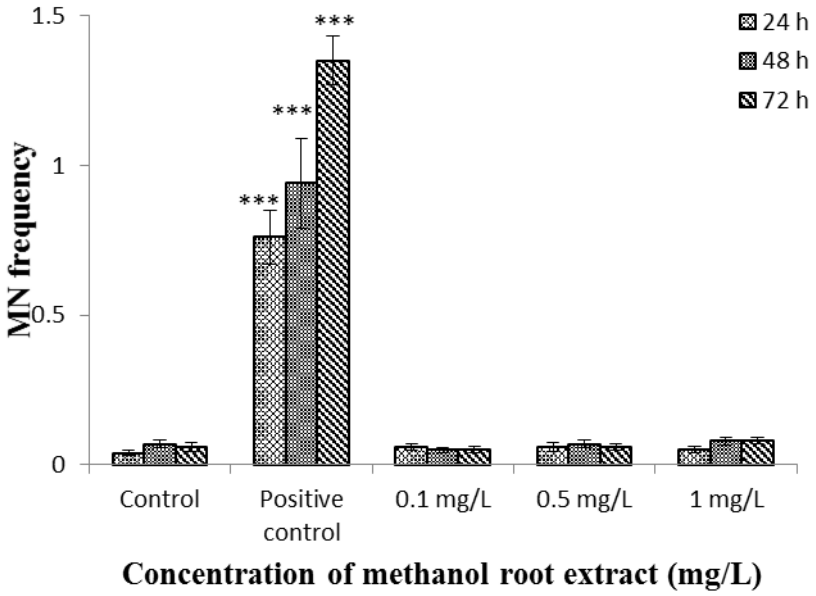


Fig. 6- f: Frequency of micronucleated blood cells of zebra fish exposed to various concentration of plant extract at 24, 48 and 72 h time intervals. Data are mean ± SD.

Note: ***= P<0.001.

No reports are available about genotoxic effect of plant extract belonging to Malvaceae family on zebra fish lymphocytes and human leukocytes. Aqueous extract from bark of *Luehea divaricate* plant belonging to malvaceae family did not show any mutagenic effect on *Drosophila melanogaster* at 0.1-0.5 mg/ml concentrations (Feliciol et al. 2011).

No cytotoxic and genotoxic have been reported with medicinal plants belonging to Malvaceae family indicating that plants of this family are a valuable plant. In contrast, several other medicinal plants belongs to various families demonstrated potential genotoxic effects on animal models. Tylor et al. (2003), investigated total 51 medicinal plant species belong to various families for genotoxic effect using comet and micronuclease assay. The results of the study indicated that *Kigelia africana*, *Merwilla*

plumbea, *Boophane disticha*, *Celtis africana*, *Crinum macowanii*, *Erythrina caffra*, *Ochna serrulata*, *Sclerocarya birrea* and *Tulbaghia violacea* depicted genotoxicity in the micronucleus test. Further extracts of *Acokanthera oblongifolia*, *Afzelia quanzensis*, *Bersama lucens*, *Ocotea bullata*, *Siphonochilus aethiopicus* and *Tetradenia riparia* caused DNA damage as detected by comet assay.

In an another study, carried out by Varalakshmi et al. (2011), five Indian medicinal plants viz. *Phyllanthus niruri, Coleus aromaticus, Azadirachta indica, Camellia sinensis, and Garcinia indica* were evaluated for cytotoxic effect on human peripheral blood cells using trypan blue assay. Results of the study indicated that extracts of *P. niruri, C, aromaticus, A. indica,* and *G. indica* were cytotoxic to human peripheral blood cells.

The combination of these two (comet & micronuclease) assays in the present study successfully justified the possible risk/benefits of MRE of *G. nervosa* for the purpose of evaluating the plant for phytotherapy. The effects of various doses of MRE on zebra fish blood cells and on human leukocytes demonstrated no evidence of cytotoxicity and genotoxicity effects. Results of the present study are sustained with the traditional belief of people that medicinal plants are nature's gift to them for curing various ailments rather than their harmful effects.

CHAPTER 7:

PARTIAL PURIFICATION & IDENTIFICATION OF BIOACTIVE COMPOUNDS FOR A SELECTED PHARMACOLOGICAL ACTIVITY

Bioprospecting of medicinal plants provides opportunities to explore for novel compounds that can be evaluated against diseases. Due to an urgency for discovering novel therapeutic drugs from natural products, different techniques for purification and identification of potential lead compound/s have been accomplished. The crude herbal extracts contains various types of bioactive compounds, out of which one or few compounds are responsible for a given pharmacological activity.

The focus of this chapter would be on partial purification and identification of the anti-diabetic compounds that inhibit α-amylase & α-glucosidase from the methanol extracts of *G. nervosa,* using various analytical techniques. The MLE & MRE of *G. nervosa* have demonstrated promising α-amylase & α-glucosidase inhibition activity. Thus the present study deals with separation & identification of the compound/s present in the MLE & MRE using TLC, column chromatography, NMR, IR, GC-MS and LC-MS.

7.1 METHODOLOGY

7.1.1 Separation & identification of compound from MLE that inhibit α-amylase activity

7.1.1.1 Thin layer chromatography

Since, MLE of *G. nervosa* demonstrated inhibition of α-amylase activity, separation of compound from MLE was undertaken. The compounds from MLE were separated on TLC (Silica gel 60, Merk, India) using hexane: ethyl acetate (9.5:0.5), Ethyl acetate: hexane (9.5:0.5), 100% chloroform, 100% benzene and benzene: hexane (50:50) as solvent system.

7.1.1.2 α- amylase inhibition potential of TLC scrapped compounds

The spots, detected on TLC plate were scraped carefully and resuspended in methanol followed by centrifugation at 2000 rpm for 2 min. the supernatant of the sample was retained and the pellet was discarded. DNS assay was used to determine the inhibition of α-amyalse activity. All parameters & conditions of DNS assay were same as described in chapter 4.

7.1.1.3 GC-MS analysis

Spot correspond 'E' exhibited α-amylase inhibition activity. Therefore, sample was concentrated and dissolved in methanol and further analyzed by using GC-MS were done in collaboration with Ram Narayan Ruia College, Mumbai. The parameters used in GC-MS analysis were same as described in earlier chapter 5.

7.1.2 Separation & identification of α-glucosidase inhibitory compound from MRE

7.1.2.1 Fractionation of MRE

Bioactivity guided fractionation of crude MRE (4 g) of *G. nervosa* was achieved using various four different solvents with increasing polarity namely petroleum ether (4×50 mL), ethyl acetate (2×50 mL), n-butanol (1×50 mL) & water. Each fraction, thus obtained, was concentrated to remove solvent and then analyzed for inhibition of α-glucosidase activity. The butanol fraction that demonstrated inhibition activity was further subjected to purification using column chromatography. The schematic representation of fractionation is depicted in Fig. 7-a.

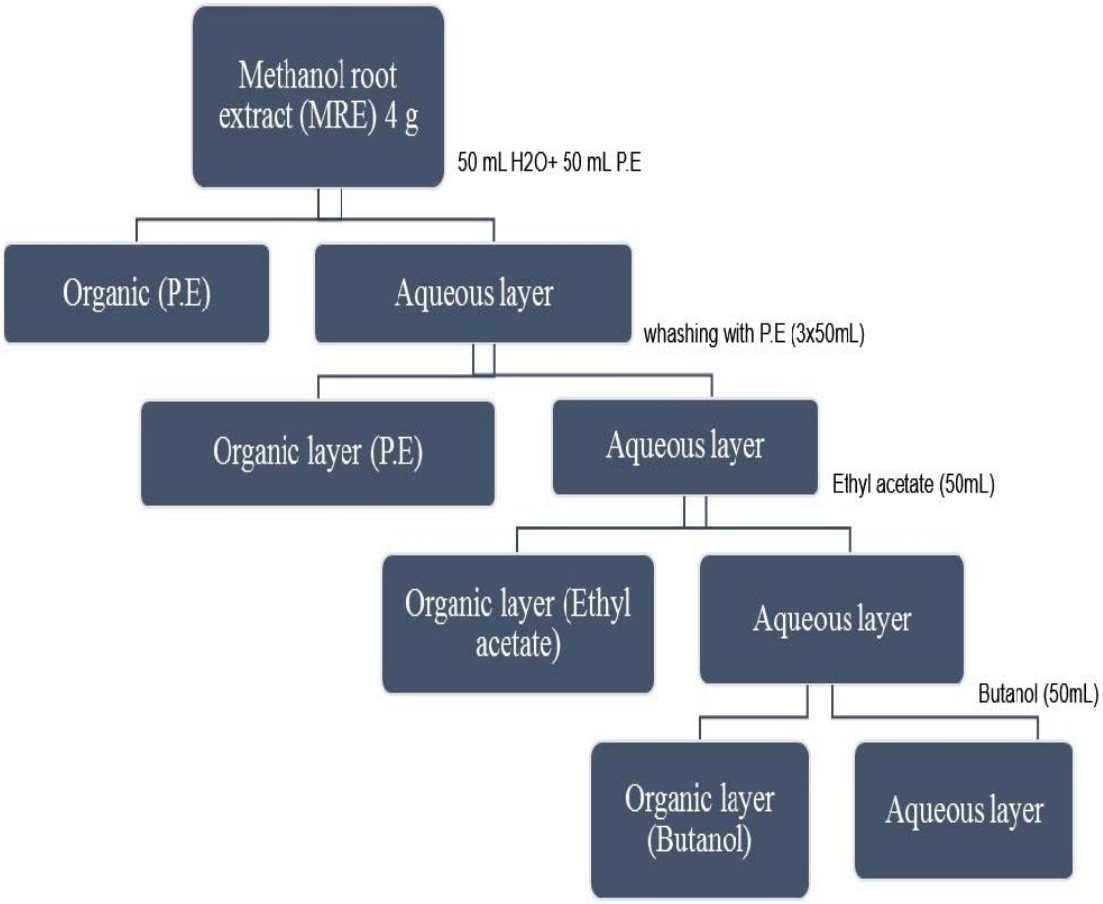


Fig. 7- a: Fractionation of methanol root extract using various solvents

7.1.2.2 Thin layer chromatography

TLC pattern of butanol and aqueous fractions of MRE of *G. nervosa* were also checked using different solvents systems like petroleum ether, ethyl acetate, methanol, petroleum ether: ethyl acetate, methanol: water, BAW (5:1:4). Separated compounds were visualized by spaying 5% H_2SO_4 in methanol.

7.1.2.3 Packing of Sephadex LH 20 column

For normal phase chromatography, column with sephadex LH 20 (Sigma-Aldrich Pvt. Ltd.) was used. A piece of cotton was put at the end of the dried clean column (2 cm X 1 m). Sephadex LH 20 gel was pre-soaked in methanol: chloroform (1:1) and slurry was added to the column slowly with the help of funnel and glass rod. Slurry was slowly added and allowed to settle each time. The excess solvent was removed by opening the knob of the column. The column was packed till it reached a desired height (95 cm).

7.1.2.4 Loading of sample on Sephadex LH 20 column

Butanol fraction of MRE was loaded carefully on the top of the column with the help of graduated pipette. Initially, little amount of extract was added and it was allowed to percolate in the column and then the remaining fraction portion was added and again allowed to percolate. After that methanol solvent was added drop wise with the help of pipette so that the bed doesn't get disturbed. After adding sufficient amount of methanol drop wise (5 cm above the bed), it was poured from the top and compounds were eluted. Column eluents (3 ml x 20) were collected in different test tubes and TLC pattern was checked.

7.1.2.5 Packing of ODS column

For reverse phase column chromatography, column with octadecylsilane (ODS) (Sigma-aldrich Pvt. ltd.) was used. A cotton plug was put at the end of the clean & dry column. A slurry of ODS prepared in methanol was filled in the column slowly with the help of funnel and glass rod. Slurry was added slowly and was allowed to settle each time. Extra

solvent was removed by draining. The column was packed till it reached a desired height (30 cm).

7.1.2.6 Loading of sample in the ODS column

Before loading the compound with extract, the column was washed with distilled water. The butanol fraction of MRE (93 mg) was resuspended in distilled water (2-3 drops of methanol was also added) and with the help of graduated pipette it was loaded on the column. First little amount of butanol fraction was added and allowed to percolate in the matrix followed by addition of remaining fraction. After that, distilled water was added drop wise with the help of pipette so that the bed doesn't get disturbed. After adding sufficient amount of distilled water drop wise, then it was poured from the top and the compounds were eluted. Column eluents (10 ml, 20-30 nos.) were collected in different test tubes and TLC pattern was checked.

7.2 RESULTS & DISCUSSION

7.2.1 Separation & identification of compound from MLE that inhibit α-amylase activity

7.2.1.1 Thin layer chromatography

Best separation on TLC plate was achieved when ethyl acetate: hexane (9.5:0.5) was used as a solvent system. Five spots (A, B, C, D, E) were detected under visible range at 363 nm and six spots (A1, A2, B, C, D, E) were observed in UV range at 254 nm (Fig. 7-b).

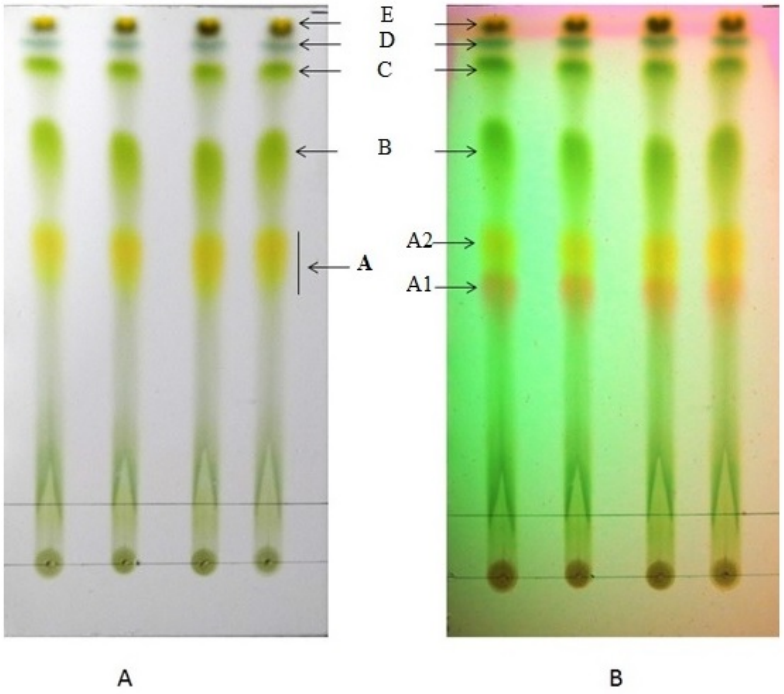


Fig. 7- b: Thin layer chromatography of methanol root extract from *G. nervosa* (A) compounds observed under visible range and (B) compounds observed under UV range.

7.2.1.2 α- amylase inhibition activity TLC spots

All separated spots were evaluated for α-amylase inhibition activity by using standard DNSA method. The compounds recovered from spot E demonstrated inhibition of α-amylase activity, whereas, no inhibition of α- amylase activity was in presence of compounds recovered from spots A1, A2, B, C and D respectively. α- amylase activity was assumed 100 % in control (without any compound from TLC spots) (Fig. 7-c).

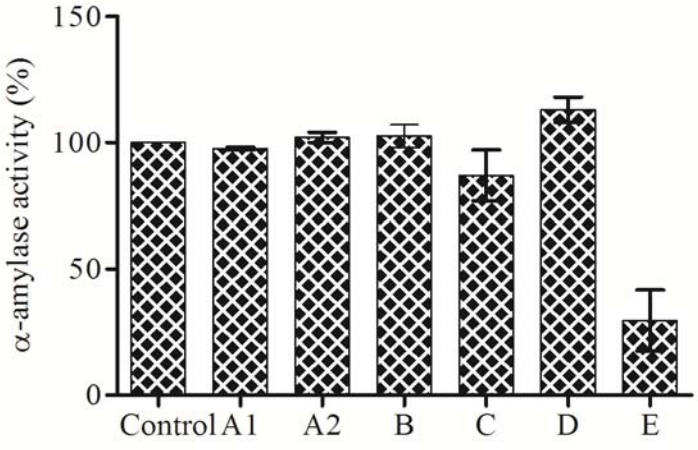


Fig. 7- c: α- amylase activity in presence of compounds recovered from TLC plate (Control-without any TLC spots, A1, A2, B, C, D and E are compounds recovered from TLC plate as seen in Fig. 7-b).

7.2.1.3 GC-MS identification of compounds recovered from spot E of TLC

The compounds recovered from spot E separated by TLC demonsrated inhibition of α-amylase activity. The GC-MS chromatogram of spot E is shown in the Fig. 7-d and the respective compound identified shown in Table 7-I. Three compounds were identified by GC-MS technique. 3-Methyl-1, 8-bis [(trimethylsilyl)oxy]anthra-9,10-quinone was identified as the major compound (24.62%), whereas, tetratriacontane (20.74%) and hexatriacontane (23.51%) were detected as minor compounds. Hexatriacontane has already also been reported in the stem bark extract of *Ailanthus excels* and stem bark extract is reported to demonstrate anti-diabetic property in rats (Lavhale & Mishra, 2007). Thus, hexatriacontane detected in the methanol leaves extract of *G. nervosa* is probably involved in inhibition of α-amylase activity. Hexatriacontane is reported in other

medicinal plants such as *Epaltes divaricata* (L.), *Rosa damascena* and *Achyranthes aspera*.

No medicinal property could be associated with tetratriacontane and 3-Methyl-1, 8-bis[(trimethylsilyl)oxy]anthra-9,10-quinone that were coming with hexatriacontane during TLC.

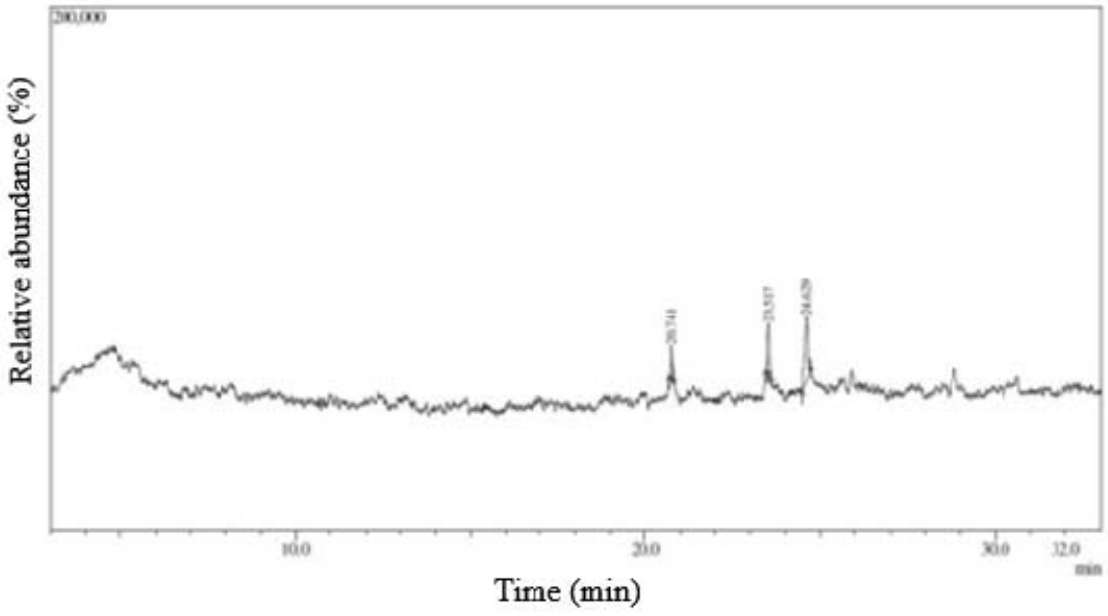


Fig. 7- d: GC-MS chromatogram profile of compounds recovered from spot E separated on TLC plate.

Table 7- I: Compounds identified by GC-MS in spot E.

Peak	**R. time**	**Area**	**Area %**	**height**	**Name**	**Formula**	**Mass**
1	20.741	43471	15.28	13162	Tetratriacontane	$C_{34}H_{70}$	478
2	23.517	75669	26.59	20068	Hexatriacontane	$C_{36}H_{74}$	506
3	24.629	165440	58.13	24727	3-Methyl-1,8-bis[(trimethylsilyl)oxy]anthra-9,10-quinone	$C_{21}H_{26}O_4Si_2$	398

7.2.2 α-glucosidase inhibitory property of MRE of *G. nervosa*

7.2.2.1 Thin layer chromatography

In order to identify the compound from MRE of *G. nervosa* that demonstrated inhibition of of α-glucosidase activity, crude butanol and aqueous fractions of MRE were seperatedon TLC using different solvent systems like petroleum ether, ethyl acetate, BAW and methanol: chloroform. The 10% methanol: chloroform TLC depicted the best seperation. When TLC plates sprayed with 5% H_2SO_4 in methanol, black-brown colored spots appeared at the baseline, indicating the presence of probable high polar compounds such as sugars, alkaloids and polyhydroxyl aldehyde compounds. Four spots observed above the baseline were found to be UV active. Since spots above the base line were in trace amount. So attempts were diverted to separate and characterize the spot that was not resolved and remained on the baseline (Fig. 7-e).

Fig. 7-e: Spots detected on TLC plate (A) aqueous fraction and (B) butanol fraction of methanol root extract of *G. nervosa.*

7.2.2.2 Purification of n-butanol fraction of MRE using sephadex LH 20 column chromatography

Size exclusive gel chromatography involves separation according to size and the molecular weights. For gel chromatography Sephadex gel (G and LH) is routenly used matrix.

In order to separate the compounds detected on the baseline of TLC plate, Fractions (3 ml, 20 nos.) eluted from sephadex LH 20 were collected in the test tubes and subjected to TLC. Only one spot was noticed on TLC plate and was assumed to be a pure compound. All the fractions (2-26) were mixed together and concentrated on rotary evaporator (Fig. 7-f).

Fig. 7-f: TLC of all fractions obtained after SEC (size exclusion charomatography, LH20 column)

7.2.2.3 Octadecyl silane (ODS) column chromatography

Reverse phase column chromatography involves hydrophobic stationary phase that has a strong affinity for hydrophobic compounds. Octadecyl silane, a non-polar (hydrophobic) stationary phase is usually used in reverse phase column chromatography. The fractions (2-26) obtained from SEC were combined & loaded on ODS column and was eluted with different solvents systems with decreasing polarity. The eluted fractions were collected in test tubes. The TLCs of these extracts were checked in 10% methanol: chloroform & BAW (visualizing agent: 5%H_2SO_4 in methnol). Finally, 9.9 mg of patially purified compound was recovered from column chromatography obtained was about ~9.9 mg using chromatography.

7.2.2.4 α- glucosidase inhibition potential of partially purified compound

The compound that was obtained from MRE by column chromatography purification evaluated for inhibition of α-glucosidase activity. The partially purified compound demonstrated dose dependent inhibition of α-glucosidase activity in comparison to control (t-test) (Fig. 7-g). Dose dependent inhibition of α-glucosidase activity by partially purified compound of *G. nervosa* ranged from 18.18± 0.85% at 0.06 µg/ml to 97.48 ±0.7% at 0.3 µg/ml. Further, one way ANOVA test of the % inhibition of α-glucosidase observed in control in comparision to different doses of partially purified compound showed significant difference at $P < 0.0001$ ($F = 2448$).

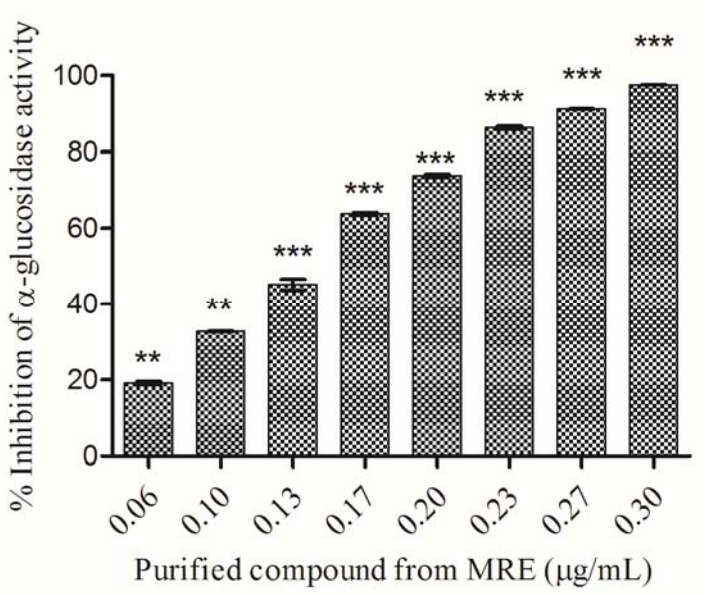


Fig. 7- g: Percentage inhibition of α-glucosidase activity at various doses of partially purified compound obtained from the methanol root extract of *G. nervosa*. Data are mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001 denotes statistically significant difference from the test control as determined by student's t-test).

7.2.2.5 Characterization of partially purified compound from MRE that inhibit the α-glucosidase activity

7.2.2.5.1 LC-MS

The LC-MS technique is highly sensitive technique used for purification & characterization of compounds. The TLC of partially purified compound obtained after normal phase column packed with sephadex and ODS column chromatography, indicate the presence of single spot.

The LC-MS profile depicted 3 peaks in its chromatogram. Hence, MS for individual compound, correspondings to peaks at the retention time of 0.19, 0.26 and 0.67 min was recorded (Fig. 7-h).

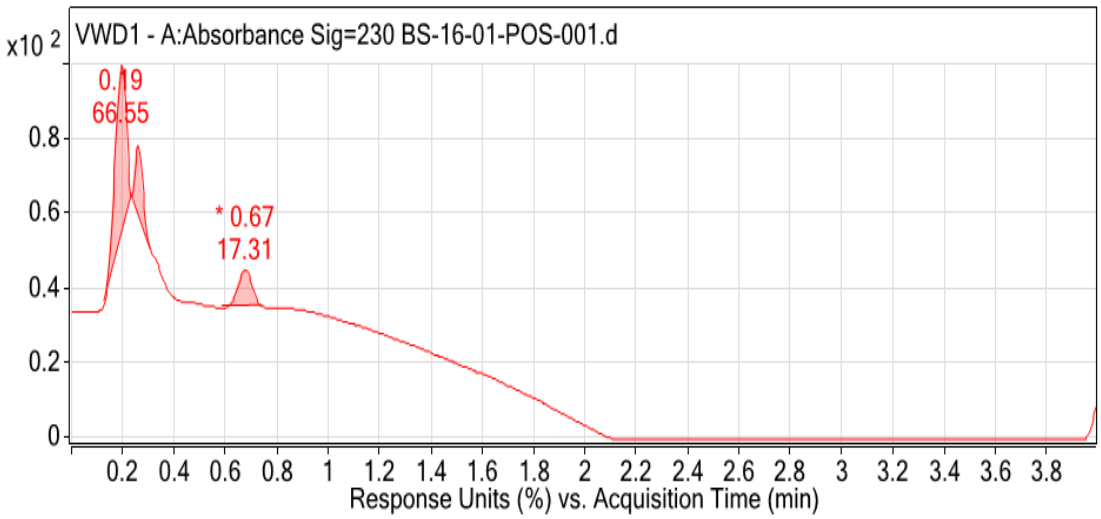


Fig. 7-h: LC-MS chromatogram of partially purified compound from the MRE of *G. nervosa*

LC-MS analysis indicated that out of three compounds detected, peak with with retention time of 0.19 min is the major compound i.e. 66.55%, whereas peaks with

retention time 0.26 and 0.67 min were minor compounds having composition i.e. 16.14 and 17.31% respectively (Table 7-II).

Table 7-II: Identification of peaks by total ion chromatogram scan in positive mode (+ TIC scan) from partially purified compound from the MRE of *G. nervosa*.

Peak	Start	RT	End	Height	Area	Area%
1	0.13	0.19	0.23	183.03	580.78	66.55
2	0.23	0.26	0.31	73.69	140.82	16.14
3	0.59	0.67	0.74	40.02	151.09	17.31

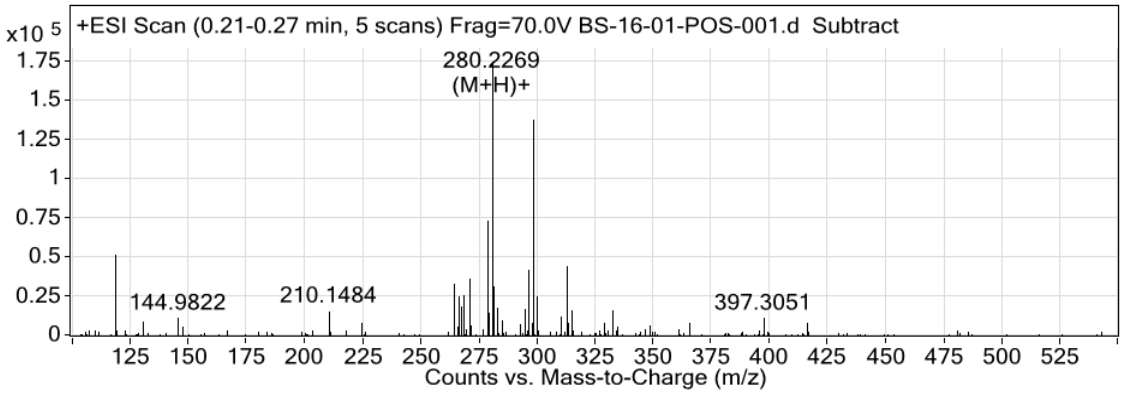


Fig. 7-i: Peaks detected by mass spectrum between retention time of 0.21-0.27 min.

Amongst the three detected compounds with m/z, compound with m/z 280.2269 is the most abundant. (Fig. 7-i & Table 7-III). The molecular formula of the compound based on its given mass was calculated using formula calculator element limits table. All possible formulas of compound with m/z 280.2269 were predicted and is shown in Table 7-IV. The $C_{17}H_{30}NO_2$ formula was confirmed, since this formula has 4 double bond equivalent.

Table 7-III: Masses of all peaks with their relative abundance detected by LC-HR/MS with retention time of 0.21-0.27 min.

m/z	z	abundance
64.0168	1	48127
81.0318	1	83323
118.0869	1	51747
264.2311	1	33147
270.2423	1	36993
278.2113	1	73300
280.2269	1	174311
296.2228	1	42309
298.2375	1	137629
312.2516	1	44217

Table 7- IV: All possible formulas of most abundant compound with m/z 280.2269 using formula calculator limit index.

Formula	Best	Mass	Tgt Mass	Diff (ppm)	Ion Species	Score	DBE	m/z
C_{17} H_{29} NO_2	TRUE	279.2196	279.2198	0.79	C_{17} H_{30} NO_2	75.39	4	280.2269
C_{12} H_{30} Cl N_5		279.2196	279.219	-2.32	C_{12} H_{31} Cl N_5	60.88	0	280.2269
C_{13} H_{25} N_7		279.2196	279.2171	-8.85	C_{13} H_{26} N_7	55.66	5	280.2269

Mass spectrum of second peak with the retention time of 0.26-030 min revealved the presence of compound with m/z 264.2316 (Fig. 7-j). All minor and major peaks detected

by mass spectrum with their m/z are summarized in Table 7-V. Compound with m/z 264.2316 was observed in abundance compare to other compounds with the same m/z. Based on m/z, two formulas were predicted namely $C_{17}H_{29}NO$ & $C_{12}H_{29}N_3O_3$. Compound with molecular formula $C_{17}H_{29}NO$ was confirmed as a true compound present in MRE (Table 7-VI).

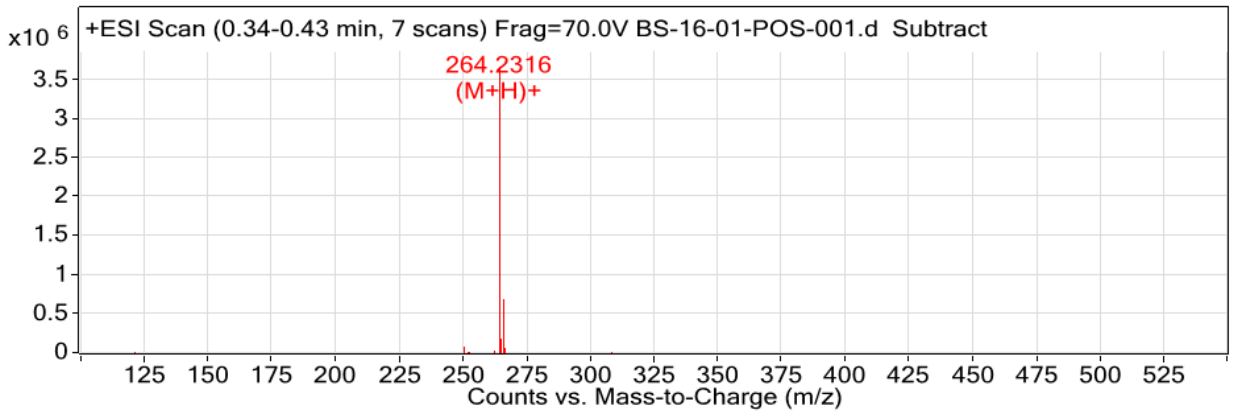


Fig. 7-j: Mass spectrum of peaks detected by LC-HR/MS between 0.34-0.43 min retention time.

Table 7-V: M/Z of compounds with their relative abundance scanned between 0.34-0.43 min.

m/z	**z**	**Abundance**
264.2316	1	3666924
264.3756	1	204456
265.2354	1	696022
563.4335	1	211868

Table 7-VI: Possible formulas of most abundant compound (m/z- 278.2473) detected in + ESI scan between 0.34-0.43 min

Formula	**Best**	**Mass**	**Tgt Mass**	**Diff (ppm)**	**Ion Species**	**Score**	**DBE**	**m/z**
$C_{17}H_{29}NO$	TRUE	263.2243	263.2249	2.35	$C_{17}H_{30}NO$	98.06	4	264.2316
$C_{12}H_{29}N_3O_3$		263.2243	263.2209	-12.94	$C_{12}H_{30}N_3O_3$	64.72	0	264.2316

Similarly, mass spectrum of compound detected at 0.67 min retention time is identified with $(M+H)^+$ of 278.2473 (Fig. 7-k). All other minor peaks along with major peak detected by mass spectrum with their m/z are summarized in Table 7- VII. Compound with m/z 278.2473 was observed in abundance compare to other compounds with same m/z. Amongst all predicted molecular formulas of compound with m/z 278.2473, $C_{18}H_{31}NO$ was confirmed based on different chemical criteria and information from the literature (Table 7- VIII).

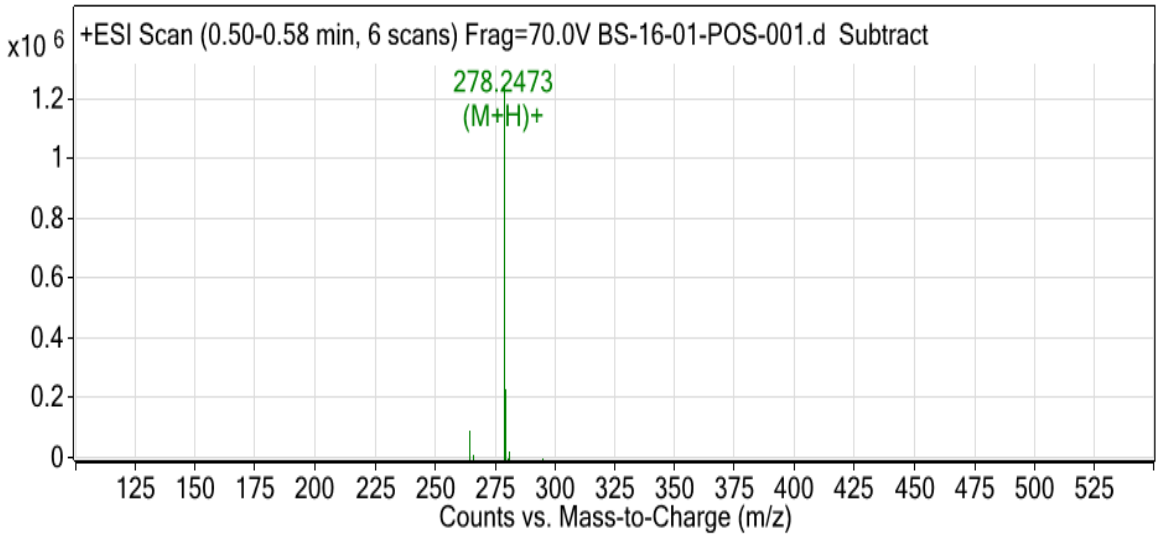


Fig. 7- k: Peaks detected by HR/MS between 0.50 to 0.58 min.

Table 7- VII: m/z of compounds with their relative possible abundance scanned between 0.50 to 0.58 min + ESI Scan

m/z	z	Abundance
264.232	1	98504
278.2473	1	1250142
279.2511	1	239013

Table 7- VIII: Possible formulas of compound with m/z- 278.2473.

Formula	**Best**	**Mass**	**Tgt Mass**	**Diff (ppm)**	**Ion Species**	**Score**	**DBE**	**m/z**
$C_{18}H_{31}NO$	TRUE	277.2401	277.2406	1.81	$C_{18}H_{32}NO$	97.64	4	278.2473
$C_{13}H_{31}N_3O_3$		277.2401	277.2365	-12.71	$C_{13}H_{32}N_3O_3$	65.91	0	278.2473

7.2.2.5.1 Infrared spectroscopy (IR)

IR spectrum of the partially purified compound from the MRE of *G. nervosa* is shown in Fig. 7-l. Following are the interpretations of the bands observed in IR spectrum.

- 3242-3600 cm^{-1}: Bands observed in this range are attributed to OH (stretching) that indicates the presence of free –OH group of alcohol.
- 2953.02 cm^{-1}: strong peak of C-H stretching CH structure in CH_3.
- 1450.47 & 1367.53 cm^{-1}: May be due to CHx deformation (C-H deformation in CH_3).
- 1612.49 cm^{-1}: may be due to C=C stretching.

- 1263.37 cm^{-1}: This peak could be attributed to C-O stretching.
- 1101.35, 1066.64 & 1002.98 cm^{-1}: May be due to C-O stretching.

Thus, IR spectrum indicating the presence of hydroxyl functionality and double bond in the compound.

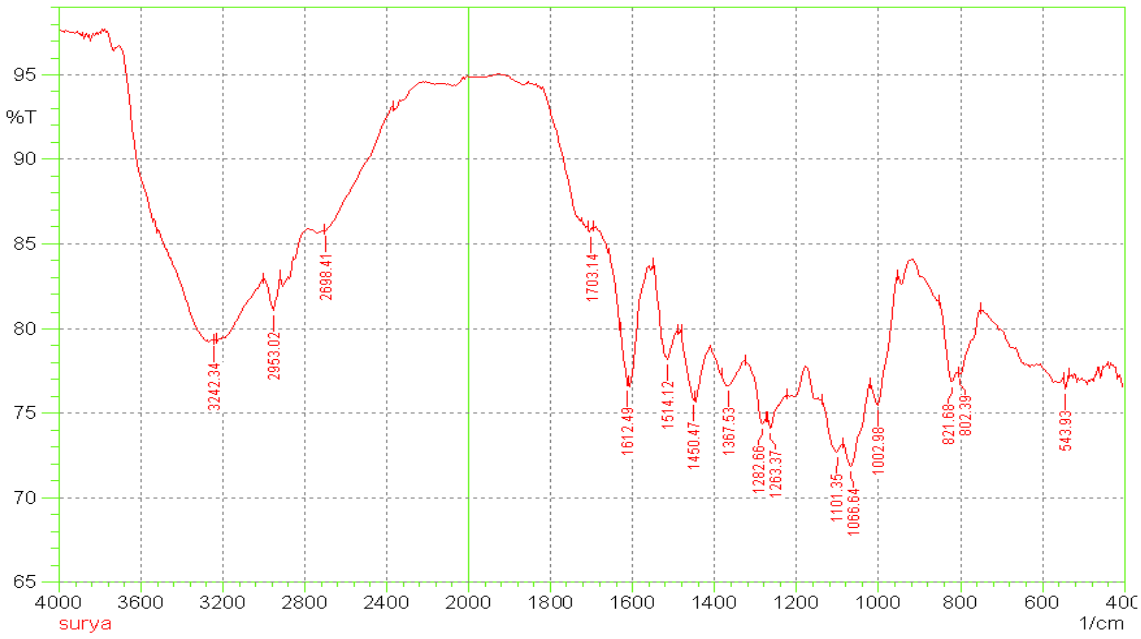


Fig. 7-l: Infra-Red spectroscopy of purified compound from MRE of *G. nervosa*.

7.2.2.5.2 Proton NMR

NMR spectrum of MRE from *G. nervosa* (Fig. 7-m) has been matched with NMR of compound isolated from *Microcos paniculata* (Table 7-IX).

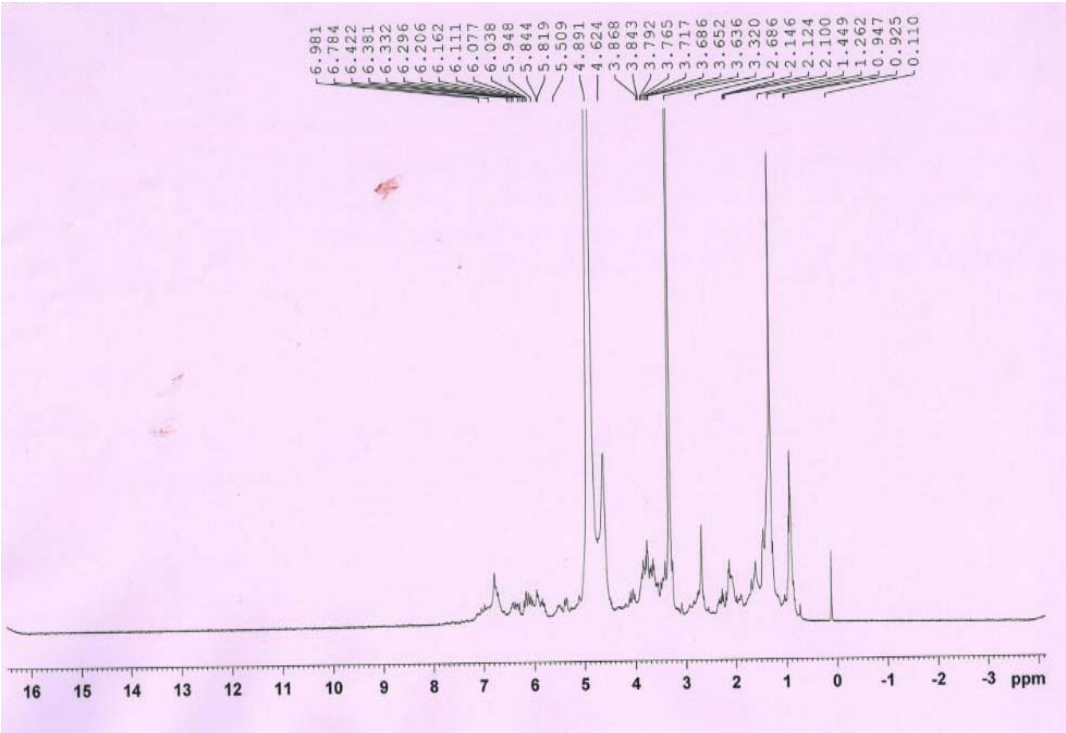


Fig. 7-m: Proton NMR spectrum of purified compound from the methanol root extract of *G. nervosa*.

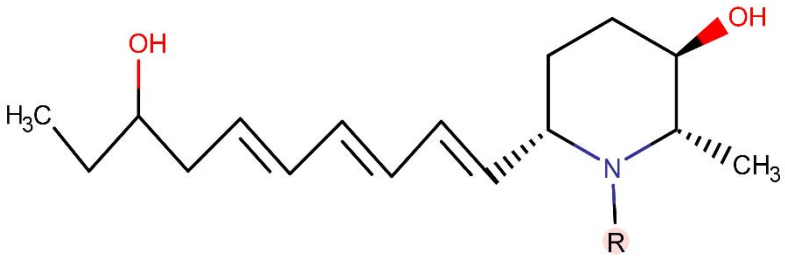


Fig. 7-n: Structure of (1) Microcosamine B (R=H, reported by Feng et al. 2008) and (2) major compound (R=CH3, isolated in present study) detected in partially purified compound from methanol root extract of *G. nervosa.*

Table 7-IX: Comparisons of ^{1}H NMR spectrum between Microcosamine B (Feng et al. 2008) and compound purified from the methanol root extract of *G. nervosa*.

Position	Microcosamine B Reported (Fig. 7-n-1)	Compound purified from MRE of *G. nervosa* (Fig. 7-n-2)
	In $CDCl_3$	**In CD_3OD**
2	2.50 (dq)	2.3- 2.4 (m)
3	3.12 (m)	3.12 (m)
4	1.33 (m), 2.00 (m)	1.26 (m), 2.10-2.2 (m)
5	1.33 (m), 1.69 (m)	1.26 (m), 1.6- 1.70 (m)
6	3.16 (m)	2.7- 3.0 (m)
1 '	5.58 (dd)	5.2-5.4 (m)
2 '	6.13 (m)	6.3-6.5 (m)
3 '	6.02 (m)	-
4 '	6.12 (m)	-
5 '	6.08 (m)	5.8-6.2 (m)
6 '	5.68 (dt)	5.4-5.6 (m)
7 '	2.15 (m), 2.27 (m)	2.10-2.2 (m)
8 '	3.54 (m)	3.4-3.5 (m)
9 '	1.44 (m)	1.44 (m)
10 '	0.90 (t)	0.92-0.94 (m)
Me-C(2)	1.15 (d)	1.2 (m)
$N(CH_3)$	-	2.68 (s)

The data obtained from HRLC-MS, IR and ^{1}H-NMR of the compound from the root of *G. nervosa* followed by the information from the literature search indicated that partially purified sample is a mixture of three compounds. Molecular formulas of these compounds are (1) $C_{17}H_{29}NO_2$, (2) $C_{17}H_{30}NO$ & (3) $C_{18}H_{30}NO$ with molecular weights of 280.2269, 264.2327 & 278.2473 respectively. Structure of all three compounds revealed that these are isomeric compounds, hence appeared as a single spot on TLC and difficult to separate by column chromatographic methods.

Several species of medicinal plants has been analyzed for their chemical profiling. The literature revealved that presence of similar mass spectrum data of compound isolated from the leaves of *Microcos paniculata* (Feng et al. 2008). Here, it would be well-meaning to note that scientific name *Microcos paniculata* is nothing but old synonym of *G. nervosa* only. The IR and NMR data of our compound exactly matches with the reported data confirm the presence of compounds N-methyl-microcosamine, Microgrewiapine A & Homomicrogrewiapine in 6.7: 1.6:1.7 ratio.

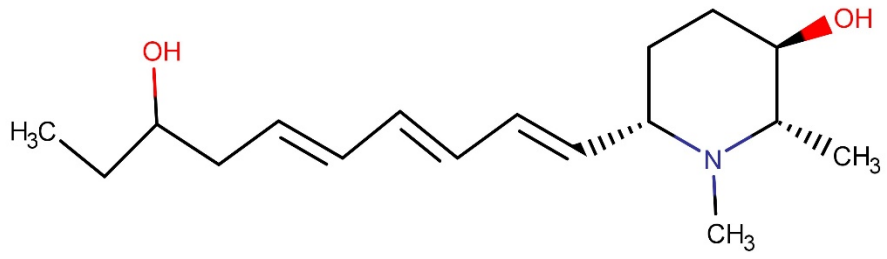


Fig.7-o: Structure of major compound (2) N-methyl-microcosamine/ homomicrocosamine in MRE of *G. nervosa*.

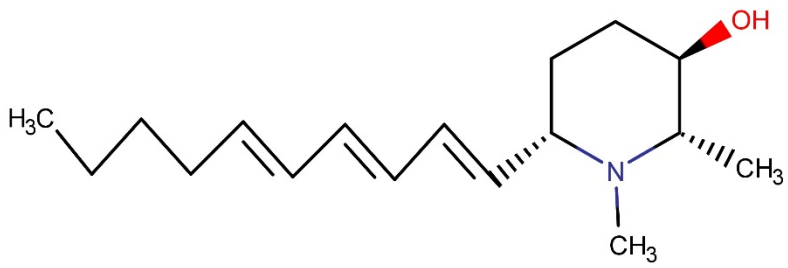


Fig. 7-p: Structure of microgrewiapine A (3) detected in MRE of *G. nervosa.*

Microgrewiapine A (Fig.7-p) is a known compound identified from the leaves of *Microcos paniculata.* The PubChem ID of microgrewiapine A is 71576920. IUPAC name of Microgrewiapine A is (2S, 3R, 6S)-6-[(1E, 3E, 5E)-deca-1, 3, 5-trienyl]-1, 2-dimethylpiperidin-3-ol. Microgrewiapine A (3) is reported to have cytotoxic effects on human HT-29 colon cancer cells (Still et al. 2013).

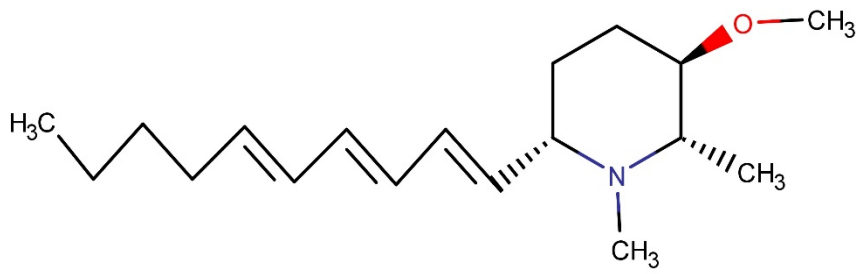


Fig. **7**-q: Structure of minor compound homomicrogrewiapine / methoxylated microgrewiapine (4) detected in MRE

Compounds namely N-methyl-microcosamine, Microgrewiapine A & Homomicrogrewiapine were isolated from methanol root extract of *G. nervosa* by comparing their NMR and MS data with the reported in the literature (Feng et al. 2008; Still et al. 2013). The chemical structures of the identified compounds are depicted in fig. Fig.7-o, Fig.7-p & Fig.7-q respectively. The observed α-glucosidase inhibition activity

could be due to the major metabolite or due to synergistic effect of all the three compounds. This stand to be the first report on isolation of N-methyl-microcosamine (2), however, Microcosamine B (1) is already reported from *Microcos paniculata* (Feng et al. 2008).

SUMMARY & CONCLUSION

- ✓ Selection of the *G. nervosa* for the present study was based on the criteria that plant is reportedly used in traditional medicine but lack scientific justification.
- ✓ Identification of the *G. nervosa* based on morphological characters was further reconfirmed using molecular markers assisted identification viz, *ITS2* and *rbcL*.
- ✓ *In vitro* callus induction was achieved using immature leaves of the *G. nervosa* as an explant. Further, using callus, production of single cell suspension culture was also achieved.
- ✓ Methanol & aqueous extracts of leaves, bark, roots and callus were prepared using soxhlet apparatus and later concentrated using Rota evoparator to prepare MLE (methanol leaves extract), MBE (methanol bark extract), MRE (methanol root extract), ALE (aqueous leaves extract), ABE (aqueous bark extract), ARE (aqueous root extract), MCE (methanol callus extract) and ACE (aqueous callus extract).
- ✓ MLE , ALE and MCE demonstrated antioxidant potential i.e. 5.41, 3.32 and 0.07 mmol/g (dw) respectively, that is much higher than the antioxidant activity reported in the fruit extract of *G. nervosa* i.e. 0.028 mmol/g (dw).
- ✓ Amongst all extracts, only MBE depicted antiinflammatory activity and is comparable to the antiinflammatory activity depicted by standard ascorbic acid.
- ✓ Based on colorimetric assays, although MLE of *G. nervosa* was confirmed to demonstrate the antilithiatic activity in dose dependent manner, but did not decrease the size and shape of *in vitro* synthesized calcium crystals when incubated with MLE.
- ✓ Amongst all the extracts, ABE depicted the potential of tyrosinase inhibition in dose dependent manner with maximum inhibition of 42.24% at 600µg/ml. This property of ABE indicated the skin whitening potential of *G. nervosa* extract.

- ✓ All methanol extracts (MLE, MBE, MRE) depicted antiproliferation & cytotoxic activity on MCF-7 breast cancer cell line. The IC_{50} values depicted by MLE, MBE & MRE are 1100, 850 & 1200 µg/ml respectively. Aqueous extracts did not show antiproliferation activity of MCF-7 call line.
- ✓ In order to identify phytoconstituents, especially the anticancer compounds present in methanol extracts (MLE, MBE, MRE) GC-MS analysis was carried out.
- ✓ The α-tocopherol, phytol & γ-sitosterol were detected in MLE and are reported as antiproliferation agent inhibiting several cancer cell line. β- sitosterol detected in MRE is also reported as antiproliferative agent of MCF-7 breast cancer cell line. GC-MS analysis did not reveal any anticancer compound from MBE of *G. nervosa.*
- ✓ Other than anticancer compounds, methanol extracts of *G. nervosa* depicted several other important chemical compounds that are included in the major class of phytochemicals such as fatty acids, sterols, alkaloids, alkanes, vitamins and diterpenes.
- ✓ Only MLE depicted α-amylase inhibition activity in dose dependent manner and the maximum inhibition of 97.5% was observed at 1 mg/ml of MLE. Whereas, α-amylase inhibition activity shown by acarbose is 91.3% at 100 mg/ml.
- ✓ MLE, MBE, MRE & ARE depicted α-glucosidase inhibition activity i.e. 90.87, 94.13, 95.63 & 90.70 % inhibition at 60, 60, 0.9 & 1.6µg/ml respectively.
- ✓ Enzyme kinetic study of α-glucosidase was carried out in presence and absence of MRE. Results of enzyme kinetic study revelaved that MRE showed no-competitive type of inhibition.
- ✓ Cytotoxic, mutagenic and genotoxic effect of MRE of *G. nervosa* was evaluated on adult zebra fish *(Danio rerio)* blood cells and human blood leukocytes. The effects

of various doses of MRE on zebra fish blood cells and on human blood leukocytes demonstrated no evidence of cytotoxicity and genotoxicity effects.

- ✓ The α-amylase inhibitory compounds present in MLE of *G. nervosa* was separated and isolated using TLC. Further compound was identified by GC-MS analysis. Hexatriacontene, a long carbon chain compound was identified as a possible α-amylase inhibitory compound as per literature.
- ✓ The α-glucosidase inhibitory compound present in the MRE was separated and partially purified using TLC, normal as well as reverse phase chromatography.
- ✓ Partially purified α-glucosidase inhibitory compound was charatcterized using IR, H^1-NMR and HRLC-MS.
- ✓ Three peaks identified by HRLC-MS indicated the presence of three compounds in partially purified sample.
- ✓ N-methyl-microcosamine is the major novel compound identified in the partially puried sample of MRE. Other two minor compounds identified are microgrewiapine A & homomicrogrewiapine which have been already reported from the *Microcos paniculata*.

FUTURE PROSPECTS

- ✓ MBE of *G. nervosa* has shown promising antiinflammatory activity depicted. Till now there is no any report describing the isolation, purification and characterization of antiinflammatory compound/s from *G. nervosa*. So, there are chances of getting novel compound with antiinflammatory activity.
- ✓ There is need to justify the antilithiatic properties of *G. nervosa* by further studies.

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APPENDIX

Appendix 1: TE buffer

Sl. No.	Chemicals	Concentration
1	Tris/HCl,	10 mM
2	EDTA	1 mM
	pH 8.0	

Appendix 2: Extraction buffer

Sl. No.	Chemicals	Concentration
1	sucrose	15% (w/v)
2	Tris-Cl (pH 8.0)	50 mM
3	EDTA (pH 8.0)	50 mM
4	NaCl	500 mM

Appendix 3: Re-suspension buffer

Sl. No.	Chemicals	Concentration
1	Tris-Cl (pH 8.0)	20 mM
2	EDTA (pH 8.0)	10 mM

Appendix 4: Murashige and Skoog's medium (MS)

Sl. No.	Chemicals	mg/L	mg/L
Stock solution 1st (20X)			
1	NH_4NO_3	1650	33000
2	KNO_3	1900	38000
3	$CaCl_2.2H_20$	440	8800
4	$MgSO_4.7H_20$	370	7400
5	KH_2PO_4	170	3400
Stock solution 2nd (200X)			
1	KI	0.83	166
2	H_3BO_3	6.3	1260
3	$MnSO_4.4H_20$	22.3	4460
4	$ZnSO_4.7H_2O$	8.6	1720
5	$Na_2MoO_4.2H_2O$	0.25	50
6	$CuSO_4.5H_2O$	0.025	5
7	$CoCl_2.6H_2O$	0.025	5
Stock solution 3rd (200X)			
1	$FeSO_4.7H_2O$	27.6	5520
2	$Na_2.EDTA.2H_2O$	37.3	7460
Stock solution 4th (200X)			
1	Inositol	100	20000
2	Nicotinic acid	0.5	100
3	Pyridoxine HCl	0.5	100
4	Thiamine HCl	0.5	100
5	Glycine	2	400
6	Biotin	0.5	100
1	Sugar stock solution	300 g/L	
2	2,4-D	2 mg/ml	
3	Kinetin	4 mg/ml	