Isolation, Structural Elucidation and Biological Evaluation of Labdane Diterpenes from Seeds of *Alpinia nigra* (Gaertn.) B.L. Burtt

A thesis submitted by

SUDIPTA GHOSH

For the award of degree of

Doctor of Philosophy

Department of Biotechnology

Indian Institute of Technology Guwahati
Guwahati-781039, Assam, India

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STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the guidance of Dr. Latha Rangan and Dr. Utpal Bora.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

November, 2013 SUDIPTA GHOSH

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November, 2013

CERTIFICATE

It is certified that the work described in this thesis, entitled "**Isolation, structural elucidation and biological evaluation of labdane diterpenes from seeds of *Alpinia nigra* (Gaertn.) B.L. Burtt**", done by Mr. Sudipta Ghosh for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

Dr. Latha Rangan
Associate Professor
(Thesis Supervisor)

Dr. Utpal Bora
Associate Professor
(Thesis Supervisor)

Dedicated to my beloved parents

A C K N O W L E D G E M E N T S

Fulfillment of this doctoral thesis was possible with the continuous help and encouragement of several people during my PhD tenure. Firstly, I am truly thankful to Dr. Latha Rangan and Dr. Utpal Bora for their scientific and moral guidance as mentor throughout my doctoral research. I express my deep gratitude to both of them for their solemn care and all support during the ups and downs of my stay at IITG.

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Last but not least, I pay my sincere thanks, love and respect to all who have directly or indirectly helped during my doctoral thesis work. Finally, I would like thank almighty God for his relentless help during my life at IIT Guwahati.

November, 2013 (SUDIPTA GHOSH)

C O N T E N T S

A B S T R A C T

The current investigation unveils the potential uses of *Alpinia nigra* (Gaertn.) B. L. Burtt as a source of bioactive compounds towards development of therapeutic agent against various diseases. The plant is used as vegetable and food flavouring agent in various parts of Northeast India apart from other uses in folk ethnomedicines.

In the present study, the mature seeds of *A. nigra* were collected, processed and subjected to soxhlet extraction in a polarity gradient with three organic solvents *viz*. n-Hexane, Ethylacetate and Methanol respectively. All the three extracts were characterized by GC-MS and chemical fingerprints (NMR and FTIR) were recorded. Qualitative phytochemical screening was performed to detect the presence of secondary metabolites (saponins, alkaloids, phenolics and terpenoids) in each extract. Moreover, study on DPPH free radical scavenging activity suggested ethyl acetate and methanolic extracts as most potential ones to explore further.

Principal components from these extracts were further isolated and characterized with spectroscopic techniques *viz*. ^{1}H NMR, ^{13}C NMR, FT-IR and HRMS that established the identity of compounds as (*E*)-labda-8(17),12-diene-15, 16-dial (**I**) and (*E*)-8β,17-epoxylabd-12-ene-15,16-dial (**II**). From the hexane extract another major compound was purified and established as 1,2-dihexadecanoyl-3-(9Z-hexadecenoyl)- sn-glycerol (**III**). All the purified compounds were tested for Lipinski's drug likeliness properties, where, compound **I** and **II** were found as suitable candidate for biological uses. Further, biocompatibility of these compounds (**I** and **II**) were determined by using RBC hemolysis assay.

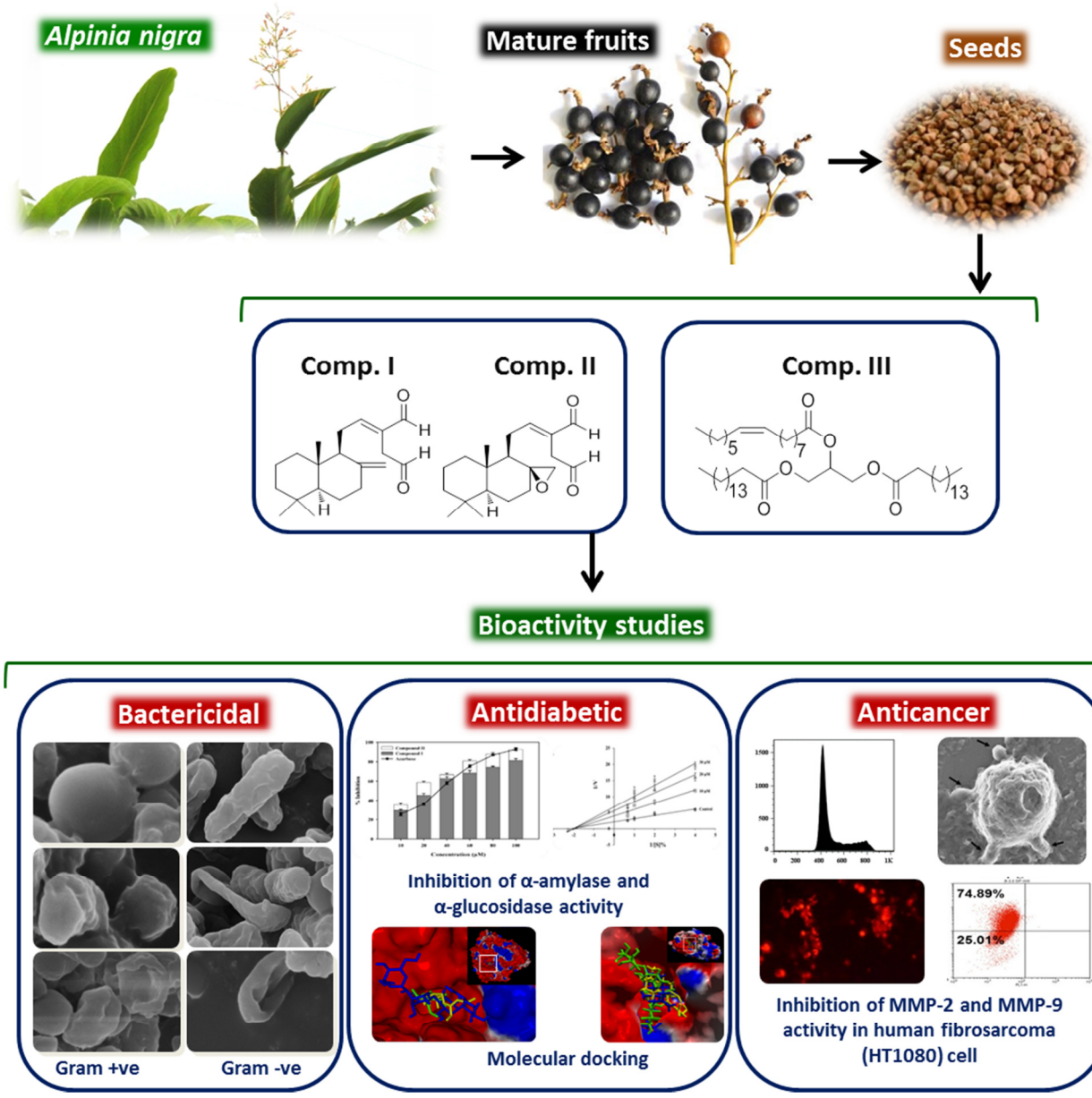
These two labdane diterpenes were critically evaluated for their antibacterial activities and tested in seven pathogenic bacterial indicators. Minimum inhibitory concentration (MIC) was determined for each compound for respective bacteria. Among the Gram positive bacteria, *Staphylococcus aureus* (ATCC6538) and among the Gram negative bacteria, *Yersinia enterocolitica* (MTCC 859) were found most susceptible to these compounds. Flow cytometry (FC) and cell leakage analysis showed the compromised state of bacterial cell membrane. Pore formation and damage of bacterial cells were confirmed by field emission scanning electron microscope (FESEM) imaging.

Antidiabetic activity of **I** and **II** were investigated which showed dose dependant inhibition of α-amylase and α-glucosidase in a non-competitive manner. Molecular docking was performed for human pancreatic α-amylase (HPA) and maltase-glucoamylase (MGAM) with both the compounds and standard drug acarbose. In case of HPA and MGAM, both the compounds interacted in the active site similar to acarbose which might lead to non-competitive mode of inhibitions.

To understand the effects of **I** and **II** on matrix metalloproteinase (MMPs) activity, HT1080 human fibrosarcoma cells was used as a source of MMPs. *In vitro* treatment with **I** and **II** showed no significant modulation of MMPs activity. However, when HT1080 culture supernatants were directly incubated with **I** and **II** they were found to inhibit the MMP-2 and MMP-9 activities. These compounds also induced cell cycle arrest in the S phase and nuclear condensation leading to apoptosis in HT1080 cells.

The current research has enabled identification of two principal bioactive compounds from *A. nigra* seeds and their potential uses in diverse biological challenges against infectious and non-infectious diseases. This will promote further research and development towards future therapeutics in herbal medicines.

G R A P H I C A L A B S T R A C T



S Y N O P S I S

Currently, plant based small molecules and natural products are getting more attention owing to their ethnomedicinal uses in folk medicines and comparative easy availability. Exploration of ethnomedicinal knowledge towards drug discovery is a smart way to reduce experimental cost and improve the possibility of success in novel drug finding projects.

The ethnomedicinal practices of the tribal communities of North East India involving the Zingiberaceae family plants were critically studied and documented (Tushar et al. 2010). *Alpinia* which is the largest genus of Zingiberaceae, is emerging as the most proliferative genus in this family due to its diverse biological applications notably as anti-microbial, anti-cancer, anti-viral, neuroprotective agent (Shi et al. 2006; Elzaawely et al. 2007; Yasuharaa et al. 2009; Upadhyay et al. 2011). Diverse members of the *Alpinia* genus possess remarkable pharmaceutical potentials and future therapeutic values. Amongst these *Alpinia nigra* (Gaertn.) B. L. Burtt. is known for its ethnomedicinal importance in the treatment of bone weakness, irregular menstruation, jaundice, gastric ulcers etc. in folk medications (Tushar et al., 2010).

The present investigation was focused on the isolation and structural elucidation of principal compound(s) from *A. nigra* seeds and their biological evaluations for the development of future herbal therapeutics with the following as objectives.

- Chemical profiling and free radical scavenging activity of crude organic solvent extracts from *A. nigra* seeds.
- Isolation and characterization of the principal component(s) from crude organic extracts of *A. nigra* seeds.
- Studies on bactericidal activities of seed extracts and purified labdane type diterpenes.

- ❖ Studies on molecular docking and inhibition kinetics of labdane diterpenes on α-amylase and α-glucosidase enzymes towards combating Type 2 diabetes.
- ❖ Studies on labdane diterpenes towards matrix metalloproteinase inhibitory activity and cytotoxicity on HT1080 fibrosarcoma cells.

Overall the thesis has been divided into eight chapters as described below. Results of the present investigation are presented in five chapters (3-7). These chapters are preceded by **Chapter One** gives a brief background of the present research and **Chapter Two** which includes the detailed review of literatures emphasizing the plant based medicinal systems, natural products as a source of novel drug, labdane diterpenes and therapeutic potential of the genus *Alpinia* towards future herbal sources of novel drug leads.

Chapter Three describes the extraction of chemical constituents from *A. nigra* seeds using soxhlet extraction method in a polarity gradient with three organic solvents *viz.* n-Hexane, Ethylacetate and Methanol respectively. All the three extracts were characterized by GC-MS and chemical fingerprints (NMR and FTIR) were recorded. Qualitative phytochemical screening was performed to detect the presence of secondary metabolites (saponins, alkaloids, phenolics and terpenoids) in the extracts. Moreover, study on DPPH free radical scavenging activity suggested ethyl acetate and methanolic extracts as most potential ones to explore further.

Chapter Four describes the isolation and characterization of principal components from these extracts with spectroscopic techniques *viz.* ^{1}H NMR, ^{13}C NMR, FT-IR and HRMS. The investigation established the identity of compounds as (*E*)-labda-8(17),12-diene-15, 16-dial (**I**) and (*E*)-8β,17-epoxylabd-12-ene-15,16-dial (**II**). From the hexane extract another major compound was purified and established as 1,2-dihexadecanoyl-3-(9Z-hexadecenoyl)- sn-glycerol (**III**). All the purified compounds were tested for Lipinski's drug likeliness

properties, where, compound **I** and **II** were found as suitable candidate for biological uses. Further, biocompatibility of these compounds (**I** and **II**) were determined by using RBC hemolysis assay.

Chapter Five unveils these two labdane diterpenes (**I** and **II**) as promising antibacterial agents which were tested in seven pathogenic bacterial strains. Minimum inhibitory concentration (MIC) was determined for each compound for respective bacteria. Among the Gram positive bacteria, *Staphylococcus aureus* (ATCC6538) and among the Gram negative bacteria, *Yersinia enterocolitica* (MTCC 859) were found most susceptible to these compounds. Flow cytometry (FC) and cell leakage analysis showed the compromised state of bacterial cell membrane. Pore formation and damage of bacterial cells were confirmed by field emission scanning electron microscope (FESEM) imaging. Similar studies were also performed with all the three extracts of *A. nigra* seeds.

Chapter Six describes the antidiabetic activity of **I** and **II** which showed dose dependant inhibition of α-amylase and α-glucosidase. The inhibition kinetics studies showed the pattern of inhibition is similar to non-competitive type for both the enzymes using compound **I** and **II**. Molecular docking was performed for human pancreatic α-amylase (HPA) and maltase-glucoamylase (MGAM) with both the compounds and standard drug acarbose. In case of HPA and MGAM, both the compounds interacted in the active site similar to acarbose which might lead to non-competitive mode of inhibitions.

Chapter Seven describes the effects of **I** and **II** on matrix metalloproteinase (MMPs) activity where HT1080 human fibrosarcoma cells was used as a source of MMPs. *In vitro* treatment with **I** and **II** showed no significant modulation of MMPs activity. However, when HT1080 culture supernatants were directly incubated with **I** and **II** they were found to inhibit the MMP-2 and MMP-9 activities. Further studies revealed, these compounds also induced cell

cycle arrest in the S-phase and causes nuclear condensation leading to apoptosis in HT1080 cells similar to the standard apoptotic agent staurosporine.

Chapter Eight describes the significance and salient features of the present study with scopes of further in depth investigations in future.

The work described in the thesis and related research carried out during the period of doctoral research has been peer reviewed and resulted in following international journal publications and patent:

Published articles:

Ghosh S, Indukuri K, Bondalapati S, Saikia AK, Rangan L (2013) Unveiling the mode of action of antibacterial labdane diterpenes from *Alpinia nigra* (Gaertn.) B. L. Burtt seeds. *Eur J Med Chem* 66, 101-105. *–Research Article*

Ghosh S, Padilla-González GF, Rangan L (2013) *Alpinia nigra* seeds: A potential source of free radical scavenger and antibacterial agent. *Ind Crop Prod* 49, 348- 356. *–Research Article*

Ghosh S, Rangan L (2013) *Alpinia*: The gold mine of future therapeutics. *3 Biotech* 3, 173-185. *–Review Article*

Manuscript under review:

Ghosh S, Rangan L (2013). Inhibition kinetics and molecular docking of α-amylase against isolated labdane diterpenes from *Alpinia nigra*. *Med Chem Res*. *–Research Article*

Ghosh S, Rangan L (2013). Labdane diterpenes as non-competitive to mixed type inhibitors of α-glucosidase. *Planta Medica*. *–Research Article*

Ghosh S, Mohan CM, Hussain M, Limaye Am, Rangan L (2013). Labdane diterpenes from *Alpinia nigra* seeds inhibit matrix metalloproteinase activity and arrest the HT 1080 fibrosarcoma cells at S-phase. *Protoplasma. –Research Article*

Ghosh S, Ozek T, Tabanca N, Ali A, Rehman JU, Khan IA, Rangan L (2013). Chemical composition and bioactivity studies of essential oils from *Alpinia nigra* (Gaertn.) B. L. Burtt. *Ind Crop Prod. –Research Article*

Ghosh S, Singh RK, Indukuri K, Dubey VK, Saikia AK, Rangan L (2013). Antileishmanial labdane type diterpene compounds from the seeds of *Alpinia nigra* (Gaertn.) B. L. Burtt. *Ind Crop Prod. –Research Article*

Patent:

Ghosh S, Singh RK, Indukuri K, Dubey VK, Saikia AK, Rangan L (2013). Antileishmanial labdane type diterpene compounds from the seeds of *Alpinia nigra* (Gaertn.) B. L. Burtt. (Applied to DST, TIFAC, Government of India).

A B B R E V I A T I O N S

ANOVA	Analysis of variance
BHT	Butylated hydroxyl toluene
CBB	Coomassie brilliant blue
CC	Column chromatography
DM	Diabetes mellitus
DPPH	2, 2-diphenyl-1-picrylhydrazyl
FC	Flow cytometry
FDA	Food and drug administration
FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalents
GC-MS	Gas chromatography mass spectrometry
GMP	Good manufacturing practices
HPA	Human pancreatic α-amylase
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
IDF	International diabetes foundation
IE	Interaction energy
KI	Kovat's indices
LB	Lineweaver-Burk
MBC	Minimum bactericidal concentration
MD	MolDock
MFI	Median fluorescence
MGAM	Maltase-glucoamylase
MIC	Minimal inhibitory concentration
MMP	Matrix metalloproteinases
MTT	Methyl-thiazolyldiphenyl-tetrazolium bromide
MVD	Molegro virtual docker

NEI	North East India
NMR	Nuclear magnetic resonance
OD	Optical density
PDB	Protein data bank
PI	Propidium iodide
pNPG	*p*-nitrophenyl-α -D-glucopyranoside
RBC	Red blood cells
R-EtAc	Ethyl accetate extract of rhizomes
R-Hex	Hexane extract of rhizomes
R-Met	Methanolic extract of rhizomes
RT	Retention time
S-EtAc	Ethyl accetate extract of seeds
S-Hex	Hexane extract of seeds
S-Met	Methanolic extract of seeds
TLC	Thin-layer chromatography
TSP	Total soluble phenolics
WHO	World Health Organisation

Abbreviations for intensities of ^{1}H-NMR signals

d	doublet
dd	doublet of doublet
Hz	Hertz
m	multiplet
MHz	Mega-Hertz
s	singlet
t	triplet

U N I T S

µg	microgram
µg/µl	microgram per microlitre
µg/ml	microgram per milliliter
µl	microlitre
µM	micromolar
^{0}C	degree celsius
Å	angstrom
a.u.	arbitrary units
g	gram
h	hour
kcal/mol	kilocalorie per mole
mg	milligram
mg/ml	milligram per millilitre
min	minute
ml	milliliter
mM	millimolar
pH	negative log H^{+} ion
rpm	revolution per minute
s	second
v/v	volume/volume
w/v	weight/volume

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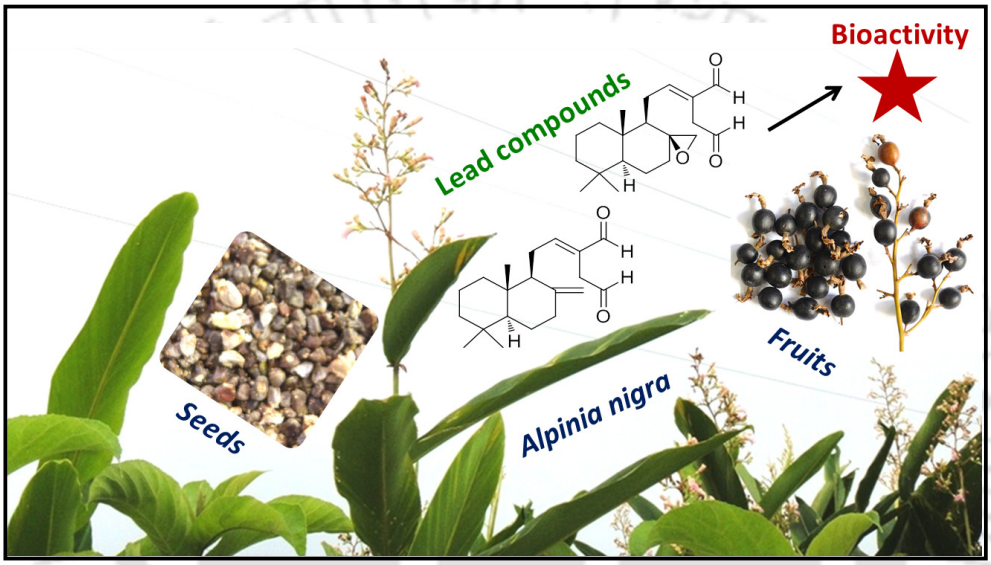
Chapter 1

Introduction



The chapter describes brief background on the role of natural product in the development of herbal medicine for curing of various diseases and ailments and finally the motivation and the specific objectives of the thesis work.

Chapter 1

Introduction

Humans have been using plants as a source of medicines to cure various ailments for centuries and ages (Solecki and Shanidar 1975; Ji et al. 2009). Over time, nature has evolved continuously to synthesize a diverse array of secondary metabolites that has emerged as one of the most sought after resources in the world. The secondary metabolites found mainly in the form of a complex mixture containing various natural compounds (crude extracts) were the first and only medicines available to mankind for a long period of time. These crude extracts were extensively used for primary healthcare in major parts of the world, however, in the western world they were mostly replaced by principal bioactive compounds or active pharmaceuticals towards the treatment of various ailments and diseases.

Initially, most of the pharmaceutical industries in the world have used plant extracts to harvest relatively crude formulations in the form of therapeutics. But, in the middle of 20th century with the development of antibiotics, formulation of drugs has become more favourable towards the use of purified bioactive compounds. These bioactive compounds are mainly derived from various natural sources, *viz.* plants, animals and microorganisms and broadly termed as natural products. Over centuries, owing to its chemical diversity, natural products have been extensively used as the preparatory materials for pharmaceutical drug discovery. In the last few decades, many of the natural products and their synthetic derivatives have been successfully commercialized towards clinical use for treating human diseases primarily in all areas of therapeutics (Baker et al. 2007).

Natural products in the form of standardized crude extracts or purified compounds offer a wide opportunity for the discovery of a novel drug or lead compound towards the

therapeutic application for a range of health related problems, including many infectious diseases (Clardy and Walsh 2004). Among several bioresources very little has been explored towards the identification of bioactive natural products, and in fact ample scope lies ahead in future, especially for plant bioresources. Plant derived and other natural products, largely secondary metabolites, are the source of a majority of drugs or lead compounds found in clinical use today. Recent reviews showed the incredible importance of natural products especially against infectious agents or diseases which represents >75% of the 97 new antibacterial drugs introduced and approved during the period from 1981-2006 (Newman and Cragg 2012; Taylor 2013). According to Newman and Cragg (2012), among all the small molecules approved in 2010, 50% were from natural sources. These statistics clearly demonstrate the continuous interest in natural product research, and its impact on drug discovery and development. Moreover, these data reflect the recent trends and highlight the emerging role of natural products as valuable lead compounds and potential new drugs. Apart from natural products and its semi-synthetic derivatives, recently natural product botanicals have also been approved as sources of novel drugs/leads by FDA (Newman and Cragg 2012). Many natural products and their semi-synthetic derivatives are in use to combat against various diseases and have emerged as potent antibacterial, antidiabetic and anticancerous drug candidates (Butler 2008; Newman and Cragg 2012). Currently, plant based small molecules and natural products are getting more attention owing to their ethnomedicinal uses in folk medicines and comparatively easy availability (Verpoorte et al. 2005; Taylor 2013). Exploration of ethnomedicinal knowledge of plants towards drug discovery is a smart way to reduce experimental cost and improve the possibility of success in novel drug finding projects (Cordell and Colvard 2005; Patwardhan 2005).

Being the largest producer of medicinal herbs, India is known as the botanical garden of the world catering to the needs of thousands of herbal medicines (Seth and Sharma 2004).

Due to the immense importance and richness of medicinal plants in North East India (NEI), a database has been created recently by Meetei et al. (2012). The database comprises information on several natural products isolated from various medicinal plants of NEI and their therapeutic uses. Previously, various other research groups have reported the uses of medicinal plants of NEI towards the curing of various diseases like malaria (Bora et al. 2007), parasitic infection (Roy et al. 2012), diabetes (Tag et al. 2012) and other traditional healing systems (Lokesh and Amitsankar 2012; Ningthoujam et al. 2013). Several other investigations showed plants of NEI can be used as cytotoxic agent against various cancer cell lines (Srivastava et al. 2009; Das et al. 2013), antibacterial (Arifullah et al. 2013). In an estimate by Dutta and Dutta (2005), NEI harbors 1350, 665 and 899 plant species under ethnomedicinal, food and other uses respectively. Similarly, in the world, around 80% population depends on the traditional medicines for their primary healthcare, largely on plant-derived drugs due to its increasing cultural acceptability and considerably lower side effects (Ghasi et al. 2000).

In the plant kingdom, Zingiberaceae, a well-recognized family, has been investigated by diverse research groups due to its versatile nature and high medicinal value. Members of this family are distributed worldwide with about 52 genera and 1,300 diverse species mainly concentrating in South and Southeast Asia (Wu and Larson 2000). In India, about 22 genera and 178 species have been reported from the North Eastern and peninsular region (Jain and Prakash 1995), whereas North East India (NEI) alone harbours 19 genera and close to about 88 diverse species (Prakash and Mehrotra 1995). Ethnomedicinal uses of Zingiberaceae plants of NEI by the tribal communities have been critically documented for future pharmacological applications. Among the various Zingiberaceae plants of NEI, many species of *Alpinia* genus were found to be useful in folk medicines with versatile biological activities (Tushar et al. 2010). *Alpinia* is the largest and most wide spread genus of the family,

constituting about 230 species distributed through tropical and subtropical climates of Asia and the Pacific (Kress et al. 2005). Members of the genus, *Alpinia*, possess complex chemical profile, bioactive potentials and prospective therapeutic values. Presence of many bioactive substances such as flavonoids, tannins and terpenoids aid to its therapeutic efficiency towards anti-inflammatory, antimicrobial, antidiabetic, anticancerous and other diseases (Ghosh and Rangan 2013). Till date most of the work has been concentrated in *A. galanga* towards the isolation and activity studies of several bioactive compounds, compared to other species in the genera (Janssen and Scheffer 1985; Khattak et al. 2005; Oonmetta-aree et al. 2006; Niyomkam et al. 2010; Rao et al. 2010; Weerakkody et al. 2011). Besides *A. galanga*, other species, *viz. A. oxyphylla*, *A. speciosa*, *A. zerumbet* and many others are also gaining attention due to the presence of diverse bioactive compounds and their natural analogue which shows multiple biological activities (Ghosh and Rangan 2013).

One such important but less explored member in the *Alpinia* genus is *Alpinia nigra* (Gaertn.) B. L. Burtt which is found in different parts of NEI and locally known as "Tora". The plant is distributed primarily in China, Thailand and other Southeast Asian countries including NEI (Wu 1981). Ethnomedicinally important but less explored *A. nigra* is known to be used for the treatment of dyspepsia, gastric diseases, insect bites, trematocidal etc. in folk medications (Roy et al. 2009). Besides these, *A. nigra* has now been established as a notable antihelminthic source towards the possible remedy against intestinal helminth infection (Roy et al. 2012). In recent years, although extensive work on the other members of the genus *Alpinia* has revealed its chemical complexity, diverse bioactive molecules and their therapeutic properties, *A. nigra* is still unexplored with limited information about its active components and bio-efficacy even though it has versatile ethnomedicinal uses.

Therefore, the present study was undertaken with the objectives to isolate the organic solvent extracts primarily from *A. nigra* seeds, study the chemical profile and free radical scavenging activity of the isolated extracts and finally, purification and structural elucidation of principal compound(s) from these seed extracts. Further, efficacy of the crude extracts and purified bioactive compounds were investigated towards antibacterial, antidiabetic and anticancerous activities in the view of prospective herbal therapeutics.

1.1. Objectives

Based on the background study and its emerging importance in the therapeutic applications, the present investigation is carried out with the broad objectives of isolation and structural elucidation of principal compound(s) from *A. nigra* seeds. Further, the biological efficacy of isolated active compounds towards the development of future herbal therapeutics was studied. The specific objectives are outlined as follows:

[1] Chemical profiling and free radical scavenging activity of crude organic solvent extracts from *A. nigra* seeds.

[2] Isolation and characterization of the principal component(s) from crude organic extracts of *A. nigra* seeds.

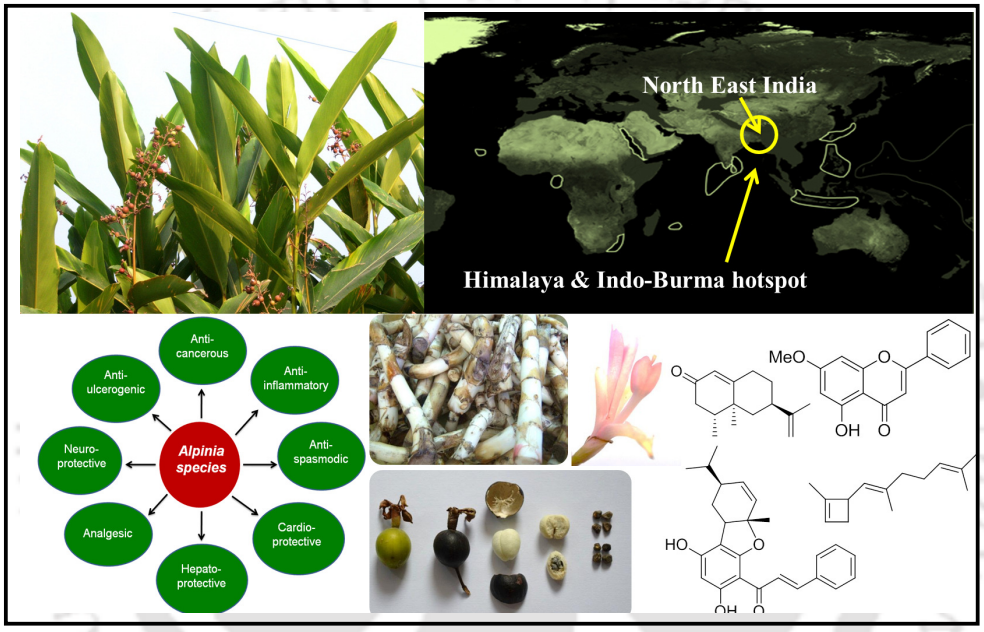
[3] Studies on bactericidal activities of seed extracts and purified labdane type diterpenes.

[4] Studies on molecular docking and inhibition kinetics of labdane diterpenes on α-amylase and α-glucosidase enzymes towards combating Type 2 diabetes.

[5] Studies on labdane diterpenes towards matrix metalloproteinase inhibitory activity and cytotoxicity on HT1080 fibrosarcoma cells.

Chapter 2

Literature review



The chapter describes the history and the current status of natural products in drug discovery. This background information further illustrates the importance of the genus *Alpinia* and its bioactive compounds towards various biomedical applications.

Chapter 2

Literature review

2.1. Introduction

Since time immemorial, besides being catering to the basic needs (food, cloth and shelter) of humans nature has served as the primary source of medicines for the treatment of a wide spectrum of diseases. Plants, in particular, for long have played a leading role in the continuous advancement of traditional systems of medicine. The first historical record of ancient Egyptian medicine is the "Ebers Papyrus", which dates back to 1500 BC, documents over 700 drugs, mostly of plant origin. Similar documentation of various uses of approximately 1000 plant derived substances in Mesopotamia date from around 2600 BC and many of these substances are in use even today for the treatment of various health related ailments (Borchardt 2002). Extensive documentation of the Chinese Materia Medica has occurred over the centuries with the first record dating from about 1100 BC (Huang and Williams 1999). Likewise, documentation of the Indian Ayurvedic system dates before 1000 BC (Charaka 341 drugs, Sushruta and Samhitas 516 drugs) which mentions about many herbal remedies against various diseases and ailments (Dev 1999; Kapoor 2000). Considering the indispensable and persistent role played by plant-based systems in the healthcare and medicines across various ethnic cultures, the same has been extensively documented for future pharmacological uses (Moerman 1986; Johnson 1999). The assessment of World Health Organization (WHO) revealed that about 65% of the world's population depends mainly on plant-derived traditional medicines for their primary health care (Cragg et al. 2009).

The emergence of modern medicinal systems and their proliferation by colonial regimes cast a shadow on traditional medicinal system across the world for over a century. Ironically, the modern medicinal system derives the majority of its medicines from natural sources. Fortunately, the importance of herbal medicines in human convalescence and the interrelationship between nature and society has recently been acknowledged widely. This has drawn attention to the fact that the loss of biodiversity and the destruction or the unscientific use of medicinal plants can have direct and indirect effects on the well-being of humans. The thirst of mankind for knowledge, coupled with recent technological advancements and scientific breakthroughs, has speeded up the discovery of synthetic medicines which has aided in the treatment or prevention of several acute/chronic/life threatening diseases. But prolonged and over-use of these drugs can lead to toxic side effects, which are becoming a major threat in the modern era. Increase in pollution, unhealthy lifestyles, stress, loss of traditional medicinal practices and decline in plant biodiversity has increased in recent times leading to an alarming disturbance in the structure and function of nature (Dubey et al. 2004; Alves and Rosa 2007). Therefore, medicinally important plants are drawing much attention in conventional healthcare system owing to its reasonably less side effects and alternative approaches. The demand for various medicines, health products and pharmaceuticals from plants sources are increasing around the world owing to their healthy interaction with physiological flora, relatively less side effects and less expensive (Dubey et al. 2004; Sharma et al. 2008).

2.2. History of herbal medicine

Over time, nature has evolved continuously to synthesize a diverse collection of secondary metabolites in plants, microbes and marine biosources which are emerging as the most sought after resources in the world. The secondary metabolites mainly in the form of natural product

extracts were the only and first medicines available to mankind for a long period of time. These crude extracts were extensively used as primary healthcare in major part of the world, whereas, in the western world they were mostly replaced by principal bioactive compounds or active pharmaceuticals towards the treatment of various ailments and diseases.

The documented evidence of human intellect towards the use of plants against various diseases has been found in history of the ancient Chinese, North African and Indian civilizations. According to Phillipson (2001), during 19th century, humans started to purify the active compounds from medicinal herbs. A notable breakthrough in history of medicinal plant was the "Quinine", isolated from Cinchona bark by two French scientists, Caventou and Pelletier. Many discoveries of plant derived bioactive compounds drew much attention from scientist of the New World and explorations began to various parts of impassable forests in search of novel medicines. Many such efforts were rewarded by the identification of several active compounds from plants and further investigated for potential uses in healthcare by the cognoscenti with leading pharmaceutical industries.

Several plant-derived bioactive compounds have been isolated before World War II which became drug candidates in clinical use and are found promising till date. The antibiotic era dawned during and after World War II due to the various antibacterial compounds extracted from species of *Cephalosporium*, *Penicillium* and *Streptomyces*. After the onset of World War II, the discoveries were relatively less in terms of new chemical entities from medicinal plants. During that time, discovery of "reserpine" from the roots of *Rauwolfia serpentine* revolutionized the uses of tranquillisers in healthcare. Similarly, other notable discoveries of that period "vinblastine" and "vincristine" used for the treatment of cancer which were isolated from *Catharanthus roseus* (Phillipson 2001). Further remarkable achievements in plant derived medicines were the discovery of potent anticancer agents,

Camptothecin and Taxol (Oberlies and Kroll 2004). Two research groups lead by Dr. M. E. Wall and Dr. M. C. Wani were identified Camptothecin and Taxol for the first time as life-saving drug in cancer chemotherapy. Camptothecin was isolated from *Camptotheca acuminate* and known to act on topoisomerase I leading to death of cancerous cells. For the treatment of ovarian cancer two analogues (first generation) of camptothecin are in use and many new analogues (second-generation) have been included in the clinical trials. Taxol was isolated from *Taxus brevifolia* and known to effect the stabilization of microtubules leading to hamper in growth of cancer cells. Taxol was clinically approved for the treatment of ovarian cancer and currently is used against various other cancers including breast cancer. Similar to earlier discoveries, morphine and codeine (from opium poppy), atropine and hyoscine (from Solanaceae plants), digoxin (from *Digitalis*), and many other plant derived new compounds are in clinical use towards the treatment of various diseases (Phillipson 2001; Newman and Cragg 2012).

2.3. Natural products in drug discovery

Natural products in the form of standardized crude extracts or purified compounds offer extensive opportunities for a novel drug or a lead compound towards therapeutic applications for a range of health related problems, including many infectious diseases (Clardy and Walsh 2004). Among the microbial, marine and plant bioresources, very little has been explored towards the identification of bioactive natural products and ample scope exists. Natural products, largely secondary metabolites, are the source of the majority of drugs or lead compounds in clinical use today.

In the areas of cancer and infectious disease, 60% and 75% of new drugs originated from natural sources between 1981 and 2002 respectively (Newman et al. 2003). According to Butler (2008), during 2001-2005, several new compounds has been isolated from natural

resources and introduced for the treatment of various ailments and diseases including bacterial infections, diabetes and cancer. Regardless of several achievements in natural product research towards the development of new drug leads, most of the mainstream pharmaceutical industries have reduced or even completely ceased their natural product based research and drug discovery program. The downfall in natural product research in major part of the world has lead to the vast decline in the discovery of novel chemical leads which possibly resulted due to the lack of economic benefit and emphasis on natural product based drug discovery (Lam 2007).

New technologies that could improve the natural product drug discovery efforts had not advanced to the degree at which the discovery rate of natural product-derived drugs could meet the demands of the industry. Lack of focus by many pharmaceutical companies led to the downfall of natural products also, since there are diverse sources of natural products and the fact that the pharmaceutical companies stretched themselves too thin by looking into too many areas. These issues led to the perception within the industry that finding a promising natural product for the treatment of any disease was an expensive and difficult challenge that was not worth the time and cost (Lam 2007). With the advent of new technologies natural product based research has revived where by screening and isolation of lead compounds have became much faster and easier than earlier. These offers major breakthrough in the natural products based drug discovery research and address the limitations of conventional screening of natural products.

Against this backdrop, recent reviews points towards the incredible importance of natural products especially against infectious agents or diseases which encompasses >75% of the 97 new antibacterial drugs introduced and approved during the period of 1981-2006 (Newman and Cragg 2012; Taylor 2013). Infectious disease is the second leading cause of

death worldwide and there is an urgent need to discover new drugs to combat drug-resistant pathogens. The anti-infective arena is experiencing a shortage of lead compounds progressing into clinical trials. Plant derived novel compounds with different mode of antibacterial action offers distinctive advantages over already established antibacterial drugs towards the treatment of multi-drug resistant bacteria and emerging pathogens. Similarly, numerous natural products of microbial origin have been identified which potentially represent new classes of antibacterial agents with unique modes of action and properties that are different from the currently used drugs (Newman and Cragg 2012). Even though some of these drug candidates are not likely to be developed as commercial antibiotics, they will certainly serve as tools to better understand targets and pathways in the disease pathophysiology (Taylor 2013). These findings might help in the development of better drugs for the treatment of infectious diseases. Nevertheless, these examples demonstrate the importance of natural products in the discovery of antimicrobial agents.

In addition to the anti-infective area, natural products have also had a major impact on cancer chemotherapy. Surveying the period from 1981 to 2002, Newman et al. (2003) reported that >60% of the approved drugs for cancer treatment are purely natural products or their derivatives. Most updated review of Newman and Cragg (2012) described that in the area of cancer, over the time frame from 1940s to 2010, of the 175 small molecules, 131 (74.8%) are other than synthetic, in which 85 (48.6%) actually being either natural products or directly derived from them. Apart from natural products and its semi-synthetic derivatives, recently natural product botanicals have also been approved as a source of novel drugs/leads by FDA (Newman and Cragg 2012). Many natural products and their semi-synthetic derivatives are in use to combat against various infectious and non-infectious diseases as

potent antibacterial, antidiabetic and anticancerous agents (Butler 2008; Newman and Cragg 2012).

2.4. Herbal medicine: An Indian scenario

Herbal medicines are having immense importance and demand in major part of the world including India. These medicines possess multiple biological applications, safer than synthetic agents and less expensive (Gadre et al. 2001). Medicinally important plants has been widely used as food supplements and also plays a key role in healthcare together with several curative bioactive compounds which have built the medicinal plant based industry as a promising sector and has enormous economic growth potential.

India harbours more than 45,000 diverse plant species and included among the twelve major biodiversity centres in the world. Among these, around 20,000 plants possess various therapeutic properties where nearly 7,500 plants are under use by Indian traditional medicines. Nearly 1400 plants were used by Ayurveda and Unani system of medicines where 600 and 30 plants were comes under the Siddha and modern system of medicine respectively. Indian system of traditional medicines consists of Naturopathy, Homeopathy, Siddha, Unani and Ayurveda as officially documented alternative medicine systems with notable usage of several herbal drugs. Gautam et al. (2003) described that a huge set of medicinal plant parts and other herbal drugs have been used in India as food condiments, in house remedies, self-medication and even prescribed alternative medicines. These systems of medicine are based on various coherent and methodical practices of diagnosis and never considered as folklore herbal practices (Vaidya 1992).

Kokate et al. (2005) has described the continuous importance of medicinal plants in Indian economy and also future potential of this sector in various pharmaceutical and

perfumery industries in India. The country is considered as golden hub for knowledge and record of vast collection of traditional herbal medicine. Continuous advancement in standardization of herbal products became needful which reveals the phyto-constituents of herbal products and can be interlinked with possible therapeutic applications (Sapna and Ravi 2007). The major traditional pharma sectors, namely Himalaya, Zandu, Dabur, Hamdard, Maharishi, etc, are standardizing their herbal formulations by chromatography techniques like TLC/HPTLC finger printing, etc to maintain the global standard according to WHO and FDA guidelines (Borris 1996). According to Chaudhri (1996), herbal based technology will be the highest revenue generator in India followed by information technology. India has tremendous market potential to fulfil the domestic as well as international demand of plant based medicines and herbal products by judicial exploitation of its diverse plant resources.

Due to the diverse collection of medicinal plants in India, often it has been named as the "Medicinal Garden of the world". Since prehistoric era, several medicinal plants of Indian origin have been used worldwide. Many historical records revealed the uses and trade of Indian medicinal plants. Srivastava and Singh (1996) described the potential of Indian Aloe which has been extensively used in preparation of cosmetics, medicinal as well as nutraceutical formulations. Likewise, the anti-aging effects of the pickled preparations are unique in India (Raina 1996; Indraji 1998). Despite the global reputation of Aloe in dermato-cosmetics, the potential as anti-aging is still untapped. At the same time, uses of *Adhatoda vasica* for the treatment of cough and colds has been documented and bioactive lead compound have been identified by extensive research efforts (Shah and Chauhan 1996). However, many other known potential of various plants towards the curing of diseases and ailments are still untapped. Certainly, there is a need for an international and also national

collaborative effort to explore on a fast track the hits provided by clinical observations of astute physicians as well as from ethnomedicinal uses.

Nowadays, there is a revival of interest in herbal medicine due to an increasing realization of the health hazards associated with the indiscriminate use of modern medicine and the fact that herbal drug industries are boomimg up in the international market. Due to lack of proper scientific management and inputs in herbal drug development, India was unable to perform well in the international trade of herbal drugs. However, the utilization of herbal drugs is on the rise and the market is growing gradually (Kamboj 2000). India has contributed significantly high amount export of medicinal plant based products and raw materials in recent years including major pharmaceuticals like vinca extracts and opium alkaloids (Kokate et al. 2005). The essential requirements for stepping into the global market includes well-documented traditional use, single-plant based medicines, medicinal plants free from pesticides and heavy metals, standardization based on chemical and activity profile, safety and stability of the herbal products. Hence, there is an enormous scope for India to become a key member in herbal product based trades in the world by judicial use of its extensive and diverse bioresources.

2.5. Plant secondary metabolites: Source of herbal medicine

Plant synthesizes primary and secondary metabolites to withstand in varying environmental conditions (Croteau et al. 2000). Primary metabolites include nucleic acids, amino acids, lipids and simple sugars that are necessary for cellular processes. Secondary metabolites encompass many compounds which are often synthesized by plants in response to external stimuli (Keeling 2006). Plants have the tremendous potential to synthesize several kinds of secondary metabolites, which in turn benefitted the mankind in terms of medicines and healthcare products (Balandrin et al. 1985). Plant based secondary metabolites sometimes

also referred as plant natural products which possess multiple biological applications and therapeutic uses. According to Croteau et al. (2000), these natural products or secondary metabolites have been classified into mainly terpenes and terpenoids (~25,000 types), alkaloids (~12,000 types), and phenolic compounds (~8,000 types).

2.5.1. Chemistry of terpenes

Terpenes and terpenoids have been considered as the largest group among all the secondary metabolites from plants and also possess defined physiological functions in plants. Chemically the basic unit of majority of secondary metabolites in plant composed simple hydrocarbon chain called isoprene subunit. Terpenes consist of a hydrocarbon backbone and when it gets modified by addition of any functional group, it is termed as terpenoids. The five carbon isoprene units can be associated with each other in several ways to build up new chemical entities and enhances the natural diversity of terpenes. The most basic group of terpenes are hemiterpenes which consist of single isoprene unit. Two isoprene unit bonded together to form a monoterpene (C10), whereas sesquiterpenes hold three isoprene units. Likewise, chemical structures of diterpenes and triterpenes comprised of two and three terpene units respectively. Further, when the chain elongates, four terpene units form tetraterpenes and above four terpene units considered as (i.e., more than eight isoprene units).

2.5.2. Therapeutic potential of diterpenes

Several natural products especially from plant sources have been found to possess remarkable biological applications and considered most capable natural product leads towards the development of new therapeutic drug candidates where majority of terpenes have emerged with great therapeutic prospect (de las Heras et al. 2007, 2009; Gurusamy et al. 2010). Among various terpenes, diterpenes have wide range of therapeutic applications ranging from

antibacterial activities to regulation of immune response against various external stimuli (Chinou 2005; Giron et al. 2008; Dong et al. 2011).

Geranyllinalyl pyrophosphate and often its C-13 allylic isomer, geranyl-geranyl pyrophosphate (GGPP) act as metabolic precursor of diterpenes. Protonation of GGPP allows a cyclization process which yielded coplyl PP and subsequently an alternative pathway produces labdadienyl PP, an enantiomeric product (Dewick 1997). The diterpene compounds are further classified according to their biogenetic origin as acyclic (phytanes), bicyclic (labdanes, halimane, clerodanes), tricyclic (pimaranes, abietanes, cassanes, rosanes, vouacapanes, podocarpanes), tetracyclic (trachlobanes, kauranes, aphidicolanes, stemodanes, stemaranes, bayeranes, atisanes, gibberellanes), macrocyclicditerpenes (taxanes, cembranes, daphnanes, tiglianes, ingenanes) and mixed compounds, in accordance with the number and the cyclization patterns displayed by their skeletal structure (Hanson 1991; Bruneton 1995; Hanson 2004; Roberts 2007; Hanson 2009).

Usually diterpenes are found in plant resins and latex where they are responsible for its sticky texture. Resins often exude from the wounds of a plant, playing an antimicrobial role. Many purified diterpenes show extensive biological activities. Phorbol and phorbol-12-myristate-13-acetate, the most potent tumor-promoting agent are found the seed oil of *Croton tiglium* (Hecker 1968). Likewise, Taxol, another important diterpenes with anticancer properties has been isolated in 1971 from *Taxus brevifolia* (Wheeler et al. 1992). Structure-activity relationship suggested that anticancer activities of Taxol are mainly due to the presence of oxetane ring (four membered) and an ester side chain in its backbone structure. Taxol principally act on the cytoplasmic microtubules towards stabilization and leads to the formation of abnormal bundles of microtubules (Schiff and Horwitz 1979; Pratt et al. 1994). Besides these, many diterpenes, in particular, labdane type diterpenes have been reported to

possess a wide spectrum of biological activities (Demetzos and Dimas 2001; Izquierdo et al. 2007; Cuadrado et al. 2011; Mahaira et al. 2011; Pertino et al. 2013).

2.5.3. Labdane type diterpenes: A promising source of bioactivity

Natural diterpenes, especially with labdane backbone are most abundant and widely distributed in nature. They are commonly found as bicyclic diterpene which known to be central core of the diterpene structure in a wide range of plant derived natural products. They are collectively termed as labdane diterpenes. The name "labdane" was derived from labdanum, a resin material of rockrose plants which was considered as the first member of that group of compounds (Cocker et al. 1956; Cocker and Halsall 1956). Many of these diterpene molecules are structurally consist of a furanoid or lactone moiety typically linked to a decalone core. Recently, many labdane diterpenes have been isolated from various plants and attracted scientific interest because of their wide range of biological activities such as antibacterial, cardiovascular, antiproliferative, anti-inflammatory as well as cytotoxic activity against various human cancer cell lines (Suresh et al. 2010; Awang et al. 2012; Chen et al. 2013a; Dey et al. 2013; Ghosh et al. 2013; Li et al. 2013a; Pertino et al. 2013). Various labdane type diterpenes have been isolated mainly from the genus of *Hedychium*, *Curcuma* and *Alpinia* which shows versatile biological applications (Itokawa et al. 1988a; Singh et al. 1991; Nakatani et al. 1994; Sirat et al. 1994; Xu et al. 1995, 1996; Ngo and Brown 1998; Matsuda et al. 2002; Abas et al. 2005; Li et al. 2011a,b; Upadhyay et al. 2011; Zhan et al. 2012; Ghosh et al. 2013).

Notably, among various labdane diterpenes, (*E*)-labda-8(17),12-diene-15,16-dial is known possess wide range of biological activities. This compound has been isolated for the first time from *Alpinia speciosa* seeds by Itokawa et al. (1980). Subsequent studies revealed the presence of this labdane diterpene molecule in many species of *Alpinia* (Itokawa et al.

1988b; Morita and Itokawa 1986, 1988; Sirat et al. 1994; Sy and Brown 1997; Ngo and Brown 1998; Kong et al. 2000; Morikawa et al. 2002; Nuntawong and Susksamrarn 2008; Hema and Nair 2009). Besides these, several other genus of Zingiberaceae family especially the genus *Hedychium* (Itokawa et al. 1988a; Nakatani et al. 1994; Nakamura et al. 2008; Chimnoi et al. 2008, 2009), *Zingiber* (Sirat 1994; Abe et al. 2006; Akiyama et al. 2006), *Aframomum* (Kimbu et al. 1987; Abreu and Noronha 1997; Duker-Eshun et al. 2002), *Renealmia* (Ramiandrasoa et al. 1986; Lognay et al. 1991; Zhou et al. 1997) and *Curcuma* (Firman et al. 1988; Roth et al. 1998; Abas et al. 2005; Tatsimo et al. 2006; Sirat and Meng 2009; Singh et al. 2010a; Sheeja and Nair 2012) have been known to possess this labdane dialdehyde as a constituent.

Recently, this labdane diterpene molecule has been investigated using *in silico* methods for its antileishmanial activity by targeting key enzymes of the respective parasites (Ogungbe and Setzer, 2013). Previous studies revealed this labdane dialdehyde as notably effective for a range of biological activities *viz.* cytotoxic (Itokawa et al. 1988; Morita and Itokawa 1988; Jung et al. 1998; Malek et al. 2011a; Igoli et al. 2012), antiplasmodial (Duker-Eshun et al. 2002), antibacterial (Tatsimo et al. 2006; Singh et al. 2010a, 2012), antitrypanosomal (Otoguro et al. 2011; Igoli et al. 2012), inhibitor of lipid peroxidation and cyclooxygenase enzymes, anticancerous (Liu and Nair 2011, 2012), anti-angiogenic (He et al. 2012), antiglycation (Chompoo et al. 2011), antiviral (González et al. 2010) and many others (Sukari et al. 2010; Upadhyay et al. 2011).

A recent patent related to this dialdehyde has been found which claimed the identification of this compound as a component of *Curcuma caesia* extracts and shown to be useful as skin-beautifying agent by acting as inhibitor of hyaluronidase (Komai et al. 2013). First patent of this compound revealed its presence in ginger and also act as inhibitor of

leukotriene formation and potent anti-inflammatory agent (Matsumoto et al. 1993). Later, Ayafor et al. (2000) has patented the extraction method of this compound from *Aframomum aulacocarpos* and *Aframomum daniellii*. They have also claimed the antiparasitic and antifungal activity of this compound for the first time. Besides these, the use of this compound in preparation of candy as an enhancer of the cooling effect in the peppermint oil has been patented by Yang et al. (2008).

Similarly, (*E*)-8β, 17-Epoxylabd-12-ene-15,16-dial, a natural epoxide analogue of (*E*)-labda-8(17),12-diene-15,16-dial, has been first time isolated from the seeds of *Aframomum daniellii* (Kimbu et al. 1979) and trivially named as aframodial. Later, this epoxide analogue was also isolated from *Zingiber mioga* and identified as the pungent principle of Myoga (Abe et al. 2002). Subsequently, the compound has been identified and isolated from various species of *Alpinia* (Morita and Itokawa 1986, 1988; Ngo and Brown 1998), *Zingiber* (Tanabe et al. 1991, 1992, 1993; Abe et al. 2002; Jang et al. 2003; Abe et al. 2004), *Aframomum* (Ayafor et al. 1994a, 1994b; Tsopmo et al. 2002; Kenmogne et al. 2006; Tatsimo et al. 2006; Wabo et al. 2006) and also from non-Zingiberaceae plant like *Platycladus orientalis* (Asili et al. 2004).

Presence of aframodial as one of the major constituents in Chinese medical prescriptions (V) has been documented previously which suggest its immense bioactive potential towards the curing of various ailments and diseases (Kano et al. 1990). This epoxide molecule has been reported as notably active in a wide range of biological application *viz*. antioxidative and anti-inflammatory agent (Kim et al. 2005), antimicrobial agent (Jang et al. 2003; Abe et al. 2004; Jang et al. 2005; Tatsimo et al. 2006), agonist of TRPA1 (Iwasaki et al. 2009), inhibitor of human platelet aggregation and human 5-lipoxygenase (Abe et al. 2006), antileukemic (Nyasse and Lenta-Ndjakou 2000), antifungal (Morita and Itokawa 1988;

Haraguchi et al. 1996; Kubo et al. 2001, 2003), cytotoxic (Morita and Itokawa 1988) and antiplasmodial (Duker-Eshun et al. 2002; Asili et al. 2004; Kenmogne et al. 2006). Moreover, this compound is also reported to have cholesterol-lowering effect and known to act as a vanilloid which suggests the multiple biological applications of this labdane diterpene towards the development of herbal medicine (Tanabe et al. 1993; Szallasi et al. 1998).

Presence of this epoxide molecule in ginger was first time patented by Matsumoto et al. (1993) together with its notable activity as inhibitor of leukotriene formation and potent anti-inflammatory agent. The method of extraction of this compound from seeds of *Aframomum daniellii* was patented by Liu and Guo (2012) and application of this molecule as agonist of TRPV1 towards the prevention of ischemic injury has also been patented (Jones et al. 2010, 2012). Several other groups have patented the use of this compound as flavoring agents (Liberati et al. 2013), skin-beautifying agent by acting as inhibitor of hyaluronidase (Komai et al. 2013), one of the component for prevention and relaxation in muscle cramps and recovery from neuromuscular irritability and tiredness after exercise (Bean et al. 2012) and flavor enhancers (Yang et al. 2008a). Furthermore, isolation of this compound from rhizomes of *Zingiber mioga* and its application against various cancer cells by inducing apoptosis has been patented previously for skin cutaneous melanoma, liver cancer breast cancer, leukemia and lung cancer (Lee et al. 2006a). The application of aframodial in the preparation of capsaicinoid gel formulations has been also patented towards the method for relieving the pre- and post-surgical pain at a site considering a human or animal (Burch et al. 2004, 2006) and also as vanilloid receptor agonist for controlling bleeding (Moore and Miller 2004).

In recent years, many novel and bioactive labdane diterpenes have been isolated from various plants and investigated for several biological activities which showed the immense

importance of this class of compound towards the future development of herbal therapeutics (**Table 2.1; Table 2.2**). Reviewing the role of above secondary metabolites suggests terpenes, especially labdane type diterpenes play a crucial role in various biological applications ranging from antibacterial to anticancer activities which in turn also revealed the tremendous potential of these compounds towards the development of plant derived therapeutics. Nevertheless, besides previous studies, many in depth investigations will be required further to establish these molecules as suitable bioactive agents with higher efficacies.

Table 2.1 List of bioactive labdane diterpene compounds isolated from various plant sources

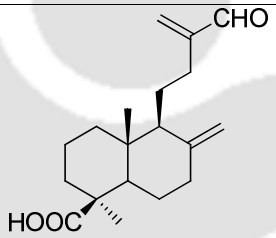
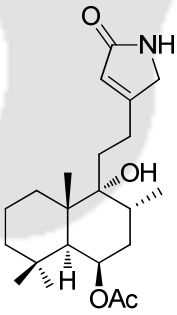
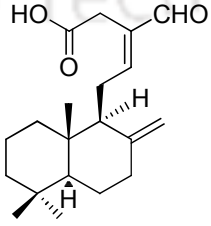
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
Thuja orientalis	Leaves and stem	 15-nor-14-oxolabda-8(17),13(16)-dien-19-oic acid	Anti-inflammatory	Kim et al. 2013
Vitex agnus-castus	Fruits	 6β-acetoxy-9α-hydroxy-13(14)-labden-16,15-amide	Chemopreventive	Li et al. 2013b
Curcuma kwangsiensis	Rhizome	 Zerumin A	$GABA_A$ receptor modulators	Schramm et al. 2013

Table 2.1 Continued

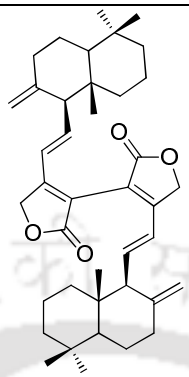
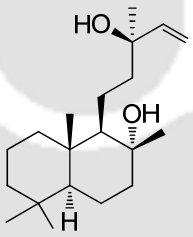
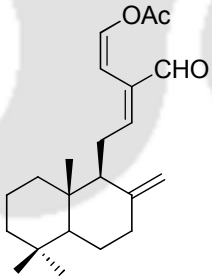
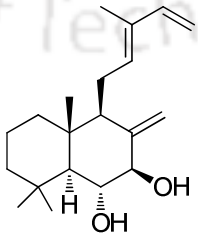
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
Alpinia pahangensis	Rhizome	 bis-labda-8(17),11,13-trien-16,15-olide	Antibacterial	Sivasothy et al. 2013
Nicotiana tabacum	Leaves	 Sclareol	Inhibit bacterial wilt disease	Seo et al. 2012
Turraeanthus mannii	Root bark	 15-acetoxy-labda-8(17),12E,14Z-trien-16-al	Antifungal and cytotoxic	Sielinou et al. 2012
Fritillaria ebeiensis	Bulbs	 6α,7β-dihydroxy-labda-8(17),12(*E*),14-triene	Neuroprotective	Xu et al. 2011

Table 2.1 Continued

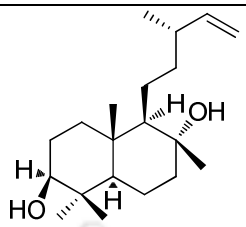
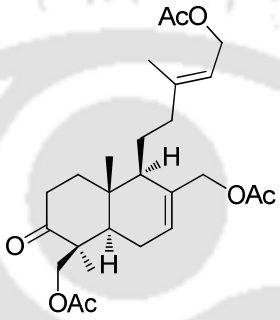
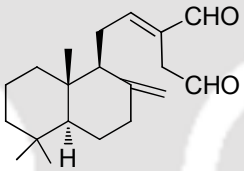
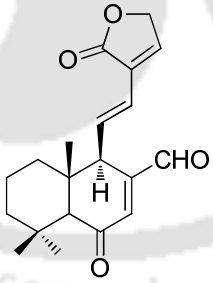
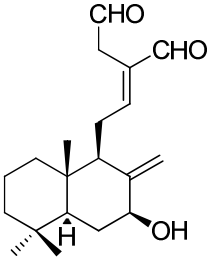
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
Ulva fasciata	Whole part	 Labda-14-ene-3α,8α-diol	Antibacterial	Chakraborty et al. 2010
Vitex cauliflora	Aerial parts	 3-oxo,15,17,18-triacetoxy-labda-7,13*E*-diene	Antiplasmodial	Rasamison et al. 2010
Curcuma amada	Rhizome	 labda-8(17),12-diene-15,16-dial	Antitubercular	Singh et al. 2010
Hedychium coronarium	Rhizome	 6-oxo-7,11,13-labdatrien-17-al-16,15-olide	Cytotoxic	Suresh et al. 2010
Hedychium coronarium	Rhizomes	 7β-hydroxy-(*E*)-labda-8(17),12-diene-15,16-dial	Cytotoxic	Chimnoi et al. 2009

Table 2.1 Continued

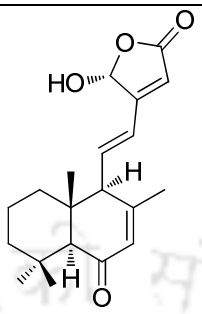
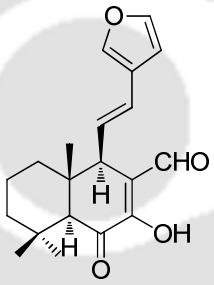
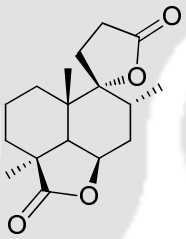
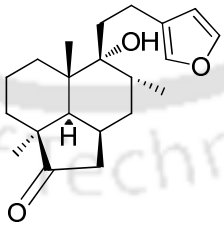
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
Hedychium spicatum	Rhizome	 Spicatanol	α-glucosidase inhibitor	Prabhakar Reddy et al. 2009
Hedychium spicatum	Rhizome	 7-hydroxy hydichinal	Cytotoxic	Reddy et al. 2009a
Marrubium cylleneum	Aerial parts	 Cyllenine C	Cytotoxic	Karioti et al. 2007
Marrubium vulgare	Leaves	 Marrubiin	Analgesic	Meyre-Silva et al. 2005

Table 2.1 Continued

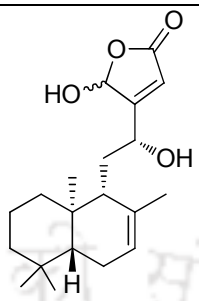
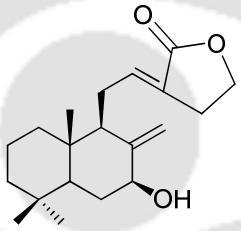
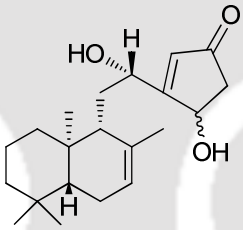
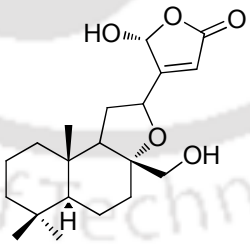
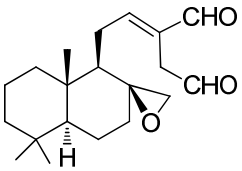
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
Alomia myriadenia	Aerial parts	 Myriadenolide	Apoptotic	Souza-Fagundes et al. 2003a
Hedychium coronarium	Rhizome	 Hedychilactone A	Inhibitory effects on nitric oxide production	Matsuda et al. 2002
Alomia myriadenia	Aerial parts	 12*S*,16-dihydroxy-*ent*-labda-7,13-dien-15,16-olide	Cytotoxic	Zani et al. 2000
Neouvaria acuminatissima	Stem bark	 Acuminolide	Cytotoxic	Lee et al. 1995
Aframomum daniellii	Seeds	 8β, 17-epoxylabd-12*E*-ene-15,16-dial	Antimicrobial	Ayafor et al. 1994

Table 2.2 List of patents on various labdane diterpene compounds and their derivatives

Title/Claim	Sources/Compound(s)	References
Anti-fatigue agent and oral composition each comprising andrographolide as active ingredient	Andrographolide	Asami et al. 2013
Beverage products having steviol glycosides and at least one acid	Steviol glycoside, Rebaudioside D	Chang et al. 2013
Andrographis paniculata extract, andrographolide and its analogue for treating inflammatory bowel disease	Andrographolide	Duan et al. 2013b
Treatment of sickle cell disease	Isocoronarin D	Essack et al. 2013
Use certain diterpene compounds in the treatment of androgen receptor-associated diseases	Isocupressic acid	Hsiao et al. 2013
Compositions and methods for including melanogenesis in a subject	Forskolin	Kleinig et al. 2013
Process for preparing water soluble diterpenes and their applications	*Coleus forskohlii*	Majeed et al. 2013
Bicyclic labdane diterpenes for use in the treatment of TRPC6 associated diseases	Bicyclic labdane diterpenes	Schaefer et al. 2013
Oncogenic-RAS-signal dependent lethal compounds	Labdane diterpene derivative	Stockwell et al. 2013
Combinations of vasoactive substances with estrogens and their use in the treatment of female sexual dysfunctions	Forskolin	Bombardelli 2012
Compositions comprising andrographis paniculata and gingko biloba extracts complexed with phospholipids	Andrographolide	Bombardelli and Giori 2012
Beverage products having steviol glycosides and at least one acid	Rebaudioside A	Lee et al. 2012
Anisic acid modified steviol glycoside sweetened beverage products	Steviol glycoside, Rebaudioside D	May et al. 2012
Diterpene modulator of macrophage phagosomal maturation	Isotuberculosinol	Peters et al. 2012
Crude extracts from *Andrographis paniculata* inhibit TNFα or IL-1β expression	*Andrographis paniculata*	Yan et al. 2012
Methods and compositions for treating urinary tract infections using agents that mimic or elevate cyclic AMP	labdane diterpenes, Forskolin	Abraham et al. 2011
Compositions for the treatment of chronic degenerative inflammatory conditions	Labdane diterpenes	Bombardelli and Morazzoni 2011
Composition of labdane diterpenes extracted from A*ndrographis paniculata*, useful for the treatment of autoimmune diseases, and alzheimer disease by activation for PPR-gamma receptors	A*ndrographis paniculata*	Hancke Orozcoand Burgos Aguilera. 2011
Methods for microbial production of terpenoids	Levopimaradiene and steviol glycoside	Leonard et al. 2011
Anisic acid modified steviol glycoside sweetened beverage products	Steviol glycoside	May et al. 2011
Beverage products	Rebaudioside A	Talebi et al. 2011
Crude extracts from *Andrographis paniculata*	Andrographolide	Yan et al. 2011
Anti-tumour compounds	Labdane diterpenes	Hortelano et al. 2010
Abietane diterpenoid compounds or terpenoid compounds isolated from *Torreya nucifera* for prevention and treatment of cardiovascular disease	*Torreya nucifera*	Jeong et al. 2010

Table 2.2 Continued

Title/Claim	Sources/Compound(s)	References
Diterpenes from the fruiting body of *Antrodia camphorata* and pharmaceutical compositions thereof (neuroprotective activity)	*Antrodia camphorata*	Lai et al. 2010
Identification of syn-stemodene synthase	Rice labdane-related diterpenoid biosynthesis	Peters and Xu 2010
Therapeutics for neurological disorders	Andrographolide	Shaw et al. 2010
Identification of syn-copalyl diphosphate synthase	Biosynthesis of labdane-related diterpenoids	Petersand Xu 2009
Compositions for rapid and non-irritating transdermal delivery of pharmaceutically active agents and methods for formulating such compositions and delivery thereof	Forskolin	Kirbyand Pettersson 2007
Identification of compositions, compositions, and methods of treatment of obesity and overweight conditions	Sclareol and sclareolide	Subbiah 2007
Pharmaceutical formulations comprising labdanes for the treatment of tumors or leukemias	Labdane diterpenes	Anastassaki et al. 2006
Compositons comprising sclareol or scareolide for the treatment of microbial infections	sclareol or scareolide	Subbiah 2003
Agent for lowering prolactin	Bicyclic labdane diterpene compounds	Wuttke et al. 2003
Antifungal and antiparasitic compounds	Labdane-dial from *Aframomum danielli*, and *Afromomum aulocacarpus*	Jackson et al. 2002
Cell growth activating composition containing compound having labdane structure	Labdane diterpene compounds	Tamai et al. 2002a
Biologically active labdane or labdene derivatives from Cistus	labdane diterpenes	Tamai et al. 2002b
Melanin inhibiting and cell growth activating compositions containing compounds having labdane structure	Labdane diterpene compounds	Tamai et al. 2001
Process for extracting sweet diterpene glycosides	*Rubus Suavissimus*	Zhou et al. 2001
Certain diterpenes and extracts or concentrates of *Curcuma amada* containing them for use as medicaments	*Curcuma amada*	Jacobsen et al. 2000
Aframonum seeds for improving penile activity	*Aframomum stipulatum* seeds, labdane diterpenoid	Allas et al. 1999
Composition based on hydrated lipidic lamelar phases or on liposomes containing at least one derivative of labdane, or a plant extract containing it; cosmetic or pharmaceutical, particularly dermatological composition containing it	Derivative of labdane, or a plant extract to reduce hair loss and promotes hair growth	Bonte et al. 1999
Purified flavonoid and diterpene 5.alpha.-reductase inhibitors from thuja orientalis for androgen-related diseases	A group of labdane diterpene compounds	Takahashi et al. 1998
Method for preparing dodecahydro-3A,6,6,9A-tetramethylnaphtho [2,1-B]furan and novel haloethyl decalin derivatives	Synthesis from sclareol	Christenson 1995
Compositions containing forskolin	Forskolin	Feigenbaum 1994
Physiologically active substance	Derivative of labdane as antitumor agent	Yoshida et al. 1994
Composition and method for treating tumors	Abietic acid	Bang et al. 1993
Process for the preparation of 6-acyl, 7-acyl, and 6,7-diacyl analogues of forskolin and intermediates thereof	Analogues of forskolin	de Souza et al. 1993

Table 2.2 Continued

Title/Claim	Sources/Compound(s)	References
Method for treating viral infection parenterally	Labdane dietrpene derivative	Herman 1993
Labdane derivatives, a process for their preparation, and their use as medicaments	Labdane derivatives lowering intraocular pressure and lowing blood pressure	Khandelwal et al. 1993b
7-aryl and heteroaryl ethers of desacetylforskolin	Novel forskolin derivatives	Kosley, Jr. et al. 1993
Abienol and sclareol producing somaclonal variants of nicotiana	*Nicotiana tabacum*	Whitaker 1993
6- and 7-deoxyforskolin and derivatives thereof	Novel forskolin derivatives	Kosley, Jr. et al. 1992
Pharmaceutical compositions comprising labdane diterpenoid derivatives and pyrimido(6,1-a)isoquinolin-4-one derivatives and their use	Pharmaceutical compositions comprising labdane diterpenoid derivatives	Lal et al. 1992
Method for treating viral infection of HIV	Labdane diterpenoid derivatives	Herman 1991
7-aryl and heteroaryl ethers of desacetylforskolin	Forskolin derivatives	Kosley, Jr. et al. 1991
Process for obtaining labdane-type diterpenes, especially forskolin, from Coleus forskohlii	Coleus forskohlii	Mandler et al. 1991
12-halogenated forskolin derivatives	Forskolin derivatives	Shutske 1991b
Synergistic intraocular pressure lowering combinations	Labdane diterpenoid derivatives	Conway and Helsley 1990
Labdane compounds, pharmaceutical compositions and use	Labdane diterpenoid derivatives	Kosley, Jr. And Cherill1990
Methods for preventing thrombosis; and surgical implant having reduced platelet deposition characteristics	Forskolin or its derivatives	Thulesius and Christenson 1990
Treatment of metastasis	Forskolin	Agarwal and Parks Jr. 1988
Method of treating inflammatory diseases with labdan derivatives	labdan derivatives	Dadkar et al. 1988
Method of treating inflammatory diseases with labdan derivatives	*Coleus forskohlii*	Dohadwalla et al. 1988
Oxolabdanes useful as pharmaceuticals for reducing intraocular pressure	Labdane diterpenoid derivatives	Hrib 1988
Method for treating allergic reactions with Forskolin	Antiallergic activity of forskolin or its derivatives	Kreutner et al. 1988
Aminoacyllabdanes, pharmacuetical compositions and use	Novel aminoacyllabdanes, intermediates	Kosley, Jr. And Cherill1987
Composition and method for treatment of glaucoma	Forskolin	Sears and Caprioli 1984
Polyoxygenated labdane derivatives	Novel polyoxygenated labdane derivatives	Bajwa et al. 1979

2.6. *Alpinia*: Gold mine of herbal therapeutics

In the plant kingdom, Zingiberaceae family is well known due to its multiple medicinal uses and several food flavouring spices of high commercial values. It is widely distributed in the world with about 50 genera and 1,300 diverse species mainly concentrating in South and Southeast Asia (Wu and Larson 2000). India comprises about 22 genera and 178 species in North Eastern and peninsular region (Jain and Prakash 1995), whereas North East region alone harbors 19 genera and close to 88 diverse species (Prakash and Mehrotra 1995).

The largest and most complex genus of the Zingiberaceae family is *Alpinia* which was classified by Charles Plumier, the famous French botanist and named after Prospero Alpino, the well-known Italian botanist of 16th century. The genus, *Alpinia* belongs to the flowering plants group (angiosperms). According to Angiosperm Phylogeny Group II (APG II) system, it comes under the umbrella of monocotyledonous plants (Angiosperm Phylogeny Group 2003), belonging to the order Zingiberales, subfamily Alpinioideae and tribe Alpinieae. The genus includes 230-250 species distributed throughout tropical and subtropical climates of Asia and the Pacific. DNA-based studies showed the genus as polyphyletic represented by six clades scattered across the tribe Alpinieae (Kress et al. 2005).

Majority of the members of the genus produces attractive inflorescence, possesses aromatic aerial and underground parts, which are generally subjected to different fractionation process for the extraction of essential oils, aqueous extract and bioactive components. Various parts of this plant have significant potential to yield bioactive components towards the development of future herbal remedies. The essential oil extracted from different parts of the plant contains diverse natural compounds having multiple medicinal properties. Because of its multipurpose utility, the genus *Alpinia* demands much attention from the researchers towards the development of potential therapeutics against

various diseases like cancer, diabetes, ulcer and many neural disorders. Current research and reviews demonstrated the importance and medical application of potential bioactive compounds isolated from different species of the genus and further research is continuing to unveil the mechanism of action of the natural bioactive compounds in regulating the disease progression and cure. The details of phyto-constituents, bioactive components and their biological applications are described in the following sections.

2.7. *Alpinia*: Source of bioactive compounds

The members of the genus *Alpinia* have complex chemical profile and possess diverse types of bioactive compounds. Many of these natural compounds have been isolated from different species of the genus *Alpinia* and investigated towards their biopharmaceutical applications. Some notable bioactive compounds are enlisted in Table 2.3.

Table 2.3 List of prospective pharmacologically important bioactive compounds isolated from different species of *Alpinia*

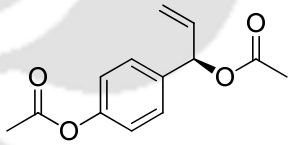
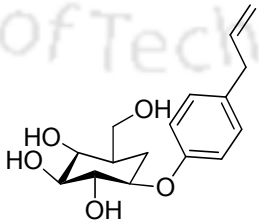
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
A. conchigera	Rhizome	 1'S-1'-acetoxychavicol acetate	Antibacterial	Aziz et al. 2013
A. conchigera	Rhizome	 Chavicol-glucopyranoside	Anti-Melanogenesis	Ujang et al. 2013

Table 2.3 (Continued)

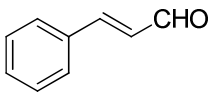
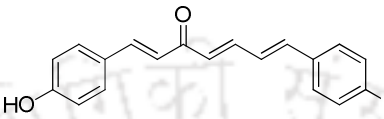
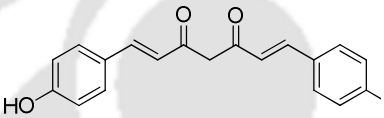
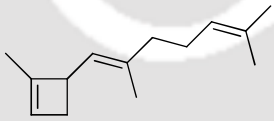
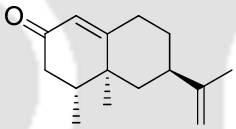
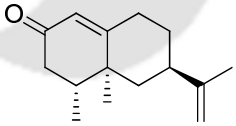
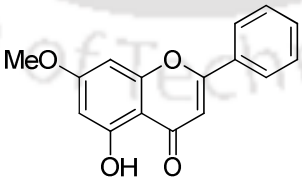
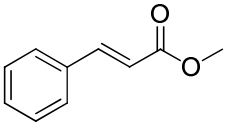
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
A. conchigera	Rhizome	 Trans- cinnamaldehyde	Anti-Melanogenesis	Ujang et al. 2013
A. galanga	Rhizome	 1,7-Bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one	Antimelanoma and Antityrosinase	Lo et al. 2013
A. galanga	Rhizome	 Bisdemethoxycurcumin	Antimelanoma and Antityrosinase	Lo et al. 2013
A. japonica	Rhizome	 (Z)-4-(2,6-dimethylhepta- 1,5-dien-1-yl)-1-methyl-cyclobut-1-ene	Inhibition of nitric oxide production	Li et al. 2013c
A. oxyphylla	Fruits	 Nootkatone	Anti-diarrheal	Zhang et al. 2013a
A. oxyphylla	Fruits	 Nootkatone	Anti-diarrheal	Zhang et al. 2013a
A. oxyphylla	Fruits	 Tectochrysin	Anti-diarrheal	Zhang et al. 2013a
A. ligulata and A. nieuwenhuizii	Rhizome	 (*E*)-methyl cinnamate	Antimicrobial	Yusoff et al. 2011

Table 2.3 (Continued)

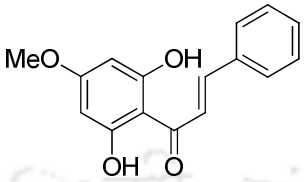
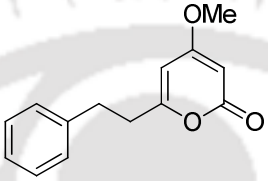
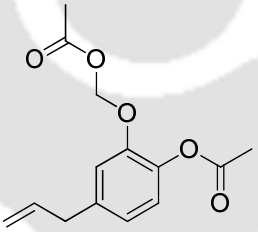
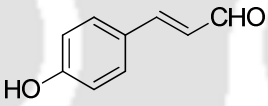
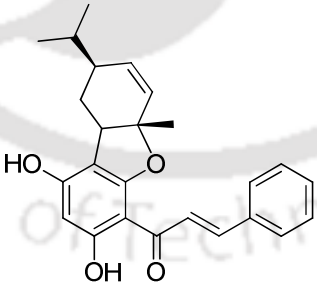
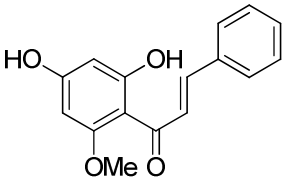
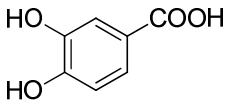
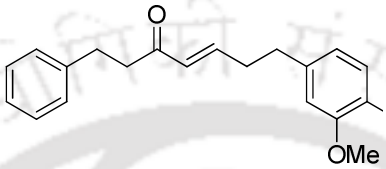
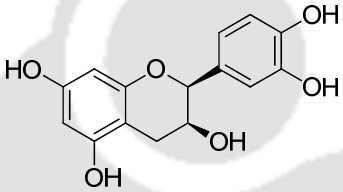
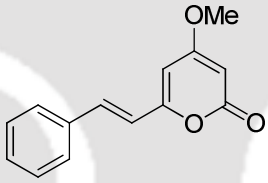
Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
A. mutica	Rhizome	 Pinostrobin chalcone	Anticancer	Malek et al. 2011a
A. zerumbet	Leaves	 Dihydro-5,6- dehydrokawain	HIV-1 integrase and neuraminidase inhibitors	Upadhyay et al. 2011
A. galanga	Rhizome	 1'-acetoxyeugenol acetate	Antileishmanial	Kaur et al. 2010
A. galanga	Rhizome	 *p*-hydroxycinnaldehyde	Treatment against osteoarthritis	Phitak et al. 2009
A. katsumadai	Seeds	 Sumadain C	Anticancer	Hua et al. 2009
A. conchigera	Rhizome	 Cardamomin	Inhibitor of NF-κB activation	Lee et al. 2006b

Table 2.3 (Continued)

Species Name	Plant parts used	Structure and name of the Compounds	Bioactivities	References
A. oxyphylla	Kernels	 Protocatechuic acid	Neuroprotective activity	An et al. 2006
A. officinarum	Rhizome	 Diarylheptanoid	Anti-inflammatory	Yadav et al. 2003
A. speciosa	Rhizome	 Epicatechin	Antioxidant	Masuda et al. 2000
A. blepharocalyx	Seeds	 5,6-dehydrokawain	Antiplatelet	Dong and Chen 1998

2.8. *Alpinia*: Bioactive potential

Review of the genus *Alpinia* showed its incredible biopharmaceutical potential as evident from earlier published reports and is gaining the attention of researchers from diverse disciplines. The presence of the bioactive substances such as flavonoids, tannins and terpenes are the key for its therapeutic efficiency. The potential biological applications of diverse species of *Alpinia* are depicted in Fig. 2.1. Brief accounts of various biological studies and efficacy of diverse *Alpinia* members towards the therapeutic uses are described below.

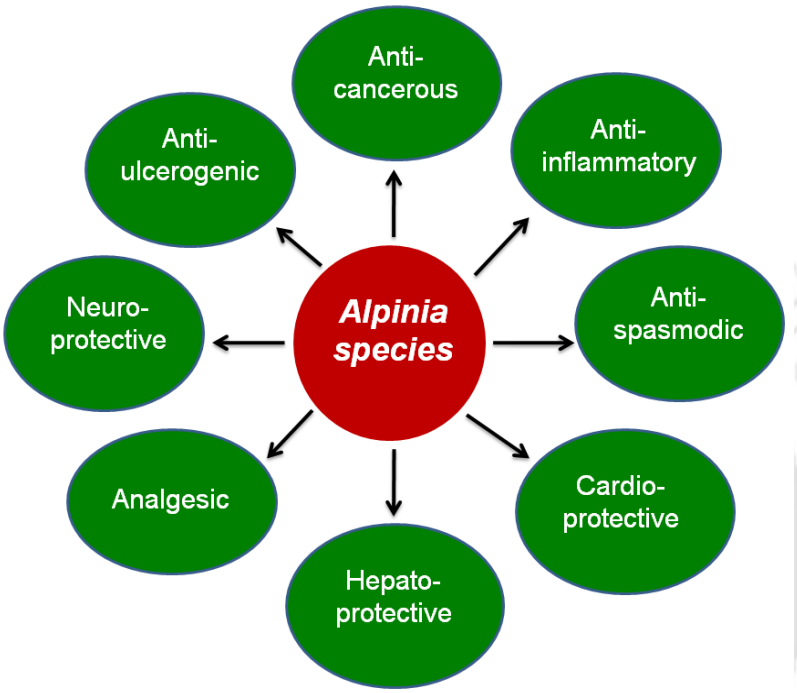


Fig. 2.1 Diverse biological applications of *Alpinia* species

2.8.1. Antibacterial activity

The approval of the sulphonamide antibiotics and penicillin during 1930s and 1940s has revolutionized the medical treatments by radically declining the mortality rates realted to bacterial infections (Drews 2000; Newman et al. 2000; Sneader 2005). Such achievements and discoveries directed to an extensive exploration towards new antibacterial lead molecules from nature which continued for many decades and resulted in the identification of many present day antibacterial drug molecules of natural origin or their direct derivatives (Finch et al. 2003; Sneader 2005; Walsh and Wright 2005). Besides these achievements, only few novel antibacterial classes like mupirocin, oxazolidinone linezolid and lipopeptide daptomycin have been approved during the year of 1985, 2000 and 2003 respectively which was notably low since 1970. During last two decades of 19th century, there has been more than 50% decline in the number of antibiotics approved annually by the FDA (Hegde and Carter 2003; FDA 2005; Graul and Prous 2005).

However, the treatment of bacterial infections is increasingly complicated due to growing resistance of bacteria to the antibacterial agents. With the exhaustive usage of antibacterial drugs, the complexity and magnitude of bacterial resistance has been increased since many years. The struggle to overcome infections continues to be more challenging as bacteria evolve ever more clever mechanisms of resistance (Krause 1992). Hence, the prevalence of natural product-derived antibacterial drugs continues as potential source due to the evolution of secondary metabolites as biologically active chemicals that conferred selectional advantages to the producing organisms. Owing to its evolving nature, many natural products have versatile chemical complexity which allowed them to cross the cell membrane barrier in bacteria and act on the various target molecules of its own specificity leading to antibacterial activities (Stone and Williams 1992).

A great depth of antibacterial activities has been reported from various members of the genus *Alpinia*. Till date most of the activity studies have been concentrated in *A. galanga* which contain several bioactive compounds (Janssen and Scheffer 1985; Khattak et al. 2005; Oonmetta-aree et al. 2006; Niyomkam et al. 2010; Rao et al. 2010; Weerakkody et al. 2011). Essential oil extracted from fresh and dried rhizomes of *A. galanga* have potential antimicrobial activities against a range of bacteria, fungi, yeast and parasite. Ethanol extract from rhizome showed cytological modification to *Staphylococcus aureus* cells by altering outer membrane integrity (Oonmetta-aree et al. 2006). However, the galangal extract, being hydrophobic in nature, could not inhibit the proliferation of Gram-negative bacteria as the extract was unable to penetrate the lipopolysaccharide monolayer of outer membrane of the cell wall. A notable compound, acetoxychavicol acetate isolated from dried rhizomes of *A. galanga*, is found potentially active against several bacteria (Janssen and Scheffer 1985).

Besides *A. galanga*, other species, *viz. A. oxyphylla, A. speciosa, A. zerumbet* and many others are gaining attention due to the presence of diverse polyphenolic compounds with complex chemical profile. Various studies showed the antibacterial potential of crude ethanolic extract, chloroform extract, hydrodistillation product and a number of purified compounds against a wide spectrum of bacterial pathogens (Table 2.4). The significant antibacterial activities of different fractions and pure components of *Alpinia* species are catalogued in Table 2.4.

Table 2.4 List of antibacterial activities of bioactive fractions and pure compounds of *Alpinia* species

Species Name	Parts used	Bioactive fractions/compounds	References
A. conchigera	Rhizome	1'S-1'-acetoxychavicol acetate	Aziz et al. 2013
A. galanga	Flower	Ethanol extract	Cadet et al. 2013
A. mutica	Rhizome	5,6-dehydrokawain, flavokawin B, pinostrobin, β-sitosterol and pinocembrin	Mustahil et al. 2013

Table 2.4 (Continued)

Species Name	Parts used	Bioactive fractions/compounds	References
A. oxyphylla	Fruits	Ethanol extract	Wang et al. 2013a
A. purpurata	Leaves	Ethyl acetate extract	Arul Raj et al. 2012
A. galanga	Rhizome	1'-acetoxy-chavicol acetate	Weerakkody et al. 2011
A. ligulata and	Rhizome	Essential oil	Yusoff et al. 2011
A. nieuwenhuizii			
A. pahangensis	Leaves and rhizomes	Hydrodistilled essential oil	Awang et al. 2011
A. galanga	Rhizome	Ethyl acetate extract (1'-acetoxychavicol acetate)	Niyomkam et al. 2010
A. galanga	Leaves and rhizomes	Methanol, acetone and diethyl ether extracts	Rao et al. 2010
A. conchigera	Leaves, stem and rhizomes	Essential oil obtained from hydrodistillation	Ibrahima et al. 2009
A. galanga	Rhizome	D, L-1-acetoxychavicol acetate	Oonmetta-aree et al. 2006
A. galanga	Rhizome	Ethanol extract	Khattak et al. 2005
A. speciosa	Leaves	Ethanol extract	Wang and Huang 2005

2.8.2. Anticancerous activity

The remarkable contribution of natural products as a source for medicine has been documented since ancient times (Farnsworth 1985; Cragg et al. 1997). In last few decades natural products from various bioresources has contributed substantially in cancer therapeutics. Further, discovery of several natural products possessing antitumor activity has opened up new avenues in the research related to cytotoxic agents where many of the plant derived products are in use currently for the treatment of cancer. Subsequent approval of various drugs like ixabepilone, trabectedin and temsirolimus are the major breakthrough in the advancement of natural products in cancer therapeutics. Regardless of notable progress and advancement in combinatorial chemistry, natural product derived candidates shared the major contribution in drug discovery research till today (Cragg et al. 1997).

For the survival in various hostile environmental conditions, plants have acquired natural system of evolution which allows them to synthesize several useful chemical entities such as terpenes and alkaloids towards combating the predators or any unwanted plant species in the vicinity. Many of these terpenoids and alkaloids have been introduced in cancer therapeutics. A notable example is vinca alkaloid, vincristine, purified from *Catharanthus roseus* and this alkaloid has been found very much effective against Hodgkin's disease and even can cure some leukemic diseases (Devita et al. 1970; Noble 1990).

Many *in vitro* studies done in diverse cancer cell lines and *in vivo* studies with animal models clearly reflect the potential of various species of *Alpinia* as anticancerous herbal source. For instance, a novel compound, pinostrobin chalcone, has been isolated from *A. mutica* which displays notable cytotoxic potential to various human carcinoma cell lines (KB, MCF7 and Caski cells) with significantly low IC_{50} values (Malek et al. 2011a). Antiangiogenic potential of *A. oxyphylla* fruits has been found in n-hexane and ethyl acetate fractions and have been tested against zebra fish model, human umbilical vein endothelial cells and tumour cell lines (He et al. 2010). Investigation of Nam et al. (2005) on the n-hexane and chloroform extract of *A. galanga* rhizome lead to the isolation of two compounds, *viz.* 1'-(S)-1'-acetoxychavicolacetate and *p*-coumaryl alcohol c-O-methyl ether. Out of the two compounds, former showed significant cytotoxic activity against human cancer cell lines like A549 (IC_{50} = 8.14 µg/ml), SNU638 (IC_{50} = 1.27 µg/ml), HT1080 (IC_{50} = 1.2 µg/ml), HL60 (IC_{50} = 2.39 µg/ml) and HCT116 (IC_{50} = 1.77 µg/ml). Whereas, the second compound revealed specific activity against SNU638 (IC_{50} = 1.62 µg/ml). In some other cancer cell lines cytotoxic activity has been screened with four different compounds isolated from *A. officinarum* and only 7-(3,4-dihydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-4-en-3-

heptanone was found to be a remarkable cytotoxic agent against HepG2, MCF-7 and SF-268 (An et al. 2008).

Lu et al. (2007) studied the effect of flavonoid constituents of *A. officinarum* on whitening effects based on melanin biosynthesis in B16 mouse melanoma cells. The flavonoid mixture and galangin exhibited a broad absorption band at 270-290 nm related to the UV-B area supporting that galangin could be a whitening agent and a capable candidate for prevention of skin cancer. The summarized anticancerous activities of the crude extract and isolated principal compounds of the genus *Alpinia* are listed in Table 2.5.

2.8.3. Anti-inflammatory and analgesic activity

Inflammation is a protective response by the organism to eliminate the injurious stimuli and to initiate the healing process. It is a complex biological response of vascular tissues to detrimental stimuli such as pathogens, injured cells or external irritants (Ferrero-Miliani et al. 2007). Therefore, anti-inflammatory drugs refer to the property of a substance that trims down inflammation. Anti-inflammatory drugs reduce inflammation without affecting the central nervous system and make up about half of analgesics available in the market. Medication towards inflammation depends on steroids, non-steroidal anti-inflammatory drugs (NSAID), immune selective anti-inflammatory derivatives (ImSAIDs) and herbal drugs. However, inhibitions of natural hormones and liver dysfunction are the common side effects of steroidal drugs (Hartgens et al. 1996; Urhausen et al. 2003). Similarly, NSAID can cause gastric erosions, leading to stomach ulcers and in extreme cases can cause severe haemorrhage, resulting in death by myocardial infarction and stroke (Trelle et al. 2011). Therefore, ImSAIDs and herbal drugs are more acceptable in treating inflammation and remedying pain. There are several bioactive compounds that have been isolated from *Alpinia* species which showed anti-inflammatory and analgesic actions.

Natural bioactive compounds and crude hydroalcoholic fractions isolated from the *Alpini*a species like *A. galanga*, *A. zerumbet*, *A. officinarum*, etc. showed potential activities as anti-inflammatory and analgesic agents. Aqueous and hydroalcoholic extracts from leaves and rhizomes of above species possess key factors responsible for antinociceptive (reducing sensitivity to painful stimuli) and antiallergic properties. Diarylheptanoids, a novel class of potent platelet activating factor (PAF) antagonists isolated from *A. officinarum* rhizome extract (Fan et al. 2007), showed antirheumatic, antipsychiatric and analgesic activities (Lee et al. 2009). A brief account of the anti-inflammatory, analgesic and other related activities of *Alpinia* are listed in Table 2.5.

Table 2.5 List of anticancerous, anti-inflammatory and analgesic activities of bioactive fractions and major compounds from *Alpinia* species

Species Name	Parts used	Bioactive fractions/compounds	Bioactivity	References
A. conchigera	Whole plant	8-9′ linked neolignans	Anticancerous	Xu et al. 2013
A. mutica	Rhizome	Crude extract	Anticancerous	Mustahil et al. 2013
A. officinarum	Rhizome	Methanolic extract	Anticancerous	Ghil 2013
A. officinarum	Rhizome	Methanolic extract	Anti-leukemic	Omoregie et al. 2013
A. officinarum	Rhizome	Galangin	Anticancerous	Zhang et al. 2013b
A. oxyphylla	Seeds	Yakuchinone A	Anticancerous	Lin et al. 2013
A. oxyphylla	Fruits	Ethanol extract	Anticancerous	Wang et al. 2013b
A. purpurata	Leaf	Ethyl acetate extract	Anticancerous	Arul Raj et al. 2012
A. mutica	Rhizome	Pinostrobin	Anticancerous	Malek et al. 2011a
A. conchigera	Rhizome	1'S-1'-Acetoxychavicol acetate	Apoptotic	Awang et al. 2010
A. oxyphylla	Fruits	Hexane and ethyl acetate fractions	Anti-angiogenic	He et al. 2010
A. scabra	Leaves and rhizome	Hexane and dicholoromethane extract	Anticancerous	Ibrahim et al. 2010
A. katsumadai	Seeds	Rubraine, isorubraine and sumadain	Anticancerous	Hua et al. 2009
A. officinarum	Rhizome	Ethanolic extract	Antinociceptive, anti-inflammatory, and anti-psychiatric	Lee et al. 2009
A. oxyphylla	Fruits	Oxyphyllone A and B	Anticancerous	Xu et al. 2009a

Table 2.5 (Continued)

Species Name	Parts used	Bioactive fractions/compounds	Bioactivity	References
A. officinarum	Rhizome	7-(3,4-dihydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-4-en-3-heptanone	Anticancerous	An et al. 2008
A. pricei	Rhizome	Ethanolic extract	Apoptotic	Yang et al. 2008b
A. officinarum	Rhizome	Hydroxy-1,7-diphenyl-4-en-3-heptanone 6,6-(2-hydroxy-phenyl)-4-methoxy-2-pyrone,1,7-diphenyl-4-en-3-heptanone, 1,7-diphenyl-5-methoxy-3-heptanone and apigenin	Platelet-activating factor (PAF) antagonists	Fan et al. 2007
A. officinarum	Rhizome	Galangin	Prevents skin cancer	Lu et al. 2007
A. conchigera	Rhizome	Cardamomin	Anti-inflammatory	Lee et al. 2006b
A. galanga	Rhizome	1'S-1'-Acetoxychavicol acetate and *p*-coumaryl alcohol γ-O-methyl ether	Anticancerous	Nam et al. 2005
A. calcarata	Rhizome	Aqueous and ethanolic extract	Antinociceptive	Arambewela et al. 2004
A. galanga	Rhizome	Alcoholic and aqueous extracts	Anti-inflammatory	Satish and Dhananjayan 2003
A. galanga	Rhizome	7-(4'-hydroxy-3'-methoxyphenyl)-1-phenylhept-4-en-3-one	Anti-inflammatory	Yadav et al. 2003
A. blepharocalyx	Seeds	Diarylheptanoids	Antiproliferative	Ali et al. 2001a

2.8.4. Neuroprotective and antioxidant activity

According to Cotran et al. (1995), neurodegeneration defined as the neuronal death condition due to the continuous disease progression for a longer period of time. Medically it involves the degradation of restricted set of neurons which may possibly be functionally or structurally linked with each other (Beal 1997). All the neural tissues undergo deterioration during the process of neurodegeneration. As a consequence, several diseases like Alzheimer's and Parkinson's disease and even loss of cognitive functions may arise as the degenerated neurons are not replaced by normal recovery process. Therefore, in recent years the field

related to neurodegeneration has become more serious concern where neuroprotection by therapeutic means has attracted much attention to add up some new insights into therapeutics and cell biology.

Neuroprotection collectively refers to prevention of neuron damage or dying by some therapeutic approach involving either any protective drug or other strategic treatment. The aim of neuroprotection is to reduce neuronal dysfunction of damaged or degenerated neurons and also it tries to retain the best possible integrity with other cellular interactions in brain ensuring normal functions of neurons. Several neuroprotective products are available in the market and many are under clinical trials. These drugs have similar mode of actions which can judicially be utilized in various types of neural disorders. The most promising type of drugs mainly includes antioxidative agents. Antioxidants are known to counterbalance free radicals and found successful in controlling or reducing the prevalence of these disorders. According to Halliwell (1994), antioxidant agent refers to any molecules which in very dilute concentrations than any oxidizeable compound significantly slows or even prevents oxidation of the substrate. On the basis of origin, these antioxidants may be of endogenous or exogenous in nature. On the other hand, on the basis of their mechanism of actions, they have classified as preventive type and chain breaking type antioxidants. In the physiological system some key antioxidant enzymes are noteworthy, *viz.* superoxide dismutase, catalase and glutathione peroxidase which represents the key intracellular protection system as antioxidants by scavenging superoxide anion and hydrogen peroxide. Current research suggests that several natural products derived from plants showed antioxidant activities and in turn limit the serious consequences of neurodegeneration. Further, among the plant derived natural compounds, phenolic substances like flavonoids showed promising antioxidant

activities (Cao et al. 1997). Morel et al. (1993) described free radical scavenging activity of flavonoids which even can reduce the lipid peroxidation process.

In Ayurvedic system, two major approaches, *viz*. disease preventive and health promotive approach are gaining much attention due to its wide acceptability. The famous 'Rasyanachikitsa' of Ayurveda based on revitalization and rejuvenation therapy. The "Rasayana drugs" known to function by modulating the nueral, endocrinal and immune systems in the body and have been identified as rich source of antioxidant molecules (Brahma and Debnath 2003; Pushpangadan 2005).

Recently, in this area *Alpinia* genus has showed remarkable contribution as herbal remedies. Report of Singh et al. (2011a) revealed the chloroform fraction of *A. galanga* as anti-amnesic where the active compound for this activity could be 1'S-1'-acetoxyeuginol acetate as it was found to be the lead compound in the fraction. On the other hand, *A. galanga* ethanol extract shows anti-amnesiac effect in Amyloid β induced neurodegeneration (Singh et al. 2011b). Likewise, *A. oxyphylla* fruit was found to have the neuroprotective activities (Koo et al. 2004) and subsequently many other *Alpinia* species have been reported till date (Table 2.4). Protocatechuic acid (PCA), a principal compound of the *A. oxyphylla*, protects against oxidative damage *in vitro* and reduces oxidative stress *in vivo* (Shi et al. 2006). It has been shown that PCA also reduces the hydrogen peroxide or sodium nitroprusside induced cell death in PC12 cells in dose-dependent manner (An et al. 2006) and this offers a valuable therapeutic strategy for the cure of oxidative stress-induced neurodegenerative disease like Parkinson's disease. Other reports revealed that *A. katsumadai* seed extract protects neurons from ischaemic damage (Li et al. 2011a) and the treatment significantly decreased the activation of astrocytes and microglia in the hippocampal CA1 region (Li et al. 2011b). Similarly, methanolic extract of *A. officinarum* rhizome showed protection against oxidative

damage in PC12 cells (Chang et al. 2011). Essential oil of *A. zerumbet* has strong potential as antipsychotic and antioxidant agent (de Arau´jo et al. 2011) which may have promising efficacy for the treatment of schizophrenia. Members of the genus *Alpinia* are found to have a remarkable antioxidant activity which in turn gives more biological efficacy towards the development of therapeutics. The neuroprotective and antioxidant activities of the genus are enlisted in Table 2.6.

Table 2.6 List of neuroprotective and antioxidant activities exhibited by various natural bioactive compounds and crude fractions of *Alpinia* species

Species Name	Parts used	Bioactive fractions/compounds	Bioactivity	References
A. galanga	Rhizome	n-hexane, chloroform and ethyl acetate	Neuroprotective	Singh et al. 2011a
A. galanga	Rhizome	Ethanolic extract	Neuroprotective	Singh et al. 2011b
A. katsumadai	Seeds	70% ethanolic extract	Neuroprotective	Li et al. 2011a
A. katsumadai	Seeds	Ethanolic extract	Neuroprotective	Li et al. 2011b
A. officinarum	Rhizome	Methanolic extract	Neuroprotective	Chang et al. 2011
A. oxyphylla	Fruits	80% ethanolic extract	Neuroprotective	Zhang et al. 2011a
A. oxyphylla	Kernel	Protocatechuic acid	Neuroprotective	An et al. 2006
A. oxyphylla	Fruits	Protocatechuic acid	Neuroprotective	Shi et al. 2006
A. oxyphylla	Fruits	Water extract	Neuroprotective	Koo et al. 2004
A. oxyphylla	Fruits	94% ethanolic extract	Neuroprotective	Yu et al. 2003
A. oxyphylla	Fruits	Ethanolic extract	Neuroprotective	Yu et al. 2003
A. mutica	Rhizome	5,6-dehydrokawain, flavokawin B, pinostrobin, β-sitosterol and pinocembrin	Antioxidant	Mustahil et al. 2013
A. oxyphylla	Fruits	1-(3',5'-dihydroxy-4'-methoxyphenyl)-7-phenyl-3-heptanone and 1-(2',4'-dihydroxy-3'-methoxyphenyl)-7-(4'-methoxyphenyl)-3-heptanone	Antioxidant	Bian et al. 2013
A. oxyphylla	Seeds	Yakuchinone A	Antioxidant	Lin et al. 2013
A. oxyphylla	Fruits	Ethanol extract	Antioxidant	Wang et al. 2013b
A. zerumbet	Leaves	Hydroethanolic extract	Antioxidant	Roman Junior et al. 2013
A. galanga	Rhizome	Ethanol extract	Antioxidant	Singh et al. 2011b
A. officinarum	Rhizome	Methanolic extract	Antioxidant	Chang et al. 2011
A. oxyphylla	Fruits	Protocatechuic acid	Antioxidant	Zhang et al. 2011b

Table 2.6 (Continued)

Species Name	Parts used	Bioactive fractions/compounds	Bioactivity	References
A. calcarata	Rhizome	Hydrodistilled n-pentane and ether extract	Antioxidant	Arambewela et al. 2010
A. officinarum	Rhizome	Hydro alcoholic extract	Antioxidant	Srividya et al. 2010a
A. densespicata	Stem and leaves	Ethanol extract	Nitric oxide inhibitory	Kuo et al. 2009
A. zerumbet	Leaves and rhizome	dihydro-5,6-dehydrokawain and other ethyl acetate and hexane extract	Antioxidant	Elzaawely et al. 2007a
A. zerumbet	Flowers and seeds	Ethyl acetate and hexane extract	Antioxidant	Elzaawely et al. 2007b
A. galanga and *A. allughas*	Rhizome	Dichloromethane and methanol extract	Antioxidant	Vankar et al. 2006
A. katsumadai	Seeds	Epigallocatechine-3-gallate, resveratrol and total extract	Antioxidant	Lee et al. 2003
A. speciosa	Rhizome	Feruloyl esters with epicatechin	Antioxidant	Masuda et al. 2000

2.8.5. Antidiabetic activity

Diabetes mellitus (DM) is widely prevalent in both developed and developing countries. An estimate from the International Diabetes Foundation (IDF) suggested that diabetes affects around 246 million people in the world and expected to reach upto 380 million by the year 2025. Diabetes encompasses a huge death worldwide (~6% of total global mortality) which is considered same as in the case of HIV/AIDS (IDF 2006).

The DM is a metabolic disorder of endocrine and characterized by persistent hyperglycaemia. Due to defects in insulin secretion or action or both, it results in abnormalities of protein, fat and carbohydrate metabolism. According to WHO classification, DM is divided I'nto insulin-dependent diabetes mellitus (IDDM) or type 1 and non-insulin dependent diabetes mellitus (NIDDM) or type 2. This type 2 is most prevalent and generally

appears later in life, though it is grabbing the young patients in an escalating manner. The main reasons of type 2 diabetes lie with the predominant resistance and relative deficiency of insulin or its predominant secretory defects with or without insulin resistance (WHO 1999).

The current medicines towards the management of type 2 diabetes lead to various side effects *viz.* hepatotoxicity, hypoglycemia, flatulence, abdominal pain and diarrhea (Fujisawa et al. 2005; Singh et al. 2008). After prolonged treatment, these medicines showed lack of effectiveness due to drug resistance and therefore, as an alternative, many medicines from herbal sources have been recommended towards the treatment of diabetes (WHO 2002). Many traditional medicines from herbal sources have been used in different parts of the world for the treatment of diabetes (Odhav et al. 2010).

In one of the approach, natural products or botanicals were used to inhibit the carbohydrate hydrolysis enzymes, *viz.* pancreatic amylase and α-glucosidase to retard the absorption of glucose. These inhibitions resulted in delaying carbohydrate digestion and prolong the overall digestion time towards reduction in glucose absorption rate to reduce the postprandial hyperglycemia (Kim et al. 2004). In the last decade, several research groups have taken some initiative focusing on medicinal plants for α-amylase and α-glucosidase inhibition activity. Till date many plants were reported to have hypoglycemic compounds, such as flavonoids, polyphenols, as well as their sugar derivatives and showed above mentioned inhibitory activities (Jung et al. 2006). The genus *Alpinia* has high medicinal impact due to its diverse biomedical and therapeutic applications (Ghosh and Rangan 2013). However, the various members in the genus, *Alpinia*, are less explored with regard to its antidiabetic potentiality except for few scanty reports (Srividya et al. 2010b; Raj et al. 2011).

2.8.6. Other activities

Besides above activities, the genus is also emerging as the prospective source for anti-ageing compound like PCA from *A. oxyphylla* (Zhang et al. 2011b). Aqueous acetone extract of *A. officinarum* rhizome showed inhibition to melanogenesis process (Matsuda et al. 2009), whereas acetone extract of *A. oxyphylla* fruits act as a potent skin permeation enhancer (Fang et al. 2003). Recent studies revealed two bioactive compounds from *A. zerumbet* rhizome and leaves, *viz.* 5,6-dehydrokawain (DK) anddihydro-5,6-dehydrokawain (DDK). These compounds were found to be potent inhibitors of HIV-1 integrase and neuraminidase (Upadhyay et al. 2011) indicating that it could be used as potent drugs against those viral diseases.

2.9. *Alpinia nigra*: The plant under study

A. nigra, an unexplored and less studied member in the *Alpinia* genus, is known for its ethnomedicinal uses in North East India (NEI). The plant is distributed primarily in China, Thailand and other Southeast Asian countries including NEI (Wu 1981). In brief, the plant is herbaceous in nature with an average height of 8-10 ft. The aerial shoot is pseudostem and underground part is stolon, has simple leaf structure, acute at the base and apex, and with alternate phyllotaxy. The inflorescence is panicle type and fruit is berry with many aromatic seeds inside the locules. The pericarp is thin and green in colour when immature and turns black and brittle when gets matured (Fig. 2.2).

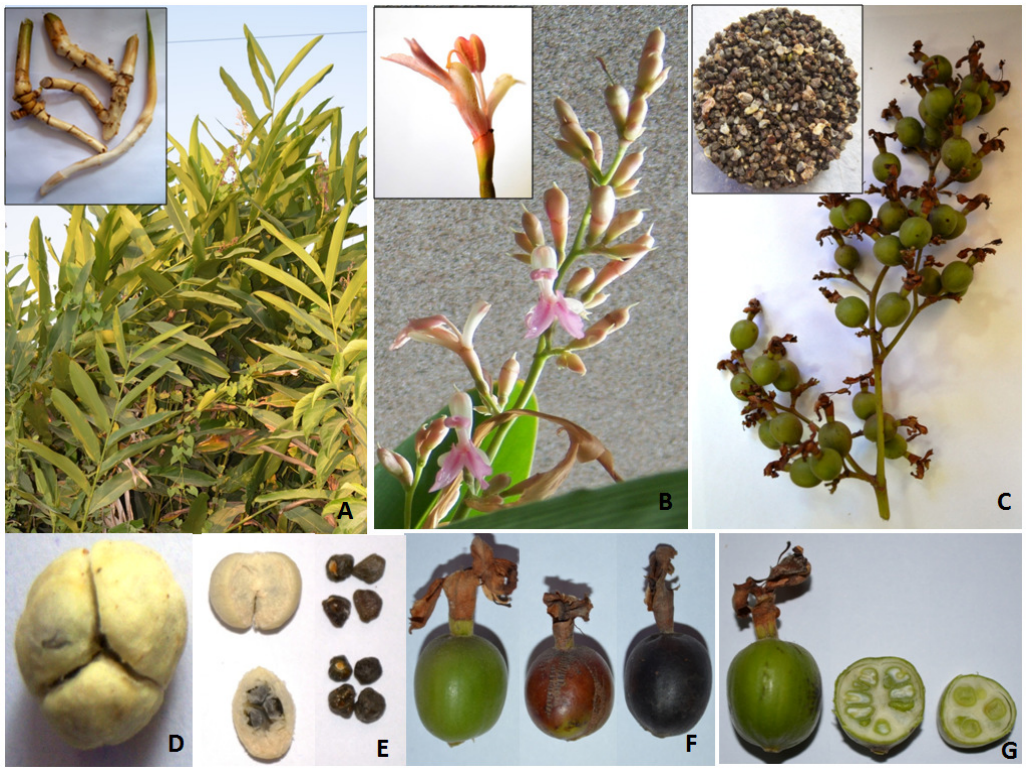


Fig. 2.2 Different plant parts of *A. nigra* used to extract bioactive compounds. (A) Alternate phyllotaxy of plants, inset depicts the stolon type of rhizome, (B) Racemose type of inflorescence, inset shows single flower, (C) Developing fruit cluster, inset shows mature seeds, (D) Pulpy dehusked fruit (trilocular), (E) Locules and mature seeds, (F) Different stages of fruit maturity and (G) Longitudinal and cross section view of the immature fruit.

A. nigra, which locally known as "Tora" in Assam (India) is also used in folk medicines to cure various health related problems like gastric ulcers, jaundice, irregular menstruation and even for bone weakness (Tushar et al. 2010). Roy et al. (2012) recently described that the aqueous juice of *A. nigra* shoots is used to cure intestinal parasitic infection, food flavouring materials and consumed as favourite vegetable diet for native peoples of Tripura, India. The rhizomes of this plant are known to be used as vegetables in Thailand. The pharmaceutical application of *A. nigra* includes mainly against stomach related problems, gastric ulcers and other related diseases. The shoot part of *A. nigra* has several traditional usages among the tribal communities Tripura, NEI. According to Roy and Tandon (1999), the green shoots of this plant were used to prepare raw juice for consumption and known to possess strong anthelminthic and antioxidant properties (Roy and Tandon1999).

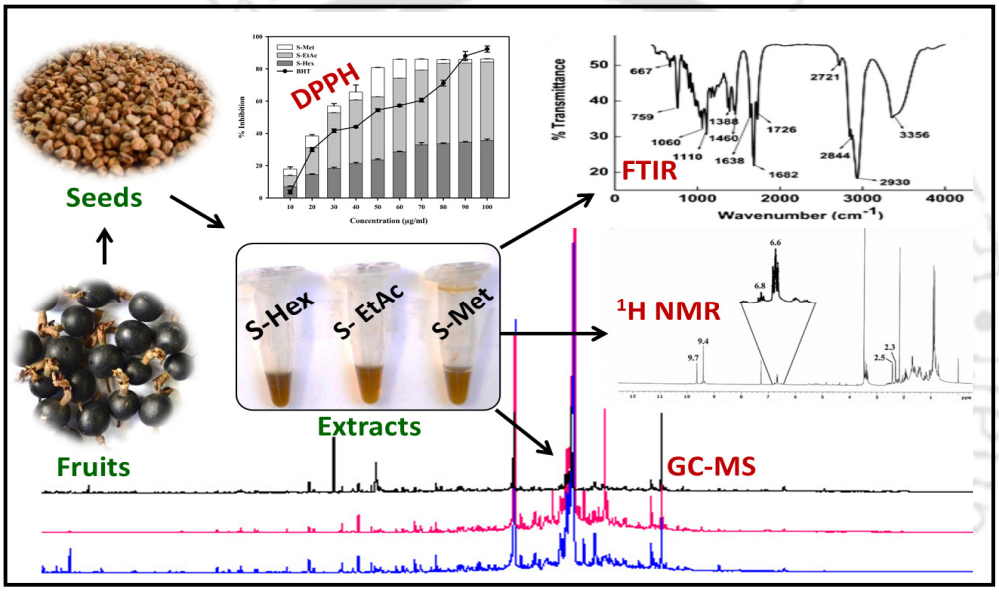
However, information about the active compounds and other phytochemicals from *A. nigra* are limited. The presence of two bioactive flavone glycosides (astragalin and kaempferol-3-O-glucuronide) in the seed clusters of *A. nigra* were described previously by Qiao et al. (2007). They have also reported kaempferol-3-O-glucuronide as the major compound in the fruit pulp of *A. nigra*. Previous study also described the presence of two volatile oils, β-Pinene and α-Pinene, in the fruits and rhizome of *A. nigra* (Qiao et al. 2000). However, GC-MS analysis of essential oil of leaf and rhizome of *A. nigra* showed 1,8-cineole as the major constituent among other phyto-volatiles (Kanjilal et al. 2010). The biological activities of any such phytochemicals or other unexplored bioactive compounds were not studied in detail till date. The molecular phylogenetic analysis suggests that *A. galanga* is closely related to *A. nigra* which could be exploited in view of unveiling the chemical profile of *A. nigra* and future application in various biological activities (Rangsiruji et al. 2000).

2.10. Future perspectives

In the current study it has been observed that various plant parts of *Alpinia* are investigated to obtain bioactive compounds and different fractions which showed remarkable biological efficacy against various biomedical challenges. Detailed examination of the cumulative data shows that rhizome and seeds/fruits are the main plant parts used for pharmacological investigation in *Alpinia* whereas other vegetative and reproductive parts were used moderately. Most of the cases it has been observed that rhizomes and seeds harbour most of the essential oil components and showed potential biological activities. It has also been observed that various solvent systems were used in the bioactivity studies and isolation of bioactive compounds from the plant parts which act as a key factor in terms of yield, number of compounds, type of compounds, etc. The bioactive compounds or crude fractions of essential oils from various species of *Alpinia* were found to be promising in various biomedical challenges like antimicrobial, anticancerous, antileishmanial and many more. From the previous reports, it has been observed that various species of *Alpinia* has ample potential to overpower biomedical threats including the most diverse microbes in the earth. Moreover, the genus *Alpinia* harbours versatile components towards its diverse biological efficacy. Much more understanding and further exploration will be needed towards the unexplored species of the genus *viz. A. nigra, A. pahangensis, A. nieuwenhuizii* and many more to circumvent the future biomedical challenges to open up new avenues in herbal therapeutics.

Chapter 3

Chemical profiling and free radical scavenging activity of crude organic solvent extracts from *A. nigra* seeds



The chapter describes the isolation of various chemical components in the form of three different organic solvent extracts from *A. nigra* seeds and their chemical profiling.

Chapter 3

Chemical profiling and free radical scavenging activity of crude organic solvent extracts from *A. nigra* seeds

3.1. Introduction

Since ancient times, human have been exploring the nature, especially plants in search of new phytochemicals towards the development of various wound healing to life saving drugs. Traditional herbal medicines have been widely used for thousands of years in both developed and developing countries due to its natural origin and lesser side effects than the synthetic drugs (Kamboj 2012). Recent herbal product development involves the application of appropriate standard and good manufacturing practices (GMP) using modern pharmaceutical techniques (Akarasereenont et al. 2010). However, the quality control and assurance of herbal preparations still remain a challenge owing to the high variability of chemical components concerned (Chitlange et al. 2008). The GMP control ensures the quality of herbal medicine which is directly related to the efficacy, safety and consistency of the preparations (WHO 1996; Rousseaux et al. 2003). The chemical composition in a specific preparation varies depending on a number of factors, such as sample origin, time of harvesting and also extraction methodology employed, which leads to the variation of chemical constituents and also their relative content. The synergism of various active components present in plant extracts usually plays an important role in bioactivities due to the specific and cross interactions with target molecules. Therefore, chemical profiling and spectroscopic fingerprinting of plant preparation is a prerequisite in

functional food, cosmetics, drug pharmacology as well as in toxicology to understand the nature of bioactive compounds in the preparation. Chemical profiling has been performed by various analytical techniques and also strongly recommended for the purpose of quality control of herbal medicines (Chitlange et al. 2008). They represent the appropriate chemical integrities of herbal medicines and therefore are in use for authentication and identification of the herbal products prior to biological applications.

In the plant kingdom, Zingiberaceae family members are known for its medicinal properties and diverse pharmaceutical uses (Li et al. 2013c; Patiño et al. 2013; Zhang et al. 2013b). The plants are widely growing in North East India (NEI), a part of Indo-Himalayan biodiversity hotspot. Tushar et al. (2010) documented the ethnomedical practices of various Zingiberaceae plants by the tribal communities of NEI. Many species belonging to the genus *Alpinia* has been reported to possess medicinal properties and versatile uses in folk medicines. The genus *Alpinia* is emerging as the most proliferative genus in Zingiberaceae family due to its diverse biological applications notably as anti-microbial, anti-cancer, anti-viral, anti-allergic, neuroprotective and as highly antioxidant agent (Shi et al. 2006; Elzaawely et al. 2007b; Yasuharaa et al. 2009; Upadhyay et al. 2011). *A. nigra* (Gaertn.) B. L. Burtt is a member in the genus *Alpinia* which distributed primarily in China, Thailand and other Southeast Asian countries including NEI (Wu 1981). Although *A. nigra* is known to be used for the treatment of dyspepsia, gastric diseases, insect bites, trematocidal etc. in folk medications (Tushar et al. 2010; Roy et al. 2012), the effectiveness of *A. nigra* towards its chemical profiling and bioactive potential has not been investigated yet.

Therefore, the present study of this chapter is focused on the isolation of various organic solvent extracts from *A. nigra* seeds, the phytochemical analysis and chemical profiling of the organic extracts. Further, the bioactive potential of all the organic extracts towards the free radical scavenging activity were determined.

3.2. Materials and methods

3.2.1. Sample collection and maintenance

The seeds and rhizome (stolon) of *A. nigra* were collected from Indian Institute of Technology Guwahati (IITG) campus (26°12.476'N to 91°41.965'E) during the period of November 2011-January 2012. The mature fruits (black color) were harvested and seeds were taken out for further processing. The rhizomes were cleaned, dried in shade before extraction. The collected plants along with the rhizome and flowers were properly tagged and maintained in the departmental green house of IITG and botanical garden of Gauhati University (GU). Hooker (1875) and Petersen (1889) were used as reference for identification of the plants. The voucher specimen is maintained as herbarium for future reference at IITG herbarium repository.

3.2.2. Preparation of the organic extracts

Various extraction methods, *viz.* conventional maceration, Soxhlet extraction, microwave assisted extraction, ultrasound assisted extraction and many other techniques are available to extract the chemical components from plant materials in the form of crude extracts. Among these, Soxhlet extraction gives significantly higher yield in terms of total phenolic content and tannins with economic investments (Aspé and Fernández 2011). Therefore, in the present study, the shade dried seeds (200 g) and rhizomes (200 g) were grounded into fine powder and

subjected for organic solvent extraction for 5 h using a Soxhlet apparatus. The extraction for the same fixed amount of the sample (200 g) was done according to the polar strength of the solvent (300 ml each) starting with non-polar (n-hexane) to polar (ethylacetate and methanol) respectively. After each solvent extraction, the same samples were dried properly to remove the traces of previous solvent so as to reduce the carry over effect of the previous solvent and again subjected for next polar solvent. The extracts were vacuum dried in rotary evaporator (BUCHI, R-210, Switzerland).

3.2.3. Determination of total soluble phenolics (TSP)

Total soluble phenolic compound (TSP) content in *A. nigra* seed extracts were estimated according to the Folin-Ciocalteu colorimetric assay (Singleton and Rossi 1965), using gallic acid as standard. To generate the standard curve, different concentrations (10-500 µg/ml) of gallic acid were used. Three different dilutions (1:1000, 1:100 and 1:10) were prepared from each extracts (1 mg/ml) and were used for the assay. Both the standard and samples were diluted in ethanol. BHT (125 µg/ml) and ethanol were used as positive and solvent control (blank), respectively. About 100 µl of 1N Folin-Ciocalteu's phenol reagent was added to all the samples, standards and controls and incubated at room temperature for 5 min. After incubation, 300 µl of sodium-carbonate (75 g/l) was added and further incubated for 2 h at room temperature. Finally, 300 µl was taken from each sample and transferred to 96-well microtitre plate and absorbance measured at 765 nm. Quantification of TSP in each extracts was determined from the gallic acid standard curve and the values were represented as milligrams of gallic acid equivalents (GAE) per g dry weight of plant extracts. All the samples were analyzed in triplicates and the whole experiment was repeated twice.

3.2.4. Phytochemical screening of organic extracts

The phytochemical tests to detect the presence of alkaloids, flavonoids, saponins, terpenoids and phenolics were performed according to the method described by Kokate (1994) and Harborne (1998). The tests were based on the visual observation of color change or formation of a precipitate after the addition of specific reagents.

Test for alkaloids (Evans 1997*)*

Each organic extract (50 mg) was taken in a test tube and stirred with few microlitre of diluted hydrochloric acid. The mixture was filtered and kept in a separate test tube. Few drops of Mayer's reagent was added on to the filtrate by the side of the test tube. Formation of cream coloured precipitate indicates the presence of alkaloids.

Mayer's reagent is prepared by dissolving mercuric chloride ($HgCl_2$) and potassium iodide (KI) in water. Firstly, 1.358 g of $HgCl_2$ is dissolved in 60 ml of water and 5 g of KI is dissolved in 10 ml of water. Both the solutions were mixed and made up the volume to 100 ml with water.

Test for flavonoids (Harborne 1998)

A few drops of aqueous 1N sodium hydroxide solution were added to the stock solution of each extract (0.5 ml) and heated. Yellow colour fluorescence indicates presence of flavonoids. The disappearance of intense yellow colour upon addition of a few drops of diluted H_2SO_4 further confirms the presence of flavonoids.

Test for saponins (Kokate 1999)

The stock solution from each crude extract of *A. nigra* (0.5 ml) was diluted with distilled water (20 ml) and then the mixture was shaken for 15 min. The formation of a foam layer on the top of the test tube showed the presence of saponins.

Test for terpenoids (Harborne 1998)

The dry crude plant extract (5 mg) was dissolved in chloroform (2 ml) and then acetic anhydride (1 ml) was added to it. One millilitre of concentrated sulphuric acid was added to the solution. The formation of reddish brown colour at the interface indicates the presence of terpenoids.

Test for phenolics (Harborne 1998; Kokate 1994)

The dry crude plant extract (5 mg) was dissolved in 0.5 ml of distilled water. Then few drops of 5% ferric chloride solution were added to it. The formation of dark green colour indicates the presence of phenolic compounds in the extracts.

3.2.5. Chemical profiling of organic extracts

3.2.5.1. Fourier transform infrared (FTIR) spectroscopy analysis

Each sample extract (1 mg) were mixed with dry KBr powders to form the pellets. The absorption spectra of the pellets (samples) were obtained with a Perkin-Elmer FTIR spectrophotometer (Norwalk, Connecticut) in the range of 450-4000 cm^{-1}.

3.2.5.2. Nuclear magnetic resonance (NMR) analysis

The ^{1}H spectra were recorded on a Varian 400 MHz FTNMR. Each extract sample (25 mg) was dissolved in 0.5 ml $CDCl_3$ and the solvent signal was used for spectral calibration. The spectral width was 6389.8 Hz, acquisition time 1.998 s, number of scans 4 and the time domain size was 32 K with relaxation delay for 1 s. The chemical shift of each spectrum was recorded as δ values.

3.2.5.3. Gas chromatography mass spectrometry (GC-MS) analysis

GC-MS analysis of the *A. nigra* seed extracts were carried out on a SHIMADZU QP 2010 which was equipped with an auto sampler and gas chromatography interfaced to a mass spectrometer instrument. The samples were analysed by employing the following conditions: sample injection volume of 1.0 µl (split ratio of 10:1), DB-5MS (30m x 0.25mm x 0.25µm) Agilent J&W GC Columns, operating in electron impact mode at 70 eV. Helium (99.99%) was used as carrier gas with a constant flow rate of 1.1 ml/min. Injector temperature was kept at 100°C and ion source temperature was 250°C. The extract components were separated on a fused silica capillary column in a temperature program from 100°C (kept 2 min) to 290°C (kept 20 min), with a rate of 3°C/min. Mass spectra were taken at 70 eV with a scan interval of 0.3 seconds and fragments were scanned from 50 to 500 Da. Total GC running time was 83.33 min.

Identification of compounds was achieved by comparing the retention time with those of standard compound. For unidentified compounds, the spectral data were compared with Wiley and NIST libraries for identification and confirmed by Kovat's index. Kovat's indices for individual peaks were determined using a mixture of n-alkanes (C11-C37) under the same conditions towards further identification.

3.2.6. Determination of DPPH radical scavenging activity

The free radical scavenging activity of seed extracts of *A. nigra* were determined using DPPH (2, 2-diphenyl-1-picrylhydrazyl) according to the method of Shimada et al. (1992). Briefly, 0.1 mM solution of DPPH in 99.99% ethanol was prepared and 100 µl of this solution was mixed with 200 µl of the ethanolic solutions of n-hexane, ethyl acetate and methanol extracts from *A. nigra* seeds at concentrations ranging from 10 to100 µg/ml. The mixture was shaken vigorously and allowed to incubate at room temperature in dark for 30 min. Butylated hydroxyl toluene (BHT) (Sigma-Aldrich, USA) was used as positive reference while ethanol was used as negative reference. The absorbance was measured at 517 nm using multimode microplate reader (Tecan, Infinite M-200, Switzerland). The DPPH radical concentration was calculated using the following equation:

DPPH Scavenging Effect (%) = 100- $[(A_0-A_1/A_0) \times 100]$,

where A_0 was the absorbance of the control reaction (DPPH + ethanol) and A_1 was the absorbance in the presence of the sample (DPPH + sample in ethanol).

3.3. Results and discussion

3.3.1. Plant material and herbarium

The materials for herbarium were collected during the full bloom and fruiting stage of *A. nigra*. The rhizomes and black colour mature fruits were collected and dried in shade for 10-15 days prior to further study.

Collected plants were replanted under green house conditions in pots with alluvial soil and sand mixture (3:1) and maintained as live specimen. These were also maintained as herbarium specimen for future reference (Fig. 3.1).

3.3.2. Preparation of the organic extracts

Organic solvents are frequently used for the extraction of phenolic compounds from the plant samples to be used as antioxidants (Pokorny and Korczak 2001). Moreover, the extraction yield and biological activity of the extracts have a strong relationship with the solvent used, mainly due to the different polarity of the chemical compounds (Moure et al. 2001). Therefore, selection of the most appropriate solvent is a determinant factor on extract properties and due to the diverse structure and composition of the sample (Al-Farsi and Lee 2008). Here, we have fractionated the chemical components of the seeds and rhizomes during subsequent steps of extraction by using gradient polarity of solvent system. Initially, defatting of samples was done with non-polar n-hexane which yielded non-polar compounds and smaller fraction of polar compounds during the continuous extraction process. The same seeds when subjected to ethyl acetate and later methanol, yielded with fractions containing more polar compounds. After each extraction, approximately 230-250 ml of solvent was recovered using rotary evaporator and was subsequently used for further extraction process. Therefore, only 100-150 ml of total amount of solvents was used to rationally fractionate the chemical components of the sample during the extraction process. The solvent extraction process yielded 0.7, 1.8 and 3.7 g for S-Hex, S-EtAc and S-Met, respectively for the seed samples and 0.12, 0.23 and 0.35 for R-Hex, R-EtAc and R-Met respectively for the rhizome samples (Table 3.1). Due to very low yield of rhizome extracts, further compound isolation and biological activity studies were carried out only with three

different seed extracts. As future line of work, isolation of bioactive compounds and their biological applications could be possible using rhizome extracts by optimising the yield parameters. Here, in order to use the seed extracts for biological studies, the stock solutions were prepared from the dried samples of each extract by dissolving in ethanol to make the final concentration as 10 mg/ml.

Table 3.1 Optimization of yield related parameters for extraction of organic solvent extract from seeds of *A. nigra*

Parameters	**Rhizomes**			**Seeds**		
Collection time	November- January			November- January		
Time for drying	10-15 days			10-15 days		
Solvent	Hexane	Ethyl Acetate	Methanol	Hexane	Ethyl Acetate	Methanol
Sample (g)	200	200	200	200	200	200
Initial volume of solvent (ml)	300	300	300	300	300	300
Time for 1st cycle (min)	45	60	70	45	60	70
Time for next cycles (min)	25	30	40	25	30	40
Solvent recovery (ml)	220	230	240	210	230	250
Solvent recovery time (min)	80	90	100	80	95	110
Oil color	Reddish brown	Black	Brown	Reddish brown	Dark brown	Brown
Total oil yield (g)	0.12	0.23	0.35	0.7	1.8	3.7
Yield (%)	0.06	0.115	0.175	0.35	0.9	1.85

3.3.3. Estimation of phenolic content

In plants, phenolic compounds are present as ether- and/or ester-linked molecules. Many phenolic bioactive compounds were identified from the various members of the genus *Alpinia* (Ghosh and Rangan 2013). In the current study, hexane fraction showed relatively low TSPs

compared to other extracts. Here, methanolic extract showed highest TSP (3533.91±23.66 mg GAE/g S-Met), than ethylacetate (2427.58±24.15 mg GAE/g S-EtAc) and hexane extract (530.46±38.89 mg GAE/g S-Hex) (Fig. 3.2). The current assay based on Folin-Ciocalteu reagent, determines the reducing capacity of each sample extracts relative to its content of TSP as compared with standard phenolic acid (Wang et al. 2010a). The result indicates the possibility of bioactive potential of S-EtAc and S-Met extracts which might be attributed by polyphenolic moieties in those extracts (Escuredo et al. 2012). The observed TSP in all three extracts of *A. nigra* were found much higher than various organic solvent extracts of *A. oxyphylla* fruits and methanolic extract of *A. nigra* leaves (Wang et al. 2013a; Sahoo et al. 2013). Therefore, the presence of high level of total soluble phenolics in *A. nigra* seed extracts could be useful in various biological applications with much higher efficacies.

Fig. 3.1 Herbarium of *A. nigra*

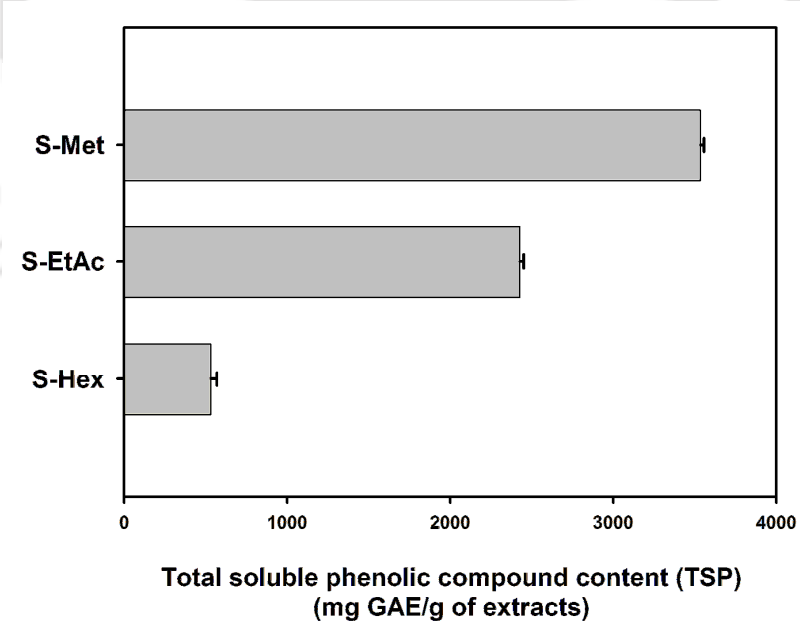


Fig. 3.2 Estimation of total soluble phenolic content (TSP) from organic solvent extracts of *A. nigra* seeds. The values were represented as milligrams of Gallic Acid Equivalents (GAE) per g dry weight of plant extracts ± SE.

3.3.4. Phytochemical screening of organic extracts

The preliminary phytochemical screening revealed the presence of flavonoides and terpenoids in all three extracts (Table 3.2). However, S-Met showed higher amount of terpenoids, alkaloids and flavonoids compared to other extracts. Both S-Met and S-EtAc showed the presence of phenolics whereas S-Hex fraction showed negative towards presence of phenolic compounds (Table 3.2). However, all the crude extracts did not contain saponins on the basis of foam test. From the present results, it could be stated that the methanolic extract (S-Met) is rich in most of the phytochemicals tested *viz*. phenolics, flavonoids, terpenoids and alkaloids respectively.

Table 3.2 Phytochemical constituent analysis of organic extract of *A. nigra*

Chemical constituents	S-Hex	S-EtAc	S-Met
Saponins	-	-	-
Phenolics	-	++	++
Flavonoids	+	+	++
Terpenoids	+	++	+++
Alkaloids	+	++	+++

- = Absent/ Not detected, + = Low, ++ = Moderate, +++ = High

3.3.5. FTIR spectral analysis

FTIR spectral analyses were performed by correlating absorbance bands with a wide range of bonds and their functional groups. FTIR spectrum can illustrate the nature of the functional groups present in the complex mixture and set the individual fingerprints for each extract. In our study, the acquired data were plotted as percent transmittance using Origin 5.0 software. Two sharp peaks at 2930.16 and 2844.03 cm^{-1} indicates the presence of alkanes (C-H stretch) and aldehyde (C-H aldehyde stretch), whereas, strong peaks at 1726.74, 1682.91 and 1638.88 cm^{-1}

refers to the signature of aldehyde (C=O stretch), α, β-unsaturated aldehydes/ketones (C=O stretch) and alkenes (C=C stretch, conjugated) in the extracts (Fig. 3.3). Similarly, sharp peaks at 1110.55 and 1060.88 cm^{-1} revealed the presence of ether linkage (C-O-C stretch, diaryl) and C-N stretch of aliphatic amines respectively. Peaks from 759 to 770 cm^{-1} refers to the presence of aromatic moieties (C-H bond, ortho) and 3432-3356 cm^{-1} indicates the existence of alcohols and phenols (O-H stretch) in all the extracts irrespectively (Prabhakaran et al. 2012) (Fig. 3.3).

3.3.6. NMR spectral analysis

In case of NMR spectral analysis, all the spectra were compared with the standard reference chart and possible mixture of compounds were determined (Silverstein et al. 2005). The spectra of each extract showed many peaks at 0-5 ppm with varying intensity and respective signatures (Fig. 3.4).

Comparison of those peaks revealed the presence of various group of molecules, *viz*. 0-2 ppm for aliphatic acyclic compounds, 1-2 represents beta substituted aliphatic compounds and 2-5 ppm describes the presence of mono substituted aliphatic compounds which is well in agreement with previous reports (Patra et al. 2012). Here, two prominent peaks at 9.4 and 9.7 ppm in S-EtAc and S-Met extracts revealed the presence of two aldehydes which are also convincing from the FTIR spectra of each extracts. Similarly, two triplets at 6.6 and 6.8 ppm characteristic to alkene which was almost absent in S-Hex whereas, very prominent in case of S-EtAc and S-Met (Fig. 3.4). Except S-Hex, another two prominent doublets at 2.3 and 2.5 ppm possibly comes from the alkene adjacent to COR and CHO respectively. A sharp peak was observed at 3.4 ppm which could be from either ether (HC-OR) or alcohols (HC-OH). It was clearly visible, that spectra possess a resemblance with one another as have been extracted in a

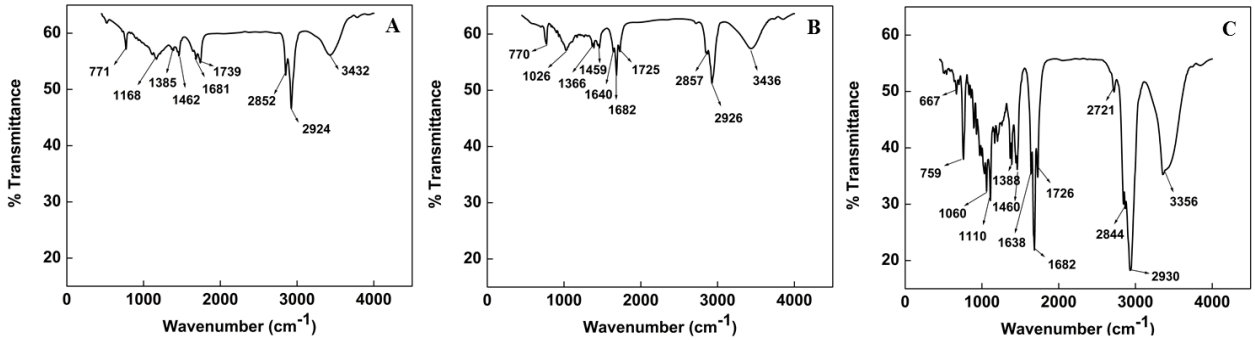


Fig. 3.3 FTIR spectra of *A. nigra* seed extracts. (A) S-Hex, (B) S-EtAc and (C) S-Met respectively.

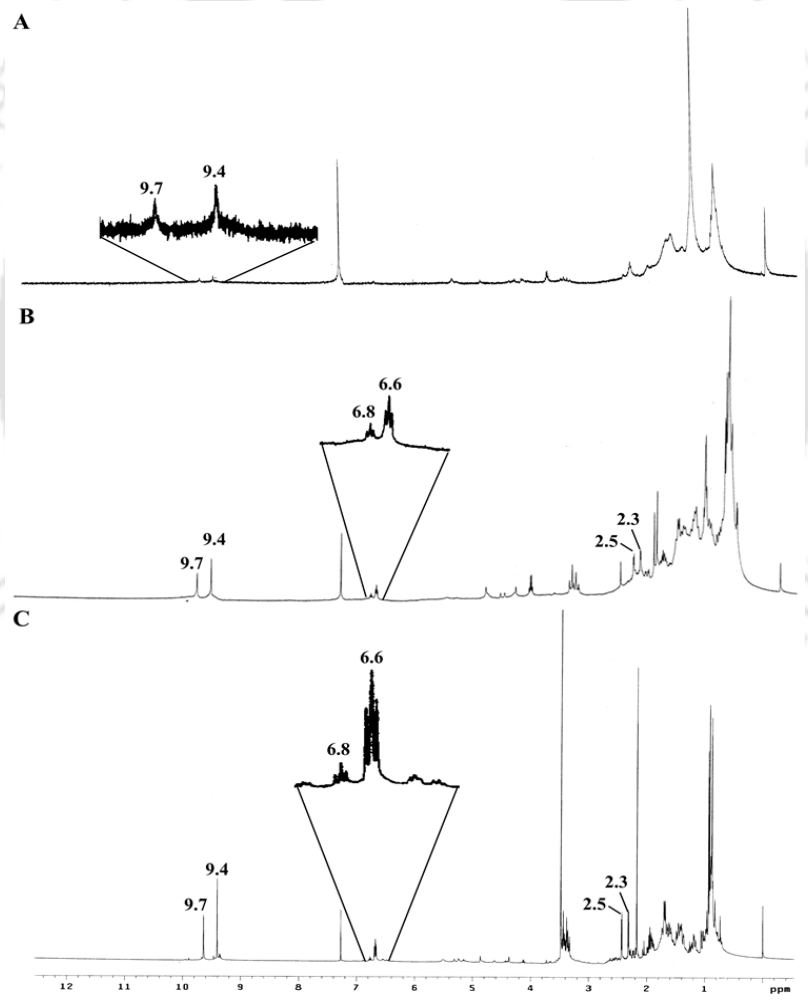


Fig. 3.4 ^{1}H NMR of crude seed extracts from seeds of *A. nigra*. Characteristic fingerprints were showed for S-Hex (A), S-EtAc (B) and S-Met (C) extracts.

gradient fractionation. Moreover, due to higher polarity S-EtAc and S-Met extracts possess a wide range of compounds which gives complex NMR fingerprints compared to the non-polar compounds from S-Hex fraction.

3.3.7. GC-MS analysis

GC-MS combines two different analytical techniques, gas chromatography and mass spectrometry. In GC, individual chemical characteristic of a compound determines how long it will take to pass through the chromatography column. The time taken by an individual compound to travel the length of the column is referred to as its retention time (RT) in that particular instrument set up condition. The RT for an individual compound is an identifying characteristic. In a combine system, the detector for the GC is the MS detector. As the compound exits the end of the GC column it is fragmented by ionization and the fragments are sorted by mass to form a fragmentation pattern. Like the RT, the fragmentation pattern for a given compound is unique and therefore is an identifying characteristic of an individual compound. Due to the higher specificity of GC-MS in a given condition, it is often referred to as the molecular fingerprint.

The compounds present in the organic solvent extracts of *A. nigra* were identified by GC-MS analysis (Fig. 3.5). Results of GC-MS showed 21, 48 and 46 compounds present in S-Hex, S-EtAc and S-Met respectively. The GC separated compounds were identified from the recorded mass spectra by comparison with the mass spectra from the NIST spectral database, Wiley library and available reports. The identified components and their respective yield from S-Hex, S-EtAc and S-Met are represented together with the RT and kovat's indices (KI) in Table 3.3, Table 3.4 and Table 3.5 respectively.

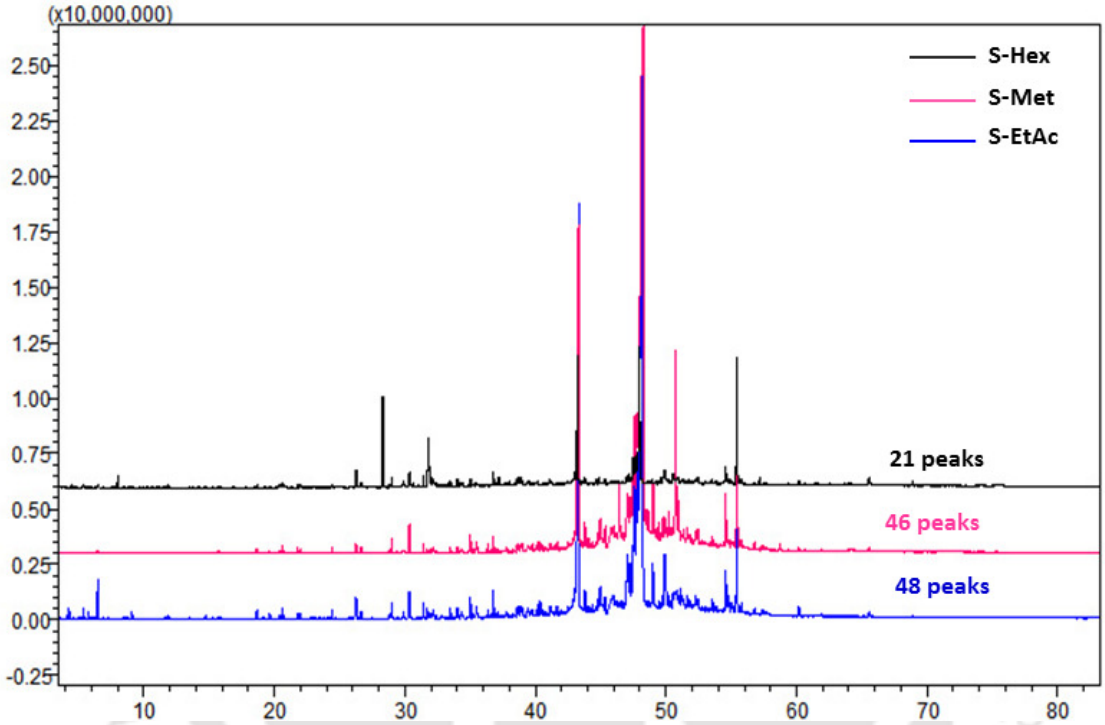


Fig. 3.5 GC-MS spectra of *A. nigra* seed extracts.

Table 3.3 List of major compounds identified from S-Hex of *A. nigra*

RT	KI	%	Compound identified
8.0	1261	0.95	trans-2-Decenal
26.3	1795	1.72	(*E*)-5-Tetradecenyl acetate
28.3	1855	10.73	(Z,Z,Z)-5,7,11-Hexadecatrienal
29.0	1875	0.80	9-Senecioylretronecine
30.3	1916	1.24	7-Senecioylheliotridine
31.4	1949	1.13	(*E*)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol
31.8	1962	8.89	n-Hexadecanoic acid
35.0	2063	0.76	(*E*)-Labda-8(17),12-diene-15-ol-16-al
36.8	2120	1.54	cis-Z-α-Bisabolene epoxide
43.0	2334	0.38	Sandaracopimar-15-en-8β-yl acetate
43.2	2343	18.74	(*E*)-labda-8(17), 12-diene-15, 16-dial
47.5	2501	2.25	Drimenol
47.7	2509	2.08	Drimenol analogue
48.0	2520	23.66	(*E*)-8β, 17-Epoxylabd-12-ene-15, 16-dial
49.8	2593	1.20	1,6-Methanonaphthalen-1(2H)-ol,octahydro-4,8a,9,9-tetramethyl-, (1R,4S,4aS,6R,8aS)
50.0	2597	0.89	Menthol, 1'-(butyn-3-one-1-yl)-, (1S,2S,5R)
54.5	2787	2.03	Aromadendren epoxide
54.7	2793	1.20	Andrographolide
55.4	2825	17.52	Cholestane (reference)
65.5	3282	1.50	β-Sitosterol
68.9	3405	0.78	Sitostenone

Table 3.4 List of major compounds identified from S-EtAc of *A. nigra*

RT	KI	%	Compound identified	RT	KI	%	Compound identified
4.3	1091	0.15	1,2,3-Propanetriol, monoacetate	41.7	2287	0.12	Caryophyllene oxide
5.4	1149	0.29	Trans-Pinocarveol	43.0	2335	0.53	Kauren-18-ol, acetate, (4β)-
5.8	1170	0.09	Pinocarvone	43.3	2347	17.12	(*E*)-labda-8(17), 12-diene-15, 16-dial
6.5	1205	0.96	Myrtenal	43.8	2363	0.46	Isoaromadendrene epoxide
9.1	1303	0.11	Perilla alcohol	44.9	2402	0.48	1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene
18.7	1581	0.17	Caryophyllene oxide	45.0	2407	0.55	Kauran-18-al, 17-(acetyloxy)-, (4β)-
20.6	1636	0.22	β-Farnesene	45.4	2421	0.34	Pregn-4-ene-1,20-dione, 12-hydroxy-16,17-dimethyl-
24.4	1742	0.19	trans-beta-Santalol	47.0	2484	2.15	3,8-α-Furanether
26.2	1792	0.18	Oxalic acid, 2-methylphenyl undecyl ester	47.2	2492	1.59	4,5-Secocholest-6-en-4-oic acid, 5-oxo-
26.3	1795	0.46	4-Butylphenol	47.5	2503	6.18	Drimenol
29.0	1875	0.33	9-Senecioylretronecine	47.7	2511	7.12	Drimenol analogue
30.3	1916	0.56	7-Senecioylheliotridine	48.2	2530	45.88	(*E*)-8b, 17-Epoxylabd-12-ene-15, 16-dial
31.4	1949	0.33	(*E*)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	49.0	2560	1.23	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-
31.7	1958	0.14	n-Hexadecanoic acid	49.4	2577	0.09	3,6,9,12-Tetraoxadocosyl acetate
33.5	2013	0.22	1,9-Dimethylphenanthrene	49.7	2587	0.24	Labdane diterpenes derivative
34.0	2031	0.21	2,4,8-Trimethyldibenzothiophene	49.9	2595	1.62	Menthol, 1'-(butyn-3-one-1-yl)-, (1S,2S,5R)-
35.0	2063	0.46	(*E*)-Labda-8(17),12-diene-15-ol-16-al	50.0	2601	1.27	Patchouli alcohol
36.8	2120	0.60	cis-Z-α-Bisabolene epoxide	50.2	2608	0.19	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-
38.9	2189	0.14	Incensole acetate	50.3	2612	0.02	Labdane diterpenes derivative
40.2	2234	0.15	trans-14-Isopropylpodocarpa-8,11,13-trien-13-ol	50.5	2620	0.21	α-Levantenolide
40.4	2241	0.31	6-(2,6,6-trimethyl-1-cyclohexenyl)-4-methyl-, (*E*)-4-Hexen-1-ol				

Table 3.5 List of major compounds identified from S-Met of *A. nigra*

RT	KI	%	Compound identified	RT	KI	%	Compound identified
26.3	1795	0.14	4-Butylphenol	47.7	2511	5.04	Drimenol analogue
29.0	1875	0.27	9-Senecioylretronecine	47.8	2514	0.72	Not identified
30.3	1916	0.56	7-Senecioylheliotridine	48.2	2529	33.73	(*E*)-8b, 17-Epoxylabd-12-ene-15, 16-dial
31.4	1949	0.15	(*E*)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	48.3	2532	18.69	Labdane diterpenes derivative
35.0	2063	0.34	(*E*)-Labda-8(17),12-diene-15-ol-16-al	48.4	2538	0.50	13-Methylpentacosane
35.5	2078	0.13	(Z,Z)-2,13-Octadecadien-1-ol	48.5	2541	0.10	3,8-α-Furanether
36.3	2105	0.14	α-Isolupanine	48.6	2546	0.51	4,5-Secocholest-6-en-4-oic acid, 5-oxo-
36.8	2120	0.29	cis-Z-alpha-Bisabolene epoxide	49.0	2561	1.33	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-
41.7	2287	0.13	Caryophyllene oxide	49.4	2577	0.52	3,6,9,12-Tetraoxadocosyl acetate
43.0	2335	0.27	Kauren-18-ol, acetate, (4.beta.)-	49.7	2589	0.43	Labdane diterpenes derivative
43.3	2346	11.12	(*E*)-labda-8(17), 12-diene-15, 16-dial	49.9	2595	0.45	Menthol, 1'-(butyn-3-one-1-yl)-, (1S,2S,5R)-
43.8	2363	0.51	Isoaromadendrene epoxide	50.0	2599	0.39	Patchouli alcohol
44.9	2402	0.48	1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene	50.2	2608	0.50	4,8,13-Cyclotetradecatriene-1,3-diol,1,5,9-trimethyl-12-(1-methylethyl)-
45.0	2408	0.52	Kauran-18-al, 17-(acetyloxy)-, (4.beta.)-	50.8	2630	4.77	Cyclohexane carboxaldehyde, 2-methyl-, dimethyl acetal
45.4	2421	0.43	Pregn-4-ene-1,20-dione, 12-hydroxy-16,17-dimethyl-	50.9	2634	0.41	Not identified
46.0	2444	0.16	Not identified	51.0	2639	1.05	Canophyllal
46.4	2461	1.27	Not identified	51.4	2655	0.14	10,10-Dimethoxy-3,7-dimethyl-deca-2,6-dien-1-ol
46.5	2465	0.31	Labdane diterpenes derivative	51.6	2664	0.21	Oct-5-en-2-ol, 8-(1,4,4a,5,6,7,8,8a-octahydro-2, 5, 5, 8a-tetramethylnaphth-1-yl)-6-methyl-
46.6	2468	0.04	Not identified	52.2	2690	0.19	Not identified
47.0	2484	1.59	3,8-α-Furanether	52.5	2699	0.26	Acetic acid, 2-(2-acetoxy-2,5,5,8a-tetramethyldecalin-1-yl)-
47.3	2492	1.29	4,5-Secocholest-6-en-4-oic acid, 5-oxo-	54.6	2788	1.21	Aromadendrenepoxide
47.4	2496	0.54	Docosenoic acid	54.7	2794	0.16	Andrographolide
47.5	2503	4.40	Drimenol	55.5	2826	3.62	Cholestane (reference)

In the S-Hex extract, presence of two major compounds was detected, *viz.* (*E*)-8β,17-Epoxylabd-12-ene-15,16-dial (>23%) and (*E*)-labda-8(17),12-diene-15,16-dial (>18%). Both the labdane diterpenes were confirmed from their characteristic fragmentation pattern. The (Z,Z,Z)-5,7,11-Hexadecatrienal and n-Hexadecanoic acid were also found to be at higher concentration in the S-Hex extract. Similarly, both S-EtAc and S-Met extracts found to be rich in labdane diterpene derivatives. Both the compounds, (*E*)-labda-8(17),12-diene-15,16-dial and (*E*)-8β,17-Epoxylabd-12-ene-15,16-dial were obtained as the major compounds in S-EtAc as well as in S-Met. Moreover, many other unidentified labdane diterpene compounds were detected in variable quantities in the S-EtAc and S-Met extracts of *A. nigra*.

Recent report of Sirat and Jani (2013) revealed the presence of 20 sesquiterpenes and 10 monoterpenes in *A. mutica* leaves essential oil among the 33 detected compounds. Similarly, GC-MS analysis of the essential oil from *A. zerumbet* leaves and rhizome showed the presence of 1,8-cineole and terpinen-4-ol as major component respectively (El-Hawary et al. 2013). Previous studies suggest that other members of the family and various Zingiberceae members contain the various derivatives of labdane diterpenes (Hema and Nair 2009; Victório 2011). Labdane diterpenes are considered as biologically active molecules and known to be potent inhibitors of a wide range of enzymes with remarkable bactericidal and fungicidal activities (Demetzos and Dimas 2001). Several analogue of labdane diterpenes, isolated from various Zingiberaceae plants were previously shown to be a potent antimicrobial, antifungal, antioxidant, anti-inflammatory and cytotoxic activity (Ayafor et al. 1994b; Morita and Itokawa 1988; Roth et al. 1998; Kim et al. 2005; Tatsimo et al. 2006; Singh et al. 2010a; González et al. 2010; Upadhyay et al. 2011). However, the present findings indicate the presence of labdane diterpene derivatives in *A. nigra* seeds for the first time.

3.3.8. DPPH assay

DPPH is a stable free radical, which loses its purple color on accepting an electron from an antioxidant molecule (Zou et al. 2004). It is evidently based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Therefore, DPPH radical scavenging is a commonly used method to evaluate free radical scavenging activity of plant extracts (Chung et al. 2006). The various concentrations of hexane, ethyl acetate and methanol fractions of seed extract (10-100 µg/ml) showed antioxidant activities in a dose dependent manner in the DPPH radical scavenging assay (Fig. 3.6).

In our investigation, a pattern of sharp increase of % inhibition was observed with initial concentration of extracts, gradually saturating after 60 µg/ml concentration depending on the availability of free OH group of the chemical constituents in each extracts. Similar reports described the saturation nature of tested sample after certain threshold concentration (Xu et al. 2009b). In general, plant extracts having polyphenolic compounds could serve as an attractive substitute of synthetic food antioxidants (Martinez-Tome et al. 2001). Various members in *Alpinia* like, *A. oxyphylla* has been shown recently as potent free radical scavenger using DPPH assay where ethyl acetate extract of fruits found more active compared to other solvent fractions (Wang et al. 2013b). Several plant extracts from various members of *Alpinia* genus were found to possess remarkable antioxidant potential which augments in various pharmacological applications (Ghosh and Rangan 2013). However, in the present study, it was found that hexane extracts showed very weak DPPH radical-scavenging activity. On contrary, ethyl acetate and methanol extracts exhibited significant free radical scavenging activity in comparison with standard drug BHT. The 50% inhibitory concentration (IC_{50}) was determined for each sample (Table 3.6).

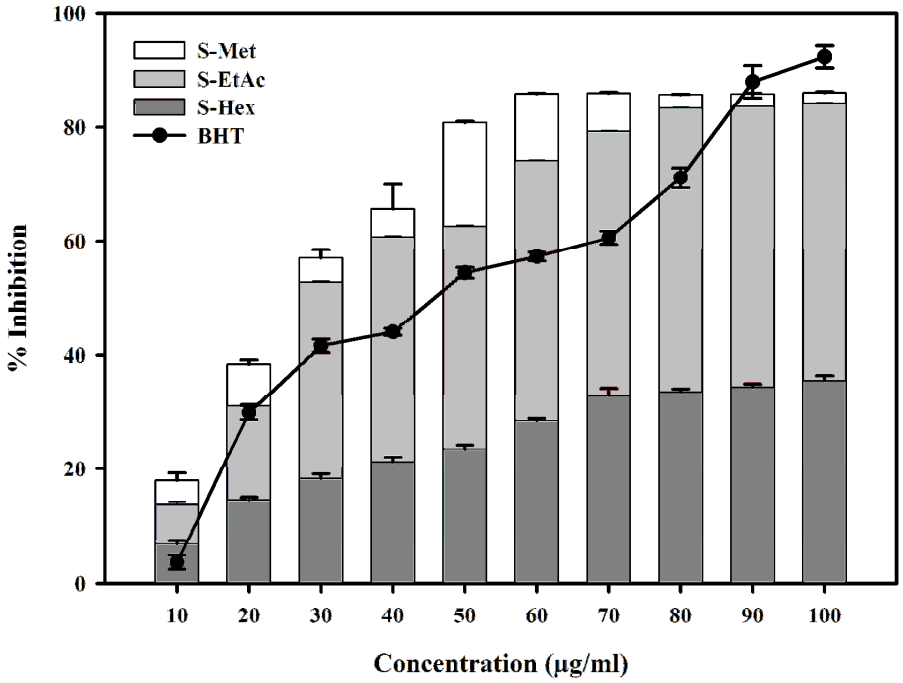


Fig. 3.6 DPPH free radical scavenging activity of three different seed extracts (S-Hex, S-EtAc and S-Met) of *A. nigra.* BHT used as positive control at varying concentration ranging from 10-100 µg/ml. Values represent means ± SE.

Table 3.6 DPPH free radical scavenging activity of *A. nigra* seed extracts

Test samples	IC_{50} of DPPH scavenging (µg/ml)[a]
S-Hex	>100
S-EtAc	27.22±0.45
S-Met	25.81±0.94
BHT	46.50±0.63

[a]IC_{50} is a measure of radical scavenging activity being the concentration required to inhibit 50% free radical activity.

Recently, free radical scavenging potential of *A. nigra* leaves were determined (IC_{50} 64.51 µg/ml) which indicates that the seed extracts (except S-Hex) were much effective than other extracts from leaves of *A. nigra* (Sahoo et al. 2013). Therefore, the current investigation on seed extracts of *A. nigra* was found to be promising in terms of free radical scavenging activity which could be due to the presence of high level of polyphenolic moieties and other polar compounds in the extracts.

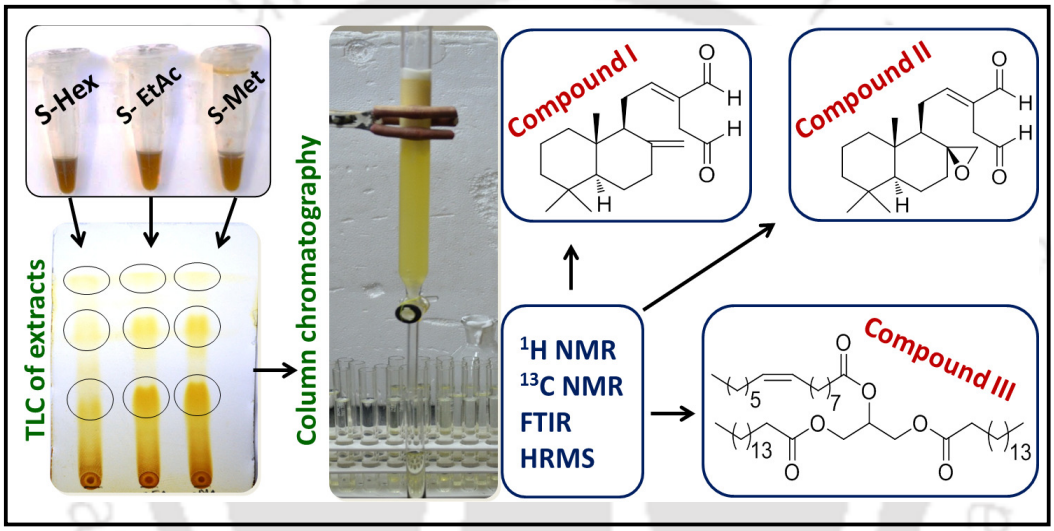
3.4. Conclusion

A. nigra, an important member of Zingiberaceae family, is widely used in ethnomedical practices of the tribal communities of NEI. Present study explored the chemical fingerprinting and free radical scavenging potential of *A. nigra* seed extracts by introducing an economically viable and effective extraction method. In agreement with the other species of *Alpinia*, *A. nigra* seed extracts were found to be rich source of various labdane type diterpenes. Among several compounds identified in S-EtAc and S-Met extracts, few labdane diterpenes were detected as major constituents. Phytochemical analysis revealed that methanolic extract is rich source of terpenoids and alkaloids compared to S-Hex extract. Similarly, highest phenolic content was obtained from S-Met extract compared to other two extracts. Spectroscopic investigation suggests the chemical complexity of all the extracts and establishes the characteristic fingerprint of each extract. Further, free radical scavenging

activity was determined and two polar extracts were found highly effective than the standard antioxidant BHT. Therefore, *A. nigra* seed extracts could be considered as bioactive extracts and also effective natural antioxidants which need more attention in the future as these efforts will also significantly contribute to a better understanding of the use of non-food crop and its application in pharmaceutical industries.

Chapter 4

Isolation and characterization of the principal component(s) from crude organic extracts of *A. nigra* seeds



The chapter describes the isolation and identification of two labdane type diterpene compounds and a triacyl glyceride from *A. nigra* seed extracts.

Chapter 4

Isolation and characterization of the principal component(s) from crude organic extracts of *A. nigra* seeds

4.1. Introduction

Nature has been a source for diverse and unique chemical compounds, collectively known as natural products. These compounds can be obtained from prokaryotic bacteria to higher eukaryotes like plants. In due course of evolution, these compounds have also evolved accordingly to have many natural analogues toward better survival and sustainability. The understanding of natural products led to significant contribution towards the development of many present-day drugs by the use of natural products or their derivatives. The practice of using natural products or herbal therapy as alternative medicine is becoming an attractive approach for the treatment of various diseases and disorders. The majority of naturally occurring flavonoids, terpenoids and phenolics found in plants possess tremendous biological activities and have been employed in various clinical trials (Saklani and Kutty 2008; Sawadogo et al. 2012).

In the plant kingdom, Zingiberaceae family is known as an important natural resource that provides many useful products in the form of food, spices, medicines, dyes, perfume and aesthetics (Jantan et al. 2003). The largest and most complex genus of this family is *Alpinia* which has been widely used in different parts of the world in folk medicine (Itokawa et al. 1981a, 1981b, 1987), to treat gastric lesions (Hsu 1988), diuretic (Laranja et al. 1991, 1992), anticancer, antimicrobial and many others biopharmaceutical potential (Ghosh and Rangan

2013). Members of the genus *Alpinia* have complex chemical profile and possess diverse types of bioactive compounds. Diverse members of *Alpinia* genus known as rich source of various phytochemicals such as many types of flavonoids (Sirat and Jamil 1999; Masuda et al. 2000), sesquiterpenes (Miyazawa et al. 2000), labdane diterpenes (Sy and Brown 1997; Sirat and Jamil 1999), diarylheptanoids (Miyazawa et al. 2000; Ali et al. 2001b) and kava pyrone (Mpalantinos et al. 1998; Sirat and Jamil 1999). Many of these natural compounds have been isolated and investigated towards their biological applications against various diseases and disorders.

However, the plant under study, *A. nigra*, has limited information about the isolation and characterization of bioactive compounds and their application in therapeutics. The presence of two flavone glycosides (astragalin and kaempferol-3-O-glucuronide) in the seed clusters of *A. nigra* were described previously by Qiao et al. (2007). They have also reported kaempferol-3-O-glucuronide as the major compound in the fruit pulp of *A. nigra*. Previous study also described the presence of two volatile oils, β-Pinene and α-Pinene, in the fruits and rhizome of *A. nigra* (Qiao et al. 2000). In another study of Kanjilal et al. (2010), 1,8-cineole was identified as the major constituent among other phyto-volatiles found in GC-MS analysis of leaf and rhizome essential oils of *A. nigra*. The biological activities of any such phytochemicals or other unexplored bioactive compounds were not studied in seeds till date to best of our knowledge.

From the previous chapter, it was evident that *A. nigra* seed extracts are rich in various types of terpenoids, especially labdane type diterpene derivatives. These labdane diterpenes are known to be active against various infectious organisms like bacteria and fungi (Demetzos and Dimas 2001), have been shown to affect cell signaling, induce depolarization of the mitochondrial membrane, cause cell cycle arrest and apoptosis in various cancer cells

(Dimas et al. 2001; Souza-Fagundes et al. 2003b; Dimas et al. 2006; Kunnumakkara et al. 2008). Therefore, the study of this chapter is focused on isolation and structural elucidation of major compounds from the crude extracts of *A. nigra* seeds. Furthermore, the purified compounds were investigated towards their drug likeliness properties and subsequently biocompatibility of those bioactive compounds was determined using human erythrocytes.

4.2. Materials and methods

4.2.1. Isolation of compounds

In order to find out the principle compounds, the hexane, ethyl acetate and methanol extracts from seed samples were subjected to analytical chemistry techniques mentioned below. These fractions were identified and characterized further. The schematic representation of isolation of compound from crude extract is depicted in Fig. 4.1.

4.2.1.1. Thin-layer chromatography (TLC)

A slurry was prepared from 60 g of TLC silica gel (SRL) containing 13% $CaSO_4 \cdot 1/2H_2O$ as binder with 5% methanol in ethyl acetate (120 ml). Thin glass plates of 2 × 20 cm were coated uniformly with the slurry. The coated plates were allowed to dry for an hour at room temperature and dried at 120°C in an oven. The crude extracts were diluted in ethyl acetate and added on the activated silica gel TLC plates with a capillary tube. The major components of the crude extracts were separated on plates using pure n-Hexane and mixture of n-hexane: ethyl acetate mixture (99:1 to 85:15). The spots related to individual components were visualized by exposing the TLC plate to iodine fumes. Fractions having the same number of spots with similar retardation factor (R_f) values on the TLC plate were pooled and numbered accordingly. After optimization of the solvent system polarity for TLC, the crude extracts were subjected to column chromatography.

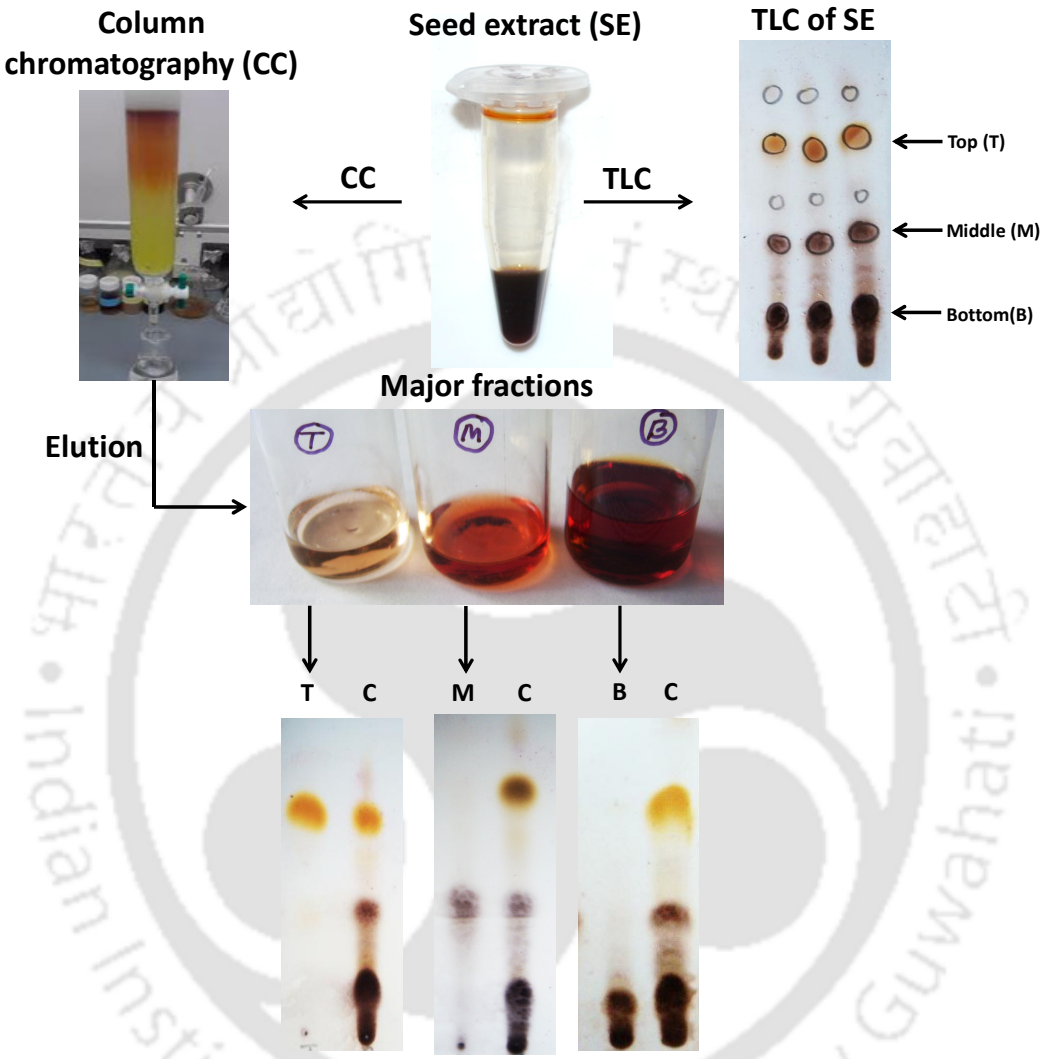


Fig. 4.1 Scheme for compound(s) isolation from seed extract of *A. nigra*. Seed extract (SE) was subjected to thin layer chromatography (TLC) and three major spots (T, M and B) were selected for further isolation and column chromatography (CC) was performed. Individual fractions were again subjected to TLC in order to confirm the separation of each spots.

4.2.1.2. Column chromatography (CC)

The glass column was plugged with cotton at the constricted end and dry silica gel (60-120 mesh) was added onto the column. The column (450 x 40 mm) was packed using n-Hexane solvent. Dry method of loading the samples was followed for this chromatographic separation. Initially, about 0.50 g of sample was uniformly dissolved in 50 ml of dichloromethane (DCM) and silica gel was added further into the solution to prepare the sample slurry. After proper mixing, sample slurry was subjected to rotary evaporator and collected after removal of DCM. The dried slurry was pressed to make fine powder and added onto the packed silica column. The column was eluted stepwise at a flow rate of 1 ml/min with 200 ml of n-Hexane and 1000 ml of n-hexane: ethyl acetate (99:1 to 90:10 v/v) gradient to collect the eluting fractions. From the S-Hex extract, one major fraction was collected, and from S-EtAc and S-Met extracts two major fractions each were collected. The fractions were concentrated using the rotary evaporator. An aliquot of all these fractions were loaded on TLC plate, fractions having similar retardation factor (R_f) values were pooled and named. Among these, the five fractions showing single spots in TLC were selected for further structural elucidation studies. The entire process of separation was repeated for few times to get the optimum amount of the desired fractions. After isolation of the fractions, they were subjected to further purification and characterization.

4.2.1.3. Purification of the selected fractions

The individual fraction was tested in TLC to determine the purity of the fraction. If impurities were detected, then the fraction was subjected to preparative TLC to collect the single spot material of desired fraction by scrapping the content from TLC plate. Subsequently, collected fraction was filtered and stored as concentrated material in refrigerator (-20°C) till further use. All the purified fractions were subjected to analytical high performance liquid

chromatography to find out the purity of the fractions before spectroscopic investigation towards its structural elucidation.

4.2.1.4. High-performance liquid chromatography

Each fraction was analysed for its purity using Varian Prostar HPLC system (Varian, USA) equipped with C-18 column (300 x 4.6 mm and 5 μm particle diameter, Thermo Hypersil) and Varian HPLC software. Water and acetonitrile of HPLC grade were used as a mobile phase for the chromatographic separation. Elution was carried out in a gradient solvent system (0-100% acetonitrile) at a temperature of 25°C, a flow rate of 0.5 ml/min. The concentration of the compounds was 250 ppm and injection volume was 25 μl. Ultraviolet (UV) detection was carried out with a diode array detector (Shimadzu) and the detection wavelength was 254 nm. The purified middle and bottom fractions were eluted at the retention time of 14.13 and 12.7 min respectively.

4.2.2. Characterization of purified compounds

4.2.2.1. Nuclear magnetic resonance (NMR) analysis of compounds

Nuclear magnetic resonance (NMR) spectra for both ^{1}H and ^{13}C were recorded for each of the isolated fraction using $CDCl_3$ with Mercury Plus 400 MHz NMR Spectrometer (Varian, USA), operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C at room temperature. Chemical shifts were recorded in parts per million (ppm) on the scale. A region from 0 to 12 ppm for ^{1}H and 0 to 200 ppm for ^{13}C was employed. Signals were referred to internal standard tetramethylsilane (TMS). About 45 mg of the isolated compound dissolved in 0.75 ml of $CDCl_3$ was used for recording the spectra.

4.2.2.2. Fourier Transform Infrared (FTIR) analysis of compounds

Each sample fraction (1 mg) was mixed with dry potassium bromide (KBr) and respective pellets were prepared. The absorption spectra of the samples were obtained with a Perkin-Elmer Fourier Transform Infrared (FTIR) spectrophotometer (Norwalk, Connecticut) in the range of 450-4000 cm^{-1}.

4.2.2.3. High Resolution Mass Spectrometry (HRMS) of compounds

Mass spectrum of the isolated compounds was recorded on instrument Water Q-TOF premier mass spectrometer (USA) by electro-spray ionization (ESI) technique with a flow rate of 0.2 ml/min) on the C-18 column and at a total run time of 30 min. Diode array was used as a detector and the ESI probe served as the positive ion mode in analysis. About 1 mg of each of the isolated and purified compounds was dissolved in 5 ml of methanol used for recording the spectrum. An aliquot of formic acid was used as an ionizing agent.

4.2.3. Physico-chemical properties

To understand the suitability of the characterized compounds as drug, analysis of the Lipinski descriptors (Lipinski et al. 2001) for bioavailability estimation using SciFinder® program "Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1994-2011ACD/Labs)" (https://scifinder.cas.org/scifinder/view/scifinder/scifinder Explore.jsf) was carried out for all the compounds. The software considers the parameters which describe molecular properties of the compounds important for drug pharmacokinetics in the human body. As per "Lipinski rules" of drug-likeliness, an orally active drug must not overrule more than one of the subsequent criteria: hydrogen donors: ≤5, hydrogen acceptors: ≤10, MW: ≤500 and log*P*: ≤5.

4.2.4. RBC hemolysis assay

RBC hemolysis assay (Kazi et al. 1994; Shukla et al. 2011) was performed to assess the suitability of the isolated labdane derivatives. Fresh blood sample was collected from 28-30 years old, healthy and non-medicated male donor. Erythrocytes were separated by centrifugation at 1000 rpm for 5 min, washed 3 times with isotonic phosphate buffer (pH 7.4) and resuspended in 10 mM PBS. RBC suspension was incubated for different time intervals as 6, 12 and 24 h with varying concentrations of isolated labdane derivatives ranging from 0.05-1000 µM. RBC suspension containing 1% Triton X-100 and PBS were taken as positive control and negative control respectively. Subsequently, after each time interval, cell were centrifuged for 5 min at 2,000 rpm and supernatant was pipetted to 96-well microtitre plate. Considering the absorbance of haemoglobin, all the samples were subjected to spectrophotometric analysis and absorbance was recorded at 540 nm. Percentage of RBC hemolysis was determined using the following formula:

$$\% \text{ RBC Hemolysis} = \frac{\text{Abs of Sample - Abs of Negative Control}}{\text{Abs of Positive Control}} \text{ X } 100$$

4.2.5. Statistical analysis

Experimental set up was repeated thrice with a minimum of three replicates for each treatment and the data shown as mean ± SE. Significance analysis performed by *t*-test and also employing one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS Statistics 17.0. Differences were considered significant at $p<0.05$.

4.3. Results and discussion

4.3.1. Identification of compounds

The major compound isolated from hexane extract was named as SH-1, ethyl acetate extract were named as SE-1 and SE-2 and methanol extract were named as SM-1 and SM-2 respectively. The structural elucidations for all the five compounds were performed after analysis and comparison of spectroscopic data (NMR, FTIR and HRMS) with the available reports.

From ethyl acetate extract of seeds, a labdane diterpene dialdehyde (SE-1) and its epoxide analogue (SE-2) were isolated. However, the compounds isolated from methanol extract (SM-1 and SM-2) were found to be the same as obtained from ethyl acetate extract. Finally, two isolated compounds were named as compound **I** and compound **II**. The isolated compound from hexane extract was identified as 1,2-dihexadecanoyl-3-(9Z-hexadecenoyl)-sn-glycerol (compound **III**) (Fig. 4.2).

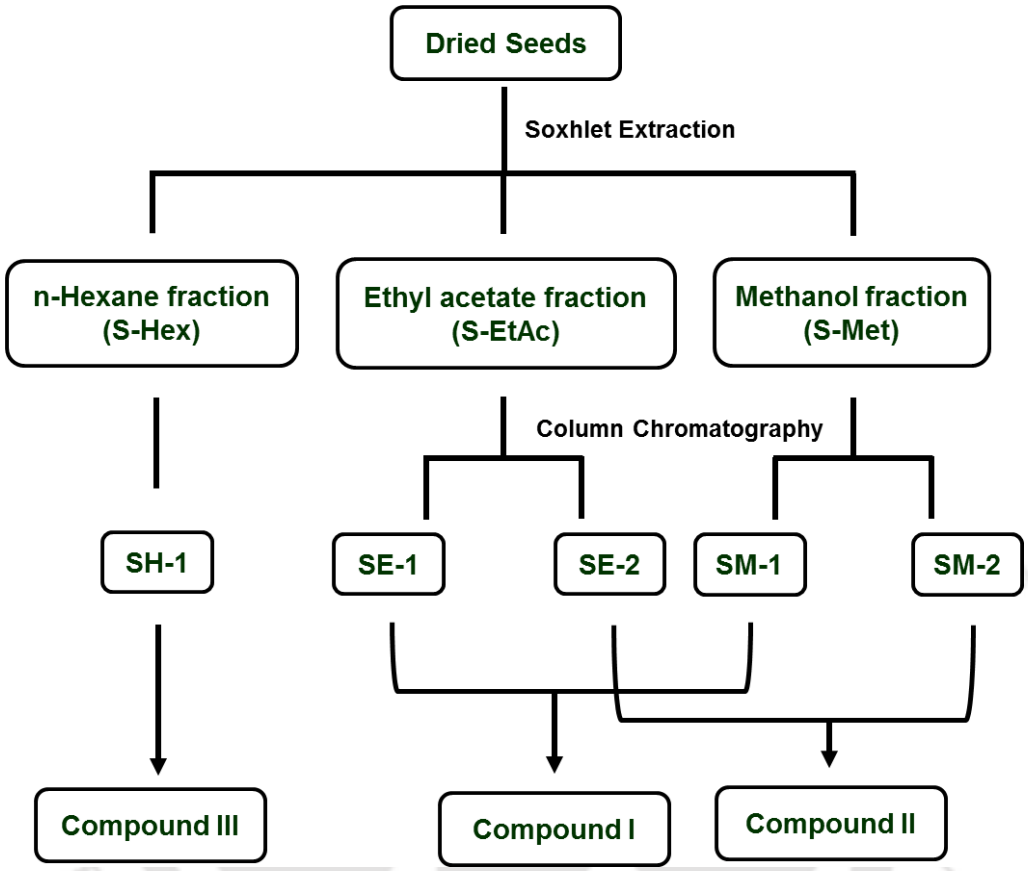


Fig. 4.2 Schematic illustration depicts the isolation of compound **I**, **II** and **III** from *A. nigra* seed extracts.

4.3.2. Identification of compound I

Compound **I** appeared as a light golden colour sticky oil. On the basis of structural elucidation of ^{1}H NMR and ^{13}C NMR, the molecular formula of the compound **I** was assigned as $C_{20}H_{30}O_2$ and it was further confirmed by HRMS m/z $[M+H]^+$ at 303.2330 (found) and 303.2340 (calculated) for $[M+H]^+$.

The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum showed three singlets at δ: 0.67 (3H), 0.76 (3H), 0.83 (3H) for three individual methyl groups associated with quaternary carbons (QC). A doublet of two protons at δ: 3.29-3.42 (2H) denotes allylic methylene, two singlets at δ: 4.32 (1H) and 4.80 (1H), one triplet at δ: 6.70 (J = 6.4, 1H) signifying the olefinic proton and two singlets at δ: 9.34 (1H) and 9.57 (J = 1.6, 1H) for two aldehydic protons. The ^{13}C NMR spectrum gave 20 carbon signals indicating the presence of methyls, methylenes, methylidyne and quaternary carbons. The ^{1}H and ^{13}C NMR data of compound **I** are in agreement with the earlier published data (Sirat et al. 1994). Structure of compound **I** was established as (*E*)-labda-8(17),12-diene-15,16-dial (Fig. 4.3).

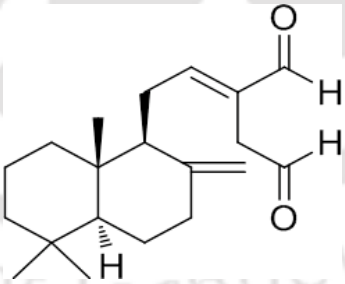


Fig. 4.3 Structure of compound **I** [(*E*)-labda-8(17),12-diene-15,16-dial]

^{1}H NMR (400 MHz, $CDCl_3$)

δ: 0.67 (s, 3H), 0.76 (s, 3H), 0.83 (s, 3H), 0.96-1.10 (m, 4H), 1.24-1.38 (m, 2H), 1.40-1.56 (m, 2H), 1.60-1.72 (m, 2H), 1.85 (d, J = 11.2 Hz, 1H), 1.97 (dt, J = 5.2 and J = 4.0 Hz, 1H), 2.23-2.58 (m, 2H), 3.29-3.42 (m, 2H), 4.32 (s, 1H), 4.80 (s, 1H), 6.70 (t, J = 6.4, 1H), 9.34 (s, 1H), 9.57 (t, J = 1.6, 1H).

^{13}C NMR (100 MHz, $CDCl_3$)

δ: 39.1 (CH_2), 19.2 (CH_2), 41.9 (CH_2), 33.5 (CH_3), 55.3 (CH), 24.1 (CH_2), 37.8 (CH_2), 147.9 (QC), 56.4 (CH), 39.5 (QC), 24.6 (CH_2), 159.6 (CH), 134.9 (QC), 39.3 (CH_2), 197.1 (CHO), 193.3 (CHO), 107.8 (CH), 33.5 (QC), 21.7 (CH_3), 14.3 (CH_3).

FTIR spectrum

IR ν_{max} showed absorption peaks at 1736 and 2722 cm^{-1} (carbonyls for two aldehydes), 1376 cm^{-1} (gem-dimethyl), 2839 and 2965 cm^{-1} (cyclohexyl ring).

FTIR: ν_{max} cm^{-1}: 2917.68, 2839.57, 2723.10, 1735.90, 1455.98, 1376.78, 1359.23, 1304.18, 1255.37, 1167.19, 1118.26, 1065.57, 997.96, 972.72 and 840.78 cm^{-1}.

HRMS spectrum

MS data showed parent molecular ion peak at 303.2330.

Theoretical value $[M+H]^+$ = 303.2340

Observed value $[M+H]^+$ = 303.2330

4.3.2.1. Spectroscopic fingerprint of compound I

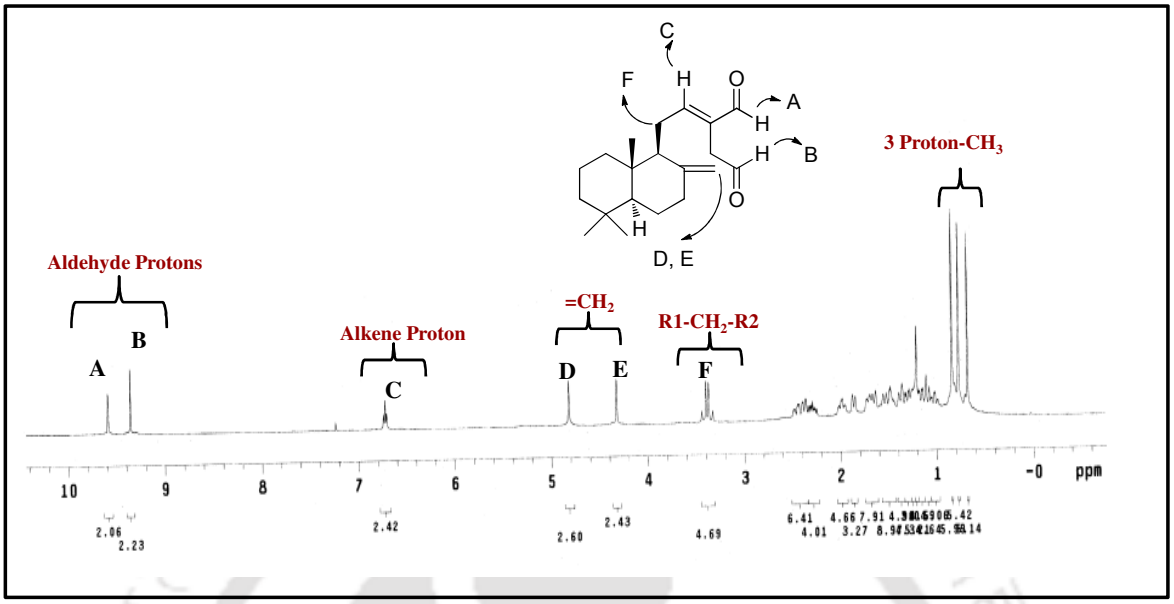


Fig. 4.4 ^{1}H NMR of compound **I** isolated from seeds of *A. nigra.*

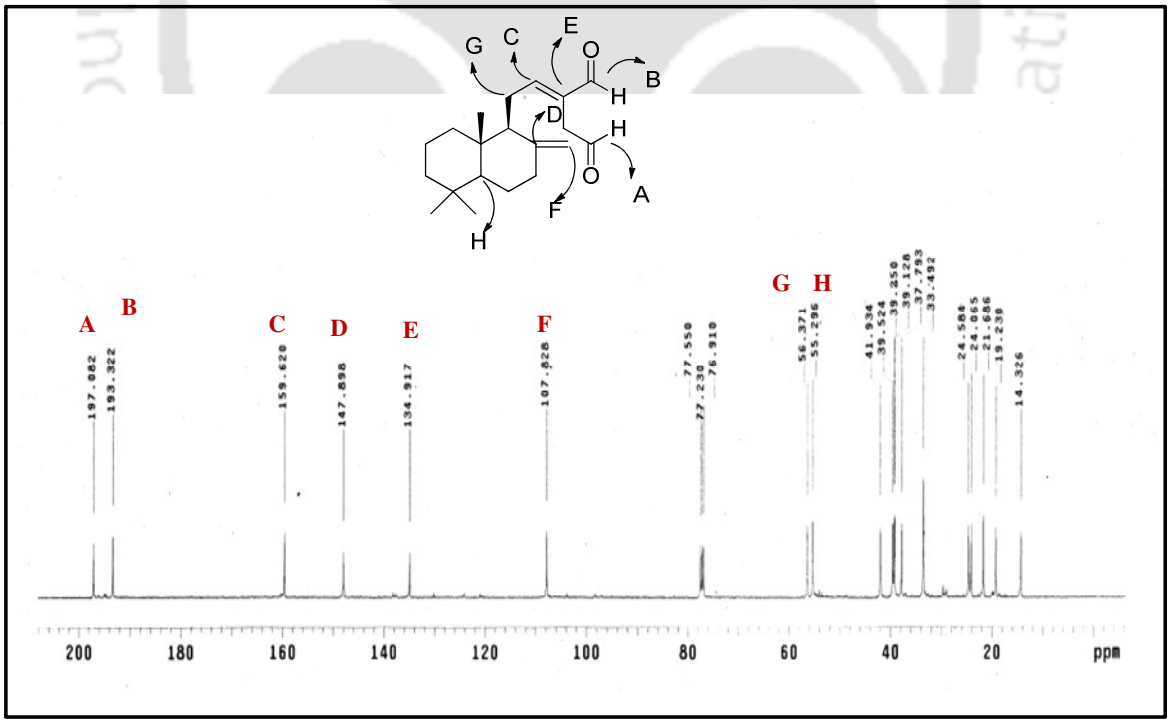


Fig. 4.5 ^{13}C NMR of compound **I** isolated from seeds of *A. nigra*.

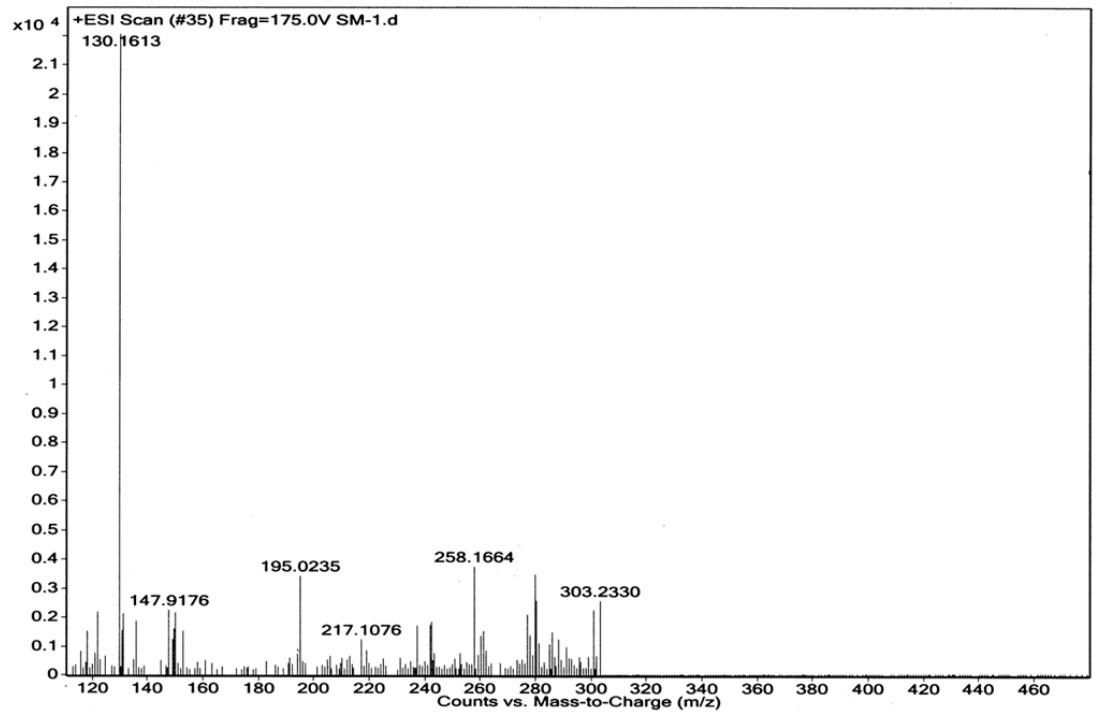


Fig. 4.6 Mass spectrum of compound **I** from seeds of *A. nigra*.

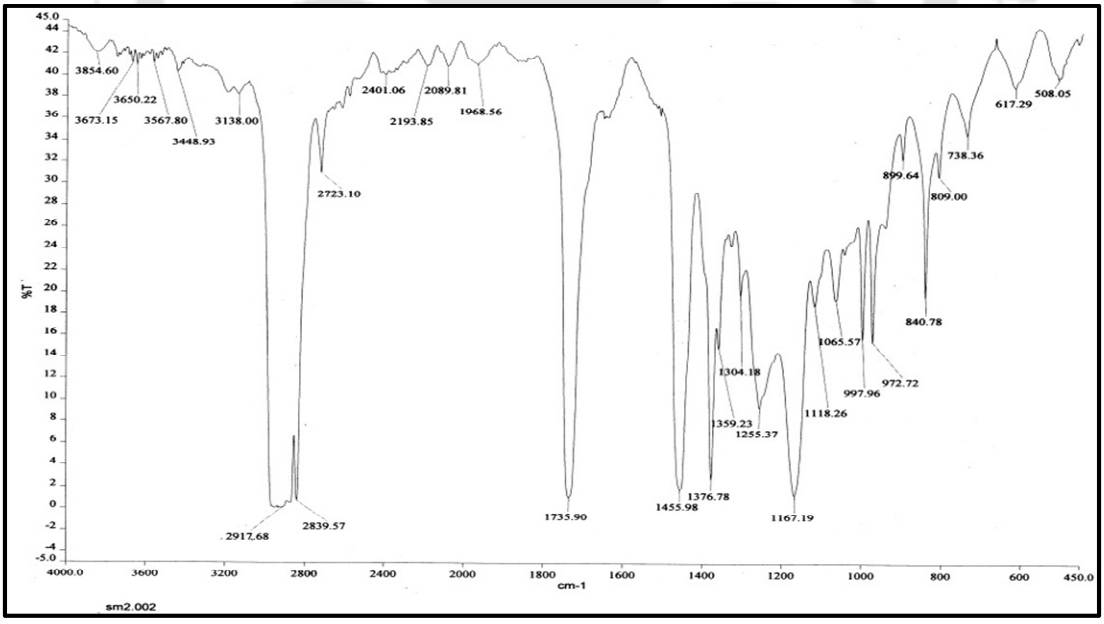


Fig. 4.7 FTIR spectrum of compound **I** from seeds of *A. nigra*.

4.3.3. Identification of compound II

Compound **II** appeared as a dark golden colour thick oil. The molecular formula of the compound was assigned as $C_{20}H_{30}O_3$ based on ^{1}H NMR and ^{13}C NMR data and further confirmed by HRMS m/z $[M+H]^+$ at 319.2178 (found) and 319.2193 (calculated) for $[M+H]^+$. The ^{13}C NMR spectrum showed the notable shifting of δ values at C-8 and C-17 positions due to presence of strained epoxide ring. The ^{1}H and ^{13}C NMR fingerprints of compound **II** were in agreement with earlier report (Haraguchi et al. 1996). Structure of compound **II** was established as (*E*)-8β,17-epoxylabd-12-ene-15,16-dial (Fig. 4.8).

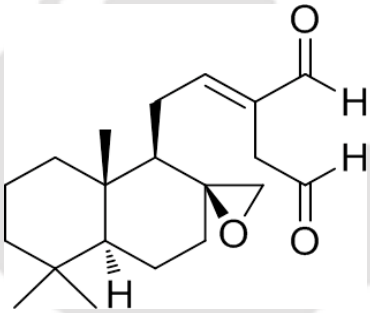


Fig. 4.8 Structure of compound **II** [(*E*)-8β, 17-epoxylabd-12-ene-15,16-dial]

^{1}H NMR (400 MHz, $CDCl_3$)

δ: 0.79 (s, 3H), 0.83 (s, 3H), 0.85 (s, 3H), 0.88-1.00 (m, 2H), 1.06-1.17 (m, 2H), 1.26-1.42 (m, 4H), 1.47-1.68 (m, 4H), 1.78-1.91 (m, 2H), 2.23 (d, *J* = 3.6 Hz, 1H), 2.35 (d, *J* = 3.6 Hz, 1H), 3.27 (d, *J* = 17.2 Hz, 1H), 3.37 (d, *J* = 17.2 Hz, 1H), 6.60 (t, *J* = 6.4 Hz, 1H), 9.32 (s, 1H), 9.55 (s, 1H).

^{13}C NMR (100 MHz, $CDCl_3$)

δ: 38.8 (CH_2), 18.1 (CH_2), 41.3 (CH_2), 33.0 (QC), 52.0 (CH), 19.6 (CH_2), 38.7 (CH_2), 56.9 (QC), 54.3 (CH), 39.2 (QC), 21.7 (CH_2), 134.7 (QC), 35.2 (CH_2), 196.5 (CHO), 192.7 (CHO), 48.2 (CH_2), 33.1 (CH_3), 21.3 (CH_3), 14.2 (CH_3).

FTIR spectrum

IR ν_{max} showed absorption bands for 1727 and 1683 cm^{-1} (two carbonyl as aldehyde), 1642 cm^{-1} (olefinic), 1388 cm^{-1} (gem-dimethyl), 2848 and 2918 cm^{-1} (cyclohexyl ring).

FTIR: ν_{max} cm^{-1}: 2918.3, 2848.9, 2721.0, 1727.6, 1683.0, 1642.3, 1459.7, 1388.1, 1366.6, 1219.1, 1164.3, 1022.5, 889.5, and 757.1 cm^{-1}.

HRMS spectrum

MS data showed parent molecular ion peak at 319.2178.

Theoretical value $[M+H]^+$ = 319.2193

Observed value $[M+H]^+$ = 319.2178

4.3.3.1. Spectroscopic fingerprint of compound II

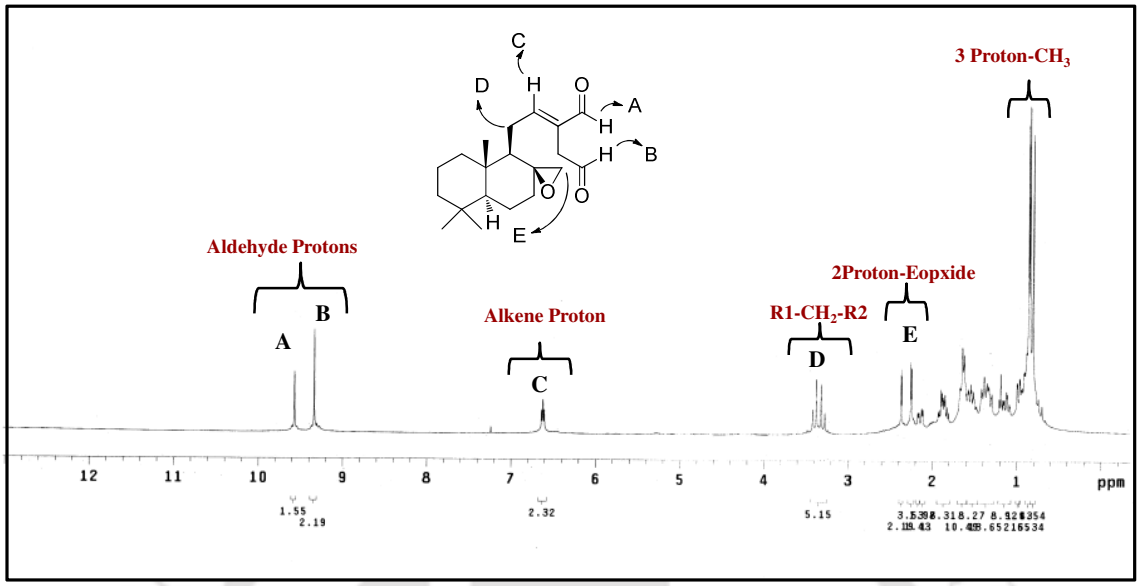


Fig. 4.9 ^{1}H NMR of compound **II** from seeds of *A. nigra.*

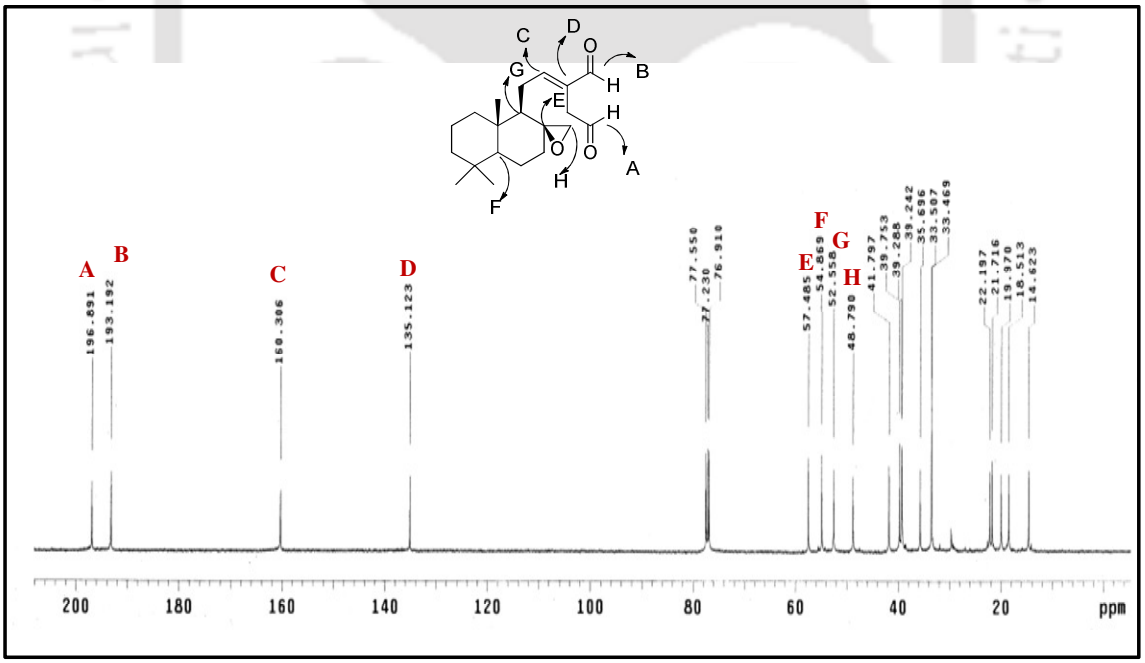


Fig. 4.10 ^{13}C NMR of compound **II** isolated from seeds of *A. nigra.*

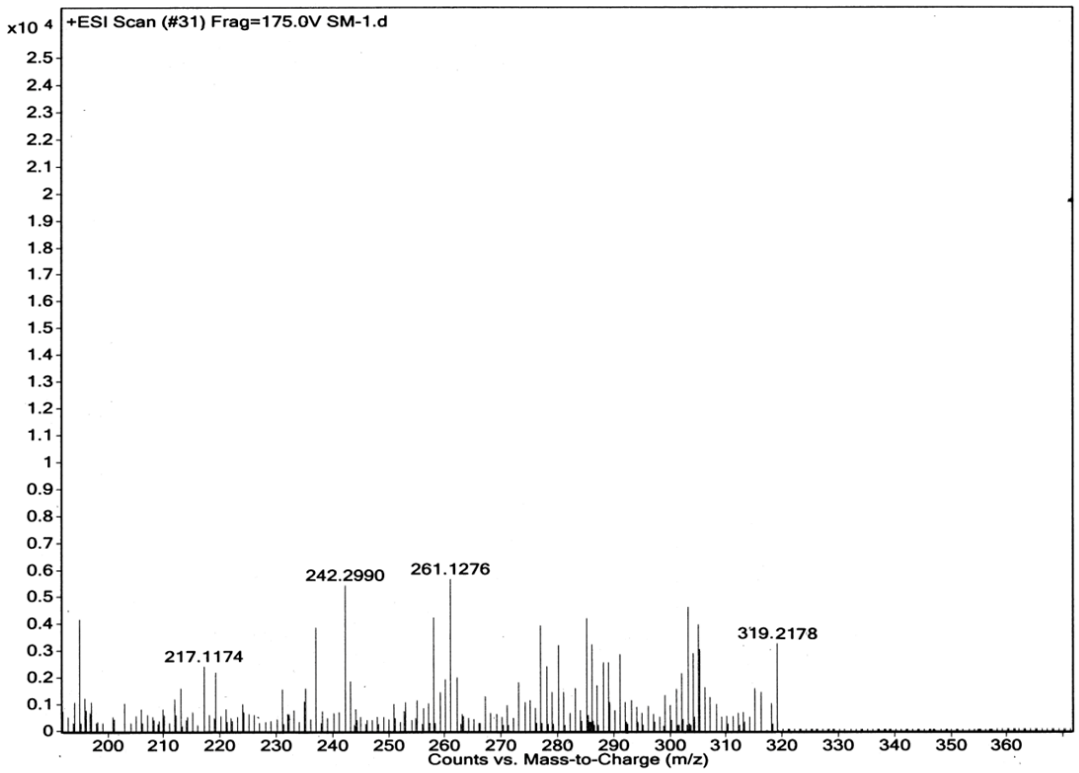


Fig. 4.11 Mass spectrum of compound **II** from seeds of *A. nigra*.

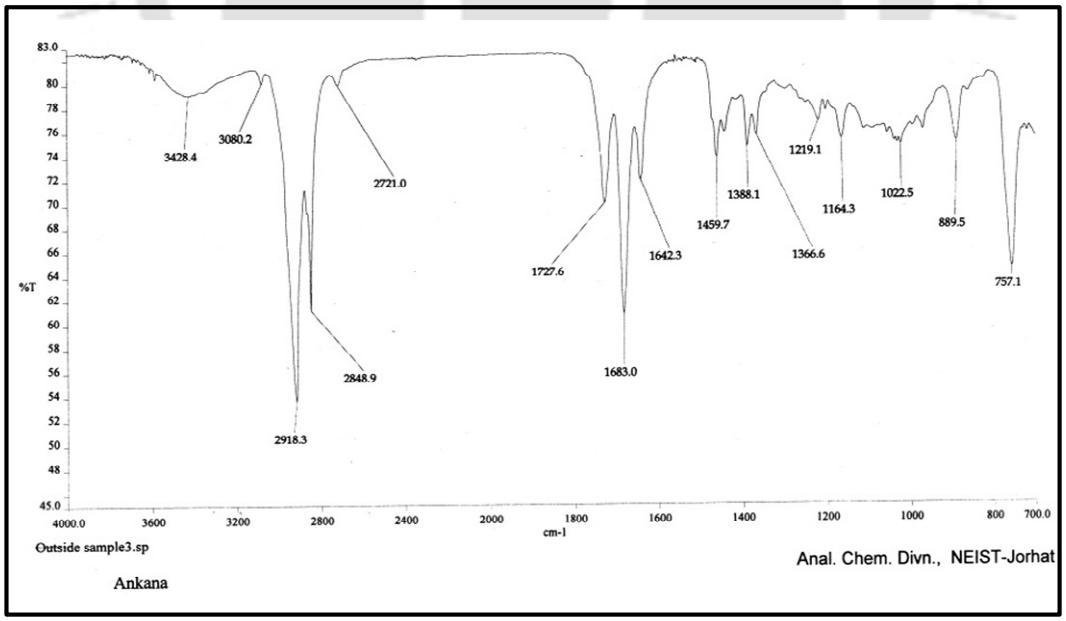


Fig. 4.12 FTIR spectrum of compound **II** from seeds of *A. nigra.*

4.3.4. Identification of compound III

Compound **III** appeared as a pale yellow colour, solid. The molecular formula of the compound was assigned as $C_{51}H_{96}O_6$ based on ^{1}H NMR and ^{13}C NMR data and further confirmed by HRMS m/z $[M+H]^+$ at 805.7279 (found) and 804.7207 (calculated) for $[M+H]^+$.

The FTIR, ^{13}C NMR and HRMS fingerprints of compound **III** were analysed and compared with earlier report (Howarth et al. 1995; Arishima et al. 1996; Vlahov et al. 1999). Structure of compound **III** was established as 1,2-dihexadecanoyl-3-(9Z-hexadecenoyl)-sn-glycerol (Fig. 4.13).

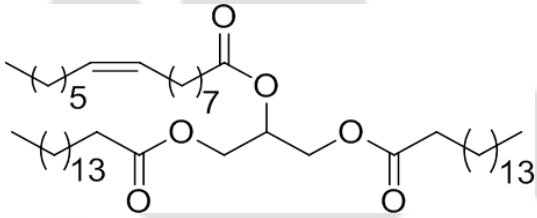


Fig. 4.13 Structure of compound **III** (1,2-dihexadecanoyl-3-(9Z-hexadecenoyl)-sn-glycerol)

^{1}H NMR (400 MHz, $CDCl_3$)

δ: 0.81 (t, J = 6.8 Hz, 9 H), 1.18 (s, 64 H), 1.53 (s, 6 H), 1.93-1.99 (m, 4 H), 2.24 (t, J = 7.6 Hz, 6 H), 4.08 (dd, J = 5.6 and J = 11.6 Hz 2 H), 4.23 (dd, J = 4.4 and J = 11.6 Hz 2 H), 5.20 (t, J = 5.6 Hz 1 H), 5.27-5.31 (m, 2 H).

^{13}C NMR (100 MHz, $CDCl_3$)

δ: 14.3 (3C), 22.9 (3C), 25.0 (3C), 27.3 (2C), 27.4, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8 (16C), 29.9 (2C), 30.0 (3C), 32.1 (2C), 34.2 (2C), 34.4, 62.3 (2C), 69.1 (1C), 129.9, 130.2, 173.4 (3C).

FTIR spectrum

IR ν_{max} showed a spectrum with vibrational bands related to the triglycerides at 1163.9 and 1745.5 cm^{-1}, respectively, attributed to the stretching of C-O and C=O groups. It also presented absorption in the wavelength range from 2852.1 and 2922.6 cm^{-1} for C-H bond stretching. The presence of a band ascribed to the group $(CH_2)_n$ is observed at 721.7 cm^{-1}.

FTIR: ν_{max} cm^{-1}: 2922.6, 2852.1, 1745.5, 1417.5, 1466.7, 1377.6, 1220.6, 1163.9, 1116.2, 759.5 and 721.7 cm^{-1}.

HRMS spectrum

MS data showed parent molecular ion peak at 805.7279.

Theoretical value $[M+H]^+$ = 805.7207

Observed value $[M+H]^+$ = 805.7279

4.3.4.1. Spectroscopic fingerprint of compound III

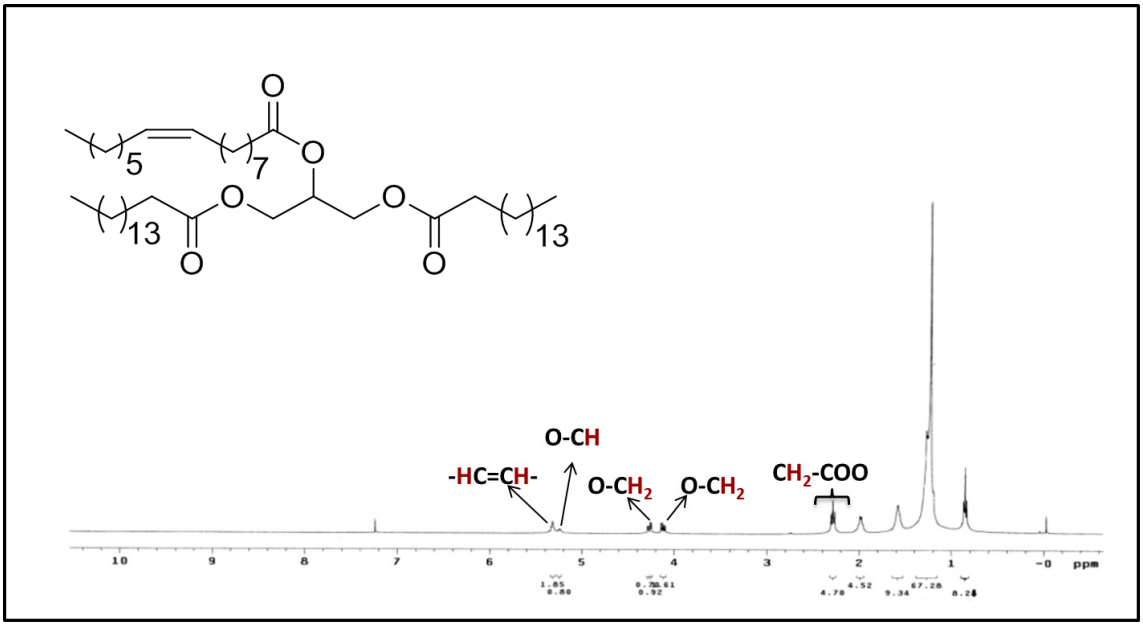


Fig. 4.14 ^{1}H NMR of compound **III** isolated from seeds of *A. nigra.*

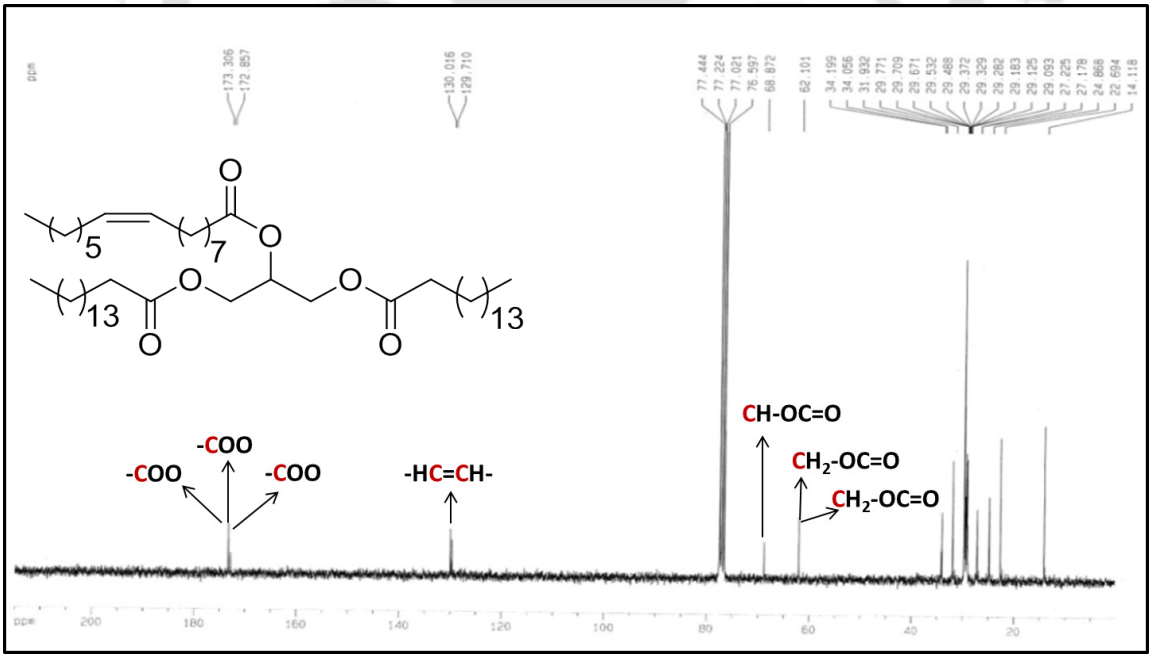


Fig. 4.15 ^{13}C NMR of compound **III** isolated from seeds of *A. nigra.*

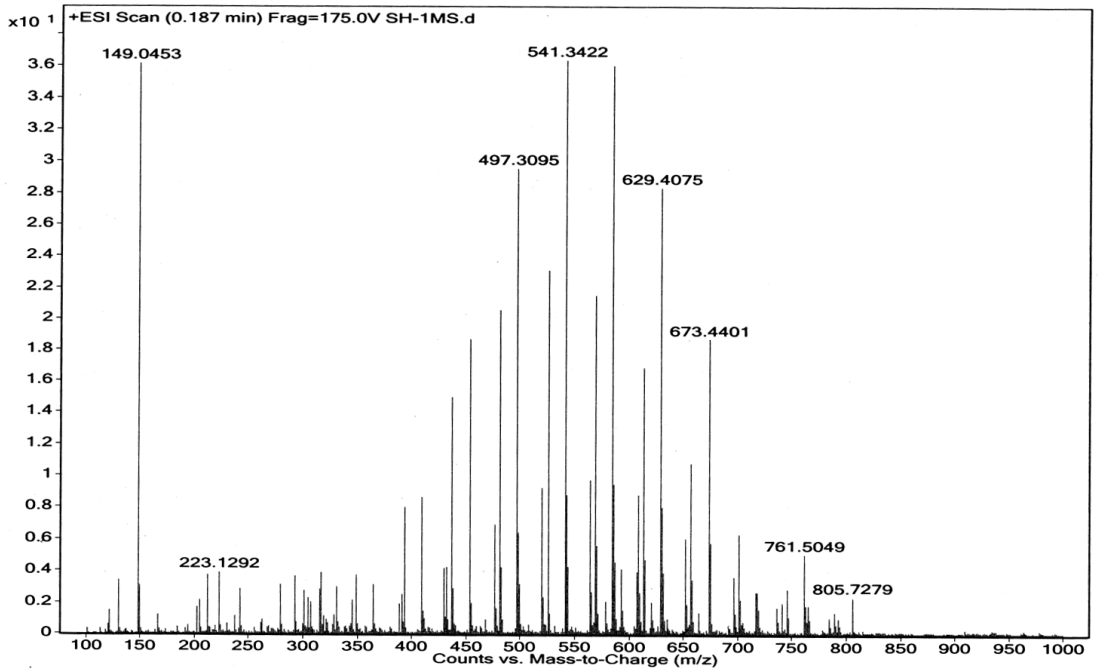


Fig. 4.16 Mass spectrum of compound **III** from seeds of *A. nigra.*

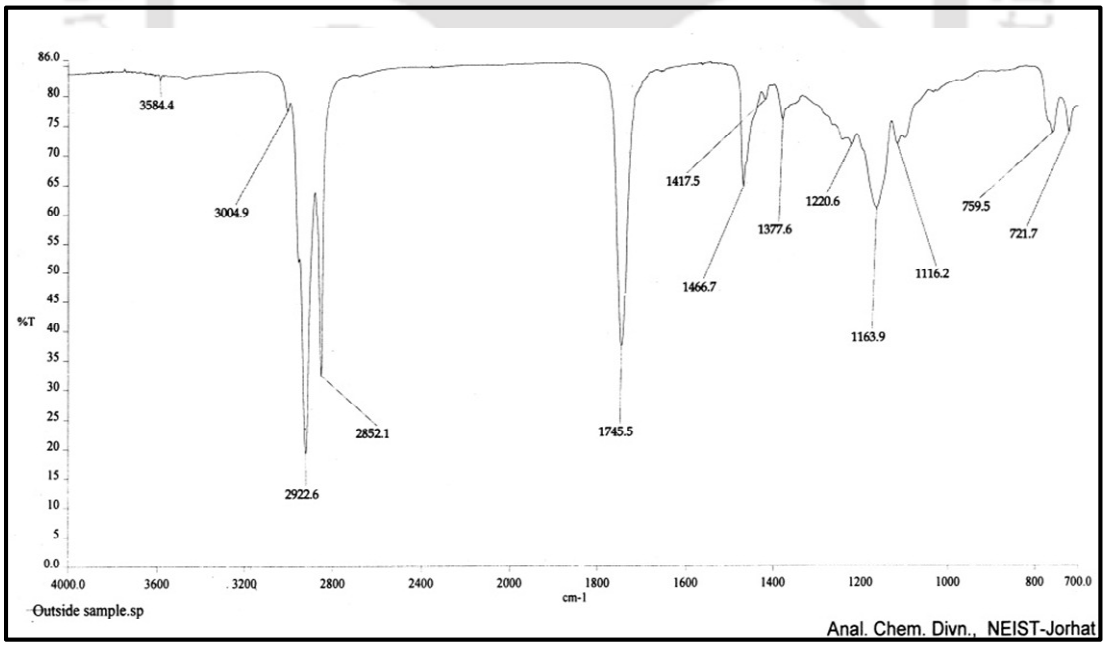


Fig. 4.17 FTIR spectrum of compound **III** from seeds of *A. nigra.*

4.3.5. Physico-chemical properties

Based on the parameters for hydrogen donors, hydrogen acceptors and MW, both the compounds (**I** and **II**) were following the claims of the Lipinski rule (Table 4.1). Considering the log*P* value (lipophilicity of the test molecule), although compound **II** meets the properties of the claim, compound **I** does not fulfil this Lipinski descriptor (Table 4.1). However, log*P* value of **I** is following the claims of modified Lipinski rule which brings back **I** under a suitable candidate drug category so as to be a prospective agent similar to compound **II** (Ghose et al. 1999). log*P* and H-bond donors basically highlights the significance of these two parameters in predicting bioavailability of any compound. Often, compounds isolated from natural sources were referred as an exception to the Lipinski "rule of five". Ganesan (2008) suggested that this violation could be observed as the nature synthesizes high molecular weight bioactive compounds with several freely rotatable bonds owing to their low hydrophobicity and intermolecular H-bond donating potential during synthesis. Beyond the exception, with the renewed interest in natural products in drug discovery studies revealed that the drug or lead-likeliness property of a natural product can be determined by using the Lipinski "rule of five" with significantly higher confidence limit (Koehn and Carter 2005; Quinn et al. 2008). They have also revealed that majority of the compounds mentioned in *The Dictionary of Natural Products*, showed no violations of Lipinski's rule and moreover, 85% of the isolated natural products in the investigation have been found as lead molecule according to the "rule of five". Therefore, similar to the previous investigations, estimation of physico-chemical properties of isolated natural products provides an unique opportunity and open up a new avenues towards the discovery of new therapeutic drug for the treatment of various diseases such as cancer, diabetes and other infectious diseases.

Table 4.1 Lipinski and drug likeliness properties of isolated compounds

Compounds	log*P*[a]	MW[b]	H Donors[c]	H Acceptors[d]	Lipinski's violation
I	5.435±0.450	302.45	0	2	0/1[#]
II	3.843±0.537	318.45	0	3	0
III	21.205±0.27	804.30	0	6	0

[#] modified range of Lipinski's log*P* value is ranging from -0.4 to +5.6

[a] compound's lipophilicity, expressed as log*P* (the logarithm of the partition coefficient between water and 1-octanol), less than 5

[b] MW-molecular weight, less than 500

[c] number of hydrogen bond donor, less than 5

[d] number of hydrogen bond acceptors, less than 10

4.3.6. Hemolytic assay

The compatibility and toxicity level of the labdane diterpenes towards application as bioactive agent against various diseases was evaluated by RBC hemolytic assay. This assay was chosen, because it has been well documented in the literature, readily available and can be easily performed with blood samples. Furthermore, the assay also included an evaluation of the denaturation of cell proteins released during the process of haemolysis, based on changes in the absorbance of oxyhemoglobin, which serves as an indicator of both damage of cell membrane and release of protein. The stability of erythrocyte membrane is considered as an indicator of *in vitro* damage towards cytotoxicity of any candidate drug molecule which can make characteristic changes in erythrocytes structure (Sharma and Sharma 2001). Red blood cells were also used as basic model to analyze the cytotoxicity or defensive activity of such candidate molecule towards any oxidative stress associated with the damage (Pape and Hoppe 1990; Aparicio et al. 2005; Lexis et al. 2006; Muñoz-Castañeda et al. 2006; Silva et al. 2009).

Here, the tested compounds displayed a concentration-dependent hemolytic effect (Fig. 4.18). The hemolytic activity was evaluated at range of 0.05-1000 µM. Fig. 4.18

showed that both the compounds did not affect RBCs much till 100 μM concentration which is far beyond the range of IC_{50} obtained in each case. However, very low hemolysis (12% and 16% for compound **I** and **II** respectively) was observed when the RBC cells were treated with 1 mM of each compound. There are many reports which showed mild to strong hemolytic activity of natural diterpenes isolated from various plants. Previously, kaurenoic acid, a diterpene molecule isolated from *Copaifera langsdorffii* showed dose dependant hemolysis of mouse and human erythrocytes (Costa-Lutufo et al. 2002). Considering the effect on human erythrocytes, both the isolated diterpenes in present study were found comparatively more suitable than kaurenoic acid towards hemolytic activity. Likewise, another diterpene (Trachylobane-360) isolated from *Xylopia langsdorffiiana* showed induction of hemolysis on mouse erythrocytes with similar experimental conditions (Pita et al. 2012). Therefore, the present results were encouraging since the highest concentrations of each compound used for hemolytic assay were found to be in the safe range for further bioactivity studies.

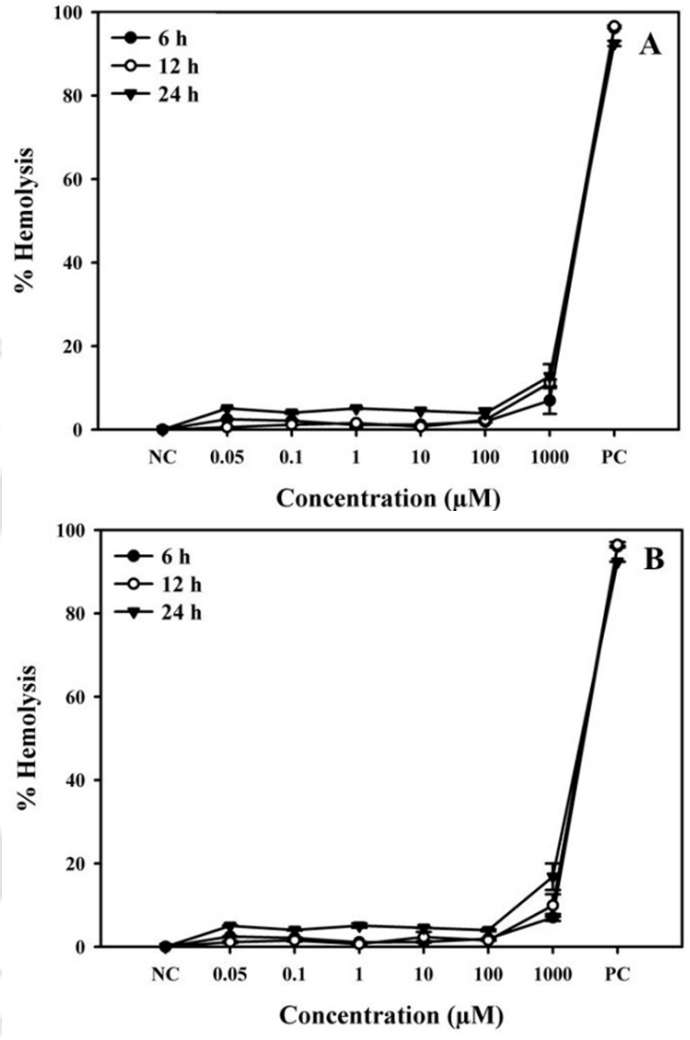


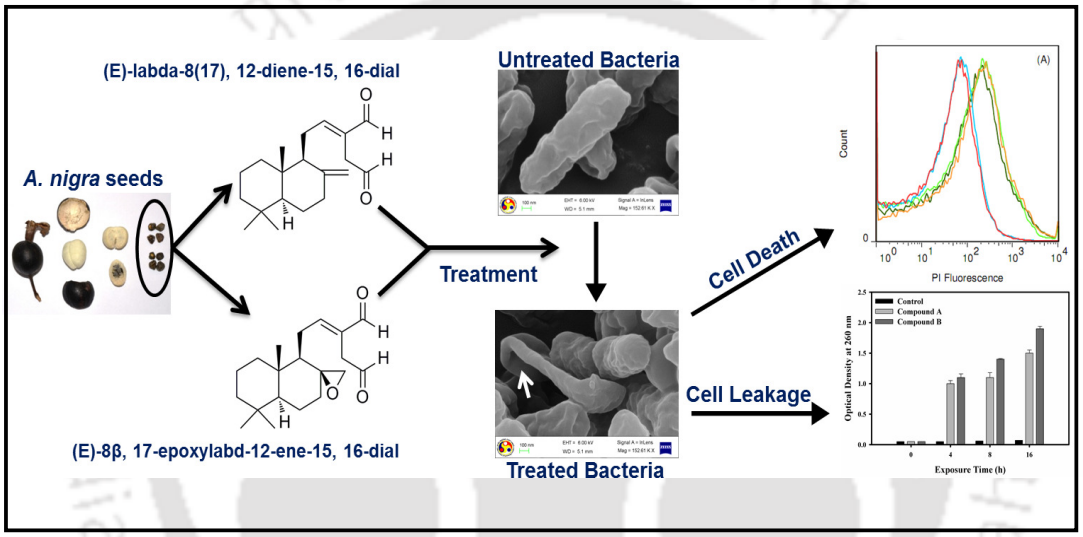
Fig. 4.18 Hemolytic activity of (A) compound **I** and (B) **II** were tested on human erythrocytes at different time intervals. Values are means ± SE from three independent experiments.

4.4. Conclusion

In this chapter, it was demonstrated that, *A. nigra* seed extracts contain three principal compounds; two labdane-type diterpene derivatives and a triglyceride. The compounds are characterised as compound **I** [(*E*)-labda-8(17),12-diene-15,16-dial], compound **II** [(*E*)-8β,17-Epoxylabd-12-ene-15,16-dial] and compound **III** [1,2-dihexadecanoyl-3-(9Z-hexadecenoyl)-sn-glycerol]. Analysis of the Lipinski descriptors for bioavailability estimation suggests both the compound **I** and **II** as potential drug candidate. Moreover, human red blood cells hemolysis experiment suggests these compounds to be fairly safe. Therefore, as this plant has received widespread use in folk medicine, this present result implies the necessity of more studies on these compounds towards their biomedical applications.

Chapter 5

Studies on bactericidal activities of seed extracts and purified labdane type diterpenes



The chapter describes the effectiveness of *A. nigra* seed extracts and its isolated bioactive compounds for antibacterial activity. Also, the mode of activity of isolated labdane diterpenes on selected pathogenic bacteria was studied.

Chapter 5

Studies on bactericidal activities of seed extracts and purified labdane type diterpenes

5.1. Introduction

Infectious diseases caused by bacteria, fungi and viruses are a threat to the mankind and leads to loss of life in both developed and developing countries around the world, more so in developing countries due to the life style such as poor sanitation, unhygienic and informal settlements (Ndhlala et al. 2013). The infections caused by bacteria remain a serious therapeutic problem with additive challenges from increasing resistance to antibiotics. It has been observed that the Gram-negative and Gram-positive bacteria isolated from hospitalized and ambulatory patients possess increasing resistance to one or multiple antibiotic classes (Heinemann et al. 2000; Spellberg et al. 2008). Many critical infections like listeriosis, yersiniosis, enteric fever and other food borne diseases caused by a wide range of bacteria are a major health concern in both developed and developing countries (WHO 2007; Freitag et al. 2009; Crump and Mintz 2010). Consequently, there is a greater need to discover and identify natural antibacterial agents from available bioresources as potent future antibacterial therapeutics (Gaspar-Marques et al. 2006; Negi et al. 2008).

As an alternative approach against bacterial infections, complementary and alternative medicines have been in use (Romero et al. 2005). This is a blend of indigenous beliefs and practices with folk herbal formulations. Recently, a growing concern and research interest observed towards the alternative means of antimicrobial compounds from nature, including the various herbal extracts (Kotan et al. 2013). The ethnomedical practices of tribal communities towards the uses of Zingiberaceae members from North East India (NEI) were

studied extensively and documented for further investigation (Tushar et al. 2010). Ethnomedicinally important but less explored *A. nigra* is known to be used for the treatment of dyspepsia, gastric diseases, insect bites, trematocidal etc. in folk medications (Roy et al. 2009). However, the effectiveness of *A. nigra* for antibacterial activity has not been investigated yet.

GC-MS analysis of organic solvent extracts of *A. nigra* seeds indicates the presence of several diterpene derivatives as major compounds. Among various types of phytoconstituents, diterpenes are known to display a wide spectrum of biological activities, including antibacterial activity (Kuzma et al. 2007; Almeida et al. 2008; Porto et al. 2009a, 2009b). Previous studies demonstrated that several classes of diterpenoids, such as pimarane, clerodane, kaurane, isopimarane, labdane and others have been used as a potential source of antimicrobial agents (Kalpoutzakis et al. 1998; Habtemariam 2003; Woldemichael et al. 2003; Wiart et al. 2005; Stavri et al. 2009; Radulovic et al. 2010). In recent years, extensive work has been performed using various plant extracts and isolated bioactive molecules towards their antibacterial properties, however, the effect of crude extracts or any isolated compounds from *A. nigra* against pathogenic bacteria are not explored yet.

Therefore, in current study of this chapter, *A. nigra* seed extracts and isolated compounds were investigated to unveil the mechanism of action as bactericidal agents against tested bacteria.

5.2. Materials and methods

5.2.1. Study materials

The organic solvent fractions (S-Hex, S-EtAc and S-Met) and isolated labdane diterpene compounds (**I** and **II**) were subjected to antibacterial studies.

5.2.2. Bacterial strains

The antibacterial activities of the study materials were evaluated against seven bacteria, *viz. Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19115), *Escherichia coli* (ATCC 25922), *Salmonella paratyphi A* (MTCC 735), *Escherichia coli* enterotoxic (MTCC 723) and *Yersinia enterocolitica* (MTCC 859). All the tested bacteria were grown and maintained on Nutrient Agar (NA) as described earlier by Kesari et al. (2010).

5.2.3. Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimal inhibitory concentration (MIC) was determined by using the microdilution method in 96 well microtitre plates (Camporese et al. 2003). Two fold serial dilutions of extracts prepared in ethanol (10-0.08 mg/ml) were added in each well containing bacterial suspension (approximately 10^6 cells/ml). Similarly, two fold serial dilutions of each compound were prepared in ethanol ranging from 100-0.8 µg/ml from which 10 µl of each concentration was pipetted to individual wells. The plate was incubated for 18 h at 37°C. Results were analyzed with multimode microplate reader (Tecan, Infinite M-200, Switzerland) at 620 nm and the lowest concentration of each compound, at which bacterial growth was inhibited, have been considered as MIC. Equal volume of ethanol was used as negative control for each experiment. The experiment was performed in triplicates and MIC recorded as the mean concentration of triplicate values.

In order to determine the MBC, 10 µl of broth medium from each well of MIC tested plate was spreaded on nutrient agar plate and incubated for 24 h at 37°C. The least concentration showing no visible growth on plate was taken as MBC value. The MBC was recorded as the mean concentration of triplicates.

5.2.4. Determination of antibacterial activity using flow cytometry (FC)

The mode of action of all three extracts from *A. nigra* seeds and the compounds (**I** and **II**) against seven tested bacteria were investigated using flow cytometry (FC) technique. To determine the effects of the extracts and compounds on bacterial cells, each bacterial culture was treated with the individual extract or compound at their respective MICs and incubated for 12 h. Heat killed bacteria (70°C for 30 min), ethanol treated bacteria and untreated bacteria were considered as positive control, negative control and vehicle control for the experiments. Each bacterial suspension was centrifuged at 10,000 rpm for 10 min at room temperature followed by washing of bacterial cell pellet thrice with PBS (phosphate buffer saline, 50 mM, pH 7.0). Finally, the cells were resuspended in PBS and adjusted to cell concentration of approximately 10^6 cells/ml. The cells were incubated with 100 µg/ml of propidium iodide (PI) (Sigma-Aldrich, USA) for 15 min in dark. To assess the effect of all the extracts on bacterial populations, the cell populations were stained with PI, a nucleic acid stain not taken up by intact live cells. The FC analysis of the cell samples were performed using FACS Flow solution (Becton-Dickinson) as sheath fluid and FlowJo software (Tree Star, Stanford, USA) was used for histogram plot analysis. The instrument is equipped with an argon ion laser (488 nm) and the cytometer was adjusted to count 50,000 fluorescent events for each sample. The FL-2 channel was used to detect the red fluorescence of PI stained bacterial cells. The antibacterial effects of extracts were determined according to the fluorescence intensity of PI which correlates with the damage of bacterial cell membrane (Paparella et al. 2008).

5.2.5. Field emission scanning electron microscopy (FESEM) study

Morphological examinations of the bacterial cells before and after the exposure of all the extracts and compounds were performed using field emission scanning electron microscope

(Carl Zeiss, Ultra 55). FESEM studies were carried out on two most susceptible bacteria *viz. S. aureus* (Gram-positive) and *Y. enterocolitica* (Gram-negative) respectively, at their respective MICs. Untreated bacterial cells were used as negative control. The bacterial samples were washed gently with 50 mM phosphate buffer solution (pH 7.2), fixed with 2.5% glutaraldehyde in PBS and rinsed with the same buffer solution. The specimen was dehydrated using sequential exposure for each ethanol concentrations ranging from 30% to 100%. Finally, the specimens were coated with gold and analyzed with FESEM (Carl Zeiss, Ultra 55).

5.2.6. Effect of extracts on bacterial cell membrane

In order to further confirm the membrane damaging efficacy of the organic seed extracts and two compounds, cell leakage analysis was performed by monitoring the absorbance at 260 nm of respective cell supernatants. The experiments were carried out for *S. aureus* and *Y. enterocolitica*, whose morphological damage were previously examined using FESEM. Overnight growth of bacterial suspension was harvested by centrifugation at 10,000 rpm for 10 min and resuspended in 0.9% sterile sodium chloride solution. Then each bacterial culture was treated with individual extract and compound at their respective MIC and incubated for 0, 4, 8 and 16 h respectively. After incubation, samples were centrifuged at 10,000 rpm for 5 min in order to separate out the additional extracts and the bacterial cells from the low molecular weight metabolites such as nucleotides, amino acids and inorganic ions which are known to leak from cells after membrane damage. Finally, the level of released material from the bacterial cell was determined by measuring optical density (OD) of the supernatant at 260 nm using UV/visible spectrophotometer (Varian Carry 50, USA).

5.3. Results and discussion

5.3.1. Antibacterial activities of extracts

All the extracts from *A. nigra* seeds were used to determine MIC and MBC by broth dilution method. The results of the MIC and MBC values of all the extracts are represented in Table 5.1. The MIC and MBC values for bacterial strains were in the range of 0.16-5.00 mg/ml. Moreover, it was clearly observed that the solvent type used in the extraction had a significant impact on MIC and MBC. The methanol extract showed relatively lower MIC and MBC values compared to other extracts against all the tested bacteria (≤0.62 mg/ml). Furthermore, it was observed that among all the tested bacteria *S. aureus* (MIC and MBC = 0.16 mg/ml) and *Y. enterocolitica* (MIC and MBC = 0.31 mg/ml) were most sensitive to the methanol extract. However, antibacterial activity of hexane extract remained low irrespectively against all the tested bacteria.

Herbal extracts are gaining importance as potent antibacterial agent in recent years and have been extensively used against various food borne pathogenic bacterial strains and many other clinical isolates (de Oliveira et al. 2013; Khan et al. 2013; Lee et al. 2013; Marathe et al. 2013; Ogihara et al. 2013; Subbiya et al. 2013). In the present study, *Y. enterocolitica* found to be most susceptible to the S-Met extract which is also found to be much effective than any other plant extracts against *Y. enterocolitica* published previously (Chauhan et al. 2007; Al-Zoreky 2009; Ahameethunisa et al. 2010; Bahador and Baserisalehi 2011). Similar to the present study, other plant extracts, particularly ethyl acetate and/or methanolic extracts have been found most effective against *S. aureus* (Chauhan and Abraham 2013; Khan et al. 2013; Madikizela et al. 2013; Marathe et al. 2013).

Table 5.1 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (mg/ml) of *A. nigra* seed extracts against selected Gram-positive and Gram-negative bacteria

Bacterial strains	**S-Hex**		**S-EtAc**		**S-Met**	
Gram (+)ve	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
S. aureus	1.25	1.25	0.31	0.62	0.16	0.16
B. cereus	0.62	1.25	0.31	0.31	0.31	0.31
L. monocytogenes	1.25	0.25	0.62	0.62	0.31	0.31
Gram (-)ve	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
E. coli	5.00	5.00	1.25	2.50	0.62	0.62
S. paratyphi	2.50	5.00	1.25	1.25	0.31	0.62
E. coli enterotoxic	2.50	2.50	0.62	0.62	0.31	0.62
Y. enterocolitica	1.25	1.25	0.31	0.62	0.31	0.31

5.3.2. Antibacterial activities of compounds

The antibacterial activities (MIC and MBC) of the two compounds (**I** and **II**) were evaluated against seven pathogenic bacteria. The MIC and MBC for bacterial strains were in the range of 3.375-25 µg/ml (Table 5.2). Compound **II** showed the greatest antibacterial activity over compound **I** in terms of MIC and MBC (except the MBC of *S. paratyphi*). Furthermore, it was observed that of all the pathogens tested, *S. aureus* (MIC = 3.375 and MBC = 6.75 µg/ml) and *Y. enterocolitica* (MIC and MBC = 3.375 µg/ml) were most sensitive to compound **II**. The susceptibility of *S. aureus* was similarly observed for various plant derived compounds such as 6-oxo-genipin, methyl gallate, kaempferol-3-O-rhamnoside, 2-hydroxytrideca-3,6-dienyl-pentanoate and octacosa-12,15-diene recently (Kouam et al. 2013; Kumar et al. 2013; Madikizela et al. 2013). Previously compound **II,** isolated from seeds of *Aframomum daniellii* (Zingiberaceae) was tested against various pathogenic fungi and bacteria including *S. aureus* (Ayafor et al. 1994). In that report, the antibacterial activity of compound **II** against *S. aureus* NCTC 8530 was found as less effective than the current observation against *S. aureus* ATCC 6538. Recent report showed Zerumbone, a major

component of *Zingiber zerumbet*, as highly active against *B. cereus* but least active against *Y. enterocolitica* (Santosh Kumar et al. 2013). Similar to the present investigation, susceptibility of *Y. enterocolitica* were also observed with various derivatives of tetrahydrocurcumin and zingerone (Manjunatha et al. 2013).

Table 5.2 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (µg/ml) of two isolated compounds from *A. nigra* seeds against selected Gram-positive and Gram-negative bacteria

Bacterial strains	**Compound I**		**Compound II**	
Gram (+)ve	**MIC**	**MBC**	**MIC**	**MBC**
S. aureus	12.5	25.0	3.375	6.75
B. cereus	12.5	12.5	6.75	6.75
L. monocytogenes	25.0	25.0	12.5	12.5
Gram (-)ve	**MIC**	**MBC**	**MIC**	**MBC**
E. coli	25.0	50.0	12.5	12.5
S. paratyphi	12.5	12.5	6.75	12.5
E. coli enterotoxic	25.0	25.0	6.75	6.75
Y. enterocolitica	12.5	25.0	3.375	3.375

5.3.3. Flow cytometric investigation

Traditional methods for assaying antibacterial activity are based on the visualization of bacterial growth in a qualitative manner. However, in the present study, along with the qualitative assays, the quantification of individual cells in the heterogeneous population has also been carried out by FC. Moreover, the sensitivity of flow cytometric analysis is significantly higher for the reason that the detectable change of the fluorescence histogram can directly correlate the bacterial cell membrane damage. In order to provide some insights into the mechanisms of action of all three extracts and isolated compounds on seven tested bacterial cells, the multiparametric FC technique was exploited.

Flow cytometric histograms and median fluorescence intensity (MFI) of PI-stained bacteria after treating with extracts are shown in Fig 5.1. Here, the negative controls (N-cell

populations in presence of respective solvents) showed the minimum relative fluorescence with respect to control cell populations (Fig. 5.1A-G). Conversely, the positive control (HK-heat killed bacterial population) showed significant increase in relative fluorescence in all tested bacteria (Fig. 5.1A-G) and confirms the major cell populations as damaged or dead. The rightward shifting of fluorescence peak in the histogram was observed when the bacterial cells were treated with extracts as compared to control populations (Fig. 5.1). The histogram peak shifting occurs due to high PI fluorescence intensity from the treated and heat killed cells. Untreated cell population has its characteristic unaltered cell membrane whereas, treated and heat killed cell population has compromised cell membrane which allows PI to enter into the cell and stain the nucleic acids. The extents of damaged and dead cells were estimated on the basis of MFI with reference to histogram peak shifting. Responses among the seven tested bacteria varied with treatment of individual extract. Interestingly it was observed that shifting of fluorescence peak in the histograms (toward right) and MFI were maximum when the cells were treated with S-EtAc and S-Met extracts which indicates significant damage and depolarization of most of the tested bacterial cytoplasmic membrane (Fig. 5.1).

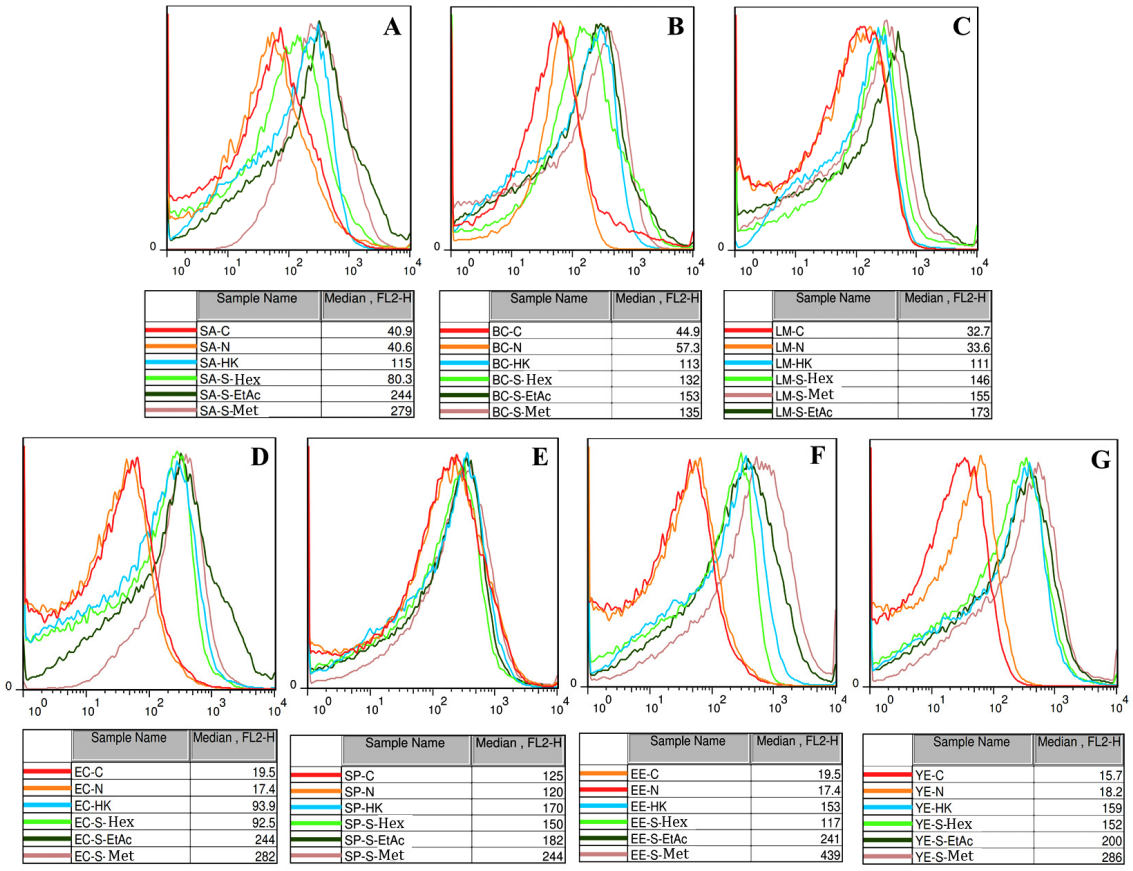


Fig. 5.1 Flow cytometric histograms of PI-stained seven tested bacteria at their respective MIC values for each extracts. Histogram analysis has been done by using FlowJo software (Tree Star, Stanford, USA) and plotted as PI fluorescence (FL2-H) against total cell counts. (A)-(G) represents overlay histograms and median fluorescence intensity (MFI) of PI (FL2-H) for *S. aureus* (SA), *B. cereus* (BC), *L. monocytogenes* (LM), *E. coli* (EC), *S. paratyphi* A (SP), *E. coli* enterotoxic (EE), and *Y. enterocolitica* (YE), respectively. C untreated bacteria (control), N bacteria treated with ethanol (negative control), HK heat killed bacteria, S-Hex, S-EtAc and S-Met are bacteria treated with seed hexane extract, ethyl acetate extract and methanol extract, respectively. Significant increase in MFI and peak shifting was clearly observed in each case with respective treatments.

Similarly, FC analysis was carried out to assess the effect of compound **I** and **II** on bacterial cell membrane integrity. Flow cytometric histograms and median fluorescence intensity (MFI) of propidium iodide (PI) stained bacteria are shown in Fig. 5.2. Alike the FC analysis with extracts, the vehicle control (cells treated with ethanol) showed minimal changes in relative fluorescence intensity with respect to control cell populations (Fig. 5.2A-G). While, the positive control (heat killed bacteria) showed significant increase in relative fluorescence intensity for all the tested bacteria (Fig. 5.2A-G) and confirmed the cell damage or death. Increase in fluorescence similar to those observed for the positive controls were also observed when the bacterial cells were treated with compounds **I** and **II**. MFI values indicated that treatment of bacteria with compounds **I** and **II** caused significant (Tukey's test, $p<0.001$) increase in fluorescence intensity compared to the vehicle control for all seven bacterial strains, with compound **II** having a significantly more pronounced effect than compound **I**, especially for the Gram-negative bacterial strains (Tukey's test, $p^{E.\ coli} = 0.001$, $p^{S.\ paratyphi} = 0.038$, $p^{E.\ coli\ \text{enterotoxic}} = 0.009$, and $p^{Y.\ enterocolitica} < 0.001$).

Flow cytometric technique has become a promising tool in wide range of application, including antibacterial activity studies by detecting the change in bacterial membrane potential, permeability and even for the development of rapid antibacterial drug discovery in recent years (Durodie et al. 1995; Caron et al. 1998; Novo et al. 2000; Gnanadhas et al. 2013; Scanlon et al. 2013; Stankovic et al. 2013). Previously, flow cytometric investigation on *L. monocytogenes* and *S. aureus* revealed the antibacterial effect by various plant essential oils (Nguefack et al. 2004). The mode of bactericidal effect of essential oil was found similar to the present study which possibly due to the permeabilization of cytoplasmic membrane of bacteria.

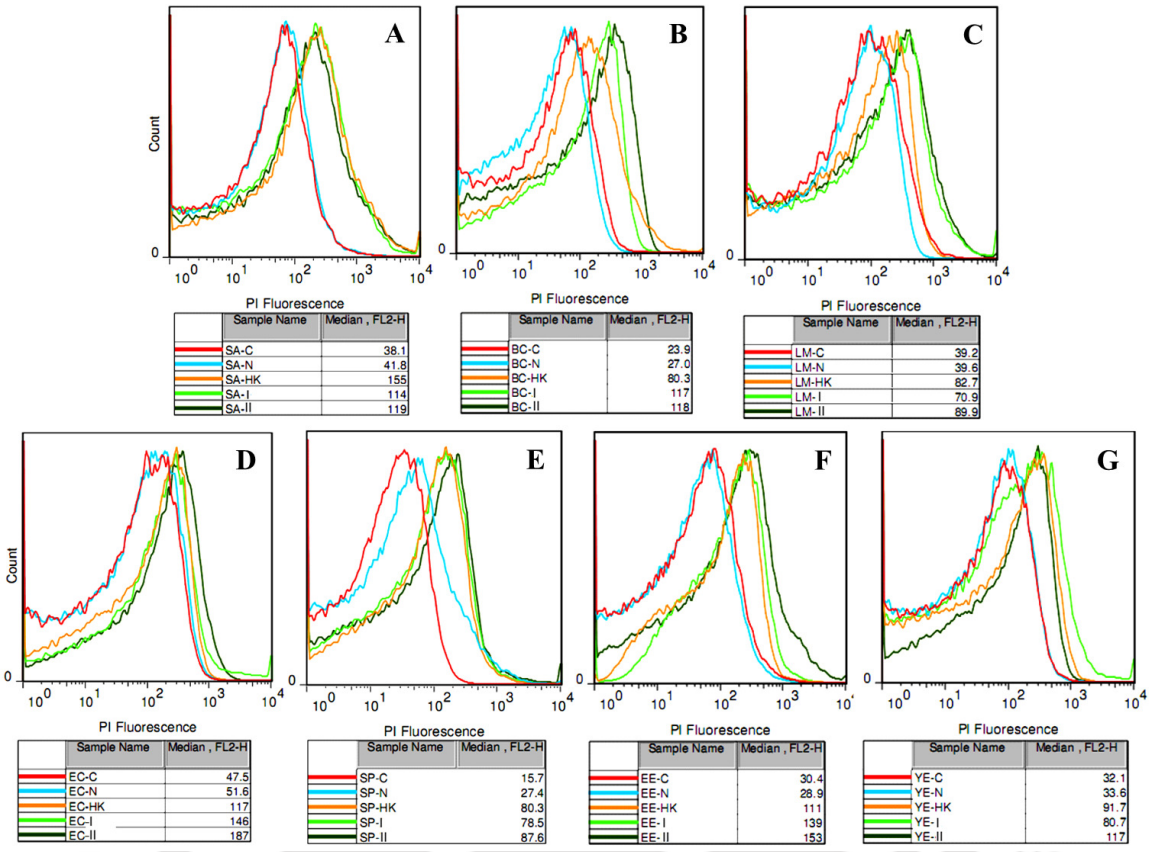


Fig. 5.2 Flow cytometric histograms of PI-stained seven tested bacteria at their respective MIC values for each compound. Histogram analysis has been done by using FlowJo software (Tree Star, Stanford, USA). The figure represents overlay histograms and median fluorescence intensity (MFI) of PI (FL2-H) for (A) *S. aureus* (SA), (B) *B. cereus* (BC), (C) *L. monocytogenes* (LM), (D) *E. coli* (EC), (E) *S. paratyphi A* (SP), (F) *E. coli* enterotoxic (EE), and (G) *Y. enterocolitica* (YE) respectively. C untreated bacteria (control), N bacteria treated with ethanol (negative control), HK heat killed bacteria. Significant increase in MFI and peak shifting was clearly observed in each case with respective treatments.

5.3.4. FESEM study

Membrane damage is found to be the key mechanism by which the plant extracts rich in phenolic compounds exerts their antimicrobial activities (Hammer and Heel 2012). However, in order to understand the mode of action of *A. nigra* seed extracts and isolated compounds on most sensitive Gram-positive and Gram-negative pathogenic bacteria, *S. aureus* and *Y. enterocolitica* was further examined by FESEM to unveil the changes in bacterial cell morphology after treating with all the solvent extracts and compounds.

FESEM study of untreated bacteria revealed characteristic morphological features (Fig. 5.3A, E), however shrinking and degradation of the cell walls were observed in bacterial cells treated with seed extracts (Fig. 5.3B-D and F-H). These findings indicate that *A. nigra* seed extracts possess antibacterial activity and they cause lysis of bacteria by degrading bacterial cell walls and damaging cytoplasmic membrane proteins.

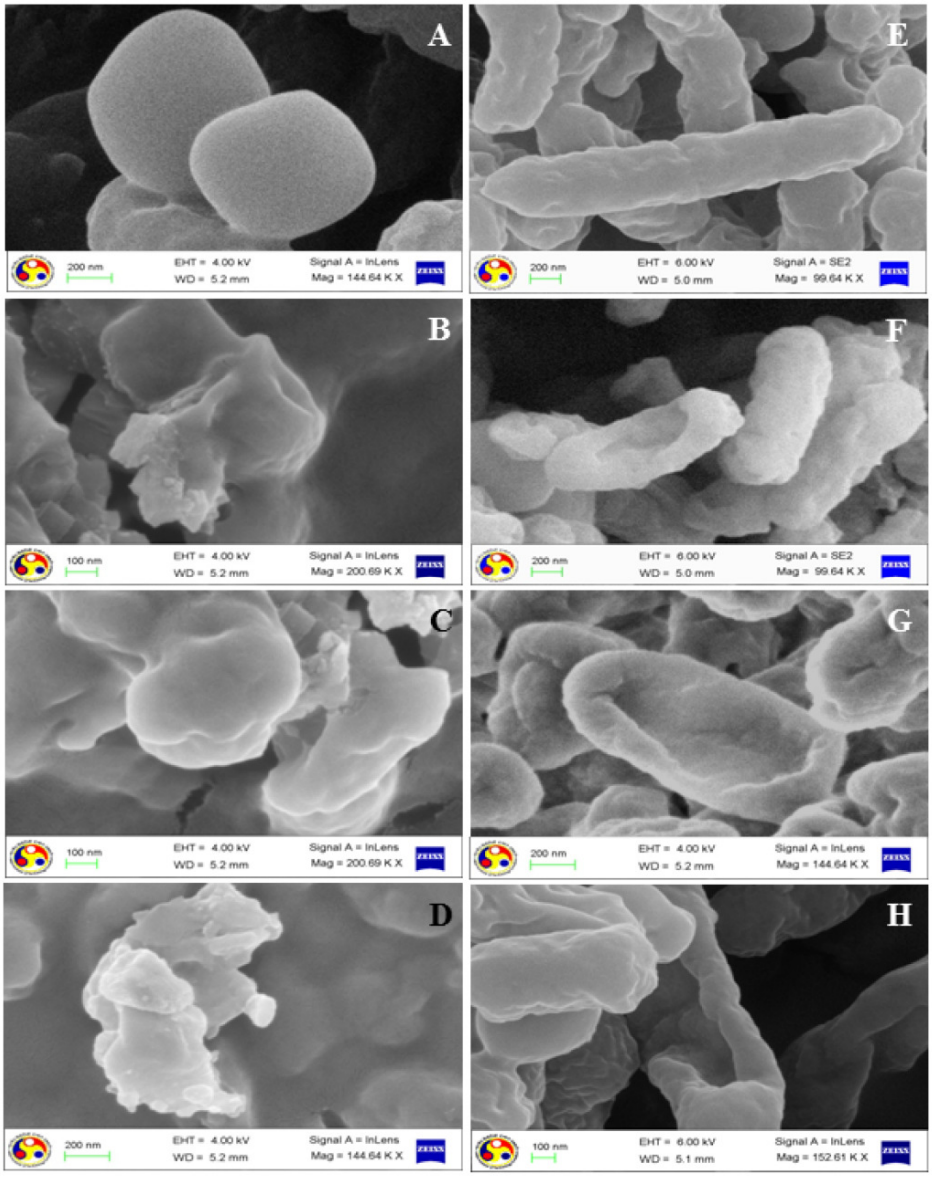


Fig. 5.3 Field emission scanning electron micrographs of *S. aureus* (A-D) and *Y. enterocolitica* (E-H). (A and E) untreated bacterial cells, (B-D and F-H) bacterial cells after treatment with S-Hex (B and F), S-EtAc (C and G) and S-Met (D and H) extracts of *A.nigra* seeds at their respective MIC.

Bacterial cultures of *S. aureus* and *Y. enterocolitica* were examined by FESEM to observe morphological changes caused by treatment with compound **I** and **II** (Fig. 5.4). FESEM images of untreated *S. aureus* showed intact, smooth cell surface with defined cell features (Fig. 5.4A), whereas membrane disintegration and prominent damage of the cell wall was observed when cells treated with each compound (Fig. 5.4B, C). Furthermore, membrane damaging effects of each compound were also observed in case of *Y. enterocolitica* cells (Fig. 5.4E, F). These findings indicate that both compounds cause lysis of bacteria by degrading bacterial cell walls. Similar type of abnormalities of bacterial cell morphology is evidential of the disruption of membrane structure, as reported previously (Koyama et al. 1997; Shin et al. 2007). Further, recent studies on antibacterial activity of a *Pseudomonas aeruginosa* derived compound against methicillin-resistant *S. aureus* strains revealed distinct altered morphology of cells after few hours of the treatment (Cardozo et al. 2013). The lead compound responsible for this activity was isolated and identified as phenazine-1-carboxamide. Recently, in another study with purified bee venom revealed antimicrobial activity on the skin bacterium, *Propionibacterium acnes*, where clear damage in the bacterial cell wall was observed in electron microscopic images (Han et al. 2013). The current observations of bacterial cell membrane damage are in accordance with previous studies which clearly indicate the altered morphology or distinct damage in bacterial cells by antibacterial agents derived from various sources including plants (Kairyte et al. 2013; Kamonwannasit et al. 2013; Ray et al. 2013; Sherry et al. 2013; Supaphon et al. 2013).

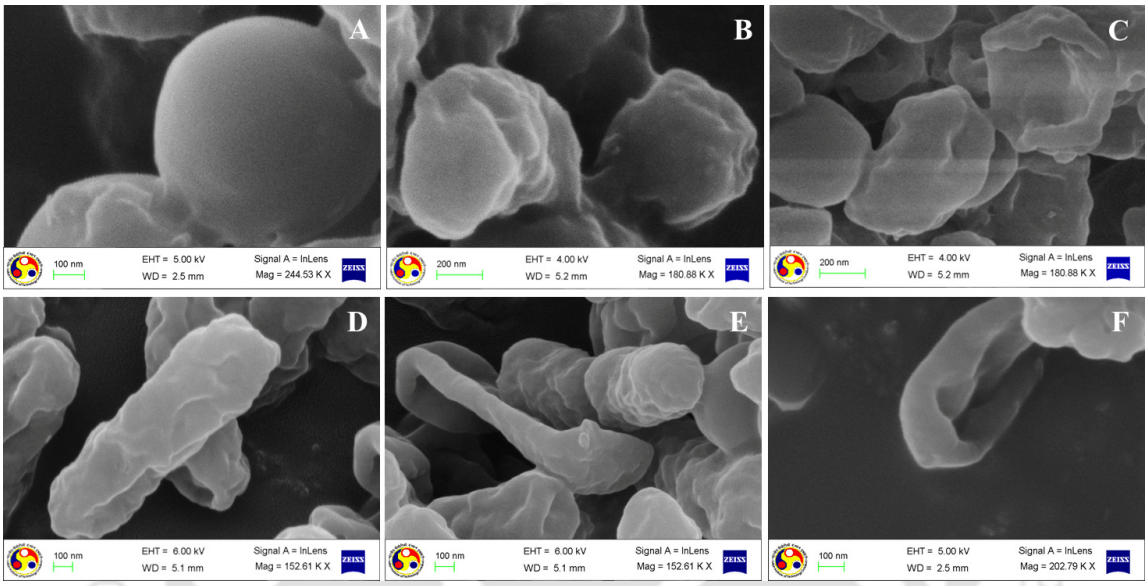


Fig. 5.4 Field emission scanning electron micrographs of *S. aureus* (A-C) and *Y. enterocolitica* (D-F). (A and E) untreated bacterial cells, bacterial cells after treatment with compound **I** (B and E) and compound **II** (C and F) at their respective MICs.

5.3.5. Effect of extracts on bacterial cell membrane

Nucleotides and their constituent building blocks (purines, pyrimidines, pentose and inorganic phosphate) are known to leak from compromised bacterial cells and the levels of leakage of these moieties were determined by measuring the optical density (OD) at 260 nm using UV/VIS spectrophotometer. When the values of OD_{260} were plotted, the amount of low molecular weight metabolites increased with increasing time of exposure due to continuous release of cellular materials through compromised cell membrane of treated bacterial strains as compared to controls. Our results demonstrate that the cell leakage increases with time of exposure and significant release of cellular material was observed in case of S-Met irrespective of the tested bacteria (Fig. 5.5). In case of compounds, the epoxide analogue (compound **II**) was found significantly more effective than compound **I** considering the results of cell leakage at 8 and 16 h treatments (Tukey's test, $p<0.05$) (Fig. 5.6). Our findings are in agreement with an earlier published report describing the role of plant derived terpenes towards cell membrane damage of diverse pathogenic bacteria (Koyama et al. 1997). Besides these, recent report also revealed similar loss of OD_{260} material from *P. aeruginosa* cells treated with silver nanoparticles and also from *S. aureus* treated with cardol, a natural compound from cashew (*Anacardium occidentale* L.) nut shell (Eid and Araby 2013; Murata et al. 2013). Therefore, a clear damage and dose-dependent release of cell material from labdane diterpenes treated bacteria is evident in the present study which has similar pattern of effects with other plant derived compounds or extracts on bacterial cells related to loss of OD_{260} materials (Álvarez-Ordóñez et al. 2013; Borges et al. 2013).

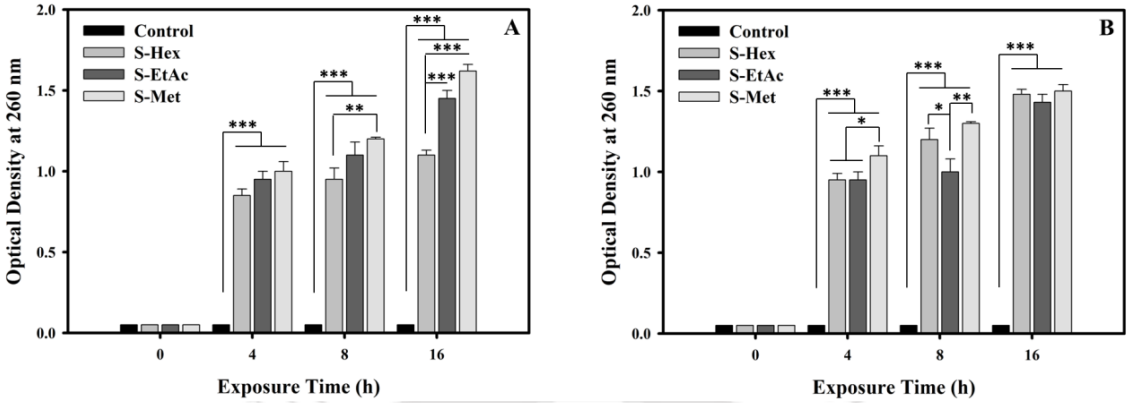


Fig. 5.5 Absorbance of the cell materials contents at 260 nm releasing from *S. aureus* cells (A) and *Y. enterocolitica* (B) after treatment with *A. nigra* seed extracts (S-Hex, S-EtAc and S-Met) at 0, 4, 8 and 16 h. The data are expressed as means ± standard errors. The asterisk denotes statistical significance (p<0.05).

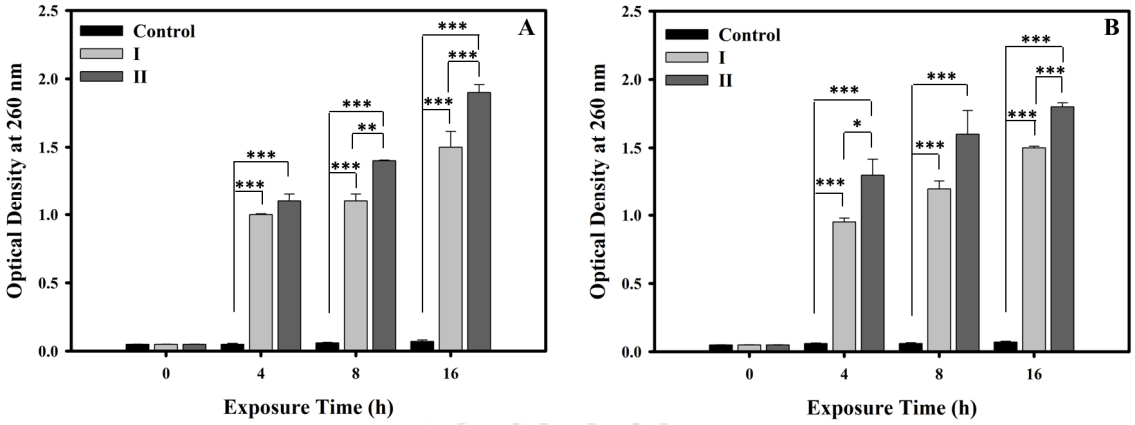


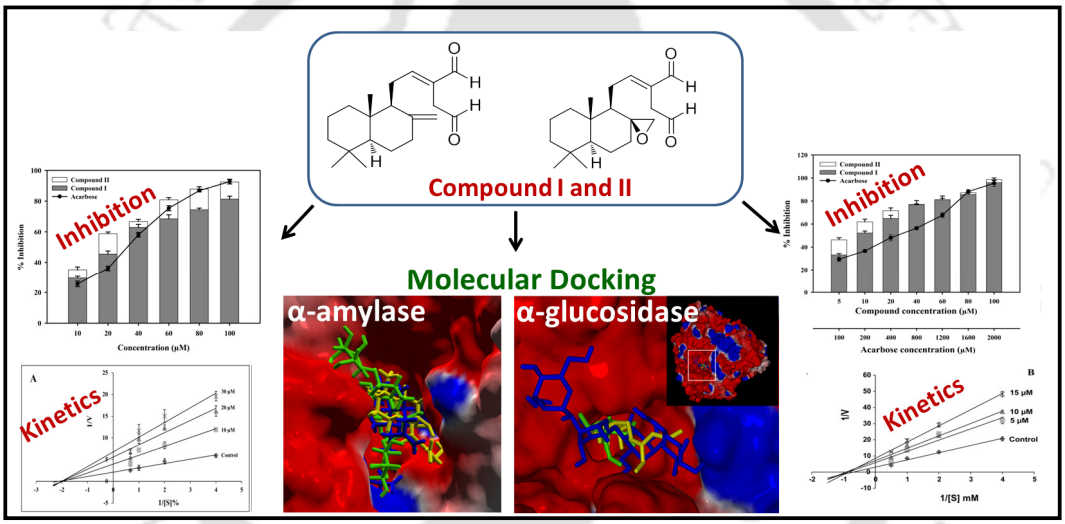
Fig. 5.6 Absorbance of the cell materials contents at 260 nm releasing from *S. aureus* cells (A) and *Y. enterocolitica* (B) after treatment with compound **I** and **II** at 0, 4, 8 and 16 h incubation period. The data are expressed as means ± standard errors. The asterisk denotes statistical significance (p<0.05).

5.4. Conclusion

Promising bactericidal activities of *A. nigra* seed extracts and isolated two bioactive labdane type diterpenes have been described in the current chapter. Among three different solvent extracts, methanol extract was found to be most active against all tested pathogenic bacteria. In case of diterpene compounds, compound **II** showed more effective bactericidal activity than **I**. Bacterial membrane damage and subsequent cell leakage were found to be the key mechanism underlying the efficacy of seed extracts and compounds. Therefore, *A. nigra* seed extracts and its purified compounds could be considered as effective bactericides which need more attention in the future as these efforts will also significantly contribute to a better understanding of the use of a non-food crop and its application in pharmaceutical industries.

Chapter 6

Studies on molecular docking and inhibition kinetics of labdane diterpenes on α-amylase and α-glucosidase enzymes towards combating Type 2 diabetes



The chapter describes inhibitory activity and inhibition kinetics for two carbohydrate hydrolysing enzymes, α-amylase and α-glucosidase using the purified labdane diterpene compounds. Further, molecular docking simulation on human pancreatic α-amylase and maltase glucoamylase illustrates the possible mode of inhibition of isolated diterpene molecules.

Chapter 6

Studies on molecular docking and inhibition kinetics of labdane diterpenes on α-amylase and α-glucosidase enzymes towards combating Type 2 diabetes

6.1. Introduction

Diabetes mellitus (DM) is a metabolic disorder of endocrine and occurs in individuals having either deficiency in insulin secretion or insulin action, or even both. This leads to abnormalities in the metabolism of foods rich in carbohydrate. The occurrence and morbidity related to type 2 DM continues to increase predominantly in developed and developing countries and is expected to rise in the next 30 years or so (Andrade-Cetto et al. 2008). In long run, DM leads to many other complications like neuropathy, microangiopathy and even high risk of heart diseases (Sales et al. 2012). To combat type 2 DM, one of available approach is to decrease the post-prandial glucose levels. This could be achieved by delaying the absorption of glucose via inhibition of the carbohydrate hydrolysing enzymes, *viz.* α-amylase and α-glucosidase (Dong et al. 2012; Ademiluyi and Oboh 2013; Chen et al. 2013b).

Pancreatic α-amylase and small intestinal α-glucosidase are the key enzymes involved in dietary carbohydrate digestion in human. These enzymes are present in the brush border of small intestine and act on the disaccharides and oligosaccharides to hydrolyse into simple sugars for easy absorption (Sales et al. 2012). Various inhibitors of these enzymes, *viz.* acarbose, miglitol and voglibose are currently in use as medicine for type 2 DM (Pogano et al. 1995; Chiasson et al. 2002; Murai et al. 2002). However, most of these drugs have several

side effects like hepatotoxicity, diarrhoea, flatulence and abdominal distension which signify the need for other alternative drugs with equal or better efficacies (Hollander 1992; Fujisawa et al. 2005; Singh et al. 2008).

Recently, various natural compounds, especially plant derived compounds have attracted lot of interest as a potential source for curing type 2 DM by inhibiting these two key enzymes linked to postprandial hyperglycemia. Therefore, screening of natural products using bioassay-guided isolation and ethnopharmacological information have provided a logical direction towards the identification of potential α-amylase and α-glucosidase inhibitors from herbal sources (Sales et al. 2012; Kumar et al. 2013; Olubomehin et al. 2013).

Bioactive molecules from the medicinal plants are the most sought after resources and also alternative means adopted to circumvent the side effects of synthetic drugs, and has been recommended for diabetic treatments (Matsui et al. 2001; Mcdougall et al. 2005). Recently, many natural compounds from various sources have been extensively studied against antidiabetic target enzymes, *viz.* α-amylase (Capocchi et al. 2013; De et al. 2013; Lordan et al. 2013; Thilagam et al. 2013) and α-glucosidase (Choo et al. 2012; Ferreres et al. 2013; Mosihuzzman et al. 2013; Phan et al. 2013). However, the labdane diterpenes under present study has not been investigated towards its antidiabetic potential, though they are well documented previously as potential bioactive agent against various diseases (Abe et al. 2004; Malek et al. 2011a).

In the previous chapter, we have investigated a medicinally important wild ginger, *Alpinia nigra* (Gaertn.) B. L. Burtt seed extracts and purified labdane diterpene derivatives against various pathogenic bacteria towards its antibacterial potential. However, the studies regarding antidiabetic efficacy of *A. nigra* crude extracts or diterpene derivatives against pancreatic α-amylase and α-glucosidase activity have not been undertaken to the best of our

knowledge. Therefore, in the current chapter, we have investigated for the first time a labdane type diterpene and its epoxide derivative from *A. nigra* seed towards the evaluation of *in vitro* α-amylase and α-glucosidase inhibitory activity, their inhibition kinetics and respective molecular docking simulations to unveil the possible mechanisms of inhibition.

6.2. Materials and methods

6.2.1. Study material

The organic solvent fractions (S-Hex, S-EtAc and S-Met) extracted from *A. nigra* seeds were subjected for α-amylase and α-glucosidase inhibition study towards antidiabetic activity. Furthermore, diterpene compounds (**I** and **II**) were investigated towards inhibition of the respective enzymes, inhibition kinetics and molecular docking simulations in the present study.

6.2.2. Inhibitory assay

6.2.2.1. α-amylase inhibitory assay

The pancreatic α-amylase inhibition assay was carried out according to the standard procedure with slight modifications (Hansawasdi et al. 2000). To determine the activity of the *A. nigra* seed extracts and the isolated compounds against α-amylase enzyme, various concentration of the extracts and compounds were pre-incubated with 10 µl of 10 µg/ml porcine pancreas α-amylase (Sigma-Aldrich, USA) for 60 min at 37°C. Acarbose was used as positive control and phosphate buffer (20 mM sodium phosphate with 6.7 mM sodium chloride, pH 6.9) in place of sample was used as blank for the experiment. One percentage of starch substrate solution, prepared with the phosphate buffer (pH 6.9) was added to each reaction and further incubated at 37°C for 20 min. The enzyme activity was monitored by

addition of 100 µl of dinitrosalicylic acid reagent (Bernfeld 1955) to the reaction mixture, and subsequently the tubes were placed in boiling water bath for the development of colour. After an incubation of 10 min, the reaction was terminated by addition of 600 µl of water to the samples. The absorbance was recorded at 540 nm using multimode microplate reader (Tecan, Infinite M-200, Switzerland). The α-amylase inhibitory activity was calculated as given below:

$$\% \text{ Inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \times 100$$

The concentration of inhibitors (acarbose, plant extracts and compounds) required to inhibit 50% of α-amylase activity under the assay conditions was defined as the IC_{50} value. The IC_{50} values were determined from plots of % inhibition versus inhibitor concentration and calculated by non-linear regression analysis from the mean inhibitory values.

6.2.2.2. α-glucosidase inhibitory assay

The α-glucosidase inhibitory assay was done according to the chromogenic method described by Matsui et al. (1996). To mimic a fluidic environment of intestine, *p*-nitrophenyl-α -D-glucopyranoside substrate (*p*NPG) solution (2 mM), prepared in a 100 mM phosphate buffer, pH 6.8 was used as substrate. To study the inhibitory effect, 0.001 U/ml of Yeast (*Saccharomyces cerevisiae*) α-glucosidase (Sigma-Aldrich, USA) was incubated with different concentration of seed extracts (10-100 µg/ml) and isolated compounds, **I** and **II** (10-100 µM) respectively. The standard drug, acarbose was used as positive control for the experiment. The absorbance was recorded at 405 nm and the percentage of α-glucosidase inhibition was determined as follow:

$$\% \text{ Inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \times 100$$

The IC_{50} values were determined from plots of % inhibition versus inhibitor concentration and calculated by non-linear regression analysis from the mean inhibitory values.

6.2.3. Kinetics of enzyme inhibition

The mode of inhibition of both the enzymes by compound **I** and **II** was determined using Michaelis-Menton and Lineweaver-Burk (LB) equations. The enzyme inhibiting activity was measured with varying range of substrate concentrations in absence or presence of studied compounds, **I** and **II**. The values of kinetic parameters (K_m, V_{max} and K_i) were determined according to the type of inhibition for both the enzyme catalyzed reaction for each compound.

6.2.3.1. α-amylase

Four different concentrations (0.25, 0.5, 1, and 1.5%) of starch were used for the inhibition study of α-amylase. The kinetics of α-amylase was studied by using 10, 20 and 30 µM of each compound, whereas the reaction without any inhibitor or test molecule was used as control for the same. Kinetic analysis was performed based on LB double reciprocal plots and the values of kinetic parameters were determined according to the type of inhibition for α-amylase enzyme catalyzed reaction for each compound.

6.2.3.2. α-glucosidase

The inhibiting activity of α-glucosidase was determined using varying range of substrate concentrations (0.5-4.0 mM) in presence or absence of test compounds. The kinetics of α-glucosidase was evaluated by using 5, 10 and 15 µM of each compound, whereas the reaction

without any inhibitor or test compound was used as control for the same. LB double reciprocal plots and Dixon plots were drawn to determine the type of inhibition and the respective values of kinetic parameters (K_m, V_{max} and K_i).

6.2.4. Statistical analysis

All tests were performed in triplicate with three independent experiments. The results expressed as a mean ± SE. The data were analyzed using one-way analysis of variance (ANOVA) and treatments were compared by employing Tukey's post-hoc test. $p<0.05$ was considered significant.

6.2.5. Molecular docking

The three dimensional structure of compound **I** (CID: 9796432), **II** (CID: 6439042) and acarbose (CID: 444254) were obtained from PubChem database (http://pubchem.ncbi.nlm.nih.gov) for docking studies. Docking studies were performed with X-ray crystal structure of human pancreatic α-amylase (HPA) and maltase-glucoamylase (MGAM) separately using Molegro Virtual Docker (MVD) 5.5.0. For many years MVD has been used extensively in a wide range of molecular docking studies (Basile et al. 2012; Yang et al. 2012; Gurubasavaraja Swamy et al. 2013; Singh et al. 2013) due to its higher accuracy compared to other available dock software (Thomsen and Christensen 2006).

6.2.5.1. α-amylase

The X-ray crystal structure of human pancreatic α-amylase (HPA) (PDB ID: 4GQR) was downloaded from RCSB Protein Data Bank (http://www.pdb.org) and processed further. The cofactors, water molecules and bound ligand were removed from the receptor (HPA) prior to

docking analysis. 3D structure of compound **I**, **II** and acarbose were used for molecular docking using Molegro Virtual Docker (MVD) 5.5.0.

For docking simulation, putative binding cavities were detected in HPA where the major cavity was obtained within the active site of the receptor (Williams et al. 2012). The binding cavity was set at the coordinate of X: 11.0 Y: 16.31 Z: 40.08 within a constraint of radius 15 Å. A grid-based cavity prediction algorithm was adopted to determine the potential binding site in HPA as described by Thomsen and Christensen (2006). In brief, the grid resolution was kept 0.3 Å and the binding site was restricted to a radius of 6 Å wherein all the residues are assumed flexible. Owing to the fact of stochastic nature of any docking engine, 100 runs for each compound were performed. Further, 50 most energetically favoured binding conformations of each ligand with target protein were considered. The docking algorithm was fixed for 10,000 iterations with 100 population size and 0.5 scaling factor. After successful docking, all the putative docking models were analysed and selected further based on MolDock (MD) score and hydrogen bonding interactions. Modified piecewise linear potential (PLP) with additional hydrogen bonding and electrostatic interactions was used for scoring of docking energy between receptor-inhibitor (Thomsen and Christensen 2006). Best docked poses for each docking experiment were subjected to LigPlot analysis (Wallace et al. 1995).

6.2.5.2. α-glucosidase

The X-ray crystal structure of maltase-glucoamylase (MGAM) for both N-terminal domain (PDB codes: 3L4Z) and C-terminal domain (PDB codes: 3TOP) was downloaded from PDB and processed subsequently prior to docking studies. The three dimensional structure of compound **I, II** and acarbose were obtained from PubChem database for further docking studies.

In brief, prior to docking putative cavities were obtained for each target molecule with a grid resolution of 0.3 Å. Ligand molecules were docked into each cavity separately. The binding cavitiy was set at the coordinate of X: 48.47 Y: 112.95 Z: 20.61 (ntMGAM) and X: 28.62 Y: 35.46 Z: 33.31 (ctMGAM) within a constraint of radius 15 Å. The radius of ligand binding site was restricted to 6 Å assuming all the residues are flexible. Here, algorithm parameters were set to 100 population size and 10,000 iterations with 0.5 scaling factor. Docking was carried out for 100 runs with each ligand for individual target cavities and thereafter, 50 most energetically favoured orientations were selected for subsequent analysis. All the putative docking models for each ligand were analyzed and selected further based on MolDock (MD) score, hydrogen bonding and interaction energy (IE) with respective target domains of MGAM. Above docking studies were performed for 3TOP and also for 3L4Z with above mentioned target ligands. Best docked poses for each docking experiment were subjected to LigPlot analysis (Wallace et al. 1995).

6.3. Results and discussion

6.3.1. The inhibitory effect on α-amylase

All three organic solvent extracts derived from *A. nigra* seeds exhibited various degrees of α-amylase inhibitory activities by *in vitro* assay. Different concentrations ranging from 10-100 µg/ml of each extract was used for the assay and IC_{50} values (the concentrations causing 50 % inhibition) were calculated respectively. Here, the results revealed that the percentage inhibition increased in a dose dependent manner for α-amylase for all the three extracts (Fig. 6.1). Moreover, based on the 50% inhibition (IC_{50}) value, the methanolic extract (S-Met) exhibited the maximum inhibitory activity, followed by S-EtAc and S-Hex (Table 6.1). Statistical analysis revealed S-Met and S-EtAc were significantly active than S-Hex ($p<0.05$, Tukey's test) against α-amylase. It was observed that the inhibitory activities against α-

amylase for all the extracts revealed a relative impact on its antidiabetic potential and were in agreement with the previous reports (Wang et al. 2010b; Ghosh et al. 2012). Among three organic extracts, polar solvent extracts (S-Met and S-EtAc) were found more effective compared to non-polar S-Hex extract against α-amylase.

Similarly, the isolated compounds, **I** and **II** were assayed for the inhibitory effect of α-amylase. As compared to the seed extracts, both the compounds revealed a remarkably higher inhibitory activity against the key enzyme under study. IC_{50} values of compound **I** and **II** were estimated to be 15.167±0.52 and 24.3±2.05 μM for α-amylase inhibition respectively (Table 6.1). Although both the compounds showed a potential inhibition for α-amylase, compound **II** exhibited the maximum inhibition percentage compared to compound **I** (Fig. 6.2). Moreover, based on IC_{50} value, it was observed that both the compounds inhibited α-amylase more significantly ($p<0.01$, Tukey's post-hoc analysis) than the standard drug acarbose (Fig. 6.2; Table 6.1).

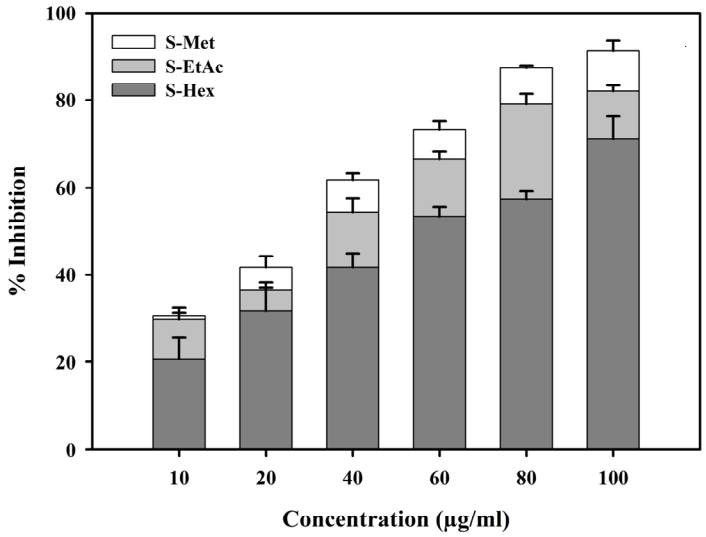


Fig. 6.1 The inhibitory effect of various concentrations of crude extracts from *A. nigra* seeds on α-amylase activity. Values are means ± SE from three independent experiments.

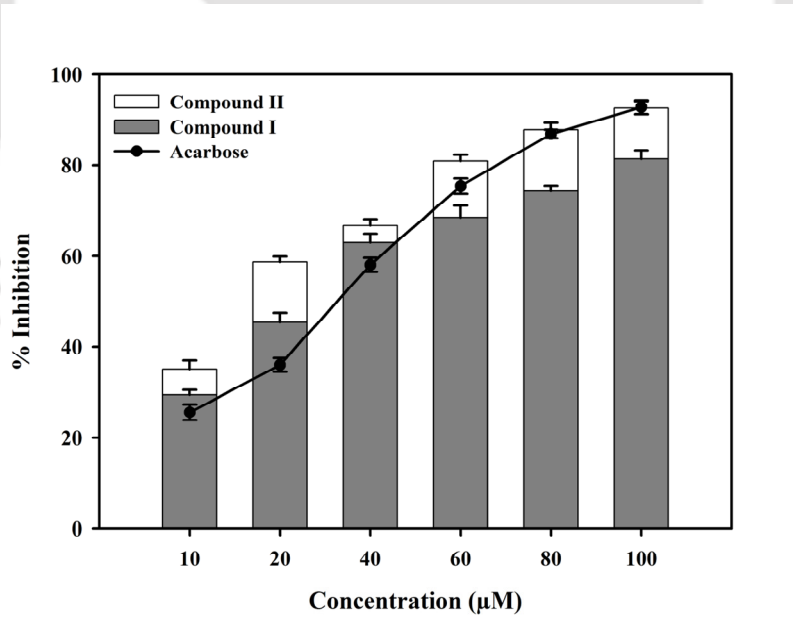


Fig. 6.2 The inhibitory effect of various concentrations of diterpene compounds (**I** and **II**) from *A. nigra* seeds and standard drug acarbose on α-amylase activity. Values are means ± SE from three independent experiments.

Table 6.1 Inhibitory activity of *A. nigra* seed extracts and isolated bioactive compounds against α-amylase.

Sample name		IC_{50}[a]	Sample name		IC_{50}[a]
Crude extracts[b]	S-Hex	53.867±0.09	**Compounds**[c]	**I**	24.3±2.05
	S-EtAc	34.8±0.15		**II**	15.167±0.52
	S-Met	27.767±0.12	**Standard**[d]	Acarbose	32.433±1.43

[a]IC_{50} value is determined as the concentration of the sample to inhibit 50% of enzyme activity under the assay condition.

[b]values for crude extracts were represented in µg/ml.

[c]values for isolated compounds were represented in µM.

[d]Acarbose is used as standard inhibitor in the assay and the value was represented in µM.

All the data were indicated as the mean ± SE.

6.3.2. The inhibitory effect on α-glucosidase

Organic extracts of *A. nigra* seeds and the purified diterpenes were further evaluated to explore the *in vitro* α-glucosidase inhibitory activities. The percent inhibitions of enzymatic activity with crude extracts and test compounds increased in a dose dependant pattern as depicted in Fig. 6.3 and 6.4 respectively. IC_{50} values of S-Met, S-EtAc and S-Hex were determined to be 25.5±2.28, 42.11±5.76 and 75.75±6.00 µg/ml respectively. Statistical analysis revealed S-Met and S-EtAc were similarly active (p>0.05, Tukey's test) where S-Hex extract was significantly less active than other two extracts (p<0.01, Tukey's test) against α-glucosidase. IC_{50} values of **I, II** and acarbose were determined as 10.17±0.094, 5.42±0.05 and 491.67±6.01µM for the test enzyme respectively (Table 6.2). Compound **II** was found significantly effective than **I** (p<0.01, t test) and both the compounds were found significantly more active than acarbose (p<0.01, Tukey's test) against α-glucosidase.

Previously, from the roots of *Gypsophila oldhamiana* various triterpenoid were isolated and reported as potent inhibitor of α-glucosidase with IC_{50} values ranging from

15.2±1.8 to 98.2±1.9 µM (Luo et al. 2008). Reddy et al. (2009b) identified spicatanol and other labdane type diterpenes from *Hedychium spicatum* rhizomes and established spicatanol as most potent labdane molecule (IC_{50} 34.1 µM). Present study suggests that the labdane diterpenes derived from *A. nigra* hold a promising ability and comparatively much effective against α-glucosidase than any previously studied terpenoids.

According to Dai and Mumper (2010), biological efficacy of crude plant extract mainly relies on the nature of compounds present and also its respective quantity in the crude mixture. In general, crude extract contains mixture of various compounds where it is certainly very difficult to understand the real mode of action of any biological study. In the current study, it was observed that two diterpene compounds were present in all the three crude extracts in varying amounts. It was also evident from the present study that these two diterpenes were notably active against both the carbohydrate hydrolysing enzymes and had also employed a remarkable impact on the antidiabetic potential of the crude extracts synergistically. However, the identification of other bioactive compounds and investigations on their individual role or synergistic effect are in progress to unfold the antidiabetic applications for future therapeutics.

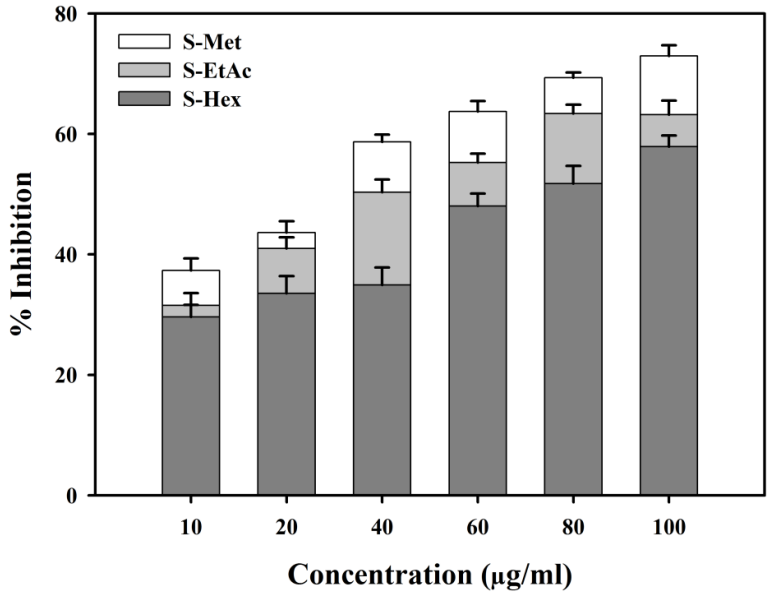


Fig. 6.3 The inhibitory effect of various concentrations of crude extracts from *A. nigra* seeds on α-glucosidase activity. Values are means ± SE from three independent experiments.

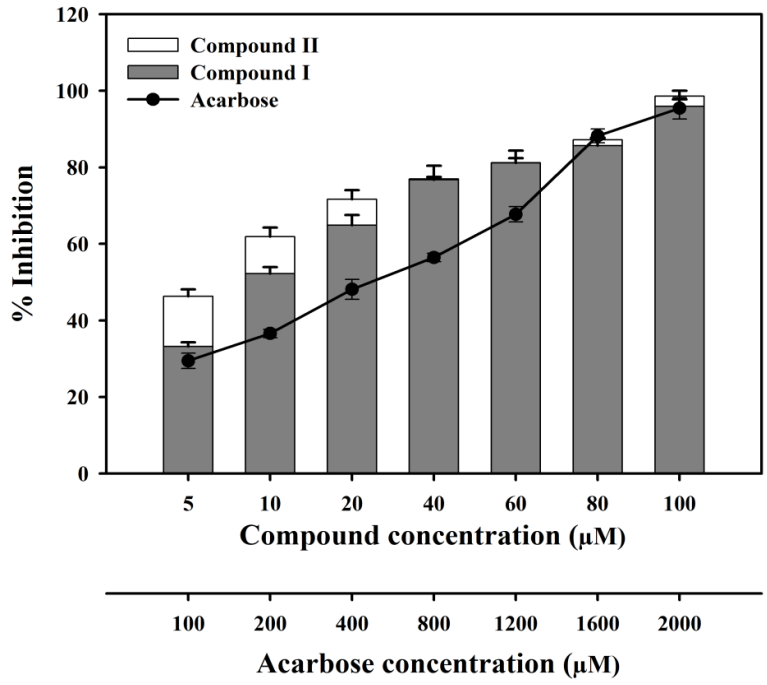


Fig. 6.4 The inhibitory effect of various concentrations of diterpene compounds (**I** and **II**) from *A. nigra* seeds and standard drug acarbose on α-glucosidase activity. Values are means ± SE from three independent experiments.

Table 6.2 Inhibitory activity of *A. nigra* seed extracts and isolated bioactive compounds against α-glucosidase.

Sample name		IC_{50}[a]	Sample name		IC_{50}[a]
Crude extracts[b]	S-Hex	75.75±6.00	**Compounds**[c]	**I**	10.17±0.094
	S-EtAc	42.11±5.76		**II**	5.42±0.05
	S-Met	25.5±2.28	**Standard**[d]	Acarbose	491.67±6.01

[a]IC_{50} value is determined as the concentration of the sample to inhibit 50% of enzyme activity under the assay condition.

[b]values for crude extracts were represented in µg/ml.

[c]values for isolated compounds were represented in µM.

[d]Acarbose is used as standard inhibitor in the assay and the value was represented in µM.

All the data were indicated as the mean ± SE.

6.3.3. Inhibition kinetic studies for α-amylase

The inhibition mode of two isolated diterpene derivative compounds, **I** and **II** were investigated against α-amylase enzyme. LB plots revealed that the mode of inhibition against the key enzyme is non-competitive for their respective substrates. Fig. 6.5 showed that the LB plots for α-amylase in absence or in the presence of various doses of compound **I** and **II** respectively, yielded straight lines which were intercepted at 1/[S] axis. Moreover, with increasing compound concentration both the vertical axis intercept and the slope were increased. This pattern indicates that the interaction of the diterpene compounds affect the velocity of α-amylase catalysed reaction, proportionately to the concentration of the compounds present in the reaction mixture, without affecting the K_m. The characteristic nature of the plots confirmed non-competitive nature of both the compounds for α-amylase with respect to substrates. Kinetic constants for the inhibition of α-amylase were evaluated using LB and Dixon plots and listed in Table 6.3. α-amylase has Michaelis-Menten constant (K_m) of 0.562±0.06% for starch and V_{max} value of 0.566±0.04 µmoles of maltose eq. released/min. The inhibitory constants (K_i), determined from Dixon plots for pancreatic α-

amylase, were 13.303±0.065 and 12.19±0.099 μM of compound **I** and **II** respectively (Table 6.3).

Inhibitory effect and mode of inhibition of α-amylase was previously reported for various plant extracts or their isolated pure compounds. The mode of inhibitions were found competitive, uncompetitive to mixed type in various reports. The nature of the inhibition depends on the structure of the inhibitor compound and catalytic environment of the enzyme. In our study, both the diterpene compounds were showing the non-competitive nature of inhibition against α-amylase enzyme under experimental conditions. Similar to the present study, non-competitive mode of inhibition were observed for α-amylase in presence of a wide range of plant crude extracts (Shobana et al. 2009; Elya et al. 2012; Ghadyale et al. 2012) and other synthetic compounds (China Raju et al. 2010; Balba et al. 2011). However, the mode of inhibition of α-amylase with natural or synthetic labdane diterpene compounds has not been investigated till date.

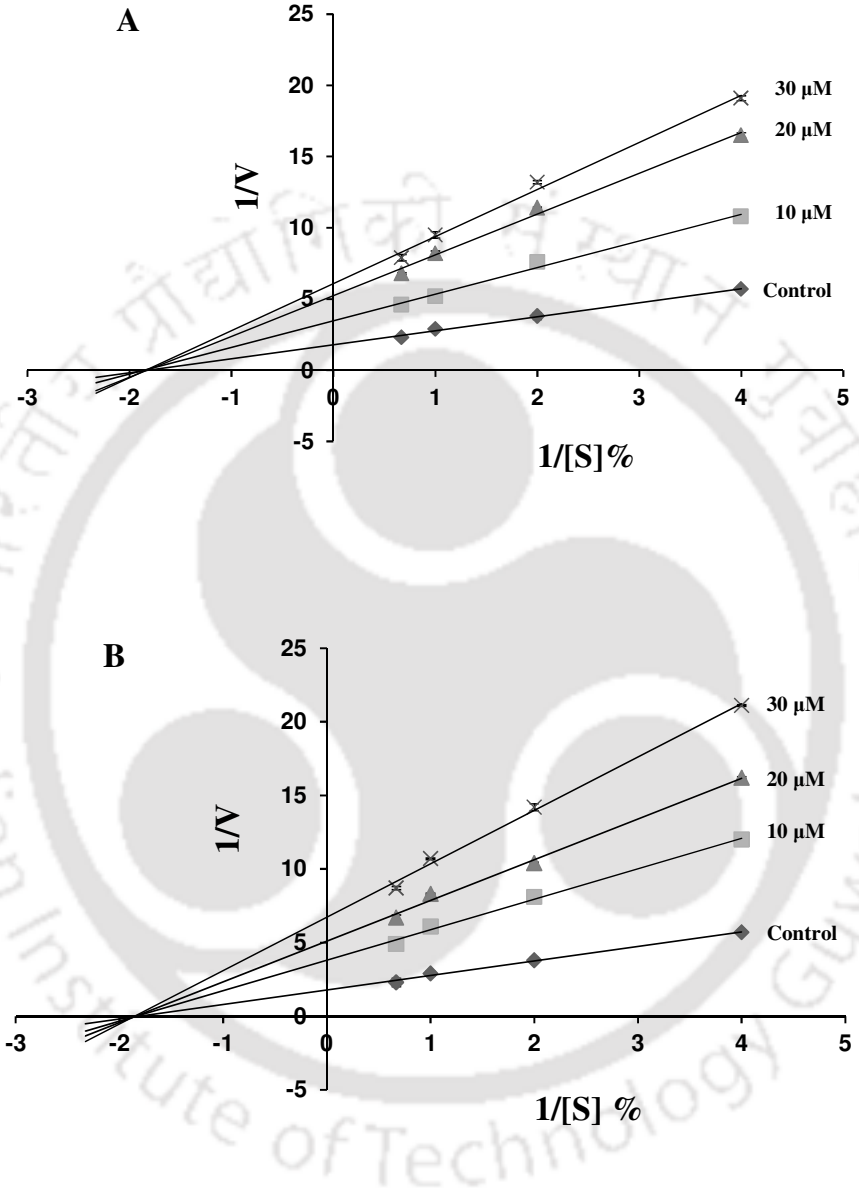


Fig. 6.5 Lineweaver-Burk plot to determine the mode of inhibition of α-amylase activity by compound **I** (A) and compound **II** (B). The kinetics was studied in absence (control) and presence of three different concentrations (10, 20 and 30 µM) of each compound. The data represents as means ± SE from three independent experiments.

Table 6.3 Kinetic properties of compound **I** and **II** on α-amylase

Parameter	I	II
V_{max} [a]	0.566±0.04	0.566±0.04
K_m [b]	0.562±0.06	0.562±0.06
K_i [c]	13.303±0.065	12.19±0.099
V_{10}'	0.323	0.311
V_{20}'	0.226	0.214
V_{30}'	0.168	0.164

[a] µmoles of maltose eq. released/min

[b] value represents Michaelis-Menten constant for starch (%)

[c] inhibitory constant (µM)

V_{10}', V_{20}' and V_{30}' represent apparent velocity of the reaction in presence of 10, 20 and 30 µM of both compounds respectively

6.3.4. Inhibition kinetic studies for α-glucosidase

In order to understand the mode of inhibition of α-glucosidase with **I** and **II**, kinetic inhibition studies were performed. Fig. 6.6 illustrates the LB plots for α-glucosidase in absence or presence of various concentrations of test compounds. Double reciprocal LB plots of enzyme kinetics revealed the intercept at [1/S] for both the compounds. The inhibitory constants (K_i) were determined from Dixon plots for α-glucosidase which indicates the non-competitive to mixed type inhibitions for **I** and **II**. Kinetic constants and other relevant parameters for the inhibition of α-glucosidase are listed in Table 6.4. The decrease of apparent V_{max} with increase of inhibitor concentrations (**I** and **II**) confirmed the characteristic nature of non-competitive inhibition (Fig. 6.6; Table 6.4).

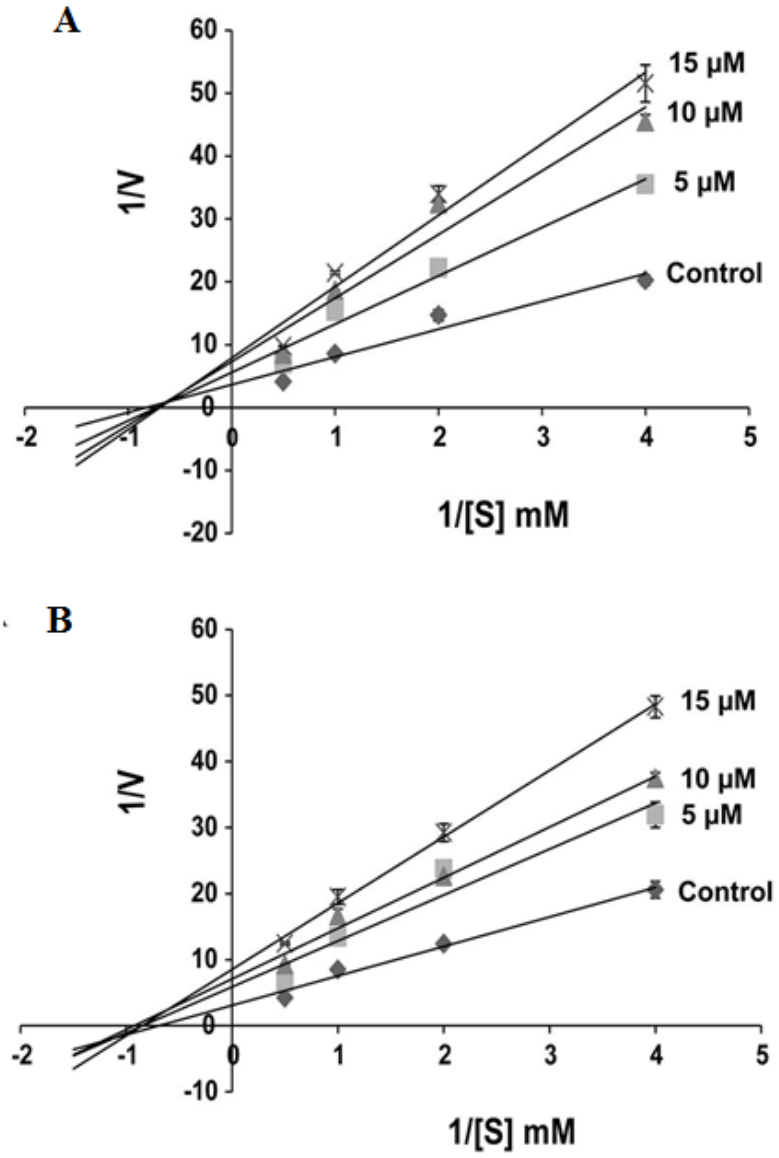


Fig. 6.6 Lineweaver-Burk plot to determine the mode of inhibition of α-glucosidase activity by compound **I** (A) and **II** (B). The kinetics was studied in absence (control) and presence of three different concentrations (5, 10 and 15 µM) of each compound. The data represents as means ± SE from three independent experiments.

Table 6.4 Kinetic properties of compound **I** and **II** on α-glucosidase

Parameter	I	II
V_{max} [a]	0.29±0.01	0.29±0.01
K_m [b]	1.23±0.009	1.23±0.009
K_i [c]	11.61±0.32	11.87±0.09
V_5'	0.178	0.167
V_{10}'	0.137	0.143
V_{15}'	0.127	0.117

[a] µmoles of *p*NPG released/min

[b] value represents Michaelis–Menten constant for *p*NPG (mM)

[c] inhibitory constant (µM)

V_5', V_{10}' and V_{15}' represent apparent velocity of the reaction in presence of 5, 10 and 15µM of both compounds respectively

Previously, a bromophenol compounds from *Grateloupia elliptica* has been studied for α-glucosidase inhibitory activity which revealed mixed type of inhibition with K_i 15.2±2.2 µM (Kim et al. 2008). Gao et al. (2008) reported a hydrolysable tannin called Chebulagic acid which showed non-competitive inhibition against yeast α-glucosidase (IC_{50} value 50 µM). Several other studies has shown the non-competitive to mixed type and even competitive mode of inhibition by various compounds against α-glucosidase (Tadera et al. 2006; Kim et al. 2008; Shobana et al. 2009).

6.3.5. Molecular docking of α-amylase

Docking simulation allowed us to understand the most potential interaction and binding affinity between ligands and the target receptor. The best docked poses of ligands with best binding affinity for HPA at the active site were selected and shown in Fig. 6.7. After post-docking analysis, best docked poses were selected on the basis of MolDock (MD) score and re-rank score as depicted in Table 6.5. Best MD score and re-rank score was obtained for **II** compared to **I**, where, the known inhibitor (acarbose) showed highest MD score and re-rank score among all the ligands under study. MD score adopted here basically depends on a

piecewise linear potential (PLP) introduced by Gehlhaar et al. (1995, 1998) which was further advanced by Yang and Chen (2004) in GEMDOCK. Furthermore, a heuristic algorithm was employed as scoring function which considers the directionality of hydrogen bonding with a re-ranking method to enhance the docking accuracy. Here, the re-rank score refers to a linear combination of E-inter (hydrogen bonding, electrostatic, steric, van der Waals) between the ligand and the receptor, and E-intra (hydrogen bonding, electrostatic, torsion, sp^2-sp^2, van der Waals) of the ligand weighted by pre-defined coefficients (Thomsen and Christensen 2006). Ligand-receptor interaction energy was calculated for each ligand and corresponding best poses were selected. The interaction energy is mainly based on both electrostatic and H-bond energy between ligand and receptor towards better understanding of the binding mode (orientation) of each ligand and possible structure-activity relationship with receptor.

In case of acarbose, the best binding confirmation was found inside the active site cleft of HPA (Fig. 6.8A) which makes 10 H-bonding interactions with various key residues of HPA as shown in Fig. 6.8B. Within the active side cleft of HPA, three important residues *viz*. Asp300, Glu233 and Asp197 are present which are principally responsible for hydrolysis of glycosidic bonds in carbohydrates. Previous report also suggested the critical functions and interactions of these residues with other inhibitors like myricetin and ethyl caffeate (Williams et al. 2012). Here, H-bonding and electrostatic interaction of acarbose within HPA active site are shown in Fig 6.8B and 6.8C respectively.

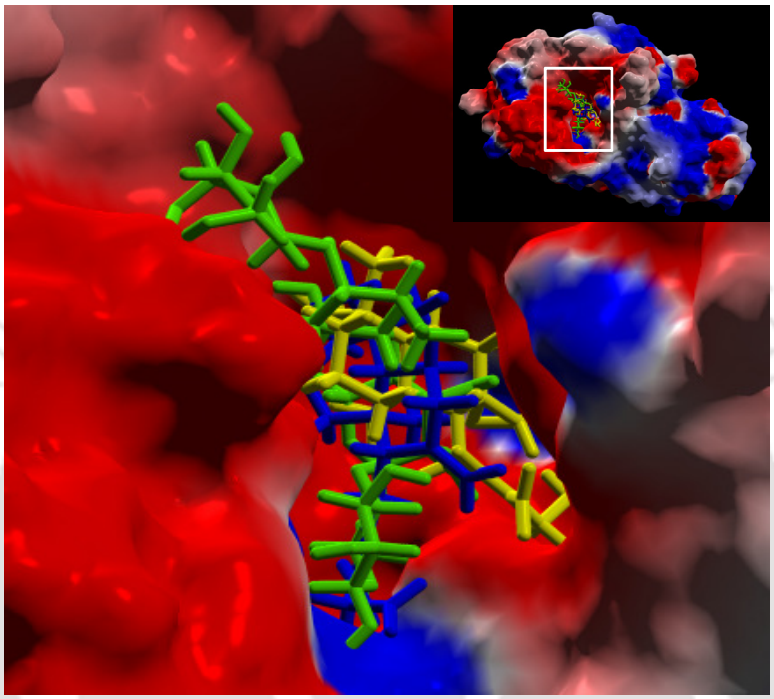


Fig. 6.7 Superimposed confirmations of best docked poses for respective ligands in the HPA active site. Acarbose, compound **I** and **II** were shown in blue, green and yellow colour respectively.

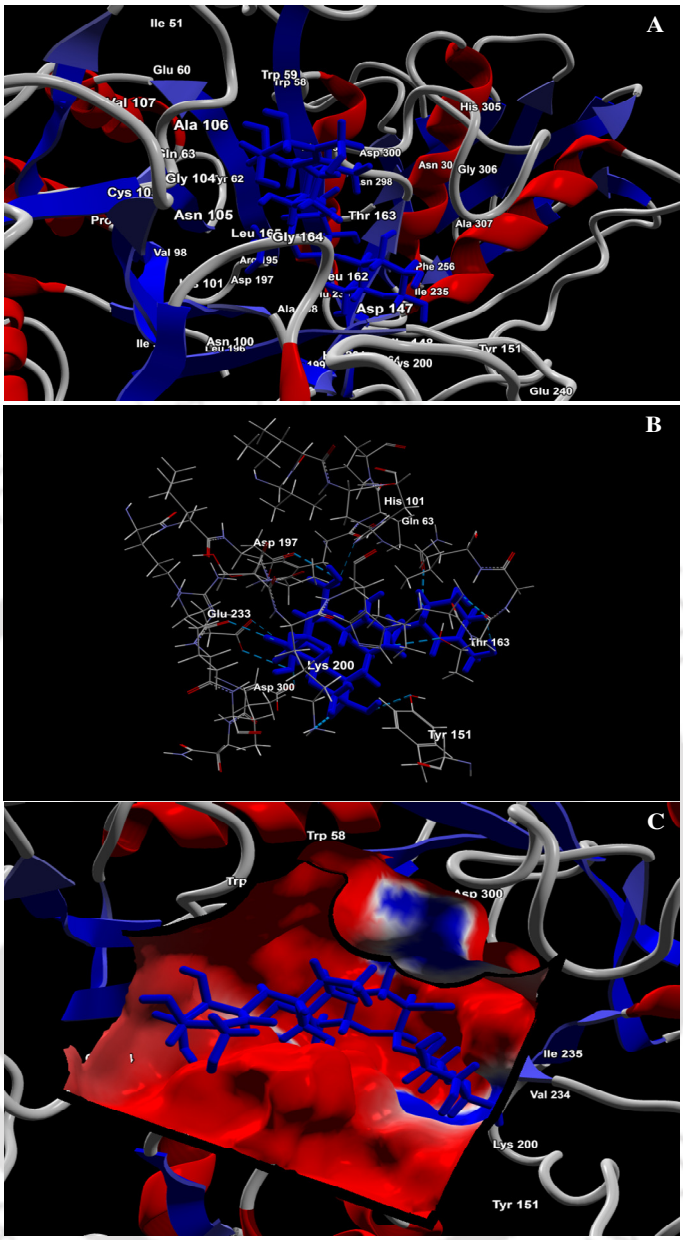


Fig. 6.8 Ligand-protein interaction for the best pose at the major binding cleft and interactive nearby residues were depicted for acarbose. (A) Predicted bonded and non-bonded interactions between acarbose and amino acid residues present in the active site of HPA. (B) Predicted ten H-bonded interaction between acarbose and the residues at the active site region. Here, blue dotted lines represent the H-bonding interactions between the ligand molecules and HPA. (C) Predicted non-bonded electrostatic interaction between acarbose and the residues at the active site region.

Both the compounds (**I** and **II**) were also docked with HPA where compound **II** showed higher MD and re-rank score as compared to compound **I** (Table 6.5). The predicted interactions between both the compounds and active site residues of HPA are shown in Fig. 6.9A and 6.9B. Notably, in these bound conformation, both the compounds showed bonded and non-bonded interactions with various key residues in the active site of HPA having two H-bond interactions. The H-bonding interaction of both the compounds (**I** and **II**) with HPA is shown in Fig. 6.10A and Fig. 6.10B where **II** were bonded with Arg195 and Asn298 residues and **I** showed H-bonding with Lys200 and Ile235 of HPA active site. The overall H-bonding interaction energy of **II** was found less than **I** which might have resulted due to the interaction distance and the individual interaction energy between ligand and interacting residues respectively. However, the overall interaction energy between the ligands and receptor revealed that compound **II** has better binding affinity to HPA than **I**. This suggests, other non-bonding interaction also plays a critical role apart from H-bonding interaction towards the binding of ligand with receptor in an energetically favoured condition. The non-bonded electrostatic interactions of both the compounds within HPA active site is depicted in Fig. 6.11.

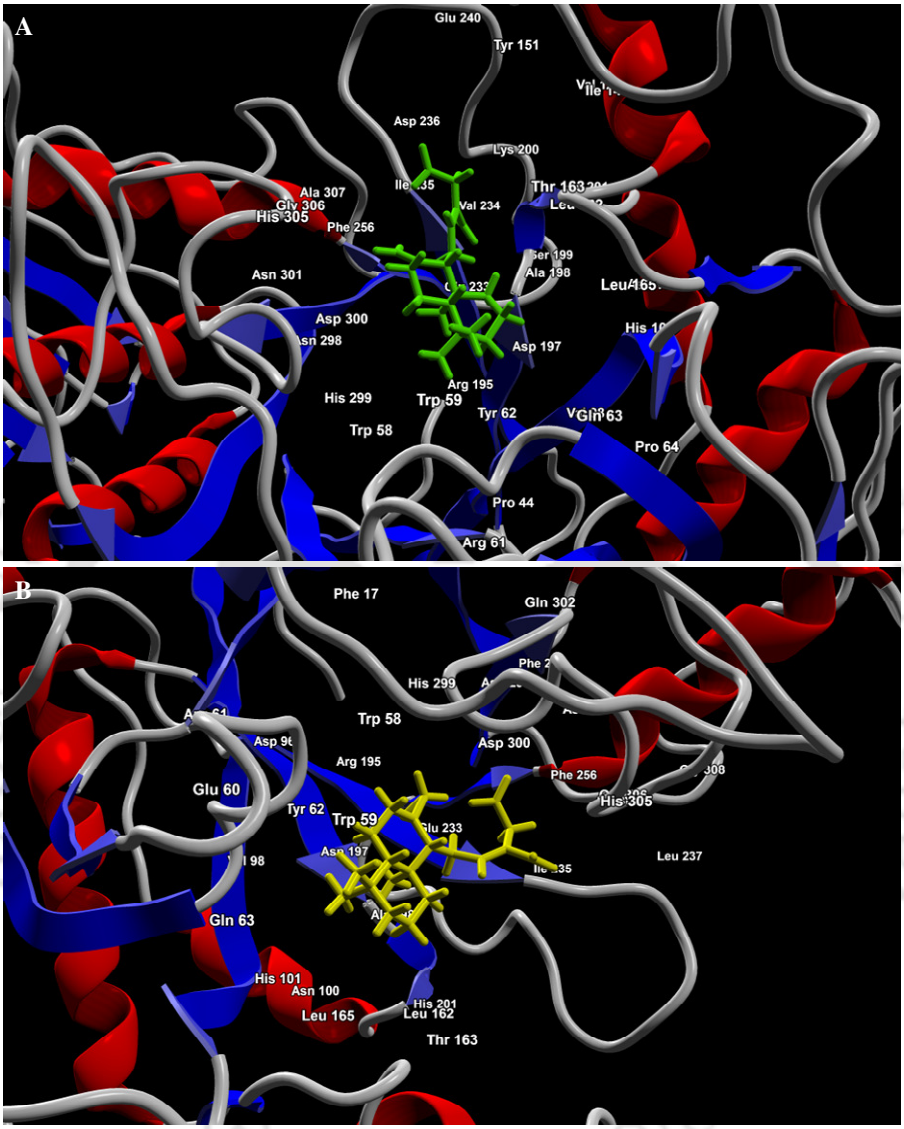


Fig. 6.9 Ligand-protein interaction for the best pose at the major binding cleft and interactive nearby residues were depicted for compound **I** and **II**. (A) and (B) Predicted bonded and non-bonded interactions between amino acid residues present in the active site of HPA and diterpene molecules (**I** and **II** respectively).

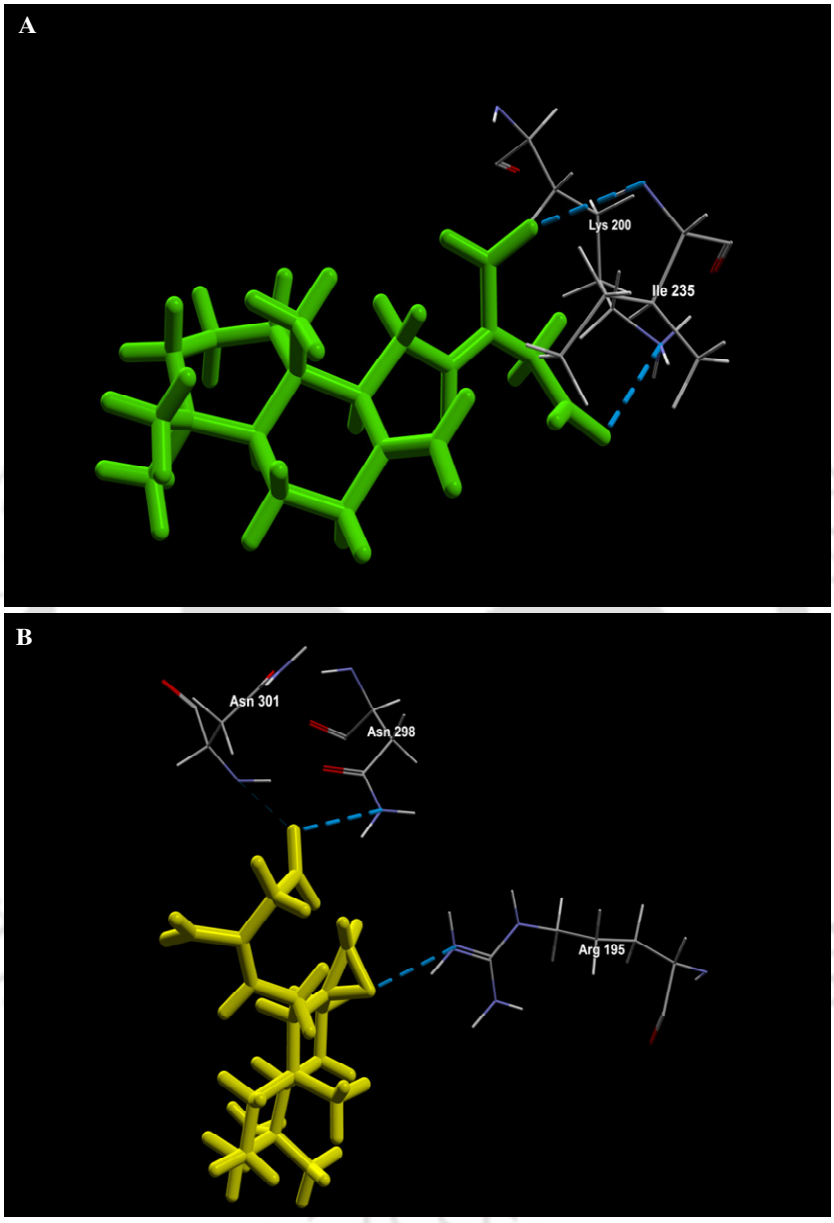


Fig. 6.10 Predicted H-bonded interaction of **I** (A) and **II** (B) with the residues at the active site region of HPA. Here, blue dotted lines represent the H-bonding interactions between the ligand molecules and HPA.

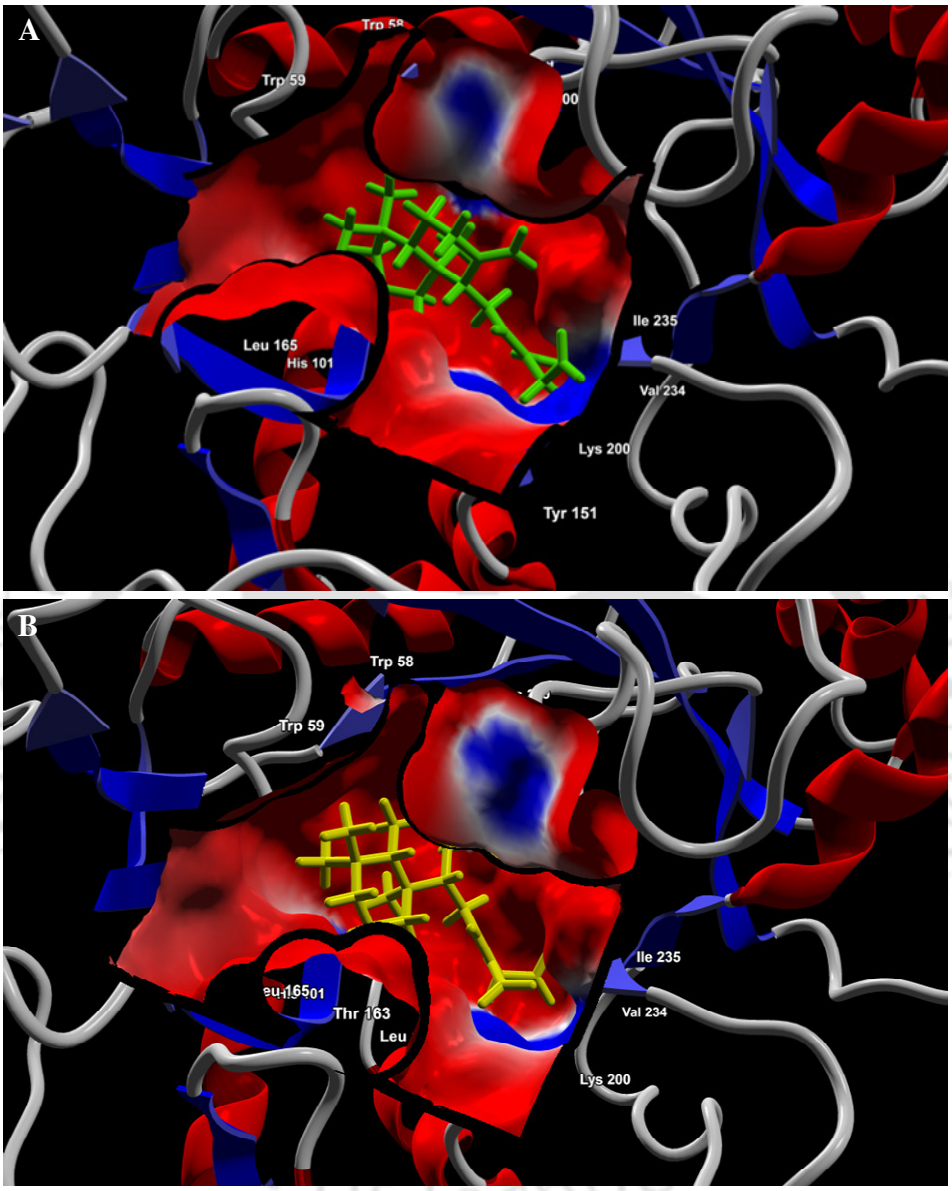


Fig. 6.11 Predicted non-bonded electrostatic interaction of **I** (A) and **II** (B) with the residues at the active site region of HPA.

Table 6.5 Theoritical affinity of best docked poses for standard inhibitor (acarbose) and diterpene compounds (**I** and **II**) with HPA

Ligand	H-bonded residues	Interaction energy (Kcal/mol)	Interaction dist. (Å)	H-Bond energy (Kcal/mol)	Interaction energy (Kcal/mol)	MolDock Score	Rerank score
Acarbose	Asp300	-1.73	4.10	-18.8607	-162.532	-121.3	-117.711
	Glu233	-1.72	3.20				
	Lys200	-2.5	2.90				
	Tyr151	-2.5	2.96				
	Asp197	-2.5	2.99				
	Thr163	-2.5/-1.72, -2.5	2.71/3.26, 3.10				
	His101	-0.86	2.40				
	Gln63	-2.5	2.75				
Compound **I**	Ile235	-2.5	2.91	-3.62972	-108.4	-102.342	-79.8406
	Lys200	-2.5	2.99				
Compound **II**	Arg195	-2.35	2.94	-2.87389	-114.369	-108.034	-88.9548
	Asn298	-2.5	3.07				

LigPlot is known as the comprehensive tool for expressing the hydrogen bonding and hydrophobic interactions involving the ligand molecule and active site residues (Wallace et al. 1995). Here, we have also confirmed by generating LigPlots for all the best fit docked poses for each compound including acarbose with HPA (Fig. 6.12). LigPlots for compound **I** and **II** revealed the same interacting residues as obtained in docking studies and both the compounds showed two H-bonding interactions with respective active site residues. LigPlot analyses were precisely advantageous in identifying the hydrophobic interaction pattern. Here for **I** and **II**, majority of the active site residues actively participated in non-bonded hydrophobic interactions in the vicinity of the ligands (Fig. 6.12A and B).

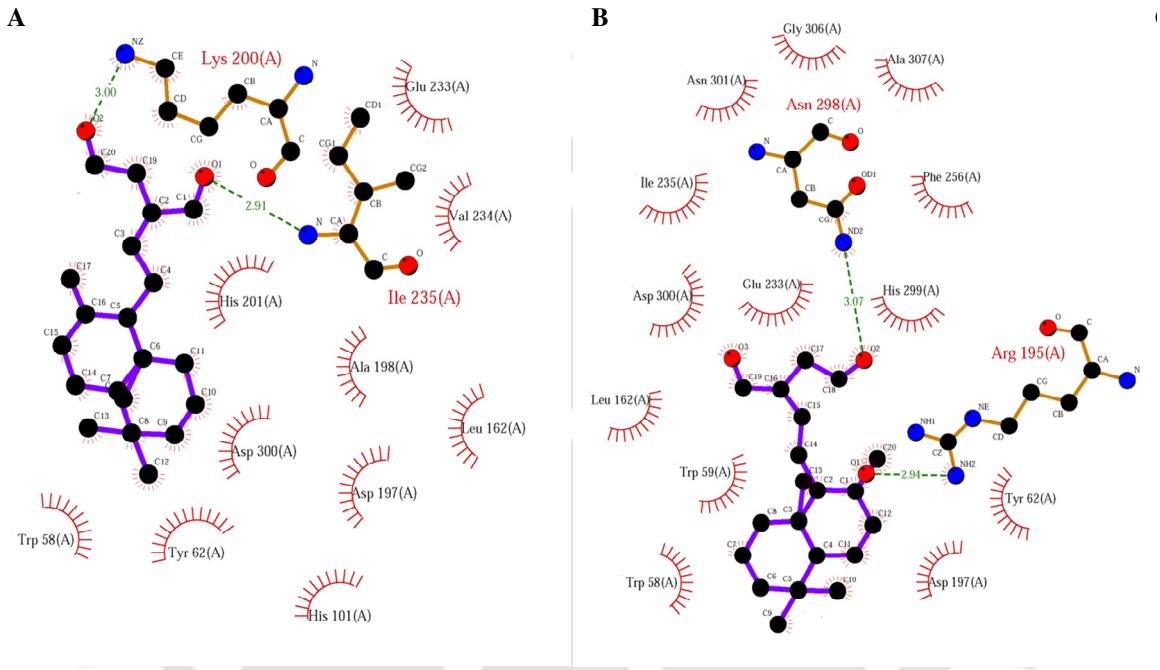


Fig. 6.12 LigPlot generated for the best poses obtained with compound **I** (A) and compound **II** (B), against HPA crystal structure. Hydrogen bonding interactions were shown as green dotted lines. Besides the H-bonding residues, other surrounding amino acids (in the vicinity of ligand) describe the hydrophobic interactions.

Similarly, clear interactions of acarbose with Asp197, Glu233 and Asp300 were observed along with other H-bonding residues in HPA active site. It is important to mention here that Asp197 of HPA was reported previously as the catalytic nucleophile in hydrolysis reactions for any polymeric substrates like dietary starch (Rydberg et al. 2002; Zhang et al. 2009). Similarly, Glu233 is known to act as an acid-base catalyst during substrate hydrolysis reactions (Williams et al. 2012). Asp300 was also previously identified as the key player for optimizing the orientation of substrate molecule using hydrogen bonding interaction and even as regulator of steric conflicts for a better binding conformation of substrate (Li et al. 2005a; Williams et al. 2012).

6.3.6. Molecular docking of α-glucosidase

To verify the observed mode of inhibitions by the isolated diterpenes, molecular docking of human maltase-glucoamylase (MGAM) was performed with both the compounds including acarbose. Here, we have taken the crystal structure of N-terminal domain of MGAM (3L4Z) and C-terminal domain of MGAM (3TOP). Prior to docking all bound inhibitors were removed from the crystal structure and subsequently docking were performed with acarbose, **I** and **II** for all putative binding cavities using MVD with above mentioned stochastic algorithms. Docking simulation revealed **I** and **II** were binding to the active site cavity of ntMGAM which was markedly similar to acarbose binding site considering the best dock poses in each case (Fig. 6.13A). Similarly, in case of ctMGAM both the diterpenes fitted themselves inside the enzyme cleft adjacent to the acarbose binding site of 3TOP allowing the best docked poses respectively (Fig. 6.13B).

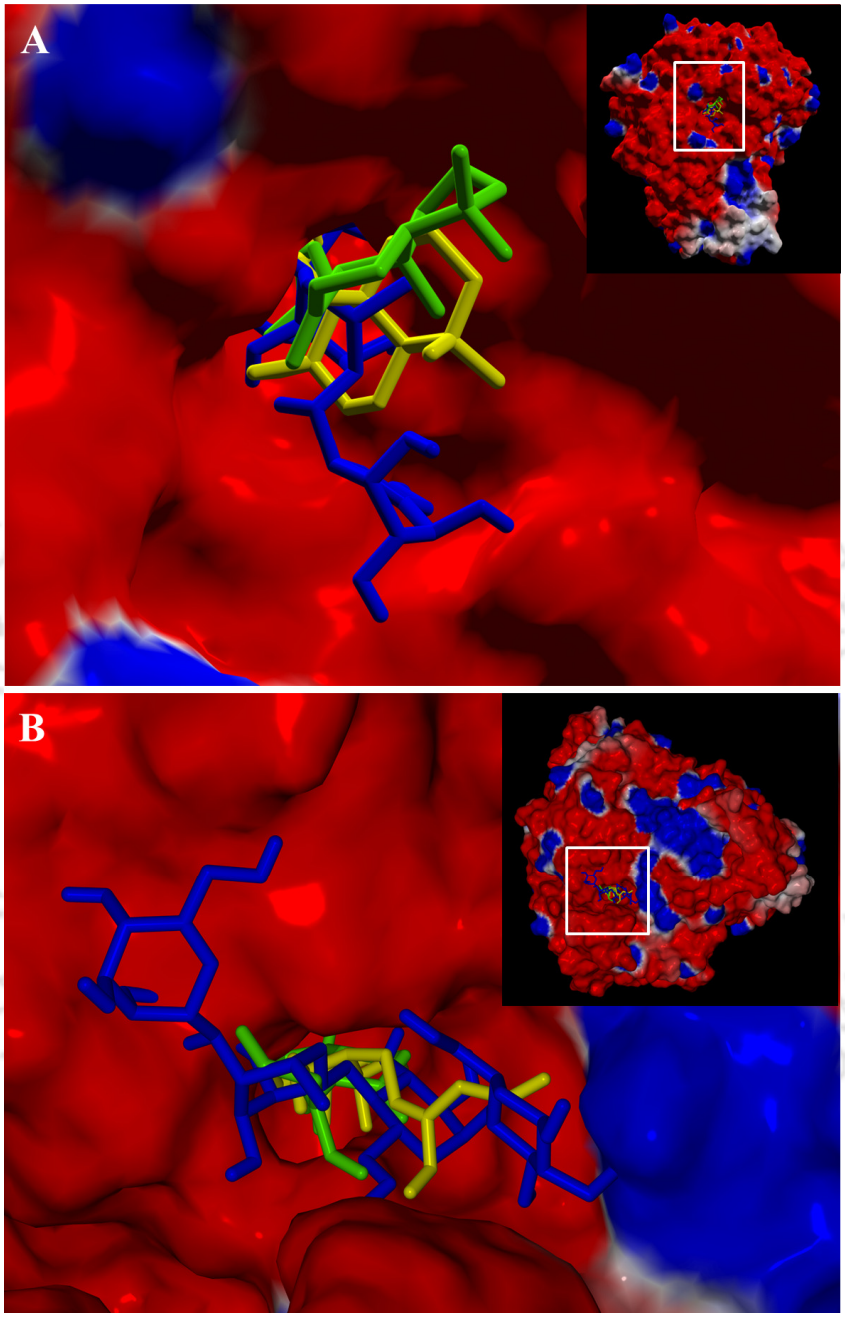


Fig. 6.13 Superimposed confirmations of best docked poses for respective ligands in the 3L4Z (A) and 3TOP (B) active sites. Acarbose, compound **I** and **II** were shown in blue, green and yellow colour respectively.

The top docked poses of ligands with best binding affinity for 3L4Z at the active site were selected and shown in Fig. 6.13A. Compound **II** was found as the strongest ligand for ntMGAM with remarkably higher MD score (-103.645), re-rank score (-82.714) and IE (-109.574 Kcal/mol) than that of **I**. However, the standard drug (acarbose) showed highest MD score and re-rank score among all the ligands under study. The docking results of ntMGAM with acarbose, **I** and **II** are shown in Table 6.6.

Table 6.6 Theoretical affinity of best docked poses for standard inhibitor (acarbose) and diterpene compounds (**I** and **II**) with ntMGAM (3L4Z)

Ligand	H-bonded residues	Interaction energy (Kcal/mol)	Interaction dist. (Å)	H-Bond energy (Kcal/mol)	Interaction energy (Kcal/mol)	MolDock Score	Rerank score
Acarbose	Thr205	-2.5	2.89	-10.9192	-114.372	-105.451	-91.632
	Tyr214	-2.5	3.07				
	Asp203	-2.32	2.58				
	His600	-2.5	3.04				
	Asp571	-2.22	2.57				
	Asp542	-2.5/-1.62	2.92/2.49				
	Trp406	-0.23	2.91				
Compound **I**	Arg526	-2.5	2.89	-1.93349	-102.261	-95.7534	-78.312
Compound **II**	Arg526	-1.9871	3.20	-2.40041	-109.574	-103.645	-82.714
	Trp406	-0.4098	3.10				

The best binding confirmation of acarbose was found inside the active site cleft of 3L4Z (Fig. 6.14A) which makes 8 H-bonding interactions with various key residues of ntMGAM as shown in Fig. 6.14B. The acarbose is held inside the active site cleft primarily through hydrogen bonding with Asp542 originating from the $(\beta/\alpha)_8$ barrel, and by Asp203, originating from the N-terminal domain loop (residues 200-217). This N terminal loop comes in close contact with the active-site region and open up the $(\beta/\alpha)_8$ barrel. The binding of acarbose at the active site involves many hydrogen bonds, particularly with the acarvosine rings similar to the earlier report (Sim et al. 2008). In the present study, majority of the interacting residues originate from the $(\beta/\alpha)_8$ barrel of ntMGAM, specifically hydrogen bonding through Asp542, Asp 571 and His600. Additional residues surrounding the other subsite of acarbose include

Asp443, Tyr299, Ile328, Ile364, Trp441, Met444 and Arg526. Previously, Asp443 has been studied and established as catalytic nucleophile of ntMGAM by mutagenesis (Nichols et al. 2003) and substrate trapping studies with sequence alignments conducted on glycosyl hydrolase family 31 (GH31) members (Lovering et al. 2005). Moreover, Asp542 is known as highly conserved amino acid among GH31 members which is nearly 6Å away from Asp443 and making it a possible candidate for the acid/base catalyst. The electrostatic interaction of acarbose within 3L4Z active site is shown in Fig 6.14C.

The best docked poses obtained for **I** and **II** with 3L4Z and interacting residues at the active site were highlighted accordingly (Fig. 6.15). Docking of **I** with ntMGAM revealed one H-bonding interaction with Arg526 (2.89 Å) whereas, Arg526 and Trp406 in the active site interacted via H-bonding with **II** at a distance of 3.2 and 3.1 Å (Fig. 6.16). The overall H-bonding interaction energy of **I** was found less than **II** which might have resulted due to the number of interaction, interaction distance and the individual interaction energy between ligand and interacting residues respectively (Table 6.6). The non-bonded electrostatic interactions of both the compounds within ntMGAM active site are depicted in Fig. 6.17. The best docked poses of **I** and **II** showed many hydrophobic interactions with bulky residues and similar to acarbose, Met444, Asp 327, Tyr299 and Phe575 residues showed energetically favoured hydrophobic interactions with both the diterpene compounds.

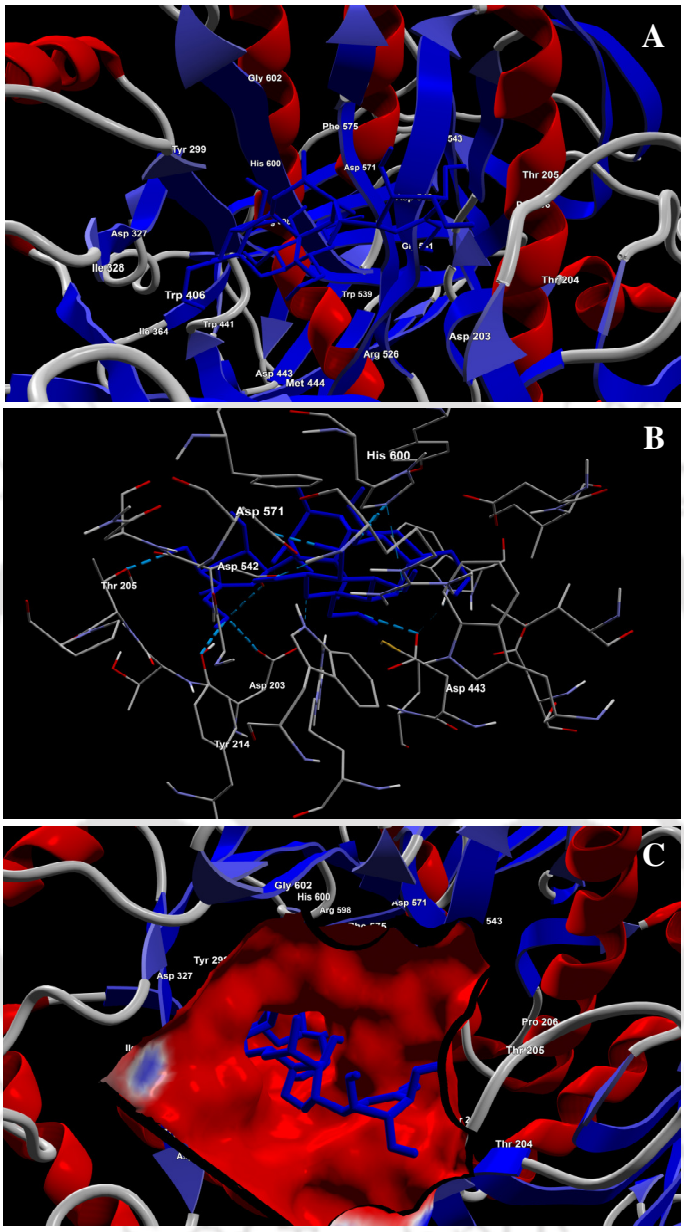


Fig. 6.14 Ligand-protein interaction for the best pose at the major binding cleft and interactive nearby residues were depicted for acarbose. (A) Predicted bonded and non-bonded interactions between acarbose and amino acid residues present in the active site of 3L4Z. (B) Predicted eight H-bonded interaction between acarbose and the residues at the active site region. Here, blue dotted lines represent the H-bonding interactions between the ligand molecules and 3L4Z. (C) Predicted non-bonded electrostatic interaction between acarbose and the residues at the active site region.

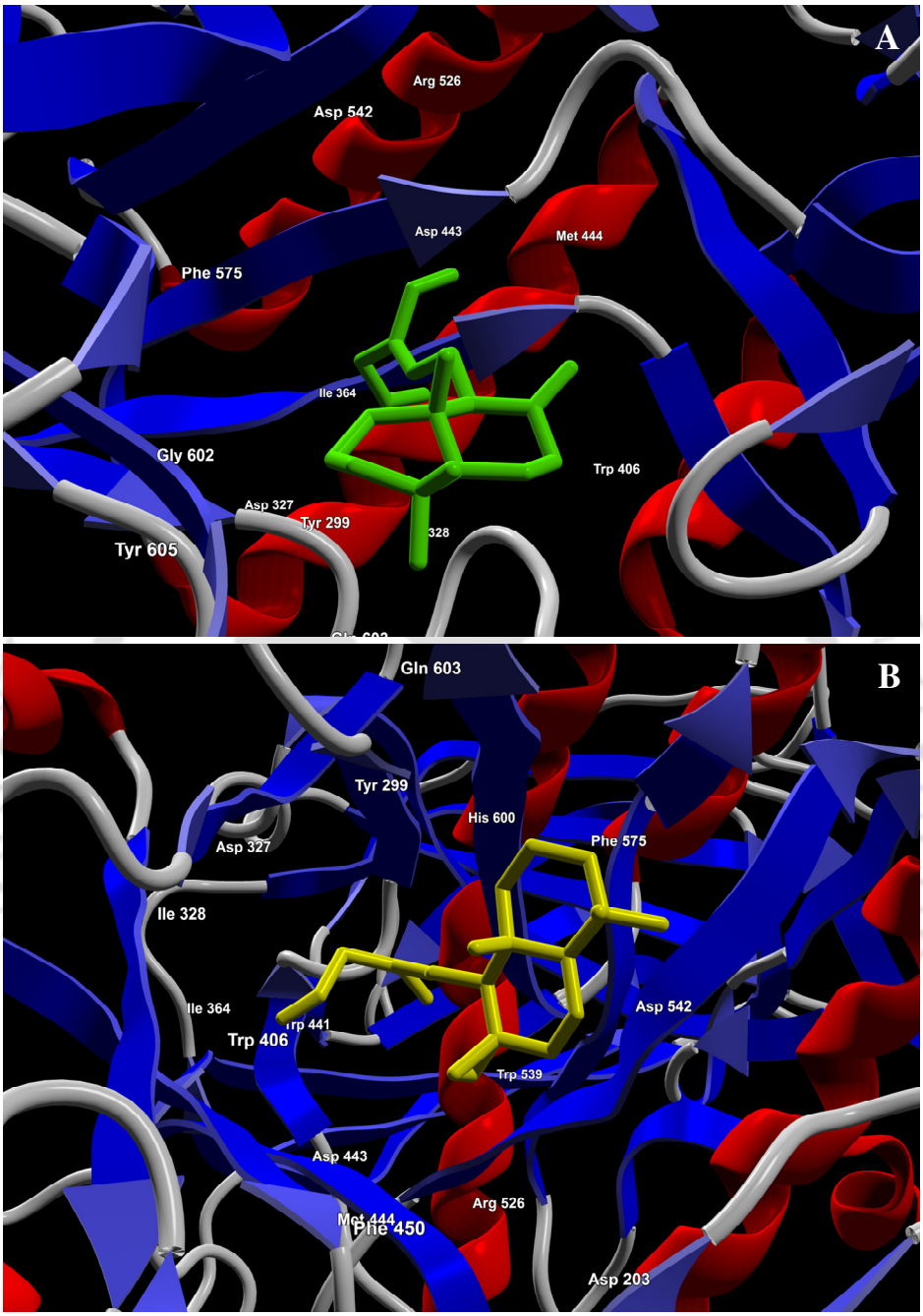


Fig. 6.15 Ligand-protein interaction for the best pose at the major binding cleft and interactive nearby residues were depicted for compound **I** and **II**. (A) and (B) Predicted bonded and non-bonded interactions between amino acid residues present in the active site of 3L4Z and diterpene molecules (**I** and **II** respectively).

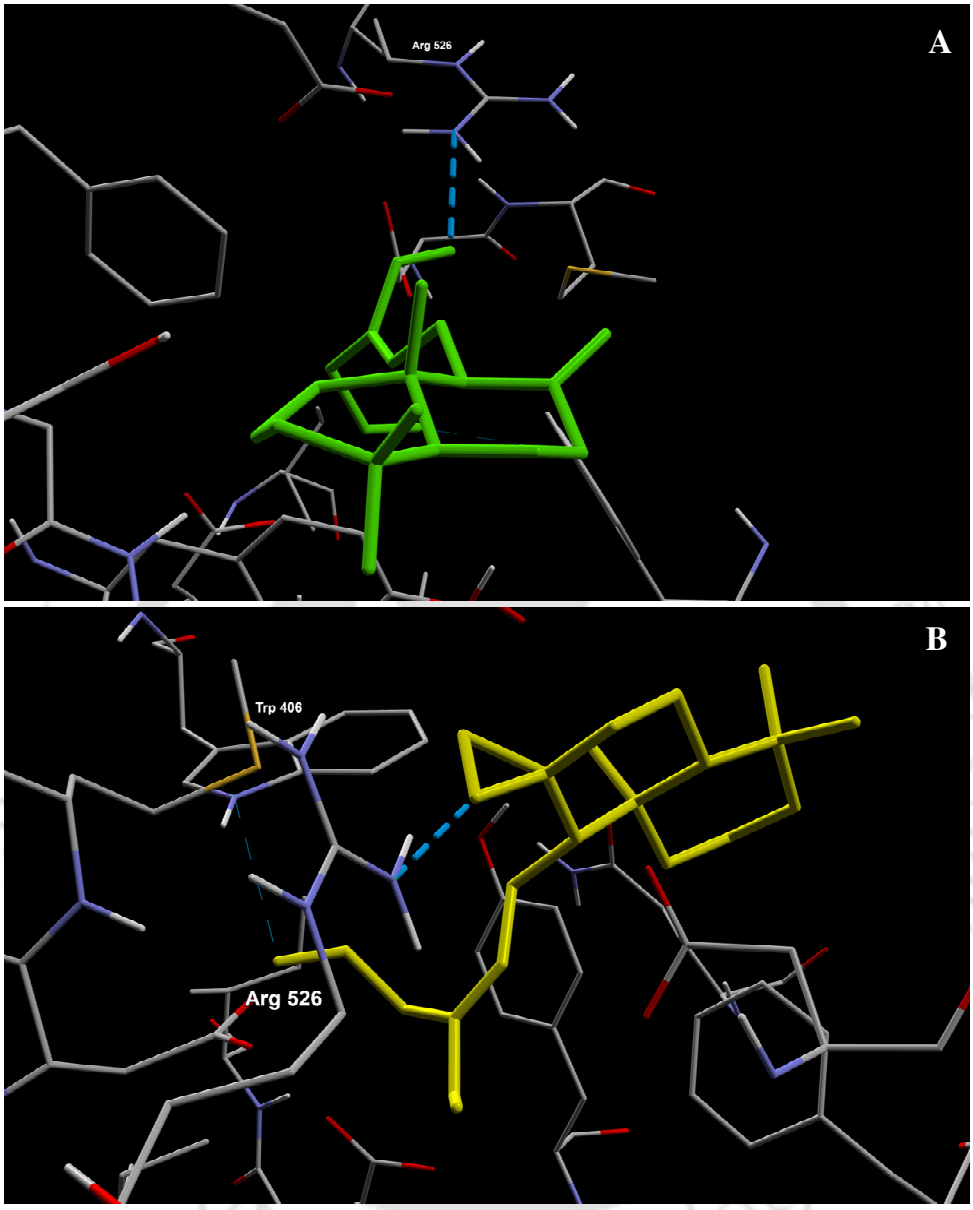


Fig. 6.16 Predicted H-bonded interaction of **I** (A) and **II** (B) with the residues at the active site region of 3L4Z. Here, blue dotted lines represent the H-bonding interactions between the ligand molecules and 3L4Z.

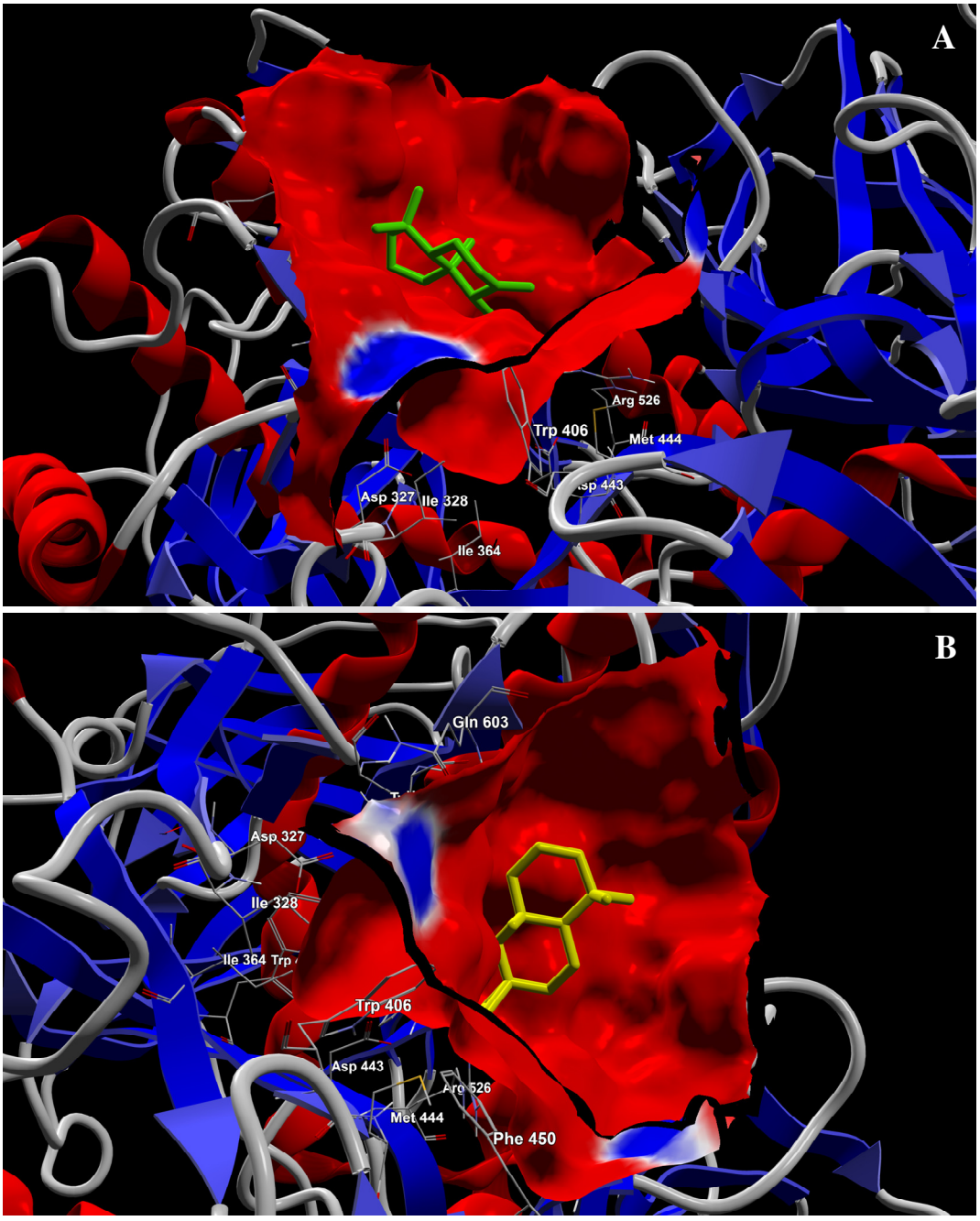


Fig. 6.17 Predicted non-bonded electrostatic interaction of **I** (A) and **II** (B) with the residues at the active site region of 3L4Z.

LigPlot analysis of the best poses for **I** and **II** with ntMGAM were in agreement with the observed docking results (Fig. 6.18). LigPlot analyses were precisely advantageous in identifying the hydrophobic interaction pattern. Here for **I** and **II**, majority of the active site residues actively participated in non-bonded hydrophobic interactions in the vicinity of the ligands (Fig. 6.18A and B). In the present study, docking simulation and LigPlot analysis revealed that the H-bonding interaction of **II** with Trp406 was in close proximity (3.1 Å) than that of hydrophobic interaction previously reported for kotalanol and de-O-sulfonated kotalanol (3.9 Å) with ntMGAM (Sim et al. 2010).

Docking results of compound **I** and **II** with ctMGAM (3TOP) revealed that both the diterpenes fitted themselves inside the enzyme cleft adjacent to the acarbose binding site on ctMGAM allowing the best docked poses respectively. In case of acarbose, the interacting residues at the active site of ctMGAM is depicted in Fig. 6.19A. Here, acarbose achieved the highest IE (-158.497 Kcal/mol) with 10 H-bonding involving seven amino acid residues at the active site of ctMGAM (Table 6.7; Fig 6.19B). The electrostatic interaction of acarbose within ctMGAM active site is shown in Fig 6.19C. Similar to acarbose, both the compounds (**I** and **II**) were docked with ctMGAM. The predicted interaction residues at the binding site of ctMGAM were shown in Fig 6.20. Here, MD score of both the compounds were found significantly higher than that of the standard drug, acarbose (Table 6.7). However, compound **II** exhibited highest MD score (-120.056) among all the ligands tested for the present study. Similarly, **II** achieved higher IE (-119.781 Kcal/mol) compared to **I** at the best docked pose. In case of re-rank score **I** was found remarkably higher (-89.0112) than other ligands.

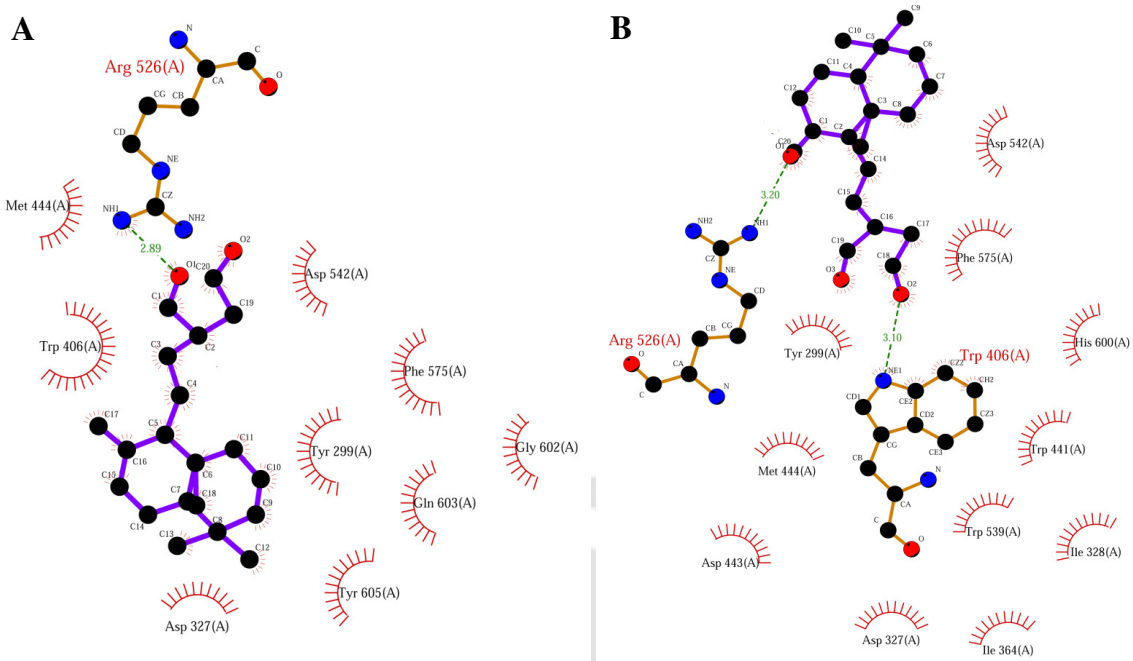


Fig. 6.18 LigPlot generated for the best poses obtained with compound **I** (A) and compound **II** (B) against 3L4Z crystal structure. Hydrogen bonding interactions were shown as green dotted lines. Besides the H-bonding residues, other surrounding amino acids (in the vicinity of ligand) describe the hydrophobic interactions.

Table 6.7 Theoretical affinity of best docked poses for standard inhibitor (acarbose) and diterpene compounds (**I** and **II**) with ctMGAM (3TOP)

Ligand	H-bonded residues	Interaction energy (Kcal/mol)	Interaction dist. (Å)	H-Bond energy (Kcal/mol)	Interaction energy (Kcal/mol)	MolDock Score	Rerank score
Acarbose	Arg1510 Asp1157 Asp1526 Lys1460 Thr1586 Trp1369 Thr1589	-2.5 -1.13 -2.26/-1.50 -2.5 -2.5/-0.62/ -2.13 -1.19 -1.95	2.92 2.98 3.15/3.30 2.91 2.13/2.60/3. 17 2.66 3.18	-11.7739	-158.497	-99.6316	-87.1888
Compound **I**	Thr1586 Trp1369	-2.5 -1.49	2.61 3.22	-1.4907	-113.963	-107.535	-89.0112
Compound **II**	Thr1586 Trp1369	-2.5 -1.27	3.03 3.10	-3.049	-119.781	-120.056	-83.39

Docking of compounds with ctMGAM revealed that both the diterpene compounds were interacting at ctMGAM binding site by two H-bonds each. The residues, Thr1586 and Trp1369 of ctMGAM were involved in the H-bond interaction with both the compounds (Fig. 6.21). Apart from H-bond interaction, several other residues were interacting via electrostatic interactions with both the compounds (Fig. 6.22). It is evident from the previous studies that lower interaction energy scores indicate better biding affinity between ligand and target protein (Thomsen and Christensen 2006). Here, it is assumed that apart from H-bonding interactions between the MGAM and diterpenes, the non-polar interactions of isoprene backbone of diterpenes with hydrophobic environment inside the active site supports the strong binding affinity.

LigPlot analysis of the best docked poses for both diterpenes further confirmed the hydrogen bonding and hydrophobic interactions involving the ligand molecules and active site residues of ctMGAM (Fig. 6.23). Considering the H- bond interaction, Trp1369 and Thr1586 residues of ctMGAM were interacting with two aldehydic oxygen molecules of compound **I**, whereas in case of compound **II**, the same residues were interacting with aldehydic and epoxide oxygen respectively. Nearly 13 other residues of ctMGAM were involved in hydrophobic interactions with both the diterpene molecules individually. Among those residues, 11 residues were common for both the compounds indicating the similar pattern of interaction with ctMGAM binding site. Stabilization of the cyclic isoprene subunit may result from hydrophobic interactions with bulk side chains of residues Tyr1251 and Trp1418 similar to the acarbose in the present study and earlier report (Ren et al. 2011). Similarly, Phe1560 also helps in stabilization of both the diterpenes and acarbose in the active site of ctMGAM by hydrophobic interactions. Residues Trp1355 and Phe1559 stack with the first and second rings of acarbose, and both the residues showed hydrophobic interaction with two diterpenes under study.

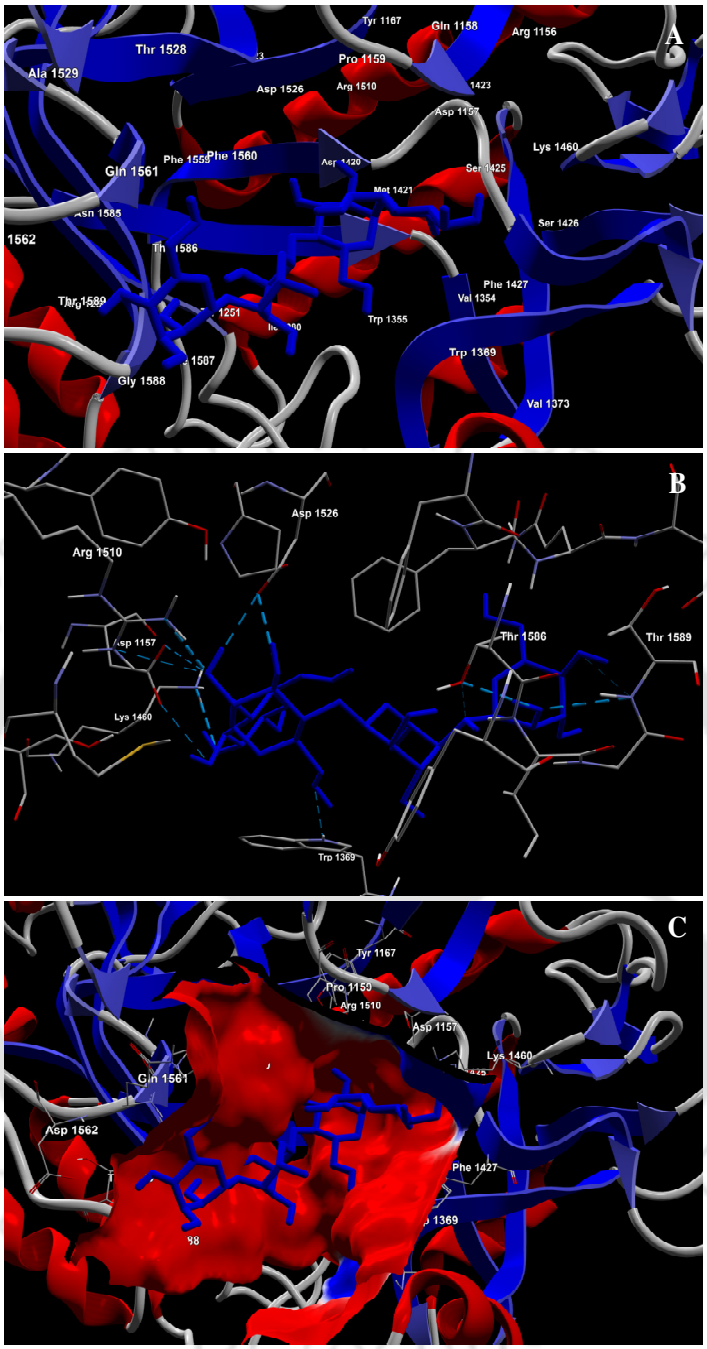


Fig. 6.19 Ligand-protein interaction for the best pose at the major binding cleft and interactive nearby residues were depicted for acarbose. (A) Predicted bonded and non-bonded interactions between acarbose and amino acid residues present in the active site of 3TOP. (B) Predicted ten H-bonded interaction between acarbose and the residues at the active site region. Here, blue dotted lines represent the H-bonding interactions between the ligand molecules and 3TOP. (C) Predicted non-bonded electrostatic interaction between acarbose and the residues at the active site region.

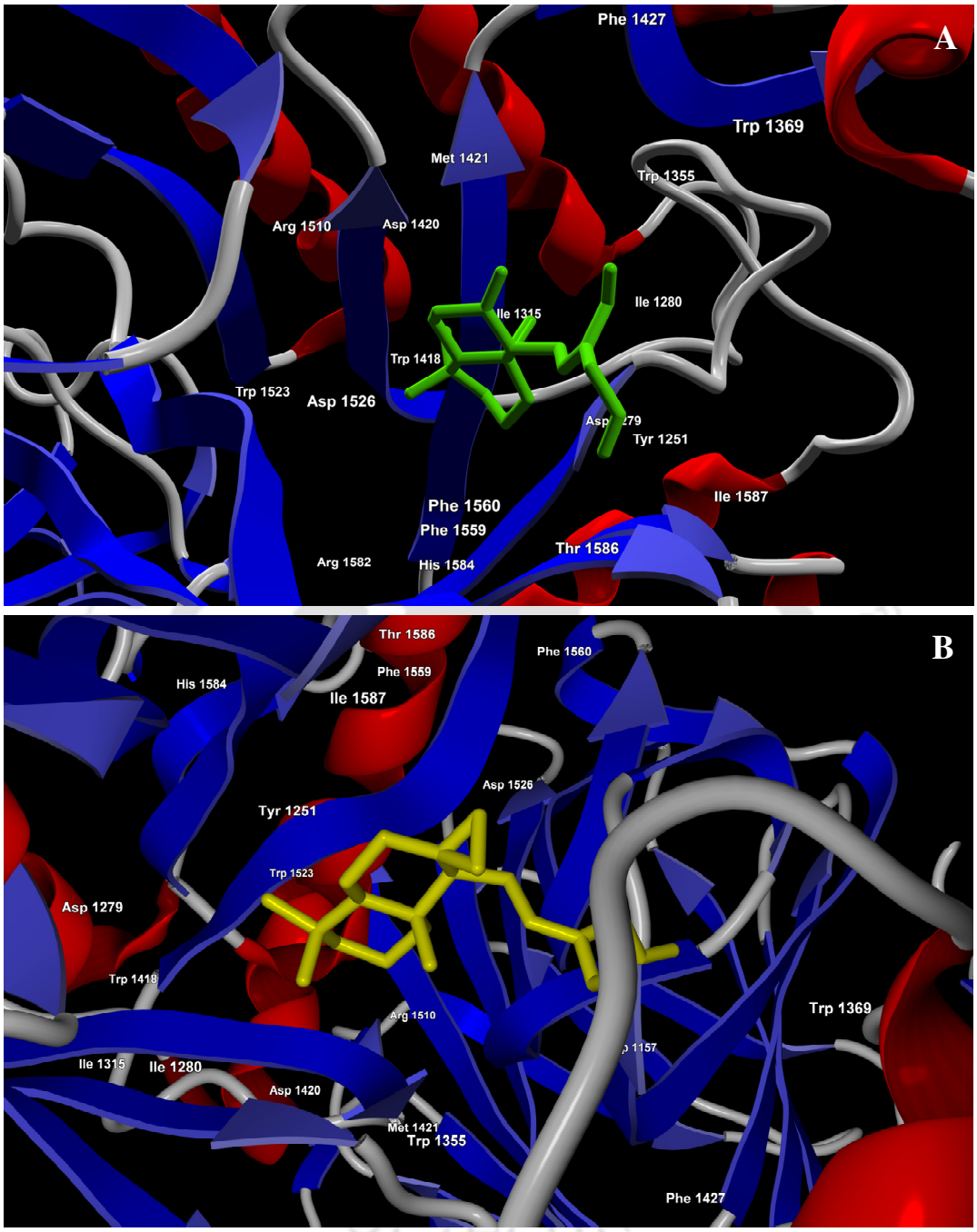


Fig. 6.20 Ligand-protein interaction for the best pose at the major binding cleft and interactive nearby residues were depicted for compound **I** and **II**. (A) and (B) Predicted bonded and non-bonded interactions between amino acid residues present in the active site of 3TOP and diterpene molecules (**I** and **II** respectively).

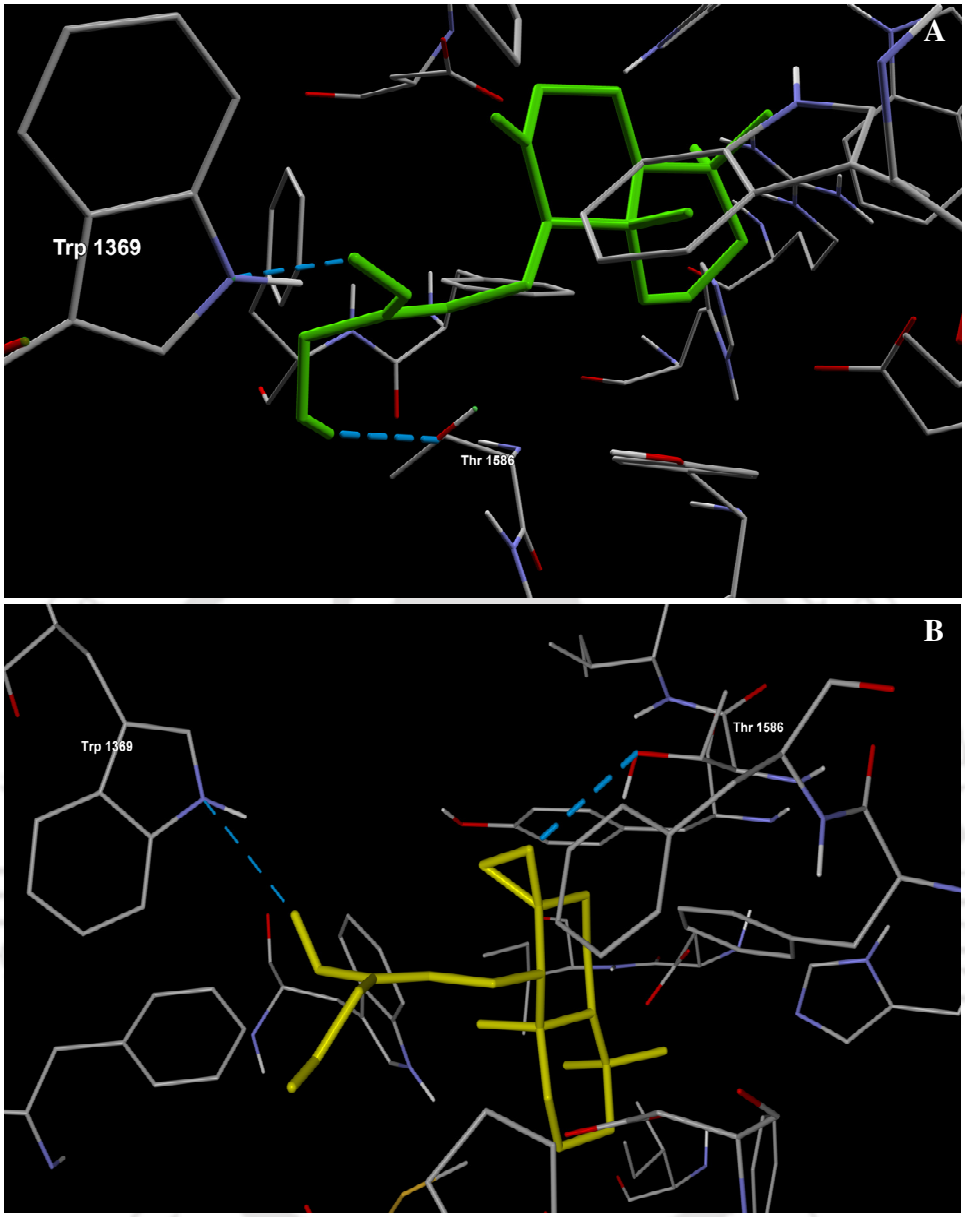


Fig. 6.21 Predicted H-bonded interaction of **I** (A) and **II** (B) with the residues at the active site region of 3TOP. Here, blue dotted lines represent the H-bonding interactions between the ligand molecules and 3TOP.

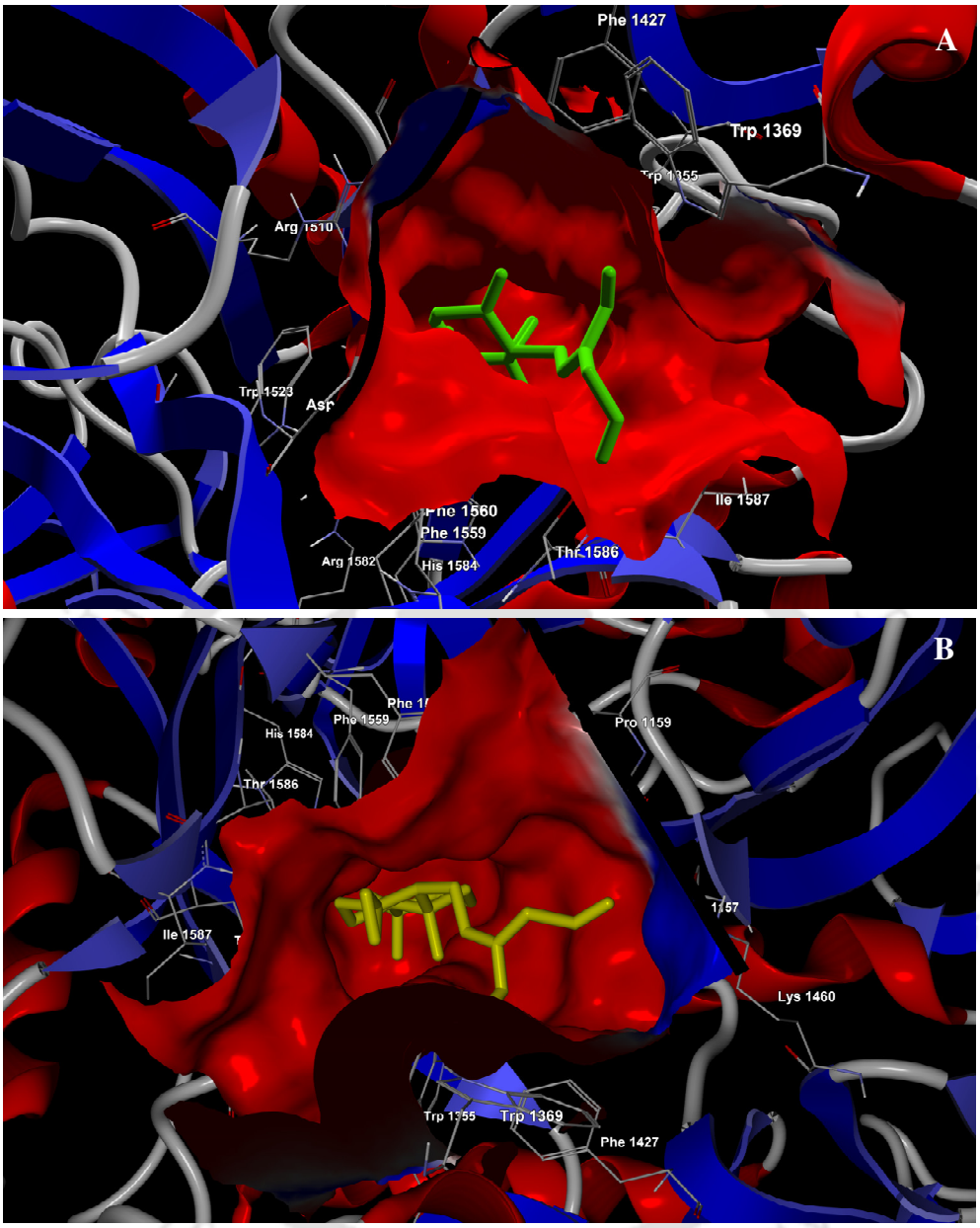


Fig. 6.22 Predicted non-bonded electrostatic interaction of **I** (A) and **II** (B) with the residues at the active site region of 3TOP.

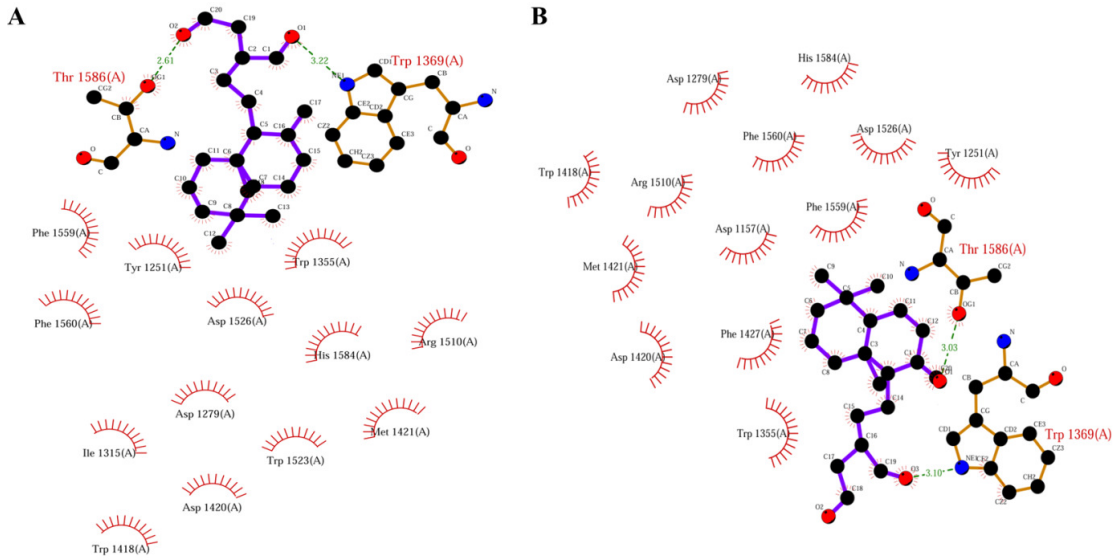


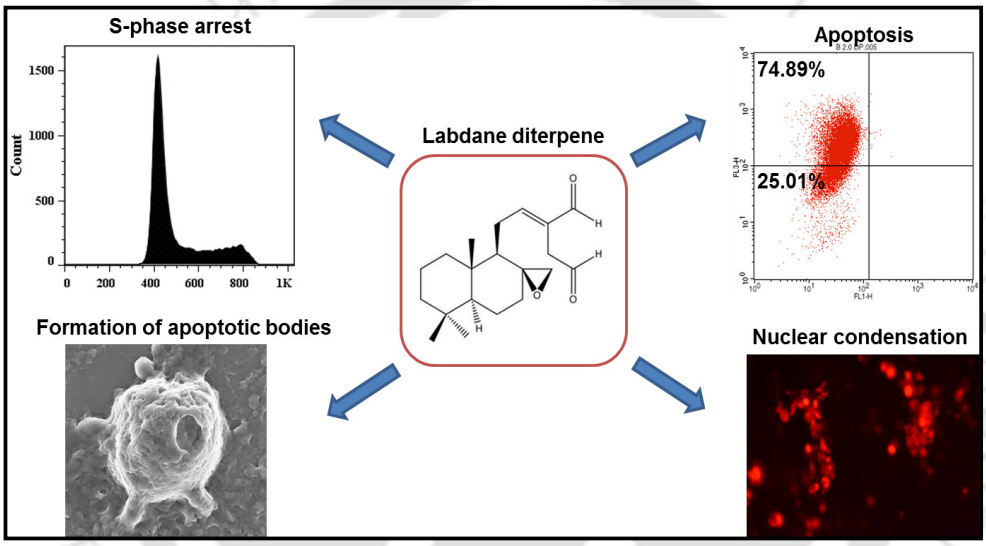
Fig. 6.23 LigPlot generated for the best poses obtained with compound **I** (A) and compound **II** (B), against 3TOP crystal structure. Hydrogen bonding interactions were shown as green dotted lines. Besides the H-bonding residues, other surrounding amino acids (in the vicinity of ligand) describe the hydrophobic interactions.

6.4. Conclusions

Although *Alpinia* genus is known for its diverse biopharmaceutical potential, there are inadequate studies and reports associated with antidiabetic property. In the present study, the efficacy of *A. nigra* seed extracts was investigated towards the inhibition of the key regulatory enzymes, α-amylase and α-glucosidase. Two labdane type diterpene molecules isolated as lead compounds showed remarkable α-amylase and α-glucosidase inhibitory effect and found to be a prominent non-competitive inhibitor in a dose dependent manner. These studies also establish the putative binding potentiality of both the labdane molecule as a candidate alternative herbal drug against HPA and MGAM to drop the spiking of postprandial blood glucose level. Current findings unveil the *A. nigra* seeds as rich source of antidiabetic compounds towards its future therapeutic application as alternative herbal means and also extended the promising candidature of these natural labdane diterpene molecules for the first time as regulator of pancreatic α-amylase and α-glucosidase.

Chapter 7

Studies on labdane diterpenes towards matrix metalloproteinase inhibitory activity and cytotoxicity on HT1080 fibrosarcoma cells



The chapter describes the apoptotic activity of these labdane diterpenes in HT 1080 human fibrosarcoma cells. Also the effect of labdane diterpenes on matrix metalloproteinase (MMPs) activity, cell cycle arrest, nuclear integrity and apoptosis were studied.

Chapter 7

Studies on labdane diterpenes towards matrix metalloproteinase inhibitory activity and cytotoxicity on HT1080 fibrosarcoma cells

7.1. Introduction

Cancer is a complex disease caused primarily by environmental factors (90-95%) and also to an extent influenced by genetic factors (Anand et al. 2008). It affects several systems in the body like circulatory, lymphatic, digestive, urinary, reproductive and even skin where it is regulated by a complex network of various transcription factors, enzymes and other key proteins in the body (Sitas et al. 2008; Sakarkar and Deshmukh 2011). Cancer is mainly characterized by uncontrolled cell division and metastasis of those abnormal cells from primary site to other parts of the body. Metastasis is a characteristic feature of malignant tumors and during this process degradation of the extracellular matrix (ECM) and basement membrane (BM) occurs. Several proteolytic enzymes are produced by tumor cells which are known to degrade the components of ECM and BM. The matrix metalloproteinases (MMPs) are members of the unique family of proteolytic enzymes which contain a zinc ion at their active sites and can degrade native collagens and other ECM components. Therefore, MMPs are believed to play a role in the invasion of the basement membrane by tumor cells (Liotta 1986).

Depending on organization of domains and specificity for substrate, the MMPs have been broadly divided into four major classes: gelatinases, membrane-type MMPs, stromelysins and interstitial collagenases. According to Nagase and Woessner (1999),

majority MMPs are known to be secreted as proenzymes with distinctive structural domains and having variation in domain composition and number. MMP-2 and MMP-9 are referred as gelatinase A and gelatinase B respectively. They are known to differ from other types of MMPs by possessing three tandem fibronectin type II repeats inside amino terminal catalytic subunit which is actually responsible for binding of gelatin (Murphy et al. 1994). Recently, these two MMPs gained much interest as putative tumour markers owing to their easy detection in body fluids. Additionally, recent reports have shown numerous roles of MMPs, rather than only degrading ECM, where they are involved in processing of surface molecules and mobilisation of various growth factors. It is evident from this background that MMP-2 and MMP-9 play a crucial role in cancer metastases and therefore, considered as potential target by many new anticancer agents (Yeh et al. 2012; Lu et al. 2013; Yang et al. 2013).

As promising and superior anticancer agents, various natural compounds have played a significant role where majority of them are from plant sources like Vinblastine and Vincristine (Johnson et al. 1963), Taxol (Wani et al. 1971), Camptothecin (Wall 1998), Epipodophyllotoxin (Canel et al. 2000) and Combrestatin (Cirla and Mann 2003). These drugs have also served and adopted as the major source of new drug candidates towards the treatment of cancer. Notably, an important group of molecules *viz.* labdane type diterpenes have been found to affect cell signalling, induce depolarization of the mitochondrial membrane, cause cell cycle arrest, inhibit MMPs and induces apoptosis in various cancer cells (Dimas et al. 2001; Souza-Fagundes et al. 2003; Dimas et al. 2006; Kunnumakkara et al. 2008).

With these backgrounds, the aim of this chapter was to investigate the effect of compound **I** and **II** on MMPs activity using HT1080 human fibrosarcoma cells. Further,

modulation of cell cycle, nuclear integrity and cytotoxicity effects were determined for both the diterpenes on HT1080 cells.

7.2. Materials and methods

7.2.1. Plant material, organic extracts and purified compounds

Three different organic solvent extracts (S-Hex, S-EtAc and S-Met) isolated from *A. nigra* seeds and two purified labdane-type diterpene compounds (**I** and **II**) were used in the present study. To prepare the stock of each extract, solvent extracts were dissolved in ethanol (10 mg/ml) and filter sterilized through 0.2 µm polytetrafluoroethylene (PTFE) membranes (PALL, USA). Similarly, the compounds (**I** and **II**) were dissolved in ethanol to prepare the stock concentration 10 mg/ml and filter sterilized. All the stocks were stored at -20°C refrigerator for further use.

7.2.2. Cell culture

HT1080 human fibrosarcoma cell line was purchased from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) (PAA, Laboratories, Austria) and 1X Pen-Strep (HiMedia, Mumbai, India). Cells were maintained in an incubator at 37°C and 5% CO_2. All the treatments were done in serum free DMEM-F12 medium (Invitrogen, India).

7.2.3. MTT assay for extracts and bioactive compounds

Approximately 2 $\times 10^6$ cells were cultured in a 96 well plate in DMEM -F12 containing 10% FBS. After 24 h cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and fed with serum free DMEM-F12 containing compounds (0-15 µg/ml) or extracts (0-30

µg/ml), or equivalent volume of ethanol. After 24 h, cells were washed with DPBS followed by addition of MTT assay reagent (5 mg/ml in culture media) and further incubated for 4 h (Mosmann 1983). The cells were again washed with DPBS and 100 µl DMSO was added to dissolve the formazan crystals. The extent of crystal formation was measured at 570 nm after the subtraction of background absorbance at 690 nm.

7.2.4. Gelatin zymography

Cells were seeded into 6 well plates in DMEM-F12 containing 10% FBS and allowed to grow for 48 h. Cells were then washed with DPBS and treated with each diterpene compound (0.01-1 µg/ml) for 24 h. Culture supernatants were collected from vehicle control and compounds (**I** and **II**) treated HT1080 cells. Total protein in the culture supernatants was quantified using the Lowry assay (Lowry et al. 1951). Proteins (250 µg) were separated on 10% non-reducing SDS-PAGE containing 1% gelatin. The gels were then washed thrice for 30 min with 2.5% Triton X-100 to remove the SDS followed by incubation in the activation buffer (1% 1M Tris, pH 8.0; 0.5% 1M $CaCl_2$; 1% 1mM $ZnCl_2$ and 1.25% Triton X-100) for 14 h. The gels were then stained with 0.25% Coomassie brilliant blue (CBB) and destained with 30% methanol-10% acetic acid till clear bands appeared against a blue background. For direct inhibition studies, equal volumes of HT1080 culture supernatants (serum free medium) were incubated with each compound at various concentrations (0.1-100 µg/ml) for 30 min at 37°C and subjected to gelatin zymography. Gel images were captured in Kodak 500 digital gel documentation system and analyzed using ImageJ software.

7.2.5. Cell cycle analysis

Cells were seeded into 6 well plates in DMEM-F12 containing 10% FBS and allowed to grow for 48 h. Further, cells were washed with DPBS and treated with each diterpene

compound (1 µg/ml) for 24 h. After the treatment cells were trypsinized, washed with DPBS, fixed and permeabilized in 70% ethanol. Pellets of ethanol fixed cells were resuspended in 25 µg/ml of propidium iodide (PI) (Sigma-Aldrich, USA) staining solution containing 2 mg/ml of RNaseA (Sigma-Aldrich, USA) and incubated for 30 min at 37°C. The relative DNA content was measured in FL2 channel (585/42 band pass) of FACS Calibur flow cytometer (BD Biosciences, USA) and analyzed using FlowJo software (Tree Star, Stanford, USA).

7.2.6. Nuclear fragmentation study

Cells were seeded into 35 mm dishes in DMEM-F12 complete medium and allowed to grow for 48 h. Cells were then washed with DPBS and treated with each diterpene compound (1 µg/ml) for 24 h. After treatment cells were washed with DPBS and fixed in 70% ethanol. Subsequently cell nuclei were stained with PI (1 mg/ml) at 37°C for 30 min in dark. The PI stained nuclei were examined under the inverted fluorescence microscope (Eclipse TS100, Nikon, USA).

7.2.7. Annexin V-FITC/PI apoptosis study

Cells were seeded into 35 mm dishes in DMEM-F12 complete medium and allowed to grow for 48 h before treatment. Cells were then washed with DPBS and treated with each diterpene compound (0.5-2 µg/ml) and 5 nM staurosporine for 14 h. After treatment cells were trypsinized and processed using Annexin V-FITC Apoptosis Detection Kit (Calbiochem) as per the manufacturer's instructions. The membrane flipping (FITC) and membrane integrity (PI) were measured in FL1 (530/30 band pass) and FL3 (670 long pass) channels of FACSCalibur flow cytometer (BD Biosciences), respectively.

7.2.8. Field emission scanning electron microscopy (FESEM) study

Cells were seeded on a sterile glass cover slip in 35 mm dishes with DMEM-F12 complete medium and allowed to grow for 48 h before treatment. Cells were then washed with DPBS and treated with each diterpene compound (1 µg/ml) and 5 nM staurosporine. After 24 h treatment, cells were washed with DPBS and dehydrated with increasing concentrations of ethanol (30-99.99%) at room temperature for 15 min each. After drying and fixation, the cells were coated with gold and analyzed under FESEM (Carl Zeiss, Ultra 55).

7.2.9. Statistical analysis

All tests were performed in triplicate with three independent experiments. The results expressed as a mean ± SE. The data were analyzed using one-way analysis of variance (ANOVA) and treatments were compared by employing Tukey's post-hoc test. $p<0.05$ was considered significant.

7.3. Results and discussion

7.3.1. MTT cell viability assay

Hexane, ethyl acetate and methanolic extracts of *A. nigra* seeds were examined for their effect on HT1080 cell viability. In the current study, the activity of each extract varied noticeably with the solvent of extraction. S-EtAc and S-Met extracts were found to be more active (Fig. 7.1) than the non-polar S-Hex extract. Higher activity conferred by S-EtAc and S-Met extracts against HT1080 cells possibly resulted due to the presence of two isolated labdane diterpenes along with other bioactive polar compounds.

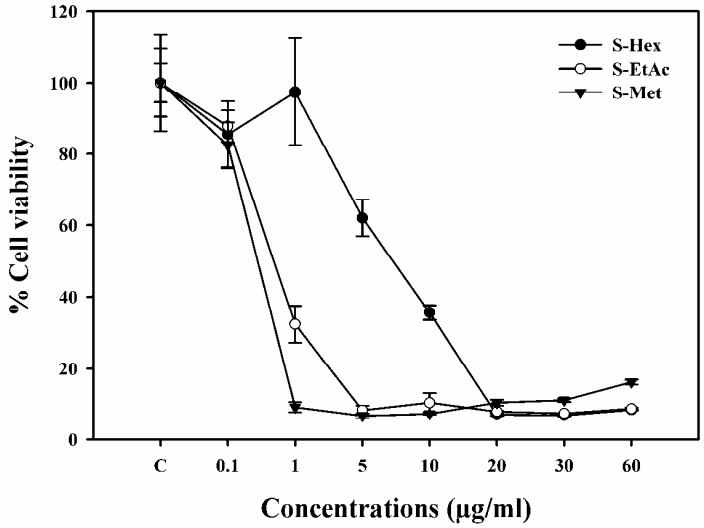


Fig. 7.1 Effect of *A. nigra* seed extracts on viability of HT1080 cells. Percent viability of HT1080 cells treated with organic extracts using MTT assay.

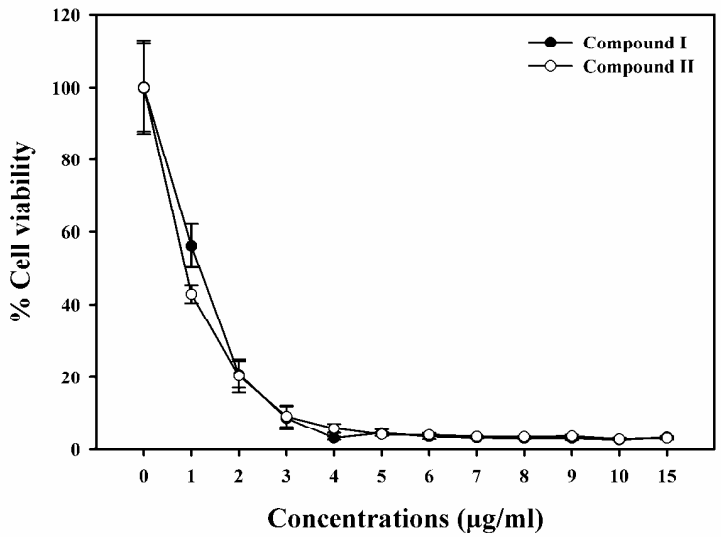


Fig.7.2 Effect of compounds on viability of HT1080 cells. Viability percentage of HT1080 cells treated with compound **I** and **II** using MTT assay.

Both the compounds showed dose dependant toxicity against HT1080 cells (Fig. 7.2). The study revealed compound **I** and **II** are remarkably active having IC_{50} 1.1 and 0.9 µg/ml respectively (Table 7.1). The bright field microscopy images confirmed the loss of HT1080 cell viability in the presence of compounds **I** and **II** (Fig. 7.3). In the present study, observed efficacy of **I** against HT1080 cells was found dose dependent and cell growth almost ceased above 4 µg/ml concentration. Compound **I** was previously reported as an anticancer agent which is active against a wide range of cancerous cell lines with a minimal toxicity to non-cancer human fibroblast cells MRC-5 (González et al. 2010; Liu and Nair 2011; Malek et al. 2011b; Igoli et al. 2012; Liu and Nair 2012). Besides its cytotoxicity, compound **I** has also been reported earlier as strong inhibitor of COX-2 enzyme which might play a critical role in regulation of skin carcinogenesis (Kundu et al. 2006; Liu and Nair 2011, 2012). Abe et al. (2002) reported this epoxide analogue (**II**) as more effective inhibitor than **I** against human 5-lipoxygenase enzyme and human platelet aggregation. Likewise, **II** has also been reported as strong cytotoxic agent against L1210 and KB cells (Morita and Itokawa 1988). In the present study, we observed the higher efficacy of **II** than **I** which could be due to the presence of reactive epoxide moiety in **II**. Our studies are in agreement with previous reports revealing the comparative efficacy of both the isolated labdane compounds towards cytotoxic activities.

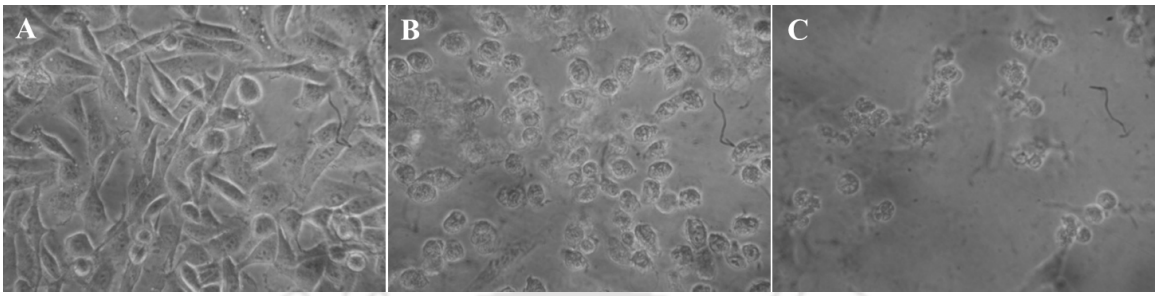


Fig. 7.3 Bright field images of HT1080 cells treated with compound **I** and **II**. (A) vehicle treated, (B and C) cells treated with 1 µg/ml of compound **I** and **II** respectively.

Table 7.1 Inhibitory activity of *A. nigra* seed extracts and isolated bioactive compounds against HT1080 cells

Sample name	IC_{50}[a] (µg/ml)
S-Hex	7.3
S-EtAc	0.7
S-Met	0.5
I	1.1
II	0.9

[a]IC_{50} value is determined as the concentration of the sample (µg/ml) to inhibit 50% of cell viability under the assay condition.

7.3.2. Inhibition of matrix metalloproteinases (MMPs)

A recent approach in cancer therapeutics involves targeting the metastatic process. Metastasis is a major cause of death in cancer patients and defined as complex, multi-step development. It involves the detachment of cancerous cells from the site of origin which invade further into new blood vessels and forms colony at distant sites. Currently, several investigations have been carried out towards the functional interference in any of the steps of metastatic process. Previous studies have revealed the fact that targeting metastatic components are advantageous than conventional treatments by involving better selectivity towards cancer cells and less chances of developing resistance (Folkman 1995; Barinag 1997). Folkman (1995) have clearly shown that anti-metastatic therapy can easily be combined with conventional chemotherapy in animal studies (Folkman 1995).

Many herbal plants have been investigated for their potential as anti-metastatic agents. For instance curcumin, a nonsteroidal polyphenolic compound from *Curcuma longa*, has been shown to suppress invasion of B16F-10 (melanoma cells) and SK-Hep-1 (hepatocellular cells) by hampering the secretion of MMP-2 and MMP-9 at non-toxic doses (Lin et al. 1998; Banerji et al. 2004). Several other natural products with minimal cytotoxic effects which have been studied for their anti-metastatic potentials include genistein, a flavone compound of soy, and resveratrol, a phytoalexin found in grapes (Shao et al. 1998; Kozuki et al. 2001). Likewise, (-)-epigallocatechin, a tea compound, has been shown to cause a marked reduction of HT1080 fibrosarcoma cell invasion by suppressing MMP secretion, with minimal effects on cell viability (Maeda-Yamamoto et al. 2003).

The property of HT1080 being aggressive cell line is mainly due to the high level of MMPs. Here, in order to check the possible role of the isolated diterpenes compounds (**I** and **II**) in regulating the MMP enzyme activity and level of secretion into supernatant gelatin

zymography was performed. In case of *in vitro* assay, the inhibitory effect of both MMP-2 and MMP-9 were studied with various concentrations of compound **I** and **II** separately. However, no significant change in the gelatinase activity was observed in treated culture supernatants with respect to their control (Fig 7.4A and B).

On the contrary, inhibitions of MMP-2 and MMP-9 activity were clearly observed in gelatin zymograph when culture supernatants were directly incubated with both the diterpene compounds (Fig. 7.5). It could be hypothesized that both the compounds were much more accessible instantly to all available MMP-2 and MMP-9 in the culture supernatant, whereas, in case of *in vitro* treated culture supernatant, most of the compounds either exhausted in various interactions with cell metabolites or other proteins during the period of treatment. The another possible reason towards less effect of both the compounds on MMPs activity in case of *in vitro* studies could be due to the uptake of the compounds inside the cells where it (**I** or **II**) might not be sufficiently available in the culture supernatant to interact and inhibit the secreted MMP-2 and MMP-9 activity. Therefore, both the compounds showed higher and dose dependant inhibitory activity on MMP-2 and MMP-9 in direct inhibition assay rather than *in vitro* cell based assay.

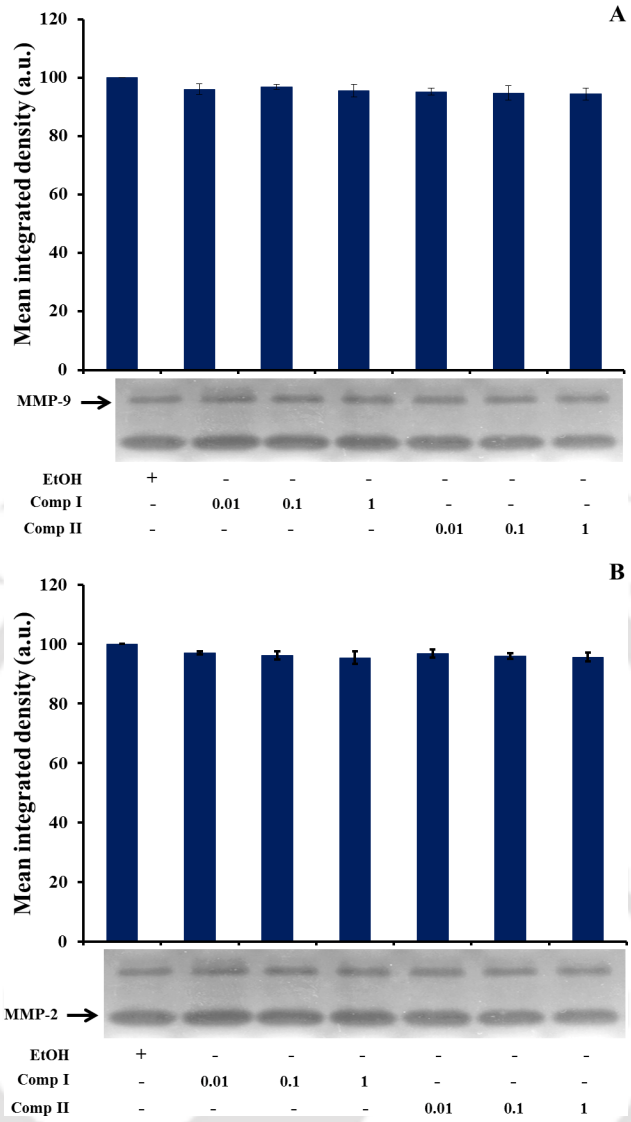


Fig. 7.4 Effect of compound **I** and **II** on MMP-2 (A) and MMP-9 (B) activities by *in vitro* assay. Diterpene compounds treated HT1080 cell supernatants with equal amount of proteins were loaded and analyzed by gelatin zymography. The experiments were performed in triplicate and gels were analyzed using ImageJ software to determine the mean integrated density (MID) for all bands with appropriate background subtractions for respective gels. The MID values for each treatment with vehicle control (EtOH) were plotted as bar diagram. No significant differences of mean values were obtained from Tukey's post-hoc test.

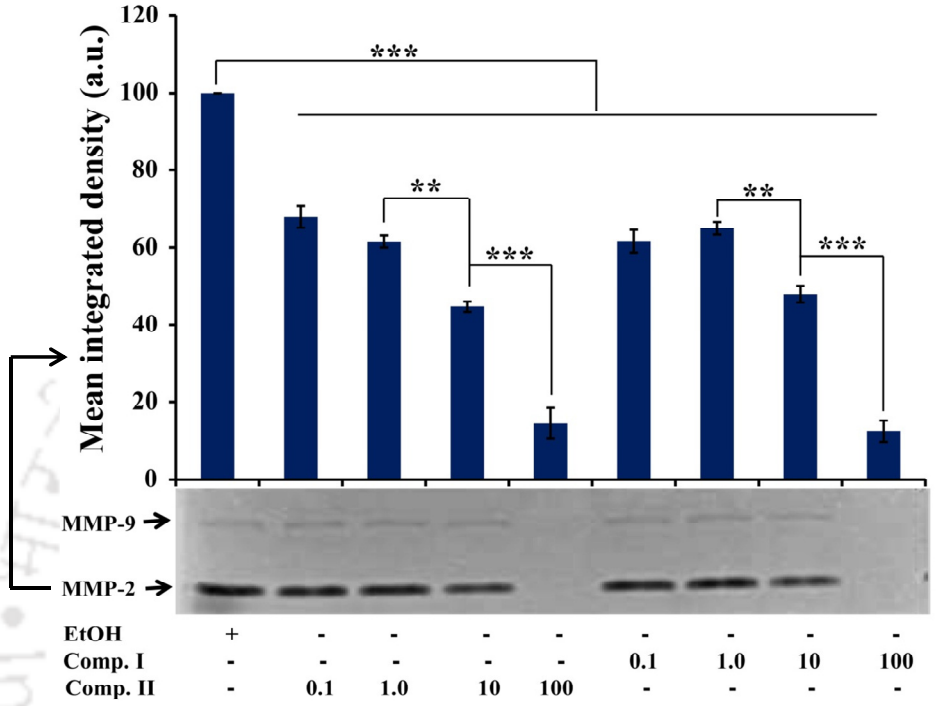


Fig. 7.5 Effect of compound **I** and **II** on MMP-2 and MMP-9 activities by direct interaction. HT1080 culture supernatant was used as a source of MMP-2 and MMP-9 and mixed with increasing concentrations (µg/ml) of compound **I** and **II** following incubation at 37°C for 30 min. Gelatin zymography of HT1080 culture supernatant with various concentrations of diterpene compounds. In each well 10 µl of reaction mixture (supernatant + compound) were loaded after incubation and analyzed by gelatin zymography. The experiments were performed in triplicate and gels were analyzed using ImageJ software to determine the mean integrated density (MID) for all bands (MMP-2) with appropriate background subtractions for respective gels. The MID values for each treatment with vehicle control (EtOH) were plotted as bar diagram. Significant differences of mean values were depicted as $^{**}p<0.01$ and $^{***}p<0.001$ obtained from Tukey's post-hoc test.

In the present study, all the treatments of **I** and **II** (Fig. 7.5) were found significantly effective even at lowest concentration of 0.1 µg/ml compared to vehicle control (p<0.001). The MMP-2 activity was clearly reduced at 10 µg/ml concentration of **II** and found highly significant compared to control (p<0.001). For both the compounds, MMP-2 and MMP-9 activity were almost diminished at concentration of 100 µg/ml. Previously, a diterpene from *Jatropha curcas* called curcusone B, showed similar type of inhibition for MMP-2 gelatinase activity in KKU-100 (cholangiocarcinoma) cell line and established as antimetastatic agent (Muangman et al. 2005). Huang et al. (2005) described the inhibitory property of a natural diterpene (carnosol) which remarkably inhibited the invasion of highly metastatic mouse melanoma B16/F10 cells *in vitro*. They have also suggested that carnosol targets mainly MMP-9 and could restrict the invasive ability of B16/F10 mouse melanoma cells by reducing MMP-9 expression towards its anticancer activity. In other studies it has been shown that MMP-9 plays a critical role in invasion and highly inhibited by labdane type diterpene, coronarin D (Kunnumakkara et al. 2008). Thus, it is assumed that both the diterpenes in the present study might play a similar role as other diterpene molecules towards its antimetastatic activity by regulating MMP-2 and MMP-9.

7.3.3. Cell cycle analysis

Two primary events in cell proliferation are DNA replication and cell division (Murray and Hunt 1993). The cell cycle has been divided into four sequential phases: G1 is the primary gap phase where the cell prepares for replication of DNA; S phase is the period for synthesis of DNA during which a sister copy of the whole genome is produced; in second gap phase (G2) cell prepares for division; and mitosis (M) is referred as the period where segregation of DNA occurs and the cell finally divides into two daughter cells of identical genetic makeup. Entry and exit of the cell cycle takes place when the cell passes through an active division

and a quiescent (G0) stage. During this phase, primary metabolism of a cell is suppressed including several active functions like replication, transcription and translation. Deficiency of essential growth factors forced cell to step into G0 stage, whereas addition of those factors give signal a cell to re-enter in the active phase. On the contrary, a cell can also way out from the normal cell cycle to undergo differentiation or even programmed cell death, known as apoptosis (Wyllie et al. 1980; Williams and Smith 1993). Therefore, cell cycle regulation plays a critical role in apoptosis and can be utilized towards the development of promising chemotherapeutic drugs against cancer.

Majority of the established antineoplastic and chemotherapeutic drugs are characterized as synthetic antimetabolites which known to block the synthesis of deoxyribonucleotides leading to hamper in elongation process. These drugs are not very specific to cell cycle but in many cases cells are more sensitive to their actions in late G1 and S phase. Representative members include flucytosine, 5-fluorouracil, 6-mercaptoguanine and cytarabine (Calabresi and Parks 1985). Several other inhibitors like hydroxyurea (inhibits ribonucleotide diphosphate reductase), aphidicolin (inhibiting DNA polymerase α by blocking expansion and elongation of the replication bubble) and mimosine (affecting the deoxyribonucleotide pool in a manner similar to hydroxyurea) are known to affect the cell cycle by arresting at S-phase (Schlegel and Pardee 1986; Raff and Glover 1988; Gilbert et al 1995).

In the present study, to elucidate the effect of compound **I** and **II** on the cell cycle progression of HT1080 cells, the DNA content of nuclei of HT1080 cells was measured by flow cytometric analysis. Cell cycle histograms for treated cells revealed a substantial increase in S-phase cell population compared to cells with only vehicle control (Fig. 7.6A-C). Both the compounds were efficient in arresting the cells significantly at S-phase, as compared

to vehicle treated groups (p<0.001). A prominent increase of cell population at S-phase were observed for compound **I** and **II** with 35% and 65% cell arrest compared to control respectively. Here, compound **II** found significantly more effective than **I** (p<0.001) in arresting the HT1080 cells at S-phase. In case of G0/G1 and G2/M, the cell population were found to be declined remarkably with the respective treatments and found statistically significant (Fig. 7.6D,E).

Previously, sclareol, a compound belonging to the labdane type diterpenes showed cell cycle arrest at G0/G1 where S-phase population diminished after treatment in the breast carcinoma MCF-7 cell line variants, MN1 (p53-expressing) and MDD2 (p53-defective) cells (Dimas et al. 2006). This diterpenes also showed G1 arrest in human T-cell leukemia lines and p53-deficient (HCT116p53−/−) human colon cancer cells (Dimas et al. 2001; Mahaira et al. 2011). In recent years, several natural compounds emerged as key player in cell cycle regulation, such as resveratrol (S-phase arrest in several human cancer cell lines), curcumin (S-phase arrest in LoVo, human colon adenocarcinoma cells), lycorine (arrest G0/G1 phase in K562, human chronic myelocytic leukemia cells), tea polyphenols (G2/M arrest in HPV16 positive human cervical cancer cells), aloe emodin (S-phase arrest in U87, human malignant glioma cells), quercetin (G2/M arrest in HeLa, human cervical cancer cells) and many other plant derived alkaloids and terpenoids have been extensively studied towards the development of chemotherapeutic agent from herbal sources (Joe et al. 2010; Singh et al. 2010b; Guo et al. 2012; Li et al. 2012; Bishayee et al. 2013; Ismail et al. 2013). Our results suggest for the first time that both the compounds have significant effect on arresting the HT1080 cells at S-phase possibly towards the repair of damaged DNA.

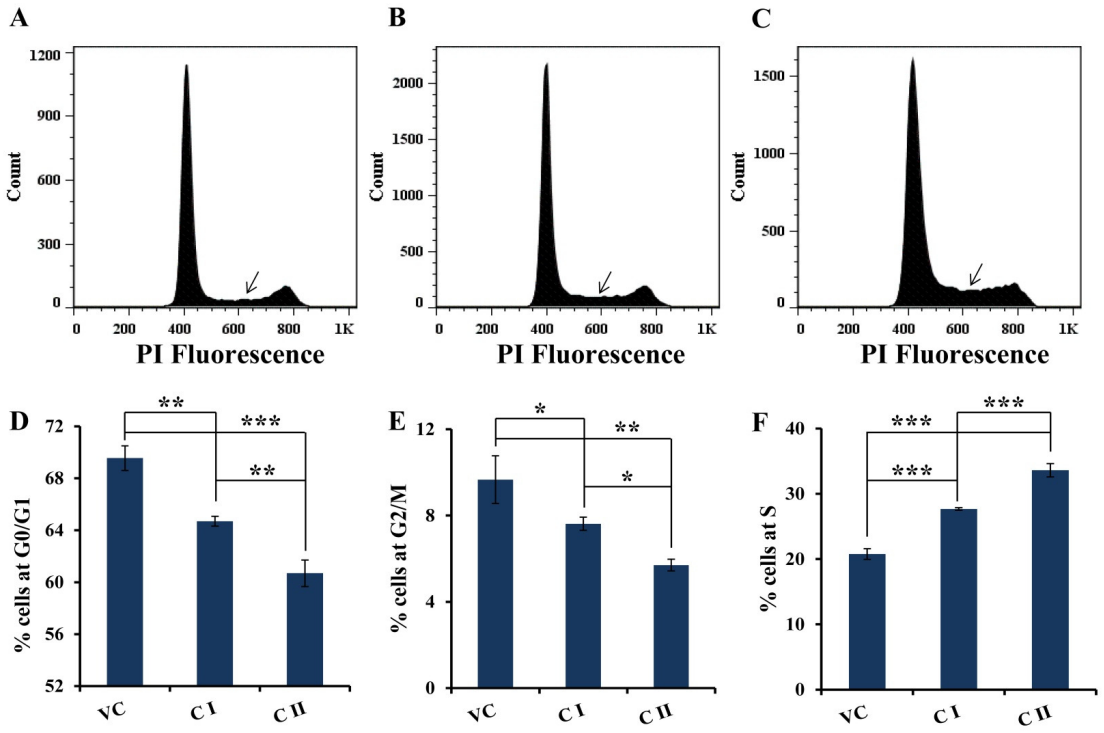


Fig. 7.6 Flow cytometric determination of G0/G1, S and G2/M population of HT1080 cells treated with compound **I** (CI) and **II** (CII) (1 µg/ml each) for 24 h. A: vehicle control, B and C: cell treated with **I** and **II** respectively. Arrow indicates increase in the S-phase cell population. D-F: Graphical representation of G0/G1, G2/M and S-phase cells treated with tested compounds respectively. All experiments were performed in triplicate and values represented as mean ± SE. Significant differences of mean values were depicted as *p<0.05, **p<0.01 and ***p<0.001 obtained from Tukey's post-hoc test.

7.3.4. Nuclear condensation and fragmentation assay

Phenotypically, a cell death via apoptosis is characterized by many morphological changes like blebbing of plasma membrane, cell shrinkage, DNA fragmentation to chromatin compaction and formation of apoptotic bodies (Green 2011). The fate of HT1080 cell nuclei after the treatment with both the diterpenes and a known apoptotic agent, staurosporine were evaluated. Results revealed that vehicle treated cells were normal with intact and uniform nuclei when stained with PI. But, a prominent shrinkage of HT1080 cells with compromised nuclear integrity was observed in case of staurosporine and two diterpenes (Fig. 7.7). This type of nuclear condensation and fragmentation were previously described when the cell were stained with nuclear dye like PI (Li et al. 2005b; Lingadurai et al. 2011). Li et al. (2005b) illustrated the morphologic evidence of apoptosis using cisplatin (standard apoptotic inducer) which is a platinum based compound that forms intra and inter-strand adducts with DNA and induce apoptosis. Similar to the present investigation, methanolic extract from leaves of *Bichofia javanica* showed clear nuclear fragmentation and condensation in U937, K562, and HL60 leukemic cell lines as evident by confocal microscopy imaging (Lingadurai et al. 2011). In the present study, apoptotic cells were identified by morphological changes (condensation and fragmentation) of the nuclei as described by Li et al. (2005b). Previously, a microbial secondary metabolite (arisostatins A) showed dose-dependent apoptosis and DNA fragmentation of human embryonic stem cell line, HN-4 where prominent morphological changes was observed in PI stained cells (Kim et al. 2003). Similar effect of extract from the needles and twigs of *Taxus cuspidata* demonstrated apoptotic fragmentation of nuclei in prostate cancer cell line, PC-3M-1E8 (Shang et al. 2011). They also showed that the extract caused G2/M cell cycle arrest and induce apoptosis. Therefore, the observed cellular morphology of HT1080 cells and their loss of nuclear integrity towards apoptosis in

the present study are in well agreement with earlier reports (Kim et al. 2003; Shang et al. 2011).

7.3.5. Annexin V-FITC/PI apoptosis study

Induction of apoptosis was monitored by dual staining of cells with Annexin V-FITC/PI to measure the amount of phosphatidylserine on the outer membranes of apoptotic cells. HT1080 cells were treated with both the diterpenes (0.5-2.0 μg/ml) and a standard apoptotic agent, staurosporine (5 nM). Dose dependant shifting of treated cell populations was observed for **I** and **II** which mimic the effect of staurosporine (Fig. 7.8). Maximum effect on HT1080 cells was obtained with staurosporine (~90% apoptotic) followed by **II** (~75% apoptotic). However, **I** was found less effective to induce apoptosis in HT1080 cells compared to **II** (Fig. 7.8D, H).

Phenomenon of apoptosis was similarly detected by annexin V-FITC/PI staining where xanthorrhizol (a sesquiterpenoid) from the rhizome of *Curcuma xanthorrhiza* induces apoptosis in human breast cancer cell line, MCF-7 (Cheah et al. 2006). The authors have also established xanthorrhizol as an antiproliferative agent which can induce apoptosis in MCF-7 by modulating various key proteins like bcl-2 (B-cell lymphoma 2), p53 (tumor suppressor) and PARP-1 (Poly [ADP-ribose] polymerase 1) levels in cells. A recent study revealed that alpinetin, a natural flavonoid widely distributed in the genus *Alpinia*, induces apoptosis and arrest the human gastric cancer cells at G2/M phase in a dose dependent manner. Alpinetin have been found to promote activation of mitochondrial dependent endogenous apoptosis pathway via Bcl-2-associated X protein (Bax) translocation (Wang et al. 2013c).

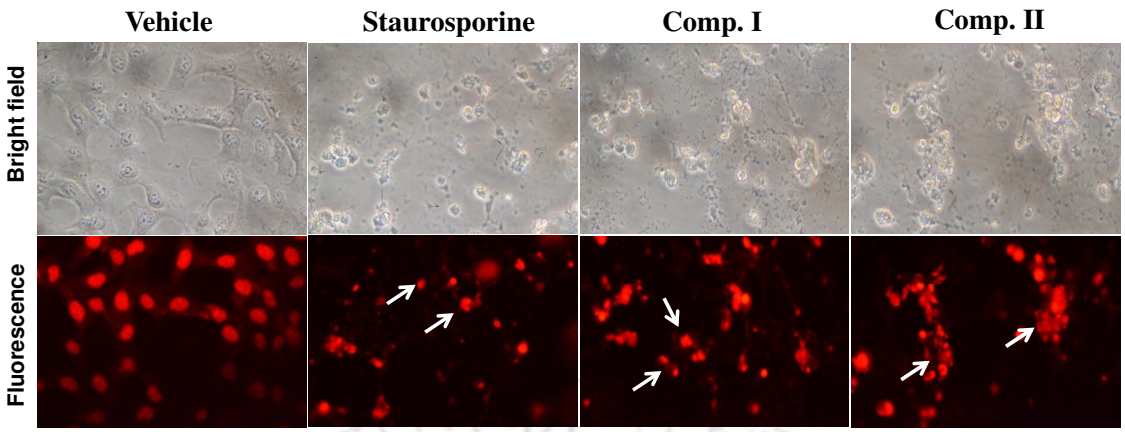


Fig. 7.7 HT1080 cells were treated with ethanol (vehicle control), apoptotic agent (staurosporine, 2 nM), compound **I** and **II** (1 µg/ml each) followed by staining with PI (25 µg/ml). Images were captured for both bright field and fluorescence in the corresponding microscopic field for each treatment. Arrows indicates the condensed and fragmented nuclei in the treated HT1080 cells.

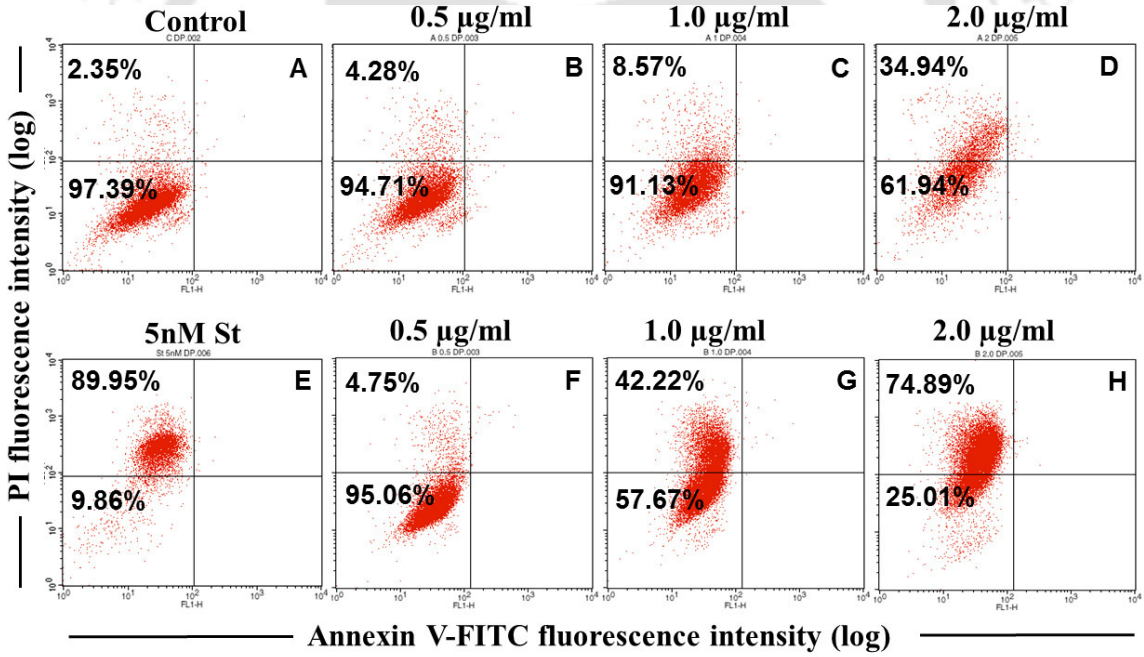


Fig. 7.8 *In vitro* assessment of apoptosis in HT1080 cells. Cells were treated with standard apoptotic agent (staurosporine, 5 nM) and two isolated labdane molecules (0.5-2.0 µg/ml) for 14 h. Here, A refers to the untreated cell population, B-D and G-I represents the cell populations treated with various concentration of compound **I** and **II** respectively. Cells treated with staurosporine taken as positive control (E).

Another compound from *Alpinia conchigera* Griff., 1'-(S)-1'-Acetoxychavicol acetate (ACA), revealed a wide activity on various human cancer cell lines where dose dependent induction of apoptosis were observed using annexin V-FITC/PI staining in flow cytometry (Awang et al. 2010). Similar to the present study, *Alpinia officinarum* extract also showed clear, dose dependent induction of apoptosis and cell cycle arrest at S-phase in the human breast cancer cell line, MCF-7 (Ghil 2013). Therefore, the observed effect of isolated labdane diterpenes on HT1080 cell towards the induction of apoptosis are in accordance with recent reports describing the apoptotic induction in various cancer cell lines by plant extracts or purified components in view of further prospects in herbal therapeutics against cancer (Ebrahimi Nigjeh et al. 2013; Mahassni and Al-Reemi 2013; Shi et al. 2013; Thakkar et al. 2013; Yedjou et al. 2013).

7.3.6. FESEM study

Here, as above we have used staurosporine (5 nM) as positive control and two diterpenes (1 µg/ml) as test compounds to determine the morphological alterations on HT1080 cells (Fig. 7.9). Treatments with **I** and **II** leads to cell shrinkage with appearance of membrane blebs (Fig. 7.9C, D). Apoptotic bodies were also observed for diterpenes and staurosporine treated cells. Similar to staurosporine, tumor necrosis factor-α (TNF-α), a protein kinase inhibitor showed extrinsic apoptosis and resulted in "sticky" aggregation, apoptotic bodies in Human Salivary Gland cell line (Wang et al. 2009). The characteristic feature of apoptotic cells and formation of apoptotic bodies were determined according to Abdel Wahab et al. (2009) and Lee et al. (2011).

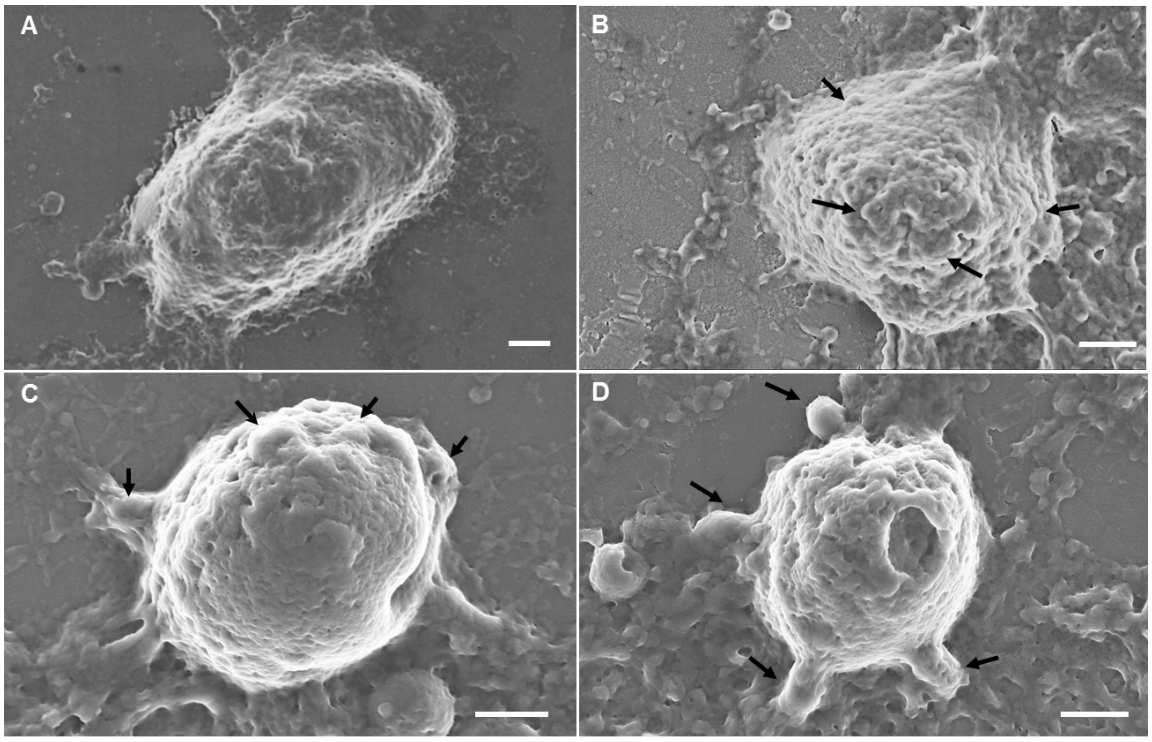


Fig. 7.9 FESEM images of treated and untreated HT1080 cells. (A) untreated, (B) treated with 5 nM staurosporine, and (C and D) treated with **I** and **II** respectively. Membrane blebbing on treated HT1080 cells were clearly depicted with black arrows. Scale bars = 2 µm.

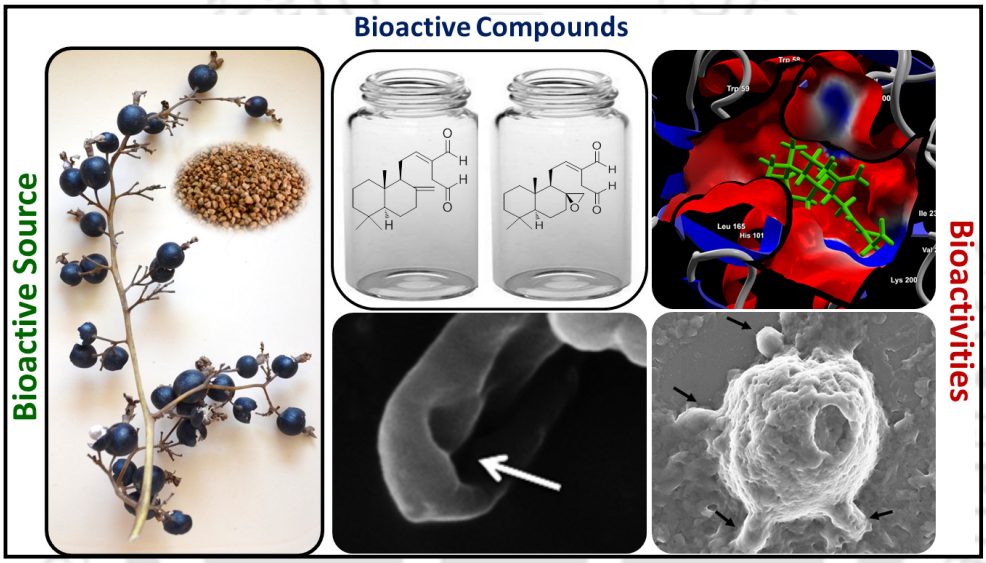
Zerumbone, a potential anticancer compound, isolated from the fresh rhizomes of *Zingiber zerumbet* also caused apoptosis in human cervical cancer cells, HeLa and revealed the clear formation of apoptotic bodies with membrane blebs (Abdel Wahab et al. 2009). Comparable morphological changes in various cancer cell lines undergone apoptosis due to external stimuli and has been documented previously which are in agreement with our present observation in FESEM studies and also confirmed the apoptotic effect of both the isolated labdane diterpenes on HT1080 cells (Dou and Li 2004; Dini 2005; Krysko et al. 2006; Wang et al. 2009; Wickman et al. 2013).

7.4. Conclusion

In summary, the study demonstrates these labdane diterpenes can modulate the activity of MMP-2 and MMP-9 by direct inhibition. Further, arresting HT1080 cells at S-phase with nuclear condensation and characteristic morphological changes suggest the apoptotic effect of labdane diterpenes on HT1080 cells. Nevertheless, further exploitation of the chemotherapeutic potential of these compounds will be required to establish them as future herbal remedies towards the treatment of cancer.

Chapter 8

Concluding remarks



The chapter describes the significance and salient features of the present work done and the future perspective of the study in the field of natural product and herbal therapeutics.

Chapter 8

Concluding remarks

8.1. Significance and salient features of the study

Herbal medicine is an emerging and attractive approach in therapeutics due to its less side effects, durability and wide acceptability. In herbal medicines Zingiberaceae plant family is known for its high medicinal value and various plant preparations towards therapeutics. Among several genus in Zingiberaceae family, members of *Alpinia* genus are drawing much attention for their complex chemical profile and versatile pharmaceutical applications. Most studied species of the *Alpinia* genus, *A. galanga* is very much popular in herbal medicines because of its wide spectrum of biological activities. Taxonomically closest relative of *A. galanga* is *A. nigra* (Kress et al. 2005) which have been used in folk medicine towards curing of many diseases and ailments in NEI (Tushar et al. 2010). However, information related to phyto-constituents, principal bioactive compounds of *A. nigra* and their uses in pharmacology is limited probably due to its geographical distribution (concentrated in Southeast Asian countries) and lack of dissemination of ethnomedicinal practices in the other parts of the globe.

In the present study, two major bioactive compounds were isolated from *A. nigra* seed extracts and the efficacies of those compounds were investigated towards various therapeutic potential. The isolated bioactive compounds were identified as the labdane type diterpenes and named as (*E*)-labda-8(17),12-diene-15,16-dial and (*E*)-8β,17-epoxylabd-12-ene-15,16-dial. The study unveiled the mechanism of bactericidal activity of the diterpene compounds against seven

different pathogenic bacteria. Apart from their bactericidal applications, the antidiabetic activity by inhibiting the major carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase were investigated. The enzyme binding affinities with those compounds were further confirmed by enzyme kinetics and molecular docking studies. The study also highlights the promising anticancerous potential of the labdane type diterpene compounds by inhibiting the activity of matrix metalloproteinase (MMP-2 and MMP-9) in HT1080 human fibrosarcoma cells. These compounds were also found to arrest the HT1080 cells in S-phase and mimic the apoptotic nature of standard drug staurosporine. Taking all into consideration, this is the first report describing the wide spectrum of biological activities of *A. nigra* seeds towards the future development of herbal remedies. The present investigation holds definite potential and promise to add up some new development in the area of herbal medicine research.

The salient features of the study are summarized below:

- The chemical profiling of *A. nigra* seed extracts (S-Hex, S-EtAc and S-Met) were studied by NMR, FTIR and GC-MS spectral analysis. Based on TSP estimation and qualitative screening for phytochemicals, S-Met was found as a rich source of phenolics, alkaloids and terpenoids. Furthermore, S-Met and S-EtAc were found as active free radical scavenger by DPPH assay.
- Two labdane type diterpene compounds and a triglyceride compound were isolated and identified for the first time from *A. nigra* seeds. Structure activity relationship suggests compound **I** and **II** could be potential for therapeutic application as a drug candidate. Furthermore, the RBC hemolysis assay confirmed that both the compounds were found to be compatible in the safe range for further bioactivity studies.

- Promising bactericidal activities have been obtained from *A. nigra* seed extracts and isolated two bioactive labdane type diterpenes. Bacterial membrane damage and subsequent cell leakage were found to be the key mechanism underlying the efficacy of seed extracts and compounds.

- The efficacy of *A. nigra* seed extracts and isolated compounds were investigated towards the inhibition of the key regulatory enzymes, α-amylase and α-glucosidase. Two labdane type diterpene molecules showed remarkable α-amylase and α-glucosidase inhibitory effect and found to be a prominent non-competitive inhibitor in a dose dependent manner. The studies also establish the putative binding potentiality of both the labdane molecule as a candidate alternative herbal drug against HPA and MGAM to drop the spiking of postprandial blood glucose level. Current findings unveil the *A. nigra* seeds as rich source of antidiabetic compounds towards its future therapeutic application.

- The study first time demonstrates that the labdane diterpenes (**I** and **II**) can modulate the activity of matrix metalloproteinase (MMP-2 and MMP-9) by direct inhibition. Arresting HT1080 cells at S-phase with nuclear condensation and characteristic morphological changes suggest the mimicking apoptotic nature of these labdane diterpenes on HT1080 cells similar to the standard apoptotic agent, staurosporine.

8.2. Future scope

A small portion of the world's biodiversity has been investigated for biological application and therapeutic development involving natural products as most promising source of drug leads. Development in analytical methods and separation techniques has eventually helped in the isolation and identification of bioactive compounds in crude extracts. However, political regulations and norms related to access to biodiversity in different source countries still need to be dealt with; the international networks such as those of Drug Discovery and the Bioresources Development and Conservation Programme provide useful frameworks for appropriate access.

With the emergence of novel screening systems and the explosion of genetic information has accelerated the need to rapidly identify novel lead structures as a vital necessity. The advent of genetic techniques that permitted the identification of biosynthetic cassettes from medicinally important plants has emerged as a new frontier for natural products lead discovery. By incorporating the appropriate metabolic pathways from unculturable organisms into convenient species, the range of accessible chemical diversity can be greatly expanded. Similar techniques can be used to 'mix-and-match' enzymes in artificial combinations, leading to even more novel structures.

As a part of the continuing study on chemical and biological characterization of different natural products from various medicinal plants, the present study was designed to investigate the antibacterial, antidiabetic and anticancerous activities of compounds isolated from *A. nigra* to search for newer, safer and more potent therapeutic agents. Work to date has concerned on one or the other type of biological activities by academic laboratories. However, the advanced technology utilized by industry with robotic high-throughput screening techniques cannot be matched by academic laboratories. Therefore, many of these need to be investigated for their clinical effectiveness by the use of well designed modern clinical trials with high throughput techniques. Moreover, the plant can be further screened against various other diseases in order to find out its unexplored efficacy which could be a potential source of chemically interesting and biologically more effective drug candidates.

In the context of the present study, much extensive screening of bioactive compounds from seeds and other parts of *A. nigra* should be carried out in view of novelty and better efficacy of the compounds. GC-MS analysis results revealed the presence of many labdane type

diterpene derivatives which could be exploited to screen the untapped candidates towards biological applications. Further, to add up into the studies on bactericidal properties of two isolated diterpenes, in depth research should be carried out to identify interacting molecule(s) responsible for membrane damage or pore formation in bacteria. Likewise, it will be interesting and much confirmative to use these two labdane diterpenes in *in vivo* system to explore their role in regulation of type 2 diabetes and establish the clear mechanism of inhibition. Moreover, application of these isolated labdane molecules should be extended to unveil the underlying molecular mechanism for apoptotic behavior observed in HT1080 cells. Further study on the effect of these compounds towards various key transcription factors in the host cells will definitely open up new directions and better applications of these molecules in future cancer therapy. Apart from extending the findings of the present study, the isolated compounds and other bioactive compounds from *A. nigra* should be investigated towards the development of herbal remedies for various diseases and ailments. Therefore, with the continuing need for novel drug-like lead compounds against an increasing number of ever-more challenging molecular targets, the diversity of chemical components derived from natural resources, especially from plants, will be practically significant towards future drug discovery and therapeutics. To look forward, it is anticipated that the natural products that have served as an important source of drugs in the past will not be overlooked in the drug discovery of 21st century.

References

R E F E R E N C E S

Abas F, Lajis NH, Shaari K, Israf DA, Stanslas J, Yusuf UK, Raof SM (2005) A labdane diterpene glucoside from the rhizomes of *Curcuma mangga*. J Nat Prod 68(7): 1090-1093.

Abdel Wahab SI, Abdul AB, Alzubairi AS, Mohamed Elhassan M, Mohan S (2009) *In vitro* ultramorphological assessment of apoptosis induced by zerumbone on (HeLa). J Biomed Biotechnol. doi: 10.1155/2009/769568.

Abe M, Ozawa Y, Uda Y, Morimitsu Y, Nakamura Y, Osawa T (2006) A novel labdane-type trialdehyde from myoga (*Zingiber mioga* Roscoe) that potently inhibits human platelet aggregation and human 5-lipoxygenase. Biosci Biotechnol Biochem 70(10): 2494-500.

Abe M, Ozawa Y, Uda Y, Yamada F, Morimitsu Y, Nakamura Y, Osawa T (2004) Antimicrobial activities of diterpene dialdehydes, constituents from Myoga (*Zingiber mioga* Roscoe), and their quantitative analysis. Biosci Biotechnol Biochem 68: 1601-1604.

Abraham SN, Bishop BL, Duncan MJ, Krishnan KRR, Song J, Li G, Zaas DA (2011) Methods and compositions for treating urinary tract infections using agents that mimic or elevate cyclic AMP. United States Patent US20110171195. Jul 14, 2011.

Abreu PM, Noronha RG (1997) Volatile constituents of the rhizomes of *Aframomum alboviolaceum* (Ridley) K. Schum. from Guinea-Bissau. Flavour Fragr J 12(2): 79-83.

Ademiluyi AO, Oboh G (2013) Soybean phenolic-rich extracts inhibit key-enzymes linked to type 2 diabetes (α-amylase and α-glucosidase) and hypertension (angiotensin I converting enzyme) *in vitro*. Exp Toxicol Pathol 65: 305-309.

Agarwal KC, Parks Jr RE (1988) Treatment of metastasis. United States Patent US 4,751,224. June 14, 1988.

Ahameethunisa AR, Hopper W (2010) Antibacterial activity of *Artemisia nilagirica* leaf extracts against clinical and phytopathogenic bacteria. BMC Complement Altern Med 10: 6.

Akarasereenont P, Thitilertdecha P, Chotewuttakorn S, Palo T, Seubnooch P, Wattanarangsan J, Phadungrakwitya R, Klumklomjit S, Chareonkij P, Sathiyos Y, Laohapand T (2010) Chromatographic fingerprint development for Quality Assessment of "Ayurved Siriraj Prasachandaeng" antipyretic drug. Siriraj Med J 62: 4-8.

Akiyama K, Kikuzaki H, Aoki T, Okuda A, Lajis NH, Nakatani N (2006) Terpenoids and a diarylheptanoid from *Zingiber ottensii*. J Nat Prod 69(11): 1637-1640.

Al-Farsi M, Lee CY (2008) Optimization of phenolics and dietary fibre extractionfrom date seeds. Food Chem 108: 977-985.

Ali MS, Banskota AH, Tezuka Y, Saiki I, Kadota S (2001a) Antiproliferative activity of diarylheptanoids from the seeds of *Alpinia blepharocalyx*. Biol Pharm Bull 24(5): 525-528.

Ali MS, Tezuka Y, Banskota AH, Kadota S (2001b) Blepharocalyxins C-E, three new dimeric diarylheptanoids and related compounds from the seeds of *Alpinia blepharocalyx*. J Nat Prod 64: 491-496.

Allas S, Ngoka V, Hartman NG, Owassa S, Ibea M (1999) Aframonum seeds for improving penile activity. United States Patent US 5,879,682. Mar 9, 1999.

Almeida LSB, Murata RM, Yatsuda R, Dos Santos MH, Nagem TJ, Alencar SM, Koo H, Rosalen PL (2008) Antimicrobial activity of *Rheedia brasiliensis* and 7-epiclusianone against *Streptococcus mutans*. Phytomedicine 15: 886-891.

Álvarez-Ordóñez A, Carvajal A, Arguello H, Martínez-Lobo FJ, Naharro G, Rubio P (2013) Antibacterial activity and mode of action of a commercial citrus fruit extract. J Appl Microbiol 115(1): 50-60.

Alves RRN, Rosa IML (2007) Biodiversity, traditional medicine and public health: where do they meet? J Ethnobiol Ethnomed 3: 1-9.

Al-Zoreky NS (2009) Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. Int J Food Microbiol 134(3): 244-248.

An LJ, Guan S, Shi GF, Bao YM, Duan YL, Jiang B (2006) Protocatechuic acid from *Alpinia oxyphylla* against MPP^+-induced neurotoxicity in PC12 cells. Food Chem Toxicol 44: 436-443.

An N, Zou Z, Tian Z, Luo XZ, Yang S, Xu LZ (2008) Diarylheptanoids from the rhizomes of *Alpinia officinarum* and their anticancer activity. Fitoterapia 79: 27-31.

Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB (2008) Cancer is a preventable disease that requires major lifestyle changes. Pharm Res 25 (9): 2097-2116.

Anastassaki T, Angelopoulou D, Kokkinopoulos D, Dimas C, Demetzos C (2006) Pharmaceutical formulations comprising labdanes for the treatment of tumors or leukemias. United States Patent US RE39,061. Apr 11, 2006.

Andrade-Cetto A, Becerra-Jim´enez J, C´ardenas-V´azquez R (2008) Alfa-glucosidase-inhibiting activity of some Mexican plants used in the treatment of type 2 diabetes. J Ethnopharmacol 116: 27-32.

Aparicio J, Fernandez-Martos C, Vincent JM, Maestu I, Llorca C, Busquier I, Campos JM, Perez-Enguix D, Balcells M (2005) FOLFOX alternated with FOLFIRI as first-line chemotherapy for metastatic colorectal cancer. Clin Colorectal Cancer 5(4): 263-267.

Arambewela LS, Arawwawala LD, Athauda N (2010) Antioxidant and antifungal activities of essential oil of *Alpinia calcarata* Roscoe rhizomes. J Ayurveda Integr Med 1(3): 199-202.

Arambewela LS, Arawwawala LD, Ratnasooriya WD (2004) Antinociceptive activities of aqueous and ethanolic extracts of *Alpinia calcarata* rhizomes in rats. J Ethnopharmacol 95: 311-316.

Arishima T, Sugimoto K, Kiwata R, Mori H, Sato K (1996) ^{13}C cross-polarization and magic-angle spinning nuclear magnetic resonance of polymorphic forms of three triacylglycerols. J Am Oil Chem Soc 73(10): 1231-1236.

Arul Raj C, Sophia D, Ragavendran P, Starlin T, Rathi MA, Gopalakrishnan VK (2012) Leaf extract of *Alpinia purpurata* (Vieill.) K. Schum screened for its phytochemical constituents and antibacterial and anticancer activities. Zhong Xi Yi Jie He Xue Bao 10(12): 1460-1464.

Asami S, Maeda A, Saito K, Tateishi N (2013) Anti-fatigue agent and oral composition each comprising andrographolide as active ingredient. United States Patent US8507547. Aug 13, 2013.

Asili J, Lambert M, Ziegler HL, Staerk D, Sairafianpour M, Witt M, Asghari G, Ibrahimi IS, Jaroszewski JW (2004) Labdanes and isopimaranes from *Platycladus orientalis* and their effects on erythrocyte membrane and on *Plasmodium falciparum* growth in the erythrocyte host cells. J Nat Prod 67(4): 631-637.

Aspé E, Fernández K (2011) The effect of different extraction techniques on extraction yield, total phenolic, and anti-radical capacity of extracts from *Pinus radiate* Bark. Ind Crops Prod 34(1): 838-844.

Awang K, Abdullah NH, Hadi AH, Fong YS (2012) Cardiovascular activity of labdane diterpenes from *Andrographis paniculata* in isolated rat hearts. J Biomed Biotechnol 2012: 876458.

Awang K, Azmi MN, Aun LI, Aziz AN, Ibrahim H, Nagoor NH (2010) The apoptotic effect of 1's-1'-acetoxychavicol acetate from *Alpinia conchigera* on human cancer cells. Molecules 15(11): 8048-8059.

Awang K, Ibrahim H, Rosmy Syamsir D, Mohtar M, Mat Ali R, Azah Mohamad Ali N (2011) Chemical constituents and antimicrobial activity of the leaf and rhizome oils of *Alpinia pahangensis* Ridl., an endemic wild ginger from peninsular Malaysia. Chem Biodivers 8(4): 668-673.

Ayafor JF, Bacchi C, Iwu MM, Jackson JE, Okunji CO, Tally Jr JD (2000) Plant-derived antiparasitic and antifungal compounds and methods of extracting the compounds. World Intellectual Property Organization WO 2000024411A2. May 4, 2000.

Ayafor JF, Tchuendem MH, Nyasse B, Tillequin F, Anke H (1994a) Aframodial and other bioactive diterpenoids from *Aframomum* species. Pure and Appl Chem 66: 2327-2330.

Ayafor JF, Tchuendem MH, Nyasse B, Tillequin F, Anke H (1994b) Novel bioactive diterpenoids from *Aframomum aulacocarpos*. J Nat Prod 57: 917-923.

Aziz AN, Ibrahim H, Rosmy Syamsir D, Mohtar M, Vejayan J, Awang K (2013) Antimicrobial compounds from *Alpinia conchigera*. J Ethnopharmacol 145(3): 798-802.

Bahador N, Baserisalehi M (2011) The effect of *Quercus castaneifolia* extract on pathogenic enteric bacteria. Anaerobe 17(6): 358-360.

Bajwa BS, Bhat SV, Dornauer H, DE Souza NJ (1979) Polyoxygenated labdane derivatives. United States Patent US 4,134,986. Jan 16, 1979.

Baker DD, Chu M, Oza U, Rajgarhia V (2007) The value of natural products to future pharmaceutical discovery. Nat Prod Rep 24(6): 1225-1244.

Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH (1985) Natural plant chemicals: Sources of industrial and medicinal materials. Science 228: 1154-1160.

Balba M, El-Hady NA, Taha N, Rezki N, El Ashry ESH (2011) Inhibition of α-glucosidase and α-amylase by diaryl derivatives of imidazole-thione and 1,2,4-triazole-thiol. Eur J Med Chem 46: 2596-2601.

Banerji A, Chakrabarti J, Mitra A, Chatterjee A (2004) Effect of curcumin on gelatinase A (MMP-2) activity in B16F10 melanoma cells. Cancer Lett 211: 235-242.

Bang SD, Johnson SK, Park JCS (1993) Composition and method for treating tumors. United States Patent US 5,248,696. Sep 28, 1993.

Barinag M (1997) Designing therapies that target tumor blood vessels. Science 275: 482-484.

Basile L, Álvarez S, Blanco A, Santagati A, Granata G, Di Pietroc P, Guccionea S, Muñoz-Fernández MÁ (2012) Sulfonilamidothiopyrimidone and thiopyrimidone derivatives as selective COX-2 inhibitors: Synthesis, biological evaluation, and docking studies. Eur J Med Chem 57: 149-161.

Beal MF (1997) Oxidative damage in neurodegenerative diseases. Neuroscientist 3: 21-27.

Bean BP, MacKinnon D, MacKinnon R (2012) Methods and compositions for preventing and relieving muscle cramps and for recovery from neuromuscular irritability and fatigue following exercise. World Intellectual Property Organization WO 2012015882A1. Feb 2, 2012.

Bernfeld P (1955) Amylases, α and β. Method Enzymol 1: 149-154.

Bian QY, Wang SY, Xu LJ, Chan CO, Mok DK, Chen SB (2013) Two new antioxidant diarylheptanoids from the fruits of *Alpinia oxyphylla*. J Asian Nat Prod Res (In press).

Bishayee K, Ghosh S, Mukherjee A, Sadhukhan R, Mondal J, Khuda-Bukhsh AR (2013) Quercetin induces cytochrome-c release and ROS accumulation to promote apoptosis and arrest the cell cycle in G2/M, in cervical carcinoma: Signal cascade and drug-DNA interaction. Cell Prolif 46: 153-163.

Bombardelli E (2012) Combinations of vasoactive substances with estrogens and their use in the treatment of female sexual dysfunctions. United States Patent US 8,211,479. Jul 3, 2012.

Bombardelli E, Giori A (2012) Compositions comprising *Andrographics paniculata* and gingko biloba extracts complexed with phospholipids. European Patent EP2523674A1. Nov 21, 2012.

Bombardelli E, Morazzoni P (2011) Compositions for the treatment of chronic degenerative inflammatory conditions. United States Patent US 7993683 B2. Aug 9, 2011.

Bonte F, Meybeck A, Marechal C (1999) Composition based on hydrated lipidic lamelar phases or on liposomes containing at least one derivative of labdane, or a plant extract containing it; cosmetic or pharmaceutical, particularly dermatological composition containing it. United States Patent US 5,891,464. Apr 6, 1999.

Borchardt JK (2002) The beginnings of drug therapy: Ancient mesopotamian medicine. Drug News Perspect 15(3): 187-192.

Borges A, Ferreira C, Saavedra MJ, Simões M (2013) Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microb Drug Resist 19(4): 256-265.

Borris J (1996) Natural products research perspectives from a major pharmaceutical company. J Ethnopharmcol 51: 29-38.

Brahma SK, Debnath PK (2003) Therapeutic importance of Rasayana drugs with special reference to their multi-dimensional actions. Aryavaidyan 16: 160-163.

Bruneton J (1995) Pharmacognosy, phytochemistry, medicinal plants; part 3. Lavoisier, London.

Burch R, Anderson TA, Lazar J (2006) Capsaicinoid gel formulation and uses thereof. World Intellectual Property Organization WO 2006058140A2. June 1, 2006.

Burch R, Carter RB, Lazar J (2004) Administration of capsaicinoids for the treatment of pain. World Intellectual Property Organization WO 2004058286A1. July 15, 2004.

Butler MS (2008) Natural products to drugs: natural product-derived compounds in clinical trials. Nat Prod Rep 25(3): 475-516.

Cadet M, Williams SK, Simonne A, Sharma CS (2013) Antimicrobial efficacy of *Alpinia galanga* (Linn.) Swartz flower extract against *Listeria monocytogenes* and *Staphylococcus aureus* in a ready-to-eat Turkey Ham product. Int J Poult Sci 12(6): 335-340.

Calabresi P, Parks RE (1985) Chemotherapy of neoplastic diseases. In: Goodman and Gilman's The Pharmacological Basis of Therapeutics. Hardman JGG, Gilman A & Limbird LL (eds), MacMillan, New York, pp. 1240-1306.

Camporese A, Balick MJ, Arvigo R, Esposito RG, Morsellino NF, De SimoneTubaro A (2003) Screening of antibacterial activity of medicinal plants from Belize (Central America). J Ethnopharmacol 87: 103-107.

Canel C, Moraes RM, Dayan FE, Ferreira D (2000) Podophyllotoxin. Phytochemistry 54: 115-120.

Cao G, Sofic E, Prior L (1997) Antioxidant and prooxidant behaviour of flavonoids: Structure activity relationship. Free Rad Bio Med 84-1: 91-94.

Capocchi A, Muccilli V, Cunsolo V, Saletti R, Foti S, Fontanini D (2013) A heterotetrameric alpha-amylase inhibitor from emmer (*Triticum dicoccon* Schrank) seeds. Phytochemistry 88: 6-14.

Cardozo VF, Oliveira AG, Nishio EK, Perugini MR, Andrade CG, Silveira WD, Durán N, Andrade G, Kobayashi RK, Nakazato G (2013) Antibacterial activity of extracellular compounds produced by a Pseudomonas strain against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Ann Clin Microbiol Antimicrob 12: 12.

Caron GN, Stephens P, Badley RA (1998) Assessment of bacterial viability status by flow cytometry and single cell sorting. J Appl Microbiol 84(6): 988-998.

Chakraborty K, Lipton AP, Paul Raj R, Vijayan KK (2010) Antibacterial labdane diterpenoids of *Ulva fasciata* Delile from southwestern coast of the Indian Peninsula. Food Chem 119(4): 1399-1408.

Chang CL, Lin CS, Lai GH (2011) Phytochemical characteristics, free radical scavenging activities, and neuroprotection of five medicinal plant extracts. Evid Based Complement Alternat Med. doi:10.1155/2012/984295.

Chang PK, Zaniewski TA, Lee T, Chen H (2013) Beverage products having steviol glycosides and at least one acid. United States Patent US 8,535,746. Sep 17, 2013.

Chaudhri RD (1996) Herbal drugs industry. Eastern Publisher, New Delhi, 1st Edn.

Chauhan AS, Negi PS, Ramteke RS (2007) Antioxidant and antibacterial activities of aqueous extract of Seabuckthorn (*Hippophae rhamnoides*) seeds. Fitoterapia 78(7-8): 590-592.

Chauhan R, Abraham J (2013) *In vitro* antimicrobial potential of the *Lichen parmotrema* sp. extracts against various pathogens. Iran J Basic Med Sci 16(7): 882-885.

Cheah YH, Azimahtol HL, Abdullah NR (2006) Xanthorrhizol exhibits antiproliferative activity on MCF-7 breast cancer cells via apoptosis induction. Anticancer Res 26(6B): 4527-4534.

Chen JJ, Ting CW, Wu YC, Hwang TL, Cheng MJ, Sung PJ, Wang TC, Chen JF (2013a) New labdane-type diterpenoids and anti-inflammatory constituents from *Hedychium coronarium*. Int J Mol Sci 14(7): 13063-13077.

Chen S, Chen H, Tian J, Wang Y, Xing L, Wang J (2013b) Chemical modification, antioxidant and α -amylase inhibitory activities of corn silk olysaccharides. Carbohydr Polym 98: 428- 437.

Chiasson JL, Josse RG, Gomis R, Hanefeld M, Karasik A, Laakso M (2002) Acarbose for prevention of type 2 diabetes mellitus: The STOP-NIDDM randomised trial. Lancet 359: 2072-2077.

Chimnoi N, Pisutjaroenpong S, Ngiwsara L, Dechtrirut D, Chokchaichamnankit D, Khunnawutmanotham N, Mahidol C, Techasakul S (2008) Labdane diterpenes from the rhizomes of *Hedychium coronarium*. Nat Prod Res 22(14): 1249-1256.

Chimnoi N, Sarasuk C, Khunnawutmanotham N, Intachote P, Seangsai S, Saimanee B, Pisutjaroenpong S, Mahidol C, Techasakul S (2009) Phytochemical reinvestigation of labdane-type diterpenes and their cytotoxicity from the rhizomes of *Hedychium coronarium*. Phytochem Lett 2(4): 184-187.

China Raju B, Tiwari AK, Kumar JA, Ali AZ, Agawane SB, Saidachary G, Madhusudana K (2010) α-Glucosidase inhibitory antihyperglycemic activity of substituted chromenone derivatives. Bioorg Med Chem 18: 358-365.

Chinou I (2005) Labdanes of natural origin-biological activities (1981-2004). Curr Med Chem 12: 1295-1317.

Chitlange SS, Bhujbal SS, Kulkarni AA, Imran M, Kulkarni PS (2008) Chromatographic fingerprint analysis for herbal medicines : A Quality Control Tool. Pharm Rev 6(2).

Chompoo J, Upadhyay A, Kishimoto W, Makise T, Tawata S (2011) Advanced glycation end products inhibitors from *Alpinia zerumbet* rhizomes. Food Chem 129(3): 709-715.

Choo CY, Sulong NY, Man F, Wong TW (2012) Vitexin and isovitexin from the Leaves of *Ficus deltoidea* with *in-vivo* α-glucosidase inhibition. J Ethnopharmacol 142(3): 776-781.

Christenson PA (1995) Method for preparing dodecahydro-3A,6,6,9A-tetramethylnaphtho [2,1-B]furan and novel haloethyl decalin derivatives. United States Patent US 5,434,300. Jul 18, 1995.

Chung Y, Chien C, Teng K, Chou S (2006) Antioxidative and mutagenic properties of *Zanthoxylum ailanthoides* Sieb & zucc. Food Chem 97: 418-425.

Cirla A, Mann J (2003) Combrestatins: from natural product to drug discovery. Nat Prod Rep 20: 558-564.

Clardy J, Walsh C (2004) Lessons from natural molecules. Nature 16: 829-837.

Conway PG, Helsley GC (1990) Synergistic intraocular pressure lowering combinations. United States Patent US 4,902,696. Feb 20, 1990.

Cordell GA, Colvard MD (2005) Some thoughts on the future of ethnopharmacology. J Ethnopharmacol 100(1-2): 5-14.

Costa-Lotufo LV, Cunha GM, Farias PA, Viana GS, Cunha KM, Pessoa C, Moraes MO, Silveira ER, Gramosa NV, Rao VS (2002) The cytotoxic and embryotoxic effects of kaurenoic acid, a diterpene isolated from *Copaifera langsdorffii* oleo-resin. Toxicon 40(8): 1231-1234.

Cotran RS, Cumar V, Collins T (1995) Pathological basis of Pharmacology. Chapman & Hall international, Thomson publishing company.

Cragg GM, Grothaus PG, Newman DJ (2009) Impact of natural products on developing new anti-cancer agents. Chem Rev 109(7): 3012-3043.

Cragg GM, Newman DJ, Snader KM (1997) Natural products in drug discovery and development. J Nat Prod 60: 52-60.

Croteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites). In: Buchanan B, Gruissem W, Jones R (Eds.), Biochemistry and molecular biology of plants. Rockville, MD: American Society of Plant Physiologists. pp. 1250-1318.

Crump JA, Mintz ED (2010) Global trends in typhoid and paratyphoid Fever. Clin Infect Dis 50: 241-246.

Cuadrado I, Fernández-Velasco M, Boscá L, de Las Heras B (2011) Labdane diterpenes protect against anoxia/reperfusion injury in cardiomyocytes: Involvement of AKT activation. Cell Death Dis 2: e229.

Dadkar NK, Souza NJD, Dohadwalla AN, Khandelwal Y, Mandrekar SS, Rupp RH (1988) Method of treating inflammatory diseases with labdan derivatives. United States Patent US 4724238 A. Feb 9, 1988.

Dai J, Mumper RJ (2010) Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules 15: 7313-7352.

de Arau´jo FY, de Oliveira GV, Gomes PX, Soares MA, Silva MI, Carvalho AF, de Moraes MO, de Moraes ME, Vasconcelos SM, Viana GS, de Sousa FC, Maceˆdo DS (2011) Inhibition of

ketamine-induced hyperlocomotion in mice by the essential oil of *Alpinia zerumbet*: Possible involvement of an antioxidant effect. J Pharm Pharmacol 63(8): 1103-1110.

De D, Bera TK, Ali KM, Mandal S, Barik B, Ghosh D (2013) Inhibitory effects of n-hexane fraction from hydro-methanolic (2:3) extract on α-amylase and α-glucosidase activities of sepal of *Salmalia malabarica*: An approach through *in vitro* study. Biomarker Genomic Med 5(1-2): 48-53.

de las Heras B, Hortelano S (2009) Molecular basis of the anti-inflammatory effects of terpenoids. Inflamm Allergy Drug Targets 8: 28-39.

de las Heras B, Hortelano S, Giron N, Bermejo P, Rodriguez B, Boscá L (2007) Kaurane diterpenes protect against apoptosis and inhibition of phagocytosis in activated macrophages. Br J Pharmacol 152: 249-255.

de Oliveira JR, de Castro VC, das Graças Figueiredo Vilela P, Camargo SE, Carvalho CA, Jorge AO, de Oliveira LD (2013) Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry. BMC Complement Altern Med 13: 208.

de Souza NJ, Desai PD, Savanur SV, Blumbach J (1993) Process for the preparation of 6-acyl, 7-acyl, and 6,7-diacyl analogues of forskolin and intermediates thereof. United States Patent US 5,268,471. Dec 7, 1993.

Demetzos C, Dimas KS (2001) Labdane-type diterpenes: Chemistry and biological activity. Stud Nat Prod Chem 25: 235-292.

Dev S (1999) Ancient-modern concordance in Ayurvedic plants: Some examples. Environ Health Perspect 107: 783-789.

Devita VT Jr, Serpick AA, Carbone PO (1970) Combination chemotherapy in the treatment of advanced Hodgkin's disease. Ann Intern Med 73: 881-895.

Dewick PM (1997) In: Medicinal natural products, 2nd Edition, John Wiley & Sons: New York, pp. 188-190.

Dey SK, Bose D, Hazra A, Naskar S, Nandy A, Munda RN, Das S, Chatterjee N, Mondal NB, Banerjee S, Saha KD (2013) Cytotoxic activity and apoptosis-inducing potential of di-spiropyrrolidino and di-spiropyrrolizidino oxindole andrographolide derivatives. PLoS One 8(3): e58055.

Dimas K, Demetzos C, Vaos V, Ioannidis P, Trangas T (2001) Labdane type diterpenes down-regulate the expression of c-Myc protein, but not of Bcl-2, in human leukemia T-cells undergoing apoptosis. Leuk Res 25: 449-454.

Dimas K, Papadaki M, Tsimplouli C, Hatziantoniou S, Alevizopoulos K, Pantazis P, Demetzos C (2006) Labd-14-ene-8,13-diol (sclareol) induces cell cycle arrest and apoptosis in human breast cancer cells and enhances the activity of anticancer drugs. Biomed Pharmacother 60: 127-133.

Dini L (2005) Apoptosis induction in DU-145 human prostate carcinoma cells. Tissue Cell 37(5): 379-384.

Dohadwalla AN, Mandrekar SS, Dadkar NK, Khandelwal Y, Rupp RH, de Souza NJ (1988) Method of treating inflammatory diseases with labdan derivatives. United States Patent US 4,724,238. Feb 9, 1988.

Dong H, Chen SX (1998) A new antiplatelet diarylheptanoid from *Alpinia blepharocalyx*. J Nat Prod 61: 142-144.

Dong HQ, Li M, Zhu F, Liu FL, Huang JB (2012) Inhibitory potential of trilobatin from *Lithocarpus polystachyus* Rehd against α-glucosidase and α-amylase linked to type 2 diabetes. Food Chem 130: 261-266.

Dong Y, Morris-Natschke SL, Lee KH (2011) Biosynthesis, total syntheses, and antitumor activity of tanshinones and their analogs as potential therapeutic agents. Nat Prod Rep 28: 529-542.

Dou CM, Li JC (2004) Effect of extracts of trichosanthes root tubers on HepA-H cells and HeLa cells. World J Gastroenterol 10: 2091-2094.

Drews J (2000) Drug discovery: A historical perspective. Science 287(5460): 1960-1964.

Duan J, Ma Z, Yan X, Zhang W, Wang T, Cai Y (2013b) *Andrographis paniculata* extract. United States Patent US 8,557,308. Oct 15, 2013b.

Dubey NK, Kumar R, Tripathi P (2004) Global promotion of herbal medicine: India's opportunity. Curr Sci 86: 37-41.

Duker-Eshun G, Jaroszewski JW, Asomaning WA, Oppong-Boachie F, Olsen CE, Christensen SB (2002) Antiplasmodial activity of labdanes from *Aframomum latifolium* and *Aframomum sceptrum*. Planta Med 68(7): 642-644.

Durodie J, Coleman K, Simpson IN, Loughborough SH, Winstanley DW (1995) Rapid detection of antimicrobial activity using flow cytometry. Cytometry 21(4): 374-377.

Ebrahimi Nigjeh S, Yusoff FM, Mohamed Alitheen NB, Rasoli M, Keong YS, Omar AR (2013) Cytotoxic effect of ethanol extract of microalga, *Chaetoceros calcitrans*, and its mechanisms in inducing apoptosis in human breast cancer cell line. Biomed Res Int. doi: 10.1155/2013/783690.

Eid M, Araby E (2013) Bactericidal effect of poly (acrylamide/itaconic Acid)-silver nanoparticles synthesized by gamma irradiation against *Pseudomonas aeruginosa*. Appl Biochem Biotechnol 171(2): 469-487.

El-Hawary SS, Kassem HA, Abdel Motaal A, Tawfik WA, Hassanein HM, El-Shamy SS (2013) GC-MS analysis of the essential oil of *Alpinia zerumbet* (Pers.) B.L. and *in vitro* hepatoprotection and cytotoxicity study. Planta Med 79: PI106.

Elya B, Basah K, Mun'im A, Yuliastuti W, Bangun A, Septiana EK (2012) Screening of α-glucosidase inhibitory activity from some plants of Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae. J Biomed Biotech. doi:10.1155/2012/281078.

Elzaawely AA, Xuan TD, Tawata S (2007a) Essential oils, kava pyrones and phenolic compounds from leaves and rhizomes of *Alpinia zerumbet* (Pers.) B.L. Burtt. & R.M. Sm. and their antioxidant activity. Food Chem 103: 486-494.

Elzaawely AA, Xuan TD, Tawata S (2007b) Antioxidant activity and contents of essential oil and phenolic compounds in flowers and seeds of *Alpinia zerumbet* (Pers.) B.L. Burtt. & R.M. Sm. Food Chem 104: 1648-1653.

Escuredo O, Silva LR, Valentão P, Seijo MC, Andrade PB (2012) Assessing Rubushoney value: Pollen and phenolic compounds content and antibacterial capacity. Food Chem 130(3): 671-678.

Essack M, Bajic V, Radovanovic A (2013) Treatment of sickle cell disease. World Intellectual Property Organization WO2013057592A3. Jun 13, 2013.

Evans WC (1997) Trease and Evans Pharmacology 14th edn. Harcourt Brace and company. Asia. Pvt. Ltd. Singapore.

Fan G, Kang YH, Han YN, Han BH (2007) Platelet-activating factor (PAF) receptor binding antagonists from *Alpinia offcinarum*. Bioorg Med Chem Lett 17: 6720-6722.

Fang JY, Leu YL, Hwang TL, Cheng HC, Hung CF (2003) Development of sesquiterpenes from *Alpinia oxyphylla* as novel skin permeation enhancers. Eur J Pharm Sci 19: 253-262.

Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z (1985) Medicinal plants in therapy. Bull World Health Organ 63: 965-981.

FDA approves Tygacil, first-in-class antibiotic. Wyeth Press Release (2005) Available at http://www.wyeth.com.

Feigenbaum JJ (1994) Compositions containing forskolin. United States Patent US 5,371,104. Dec 6, 1994.

Ferreres F, Vinholes J, Gil-Izquierdo A, Valentão P, Gonçalves RF, Andrade PB (2013) *In vitro* studies of α-glucosidase inhibitors and antiradical constituents of *Glandora diffusa* (Lag.) D.C. Thomas infusion. Food Chem 136(3-4): 1390-1398.

Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE (2007) Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol 147(2): 227-235.

Finch RG, Greenwood D, Norrby SR, Whitley RJ (2003) Antibiotic and chemotherapy: Anti-infective agents and their use in therapy. 8th ed. New York: Churchill Livingstone.

Firman K, Kinoshita T, Itai A, Sankawa U (1988) Terpenoids from *Curcuma heyneana*, Phytochemistry 27(12): 3887-3891.

Folkman J (1995) Clinical applications of research on angiogenesis. N Engl J Med 333(26): 1757-1763.

Freitag NE, Port GC, Miner MD (2009) *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat Rev Microbiol 7: 623-628.

Fujisawa T, Ikegami H, Inoue K, Kawabata Y, Ogihara T (2005) Effect of two α-glucosidase inhibitors, voglibose and acarbose, on postprandial hyperglycemia correlates with subjective abdominal symptoms. Metabolism 54(3): 387-390.

Gadre AY, Uchi DA, Rege NN, Daha SA (2001) Nuclear variations in HPTLC fingerprint patterns of marketed oil formulations of *Celastrus paniculates*. Ind J Pharmacol 33: 124-145.

Ganesan A (2008) The impact of natural products upon modern drug discovery. Curr Opin Chem Biol 12(3): 306-317.

Gao H, Huang YN, Gao B, Kawabata J (2008) Chebulagic acid is a potent alpha-glucosidase inhibitor. Biosci Biotechnol Biochem 72: 601-603.

Gaspar-Marques C, Rijo P, Simões MF, Duarte MA, Rodríguez B (2006) Abietanes from *Plectranthus grandidentatus* and *P. hereroensis* against methicillin- and vancomycin-resistant bacteria. Phytomedicine 13: 267-271.

Gautam V, Raman RMV, Ashish K (2003) Exporting Indian healthcare (Export potential of Ayurveda and Siddha products and services). Road Beyond Boundaries (The Case of Selected Indian Healthcare Systems), Export-Import Bank of India, Mumbai, pp. 14-54.

Gehlhaar DK, Bouzida D, Rejto PA (1998) Fully automated and rapid flexible docking of inhibitors covalently bound to serine proteases. Proceedings of the Seventh International Conference on Evolutionary Programming, pp. 449-461.

Gehlhaar DK, Verkhivker G, Rejto PA, Fogel DB, Fogel LJ, Freer ST (1995) Docking conformationally flexible small molecules into a protein binding site through evolutionary programming. Proceedings of the Fourth International Conference on Evolutionary Programming, pp.615-627.

Ghadyale V, Takalikar S, Haldavnekar V, Arvindekar A (2012) Effective control of postprandial glucose level through inhibition of intestinal alpha glucosidase by *Cymbopogon martinii* (Roxb.). Evidence-Based Complementary Altern Med. doi:10.1155/2012/372909.

Ghasi S, Nwobodo E, Ofili JO (2000) Hypocholesterolemic effect of crude leaf of *Moringa oleifera* in high fat diet fed wistar rats. J Ethnopharmacol 69: 21-25.

Ghil S (2013) Antiproliferative activity of *Alpinia officinarum* extract in the human breast cancer cell line MCF-7. Mol Med Rep 7(4): 1288-1292.

Ghose AK, Viswanadhan VN, Wendoloski JJ (1999) A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. J Comb Chem 1: 55-68.

Ghosh S, Ahire M, Patil S, Jabgunde A, Dusane MB, Joshi BN, Pardesi K, Jachak S, Dhavale DD, Chopade BA (2012) Antidiabetic activity of *Gnidia glauca* and *Dioscorea bulbifera*: Potent amylase and glucosidase inhibitors. Evidence-Based Complementary Altern Med. doi:10.1155/2012/929051.

Ghosh S, Indukuri K, Bondalapati S, Saikia AK, Rangan L (2013) Unveiling the mode of action of antibacterial labdane diterpenes from *Alpinia nigra* (Gaertn.) B. L. Burtt seeds. Eur J Med Chem 66: 101-105.

Ghosh S, Rangan L (2013) *Alpinia*: the gold mine of future therapeutics. 3 Biotech 3: 173-185.

Gilbert DM, Neilson A, Miyazawa H, DePamphilis ML, Burhans WC (1995) Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. J Biol Chem 270: 9597-9606.

Giron N, Traves PG, Rodriguez B, Lopez-Fontal R, Bosca L, Hortelano S, de las Heras B (2008) Suppression of inflammatory responses by labdane-type diterpenoids. Toxicol Appl Pharmacol 228: 179-189.

Gnanadhas DP, Ben Thomas M, Thomas R, Raichur AM, Chakravortty D (2013) Interaction of silver nanoparticles with serum proteins affects their antimicrobial activity *in vivo*. Antimicrob Agents Chemother 57(10): 4945-4955.

González MA, Mancebo-Aracil J, Tangarife-Castaño V, Agudelo-Goméz L, Zapata B, Mesa-Arango A, Betancur-Galvis L (2010) Synthesis and biological evaluation of (+)-labdadienedial, derivatives and precursors from (+)-sclareolide. Eur J Med Chem 45: 4403-4408.

Graul AI, Prous JR (2005) The year's new drugs. Drug News Perspect 18(1): 21-36.

Green D (2011) Means to an End: Apoptosis and other cell death mechanisms, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Guo LD, Chen XJ, Hu YH Yu ZJ, Wang D, Liu JZ (2012) Curcumin inhibits proliferation and induces apoptosis of human colorectal cancer cells by activating the mitochondria apoptotic pathway. Phytother Res 27(3): 422-430.

Gurubasavaraja Swamy PM, Ramya Sri B, Giles D, Shashidhar BV, Das AK, Agasimundin YS (2013) Synthesis, anticancer, and molecular docking studies of pyranone derivatives. Med Chem Res 22: 4909-4919.

Gurusamy N, Lekli I, Mukherjee S, Ray D, Ahsan MK Gherghiceanu M, Popescu LM, Das DK (2010) Cardioprotection by resveratrol: A novel mechanism via autophagy involving the mTORC2 pathway. Cardiovasc Res 86: 103-112.

Habtemariam S (2003) *In vitro* antileishmanial effects of antibacterial diterpenes from two Ethiopian Premna species: *P. schimperi* and *P. oligotricha*. BMC Pharmacol 3: 1-6.

Halliwell (1994) Free radicals, antioxidants and human disease : Curiosity or consequence? Lancet 344: 721-724.

Hammer KA, Heel KA (2012) Use of multiparameter flow cytometry to determinethe effects of monoterpenoids and phenylpropanoids on membrane polarityand permeability in staphylococci and enterococci. Int J Antimicrob Agents 40: 239-245.

Han SM, Lee KG, Pak SC (2013) Effects of cosmetics containing purified honeybee (*Apis mellifera* L.) venom on acne vulgaris. J Integr Med 11(5): 320-326.

Hancke Orozco JL, Burgos Aguilera RA (2011) Composition of labdane diterpenes extracted from *Andrographis paniculata*, useful for the treatment of autoimmune diseases, and alzheimer disease by activation for PPR-gamma receptors. United States Patent US 8,084,495. Dec 27, 2011.

Hansawasdi C, Kawabata J, Kasai T (2000) α-amylase inhibitors from Roselle (*Hibiscus sabdariff* a Linn.) Tea. Biosci Biotechnol Biochem 64(5): 1041-1043.

Hanson JR (1991) Diterpenoids. In: Charlwood BV, Banthorpe DV (eds) Methods in plant biochemistry. Academic Press, London, pp 263-288.

Hanson JR (2004) Diterpenoids. Nat Prod Rep 21: 785-793.

Hanson JR (2009) Diterpenoids. Nat Prod Rep 26: 1156-1171.

Haraguchi H, Kuwata Y, Inada K, Shingu K, Miyahara K, Nagao M, Yagi A (1996) Antifungal activity from *Alpinia galanga* and the competition for incorporation of unsaturated fatty acids in cell growth. Planta Med 62: 308-313.

Harborne JB (1998) Phytochemical methods. Chapman & Hall, London, pp. 60-66.

Harborne JB (1998) Phytochemical methods: A guide to modern techniques of plant analysis. 3rd eds, Chapman & Hall Pub, London, UK.

Hartgens F, Kuipers H, Wijnen JA, Keizer HA (1996) Body composition, cardiovascular risk factors and liver function in long-term androgenic-anabolic steroids using bodybuilders three months after drug withdrawal. Int J Sports Med 17(6): 429-433.

He ZH, Ge W, Yue GGL, Lau CBS, He MF, But PPH (2010) Antiangiogenic effects of the fruit of *Alpinia oxyphylla*. J Ethnopharmacol 132: 443-449.

He ZH, Gilli C, Yue GGL, Lau CBS, Greger H, Brecker L, Ge W, But PPH (2012) Anti-angiogenic effects and mechanisms of zerumin A from *Alpinia caerulea*. Food Chem 132(1): 201-208.

Hecker E (1968) Cocarcinogenic principles from the seed oil of *Croton tiglium* and from other Euphorbiaceae. Cancer Res 28: 2338-2348.

Hegde S, Carter J (2003) To Market, To Market-2003. In: Doherty AM, editor. Annual reports in medicinal chemistry. Amsterdam: Academic Press pp. 335-368.

Heinemann JA, Ankenbauer RG, Amábile-Cuevas CF (2000) Do antibiotics maintain antibiotic resistance? Drug Discov Today 5: 195-204.

Hema PS, Nair MS (2009) Flavonoids and other constituents from the rhizomes of *Alpinia calcarata*. Biochem Syst Ecol 37: 52-54.

Herman S (1991) Method for treating viral infection of HIV. United States Patent US 4,983,637. Jan 8, 1991.

Herman S (1993) Method for treating viral infection parenterally. United States Patent US 5,260,342. Nov 9, 1993.

Hollander P (1992) Safety profile of acarbose, an alpha-glucosidsase inhibitor. Drugs 44: 47-53.

Hooker JD (1875) The Flora of British India, vol. 1. L. Reeve and Co., London.

Hortelano BMS, López FR, Rodriguez GB, Beatriz De LHP (2010) Anti-tumour compounds. World Intellectual Property Organization WO 2010023347 A1. Mar 4, 2010.

Howarth OW, Samuel CJ, Vlahov G (1995) The σ-inductive effects of C=C and C≡C bonds: predictability of NMR shifts at sp^2 carbon in non-conjugated polyenoic acids, esters and glycerides. J Chem Soc Perkin Trans 2: 2307-2310.

Hrib NJ (1988) Oxolabdanes useful as pharmaceuticals for reducing intraocular pressure. United States Patent US 4,740,522. Apr 26, 1988.

Hsiao PW, Lin FM, Wang SY (2013) Use certain diterpene compounds in the treatment of androgen receptor-associated diseases. United States Patent US 8,519,007. Aug 27, 2013.

Hsu SY (1988) Protective effects of the constituents of *Alpinia speciosa* rhizomes against various gastric and duodenal lesions in rats. Chunghua Ya Hsuch Tsa Chih 40: 41-48.

Hua SZ, Luo JG, Wang XB, Wang JS, Kong LY (2009) Two novel monoterpene-chalcone conjugates isolated from the seeds of *Alpinia katsumadai*. Bioorg Med Chem Lett 19: 2728-2730.

Huang KC, Williams WM (1999) The pharmacology of Chinese herbs, 2nd ed.; CRC Press: Boca Raton, FL.

Huang SC, Ho CT, Lin-Shiau SY, Lin JK (2005) Carnosol inhibits the invasion of B16/F10 mouse melanoma cells by suppressing metalloproteinase-9 through down-regulating nuclear factor-kappa B and c-Jun. Biochem Pharmacol 69: 221-232.

Ibrahim H, Aziz AN, Syamsir DR, Ali NAM, Mohtar M, Ali RM, Awang K (2009) Essential oils of *Alpinia conchigera* Griff. and their antimicrobial activities. Food Chem 113: 575-577.

Ibrahim H, Sim KS, Syamsir DR, Nor NRM, Nurestri AMS, Awang K (2010) Cytotoxic activity of leaf and rhizome extracts of *Alpinia scabra* (Blume) Na´ves, a wild ginger from Peninsular Malaysia. Afr J Pharm Pharmacol 4(10): 708-711.

Igoli NP, Obanu ZA, Gray AI, Clements C (2012) Bioactive diterpenes and sesquiterpenes from the rhizomes of wild ginger (*Siphonochilus aethiopicus* (SCHWEINF) B.L .BURTT. Afr J Tradit Complement Altern Med 9: 88-93.

Indraji JK (1998) Vanaspati-Shastra: Kunvak, Pravin Prakashan, Rajkot, 2nd Ed., pp. 668-670.

International Diabetes Federation (2006) Diabetes atlas, 3rd Edition, Belgium.

Ismail S, Haris K, Abdul Ghani AR, Abdullah JM, Johan MF, Mohamed Yusoff AA (2013) Enhanced induction of cell cycle arrest and apoptosis via the mitochondrial membrane potential disruption in human U87 malignant glioma cells by aloe emodin. J Asian Nat Prod Res (In press).

Itokawa H, Aiyama R, Ikuta A (1981a) A pungent diarylheptanoid from *Alpinia oxyphylla*. Phytochem 20(4): 769-771.

Itokawa H, Morita H, Katou I, Takeya K, Cavalheiro AJ, de Oliveira RC, Ishige M, Motidome M (1988a) Cytotoxic diterpenes from the rhizomes of *Hedychium coronarium*. Planta Med 54(4): 311-315.

Itokawa H, Morita H, Kobayashi T, Watanabe Z, Iitaka Y (1987) Novel sesquiterpenes from *Alpinia intermedia* GAGNEP. Chem Pharm Bull 35(7): 2860-2868.

Itokawa H, Morita H, Mihashi S (1981b) Two new diarylheptanoid from *Alpinia officinarum* Hance. Chem Pharm Bull 29(8): 2383-2385.

Itokawa H, Yoshimoto S, Morita H (1988b) Diterpenes from the rhizomes of *Alpinia formosana*. Phytochemistry 27: 435-438.

Iwasaki Y, Tanabe M, Kayama Y, Abe M, Kashio M, Koizumi K, Okumura Y, Morimitsu Y, Tominaga M, Ozawa Y, Watanabe T (2009) Miogadial and miogatrial with alpha,beta-unsaturated 1,4-dialdehyde moieties-novel and potent TRPA1 agonists. Life Sci 85(1-2): 60-69.

Izquierdo R, Astudillo L, Rodríguez JA, Theoduloz C, Palenzuela JA, Schmeda-Hirschmann G (2007) Gastroprotective effect and cytotoxicity of labdenamides. Planta Med 73: 310-317.

Jackson JE, Iwu MM, Okunji CO, Bacchi C, Talley Jr JD, Ayafor JF (2002) Antifungal and antiparasitic compounds. United States Patent US 6,403,576. June 11, 2002.

Jacobsen N, Petersen MJ, Weidner MS (2000) Certain diterpenes and extracts or concentrates of *Curcuma amada* containing them for use as medicaments. European Patent EP 1045822A1. Oct 25, 2000.

Jain SK, Prakash V (1995) 22 genera and 178 species in India concentrated mainly in North eastern and the Peninsular region (Zingiberaceae in India) Phytogeography and endemism. Rheedea 5(2): 154-169.

Jang KC, Kim SC, Song EY, Kim KH, Seo HD (2005) Isolation and structure identification of antibacterial substances from the rhizome of *Zingiber mioga* Roscoe. Acta Hort 694: 325-328.

Jang KC, Kim SC, Song EY, Kim KH, Kwon HM, Kang SH, Park KH, Jung YH (2003) Isolation and structure identification of antibacterial substances from the rhizome of *Zingiber mioga* Roscoe. Han'guk Nonghwa Hakhoechi 46(3): 246-250.

Janssen AM, Scheffer JJC (1985) Acetoxychavicol acetate, an antifungal component of *Alpinia galanga*. Planta Med 6: 507-511.

Jantan IB, Yassin MSM, Chin CB, Chen LL, Sim NL (2003) Antifungal activity of the essential oils of nine Zingiberaceae species. Pharm Biol 41: 392-397.

Jeong TS, Lee WS, Kim HC, Choi YK, Kim JR, An SJ, Im KR, Jang KC, Moon OS, Son JS (2010) Abietane diterpenoid compound, and composition comprising extract of torreya nucifera, or abietane diterpenoid compounds or terpenoid compounds isolated from them for prevention and treatment of cardiovascular disease. United States Patent US 7,825,162. Nov 2, 2010.

Ji HF, Li XJ, Zhang HY (2009) Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? EMBO reports 10(3): 194-200.

Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB (2010) Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res 8(3): 893-903.

Johnson IS, Armstrong JG, Gorman M, Burnett Jr JP (1963) The Vinca alkaloids: A new class of oncolytic agents. Cancer Res 23: 1390.

Johnson T (1999) CRC Ethnobotany Desk Reference; CRC Press: Boca Raton, FL.

Jones KW, Ren X, Weintraub NL (2012) Methods of preventing ischemic injury using peripheral nociceptive stimulation. European Patent EP2427056 A1. Mar 14, 2012.

Jones WK, Ren X, Weintraub NL (2010) Methods of preventing ischemic injury using peripheral nociceptive stimulation. World Intellectual Property Organization WO 2010129845A1. Nov 11, 2010.

Jung M, Ko I, Lee S, Choi SJ, Youn BH, Kim SK (1998) A concise synthesis and *in vitro* cytotoxicity of new labdane diterpenes. Bioorg Med Chem Lett 8(23): 3295-3298.

Jung M, Park M, Lee HC, Kang YH, Kang ES, Kim SK (2006) Antidiabetic agents from medicinal plants. Curr Med Chem 13(10): 1203-1218.

Kairyte K, Kadys A, Luksiene Z (2013) Antibacterial and antifungal activity of photoactivated ZnO nanoparticles in suspension. J Photochem Photobiol B 128C: 78-84.

Kalpoutzakis E, Chinou I, Mitaku S, Skaitsounis AL, Hervala C (1998) Antibacterial labdane-type diterpenes from the resin "Ladano" of *Cistus creticus* Subsp. creticus. Nat Prod Lett 11: 173-179.

Kamboj A (2012) Analytical evaluation of herbal drugs, drug discovery research, In: Pharmacognosy, Prof. Omboon Vallisuta (Ed.), ISBN: 978-953-51-0213-7, InTech, doi: 10.5772/26109.

Kamboj VP (2000) Herbal medicine. Curr Sci 78(1): 35-39.

Kamonwannasit S, Nantapong N, Kumkrai P, Luecha P, Kupittayanant S, Chudapongse N (2013) Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall. Ann Clin Microbiol Antimicrob 12(1): 20.

Kanjilal PB, Kotoky R, Couladis M (2010) Essential oil composition of leaf and rhizome oil of *Alpinia nigra* (Gaertner) B.L.Burtt. from Northeast India. J Essent Oil Res 22: 358-359.

Kano Y, Tanabe M, Yasuda M (1990) Evaluation of the preparation of Chinese medicinal prescriptions. (V). Diterpenes from Japanese ginger "Kintoki". Shoyakugaku Zasshi 44(1): 55-57.

Kapoor LD (2000) Handbook of AyurVedic medicinal plants: Herbal reference library; CRC Press: Boca Raton, FL.

Karioti A, Skopeliti M, Tsitsilonis O, Heilmann J, Skaltsa H (2007) Cytotoxicity and immunomodulating characteristics of labdane diterpenes from *Marrubium cylleneum* and *Marrubium velutinum*. Phytochemistry 68(11): 1587-1594.

Kaur A, Singh R, Dey CS, Sharma SS, Bhutani KK, Singh IP (2010) Antileishmanial phenylpropanoids from *Alpinia galanga* (Linn.) Willd. Ind J Exp Biol 48: 314-317.

Kazi YF, Parton R, Memon BA (1994) Haemolytic assay for the detection of adenylate cyclase toxin of *Bordetella pertussis*. Pak J Pharm Sci 7: 55-59.

Keeling CI, Bohlmann J (2006) Genes, enzymes, and chemicals ofterpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol 170: 657-675.

Kenmogne M, Prost E, Harakat D, Jacquier MJ, Frédérich M, Sondengam LB, Zèches M, Waffo-Téguo P (2006) Five labdane diterpenoids from the seeds of *Aframomum zambesiacum*. Phytochemistry 67(5): 433-438.

Kesari V, Das A, Rangan L (2010) Physico-chemical characterization and microbial assay from seed oil of *Pongamia pinnata*, potential biofuel crop. Biomass Bioenergy 34: 108-115.

Khan SU, Khan AU, Shah AU, Shah SM, Hussain S, Ayaz M, Ayaz S (2013) Heavy metals content, phytochemical composition, antimicrobial and insecticidal evaluation of *Elaeagnus angustifolia*. Toxicol Ind Health (In press).

Khandelwal Y, Moraes G, Lal B, Aroskar VA, Dohadwalla AN, Rupp RH (1993b) Labdane derivatives, a process for their preparation, and their use as medicaments. United States Patent US 5,252,598. Oct 12, 1993b.

Khattak S, Rehman S, Shah HU, Ahmad W, Ahmad M (2005) Biological effects of indigenous medicinal plants *Curcuma longa* and *Alpinia galanga*. Fitoterapia 76: 254-257.

Kim HW, Murakami A, Abe M, Ozawa Y, Morimitsu Y, Williams MV, Ohigashi H (2005) Suppressive effects of mioga ginger and ginger constituents on reactive oxygen and nitrogen species generation, and the expression of inducible pro-inflammatory genes in macrophages. Antioxid Redox Signaling 7: 1621-1629.

Kim KY, Nam KA, Kurihara H, Kim SM (2008) Potent alpha-glucosidase inhibitors purified from the red alga *Grateloupia elliptica*. Phytochem 69: 2820-2825.

Kim TH, Li H, Wu Q, Lee HJ, Ryu JH (2013) A new labdane diterpenoid with anti-inflammatory activity from *Thuja orientalis*. J Ethnopharmacol 146(3): 760-767.

Kim YH, Shin HC, Song DW, Lee SH, Furumai T, Park JW, Kwon TK (2003) Arisostatins A induces apoptosis through the activation of caspase-3 and reactive oxygen species generation in AMC-HN-4 cells. Biochem Biophys Res Commun 309: 449-456.

Kim YM, Wang MH, Rhee HI (2004) A novel α-glucosidase inhibitor from pine bark. Carbohydr Res 339: 715-717.

Kimbu SF, Ngadjui BT, Sondengam BL, Njimi T, Connolly JD, Fakunle CO (1987) A new labdane diterpenoid from the seeds of *Aframomum daniellii*. J Nat Prod 50: 230.

Kimbu SF, Njimi TK, Sondengam BL, Akinniyi JA, Connolly JD (1979). The structure of a labdane dialdehyde from *Afromomum daniellii* (Zingiberaceae). J Chem Soc Perkin Trans 1: 1303.

Kirby KB, Pettersson B (2007) Compositions for rapid and non-irritating transdermal delivery of pharmaceutically active agents and methods for formulating such compositions and delivery thereof. United States Patent US 7,267,829. Sep 11, 2007.

Kleinig MJ, Kirby KB, Pettersson Jr BI (2013) Compositions and methods for including melanogenesis in a subject. United States Patent US 8,569,234. Oct 29, 2013.

Koehn FE, Carter GT (2005) The evolving role of natural products in drug discovery. Nat Rev Drug Discov 4(3): 206-220.

Kokate CK (1999) Practical pharmacognosy. New Delhi: Vallabh Prakashan, pp. 107-121.

Kokate CK (1994) Practical Pharmacognosy. Vallabh Prakashan, New Delhi, pp. 107-113.

Kokate CK, Purohit AP, Gokhale SB (2005) Pharmacognosy. Nirali Prakashan, 30th Edn.

Komai K, Naoki M, Asaka S, Suzuki K (2013) Skin-beautifying composition containing hyaluronidase-inhibiting *Curcuma caesia* extract, and cosmetic and food containing the composition. Japanese Patent JP 2013060403A. Apr 4, 2013.

Kong LY, Qin MJ, Niwa M (2000) Diterpenoids from the rhizomes of *Alpinia calcarata*. J Nat Prod 63(7): 939-942.

Koo BS, Lee WC, Chang YC, Kim CH (2004) Protective effects of *Alpina oxyphylla* fructus (*Alpinia oxyphylla* MIQ) water-extracts on neurons from ischemic damage and neuronal cell toxicity. Phytother Res 8(2): 142-148.

Kosley Jr. RW, Cherill RJ (1987) Aminoacyllabdanes, pharmacuetical compositions and use. United States Patent US 4,666,904. May 19, 1987.

Kosley Jr. RW, Cherill RJ (1990) Labdane compounds, pharmaceutical compositions and use. United States Patent US 4,963,537. Oct 16, 1990.

Kosley Jr. RW, O'Malley G, Spahl B (1991) 7-aryl and heteroaryl ethers of desacetylforskolin. United States Patent US 4,999,351. Mar 12, 1991.

Kosley Jr. RW, O'Malley G, Spahl B (1992) 6- and 7-deoxyforskolin and derivatives thereof. United States Patent US 5,093,336. Mar 3, 1992.

Kosley Jr. RW, O'Malley G, Spahl B (1993) 7-aryl and heteroaryl ethers of desacetylforskolin. United States Patent US 5,177,207. Jan 5, 1993.

Kotan R, Dadasoğlu F, Karagoz K, Cakir A, Ozer H, Kordali S, Cakmakci R, Dikbas N (2013) Antibacterial activity of the essential oil and extracts of *Satureja hortensis* against plant pathogenic bacteria and their potential use as seed disinfectants. Sci Hort 153: 34-41.

Kouam SF, Ngouompe AW, Bullach A, Lamshöft M, Kuigoua GM, Spiteller M (2013) Monoterpenes with antibacterial activities from a Cameroonian medicinal plant *Canthium multiflorum* (Rubiaceae). Fitoterapia. doi:pii: S0367-326X(13)00233-5. 10.1016/j.fitote.2013.08.025.

Koyama S, Yamaguchi Y, Tanaka S, Motoyashiya J (1997) A new substance (yoshixol) with an interesting antibiotic mechanism from wood oil of Japanese traditional tree (kisohinoki) *Chamaecyparis obtuse*. Gen Pharmacol 28: 797-804.

Kozuki Y, Yutaka M, Yagasaki K (2001) Resveratrol suppresses hepatoma cell invasion independently of its anti-proliferative action. Cancer Lett 167: 151-156.

Krause RM (1992) The origin of plagues: Old and new. Science 257: 1073-1078.

Kress WJ, Liu AZ, Newman M, Li QJ (2005) The molecular phylogeny of *Alpinia* (Zingiberaceae): A complex and polyphyletic genus of gingers. Am J Bot 92: 167-178.

Kreutner W, Green MJ, Shue HJ, Saksena AK (1988) Method for treating allergic reactions with Forskolin. United States Patent US 4,782,082. Nov 1, 1988.

Krysko DV, Denecker G, Festjens N, Gabriels S, Parthoens E, D'Herde K, Vandenabeele P (2006) Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. Cell Death Differ 13(12): 2011-2022.

Kubo I, Fujita K, Kubo A, Nihei K, Lunde CS (2003) Modes of antifungal action of (2E)-alkenals against *Saccharomyces cerevisiae*. J Agric Food Chem 51(14): 3951-3957.

Kubo I, Fujita K, Lee SH (2001) Antifungal mechanism of polygodial. J Agric Food Chem 49(3): 1607-1611.

Kumar A, Garg V, Chaudhary A, Jain PK, Tomar PK (2013) Isolation, characterisation and antibacterial activity of new compounds from methanolic extract of seeds of *Caesalpinia crista* L. (Caesalpinaceae). Nat Prod Res (In press).

Kumar D, Shah V, Ghosh R, Pal BC (2013) A new triterpenoid saponin from *Glinus oppositifolius* with α-glucosidase inhibitory activity. Nat Prod Res: Formerly Nat Prod Lett 27(7): 624-630.

Kundu JK, Shin YK, Kim SH, Surh YJ (2006) Resveratrol inhibits phorbol ester-induced expression of COX-2 and activation of NF-κB in mouse skin by blocking IκB kinase activity. Carcinogenesis 27: 1465-1474.

Kunnumakkara AB, Ichikawa H, Anand P, Mohankumar CJ, Hema PS, Nair MS, Aggarwal BB (2008) Coronarin D, a labdane diterpene, inhibits both constitutive and inducible nuclear factor-kappa B pathway activation, leading to potentiation of apoptosis, inhibition of invasion, and suppression of osteoclastogenesis. Mol Cancer Ther 7: 3306-3317.

Kuo YJ, Hsiao PC, Zhang LJ, Wu MD, Liang YH, Ho HO, Kuo YH (2009) Labdane diterpenoid glycosides from *Alpinia densespicata* and their nitric oxide inhibitory activities in macrophages. J Nat Prod 72(6): 1097-1101.

Kuzma L, Rozalski M, Walencka E, Rozalska B, Wysokinska H (2007) Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: Salvipisone as a potential anti-biofilm agent active against antibiotic resistant Staphylococci. Phytomedicine 14: 31-35.

Lai MN, Kuo YH, Shiao YJ (2010) Diterpenes from the fruiting body of *Antrodia camphorata* and pharmaceutical compositions thereof. United States Patent US 7,745,647. June 29, 2010.

Lal B, Blumbach J, Dohadwalla AN, de Souza NJ (1992) Pharmaceutical compositions comprising labdane diterpenoid derivatives and pyrimido(6,1-a)isoquinolin-4-one derivatives and their use. United States Patent US 5,141,942. Aug 25, 1992.

Lam KS (2007) New aspects of natural products in drug discovery. Trends Microbiol 15(6): 279-289.

Laranja SM, Bergamaschi CM, Schor N (1991) Evaluation of acute administration of natural products with potential diuretic effects in humans. Mem Inst Oswaldo Cruz 86(2): 237-240.

Laranja SM, Bergamaschi CM, Schor N (1992) Evaluation of three plants with potential diuretic effect. Rer Assoc Bras 38(1): 13-16.

Lee CC, Chiou LY, Wang JY, Chou SY, Lan JC, Huang TS, Huang KC, Wang HM (2013) Functional ginger extracts from supercritical fluid carbon dioxide extraction via *in vitro* and *in vivo* assays: Antioxidation, antimicroorganism, and Mice xenografts models. Scientific World Journal 2013: 210845.

Lee IS, Ma X, Chai HB, Madulid DA, Lamont RB, O'Neill MJ, Besterman JM, Farnsworth NR, Soejarto DD, Cordell GA, Pezzuto JM, Kinghorn AD (1995) Novel cytotoxic labdane diterpenoids from *Neouvaria acuminatissima*. Tetrahedron 51(1): 21-28.

Lee JH, Jung HS, Giang PM, Jin X, Lee S, Son PT, Lee D, Hong YS, Lee K, Lee JJ (2006b) Blockade of nuclear factor-kB signaling pathway and anti-inflammatory activity of Cardamomin, a chalcone analog from *Alpinia conchigera*. J Pharmacol Exp Ther 316: 271-278.

Lee JS, Kim KA, Jeong SH, Lee SG, Park HJ, Kim NJ, Lim S (2009) Anti-inflammatory, anti-nociceptive, and anti-psychiatric effects by the rhizomes of *Alpinia officinarum* on complete Freund's adjuvant-induced arthritis in rats. J Ethnopharmacol 126: 258-264.

Lee SE, Shin HT, Hwang HJ, Kim JH (2003) Antioxidant activity of extracts from *Alpinia katsumadai* seed. Phytother Res 7(9): 1041-1047.

Lee T, Chang PK, Chen H, Zaniewski TA (2012) Beverage products having steviol glycosides and at least one acid. United States Patent US 8,277,862. Oct 2, 2012.

Lee YJ, Park CE, Kim JH, Sohn HJ, Lee J, Jung SY, Shin HJ (2011) *Naegleria fowleri* lysate induces strong cytopathic effects and pro-inflammatory cytokine release in rat microglial cells. Korean J Parasitol 49(3): 285-290.

Lee YJ, Park DB, Jang KC, Jin JY, Kim SC (2006a) Composition comprising compound isolated from *Zingiber mioga* for inhibiting proliferation of cancer by inducing apoptosis of cancer cells. Korean Patent KR 2006033263A. Apr 19, 2006a.

Leonard E, Ajikumar PK, Prather KLJ, Stephanopoulos G, Thayer K (2011) Methods for microbial production of terpenoids. United States Patent US 8,062,878. Nov 22, 2011.

Lexis LA, Fassett RG, Coombes JS (2006) α-Tocopherol and α-lipoic acid enhance the erythrocyte antioxidant defence in cyclosporine A-treated rats. Basic Clin Pharmacol Toxicol 98: 68-73.

Li C, Begum A, Numao S, Park KH, Withers SG, Brayer GD (2005a) Acarbose rearrangement mechanism implied by the kinetic and structural analysis of human pancreatic α-amylase in complex with analogues and their elongated counterparts. Biochemistry 44: 3347-3357.

Li DZ, Tang C, Quinn RJ, Feng Y, Ke CQ, Yao S, Ye Y (2013a) ent-Labdane diterpenes from the stems of *Mallotus japonicus*. J Nat Prod 76(9): 1580-1585.

Li F, Meng L, Zhou J, Xing H, Wang S, Xu G, Zhu H, Wang B, Chen G, Lu YP, Ma D (2005b) Reversing chemoresistance in cisplatin-resistant human ovarian cancer cells: A role of c-Jun NH2-terminal kinase 1. Biochem Biophys Res Commun 335: 1070-1077.

Li H, Park JH, Yan B, Yoo KY, Lee CH, Choi JH, Hwang IK, Won MH (2011a) Neuroprotection of *Alpinia katsumadai* seed extract against neuronal damage in the ischemic gerbil hippocampus is linked to altered brain-derived neurotrophic factor. Lab Anim Res 27(1): 67-71.

Li H, Yoo KY, Lee CH, Choi JH, Hwang IK, Kim JD, Kim YM, Kang IJ, Won MH (2011b) Neuroprotective effects of *Alpinia katsumadai* against neuronal damage in the gerbil hippocampus induced by transient cerebral ischemia. Int J Neurosci 121(9): 490-496.

Li L, Dai HJ, Ye M, Wang SL, Xiao XJ, Zheng J, Chen HY, Luo YH, Liu J (2012) Lycorine induces cell-cycle arrest in the G0/G1 phase in K562 cells via HDAC inhibition. Cancer Cell Int 12(1): 49.

Li QM, Luo JG, Wang XB, Yang MH, Kong LY (2013c) Sesquiterpenes from the rhizomes of *Alpinia japonica* and their inhibitory effects on nitric oxide production. Fitoterapia 86: 29-34.

Li S, Qiu S, Yao P, Sun H, Fong HH, Zhang H (2013b) Compounds from the fruits of the popular European medicinal plant *Vitex agnus-castus* in chemoprevention via NADP(H): Quinone oxidoreductase Type 1 induction. Evid Based Complement Alternat Med 2013:432829.

Liberati C, Winnig M, Woehrle I (2013) N-(2,4-Dimethylpentan-3-yl)methylbenzamides and their use as flavoring agents. World Intellectual Property Organization WO 2013135511A1. Sep 19, 2013.

Lin LI, Ke YF, Ko YC, Lin JK (1998) Curcumin inhibits SK-Hep-1 hepatocellular carcinoma cell invasion *in vitro* and suppresses matrix metalloproteinase-9 secretion. Oncology 55(4): 349-353.

Lin RJ, Yen CM, Chou TH, Chiang FY, Wang GH, Tseng YP, Wang L, Huang TW, Wang HC, Chan LP, Ding HY, Liang CH (2013) Antioxidant, anti-adipocyte differentiation, antitumor activity and anthelmintic activities against Anisakis simplex and Hymenolepis nana of yakuchinone A from *Alpinia oxyphylla*. BMC Complement Altern Med 13(1): 237.

Lingadurai S, Roy S, Joseph RV, Nath LK (2011) Antileukemic activity of the leaf extract of *Bischofia javanica* blume on human leukemic cell lines. Indian J Pharmacol 43: 143-149.

Liotta LA (1986) Tumor Invasion and metastases: Role of the extracellular matrix. Cancer Res 46: 1-7.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 46: 3-26.

Liu D, Guo Q (2012) Method for purifying aframodial. Chinese Patent CN 102633752 A. Aug 15, 2012.

Liu Y, Nair MG (2011) Labdane diterpenes in *Curcuma mangga* rhizomes inhibit lipid peroxidation, cyclooxygenase enzymes and human tumour cell proliferation. Food Chem 124(2): 527-532.

Liu Y, Nair MG (2012) *Curcuma longa* and *Curcuma mangga* leaves exhibit functional food property. Food Chem 135: 634-640.

Lo CY, Liu PL, Lin LC, Chen YT, Hseu YC, Wen ZH, Wang HM (2013) Antimelanoma and antityrosinase from *Alpinia galangal* constituents. Sci World J 2013: 186505.

Lognay G, Marlier M, Severin M, Haubruge E, Gibon V, Trevejo E (1991) On the characterization of some terpenes from *Renealmia alpinia* Rott. (Maas) oleoresin. Flavour Frag J 6(1): 87-91.

Lordan S, Smyth TJ, Soler-Vila A, Stanton C, Ross RP (2013) The α-amylase and α-glucosidase inhibitory effects of Irish seaweed extracts. Food Chem 141(3): 2170-2176.

Lovering AL, Lee SS, Kim YW, Withers SG, Stry-Nadka NCJ (2005) Mechanistic and structural analysis of a family 31 α –glycosidase and its glycosyl-enzyme intermediate. J Biol Chem 280: 2105-2115.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Lu DY, Lu TR, Wu HY (2013) New insights into individualized antimetastatic therapy. Adv Tech Biol Med 1:106. doi: 10.4172/atbm.1000106

Lu YH, Lin-Tao Wang ZT, Wei DZ, Xiang HB (2007) Mechanism and inhibitory effect of galangin and its flavonoid mixture from *Alpinia officinarum* on mushroom tyrosinase and B16 murine melanoma cells. J Enzyme Inhib Med Chem 22(4): 433-438.

Luo JG, Liu J, Kong LY (2008) New pentacyclic triterpenes from *Gypsophila oldhamiana* and their biological evaluation as glycogen phosphorylase inhibitors. Chem Biodiversity 5: 751-757.

Madikizela B, Aderogba MA, Van Staden J (2013) Isolation and characterization of antimicrobial constituents of *Searsia chirindensis* L. (Anacardiaceae) leaf extracts. J Ethnopharmacol. doi:pii: S0378-8741(13)00649-1. 10.1016/j.jep.2013.09.016.

Maeda-Yamamoto M, Suzuki N, Sawai Y, Miyase T, Sano M, Hashimoto-Ohta A, Isemura M (2003) Association of suppression of extracellular signal-regulated kinase phosphorylation by epigallocatechin gallate with the reduction of matrix metalloproteinase activities in human fibrosarcoma HT1080 cells. J Agric Food Chem 51: 1858-1863.

Mahaira LG, Tsimplouli C, Sakellaridis N, Alevizopoulos K, Demetzos C, Han Z, Pantazis P, Dimas K (2011) The labdane diterpene sclareol (labd-14-ene-8, 13-diol) induces apoptosis in human tumor cell lines and suppression of tumor growth *in vivo* via a p53-independent mechanism of action. Eur J Pharmacol 666: 173-182.

Mahassni SH, Al-Reemi RM (2013) Apoptosis and necrosis of human breast cancer cells by an aqueous extract of garden cress (*Lepidium sativum*) seeds. Saudi J Biol Sci 20(2): 131-139.

Majeed M, Kumar A, Nagabhushanam K, Prakash S (2013) Process for preparing water soluble diterpenes and their applications. United States Patent CA 2537820 C. Mar 26, 2013.

Malek SNA, Lee GS, Hong SL, Yaacob H, Wahab NA, Faizal Weber JF, Shah SAA (2011b) Phytochemical and cytotoxic investigations of *Curcuma mangga* rhizomes. Molecules 16: 4539-4548.

Malek SNA, Phang CW, Ibrahim H, Norhanom AW, Sim KS (2011a) Phytochemical and cytotoxic investigations of *Alpinia mutica* rhizomes. Molecules 16: 583-589.

Mandler A, Mersinger R, Reinhard E (1991) Process for obtaining labdane-type diterpenes, especially forskolin, from *Coleus forskohlii*. United States Patent CA 1293702C. Dec 31, 1991.

Manjunatha JR, Bettadaiah BK, Negi PS, Srinivas P (2013) Synthesis of quinoline derivatives of tetrahydrocurcumin and zingerone and evaluation of their antioxidant and antibacterial attributes. Food Chem 136(2): 650-658.

Marathe NP, Rasane MH, Kumar H, Patwardhan AA, Shouche YS, Diwanay SS (2013) *In vitro* antibacterial activity of *Tabernaemontana alternifolia* (Roxb) stem bark aqueous extracts against clinical isolates of methicillin resistant *Staphylococcus aureus*. Ann Clin Microbiol Antimicrob 12(1): 26.

Martinez-Tome M, Jimenez A, Ruggieri S, Frega N, Strabbioli R, Murcia M (2001) Antioxidant properties of mediterranean spices compared with common food additives. J Food Prot 64: 1412-1419.

Masuda T, Mizuguchi S, Tanaka T, Iritani K, Takeda Y (2000) Isolation and structure determination of new antioxidative ferulic acid glucoside esters from the rhizome of *Alpinia speciosa*, a zingiberaceae plant used in okinawan food culture. J Agric Food Chem 48(5): 1479-1484.

Matsuda H, Morikawa T, Managi H, Yoshikawa M (2003) Antiallergic principles from *Alpinia galanga*: Structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF-alpha and IL-4 in RBL-2H3 cells. Bioorg Med Chem Lett 13(19): 3197-3202.

Matsuda H, Morikawa T, Sakamoto Y, Toguchida I, Yoshikawa M (2002) Labdane-type diterpenes with inhibitory effects on increase in vascular permeability and nitric oxide production from *Hedychium coronarium*. Bioorg Med Chem 10(8): 2527-2534.

Matsuda H, Nakashima S, Oda Y, Nakamura S, Yoshikawa M (2009) Melanogenesis inhibitors from the rhizomes of *Alpinia officinarum* in B16 melanoma cells. Bioorg Med Chem 17(16): 6048-6053.

Matsui T, Ueda T, Oki T, Sugita K, Terahara N, Matsumoto K (2001) Alpha glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of natural pigment with potent inhibitory activity. J Agric Food Chem 49: 1948-1951.

Matsui T, Yoshimoto C, Osajima K, Oki T, Osajima Y (1996) *In vitro* survey of α-glucosidase inhibitory food components. Biosci Biotechnol Biochem 60: 2019-2022.

Matsumoto T, Noguchi M, Matsuzaki T, Ninomya M, Kawagishi S (1993) Diterpenes as leukotriene formation inhibitors and anti-inflammatory agents. Jananese Patent JP 05085931A. Apr 6, 1993.

May R, Roy G, Lee T (2011) Anisic acid modified steviol glycoside sweetened beverage products. United States Patent US 8,084,073. Dec 27, 2011.

May R, Roy G, Lee T (2012) Anisic acid modified steviol glycoside sweetened beverage products. United States Patent US 8,337,928. Dec 25, 2012.

Mcdougall GJ, Shpiro F, Doboson P, Smith P, Black A, Stewart D (2005) Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. J Agric Food Chem 53: 2760-2766.

Meyre-Silva C, Yunes RA, Schlemper V, Campos-Buzzi F, Cechinel-Filho V (2005) Analgesic potential of marrubiin derivatives, a bioactive diterpene present in *Marrubium vulgare* (Lamiaceae). Farmaco 60(4): 321-326.

Miyazawa M, Nakamura Y, Ishikawa Y (2000) Insecticidal sesquiterpene from *Alpinia oxyphylla* against *Drosophila melanogaster*. J Agric Food Chem 48: 3639-3641.

Moerman DE (1986) Medicinal plants of Native America; Museum of Anthropology, University of Michigan: Ann Arbor, MI.

Moore BM, Miller DD (2004) Method and kit using a vanilloid receptor agonist for controlling bleeding. United States Patent US 20040202735A1. Oct 14, 2004.

Morel I, Lescoat G, Cogrel P, Sergent O, Pasdeloup N, Brissot P, Cillard P, Cillard J (1993) Antioxidant and iron chelating activities of flavonoids catechin, quercetin, and diosmetin on iron loaded rat hepatocyte cultures. Biochem Pharmacol 45: 13-19.

Morikawa T, Matsuda H, Toguchida I, Ueda K, Yoshikawa M (2002) Absolute stereostructures of three new sesquiterpenes from the fruit of *Alpinia oxyphylla* with inhibitory effects on nitric oxide production and degranulation in RBL-2H3 cells. J Nat Prod 65(10): 1468-1474.

Morita H, Itokawa H (1986) New diterpenes from *Alpinia galanga* Wild. Chem Lett 7: 1205-1208.

Morita H, Itokawa H (1988) Cytotoxic and antifungal diterpenes from the seeds of *Alpinia galanga*. Planta Med 54(2): 117-120.

Mosihuzzman M, Naheed S, Hareem S, Talib S, Abbas G, Khan SN, Choudhary MI, Sener B, Tareen RB, Israr M (2013) Studies on α-glucosidase inhibition and anti-glycation potential of Iris loczyi and Iris unguicularis. Life Sci 92(3): 187-192.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.

Moure A, Cruz JM, Franco D, Dominguez H, Sineiro J, Dominguez H, Nú˜nez MJ, Parajó JC (2001) Natural antioxidants from residual sources. Food Chem 72: 145-171.

Mpalantinos MA, de Moura RS, Parente JP, Kuster RM (1998) Biologically active flavanoid and kava pyrones from the aqueous extract of *Alpinia zerumbet*. Phytotheraphy Res 12: 442-444.

Muangman S, Thippornwong M, Tohtong R (2005) Anti-metastatic effects of curcusone B, a diterpene from *Jatropha curcas*. In Vivo 19: 265-268.

Muñoz-Castañeda JR, Muntané J, Muñoz MC, Bujalance I, Montilla P, Tunez I (2006) Estradiol and catecholestrogens protect against adriamycininduced oxidative stress in erythrocytes of ovariectomized rats. Toxicol Lett 160: 196-203.

Murai A, Iwamura K, Takada M, Ogawa K, Usui T, Okumura J (2002) Control of postprandial hyperglycaemia by galactosyl maltobionolactone and its novel anti-amylase effect in mice. Life Sci 71: 1405-1415.

Murata W, Tanaka T, Kubo I, Fujita K (2013) Protective effects of α-tocopherol and ascorbic acid against cardol-induced cell death and reactive oxygen species generation in *Staphylococcus aureus*. Planta Med 79(9): 768-774.

Murphy G, Nguyen Q, Cockett MI, Atkinson SJ, Allan JA, Knight CG, Willenbrock F, Docherty AJ (1994) Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant. J Biol Chem 269: 6632-6636.

Murray A, Hunt T (1993) The Cell Cycle: An Introduction. Nurse, WH Freeman & Co,. New York, pp 449-454.

Mustahil NA, Sukari MA, Abdul AB, Ali NA, Lian GE (2013) Evaluation of biological activities of *Alpinia mutica* Roxb. and its chemical constituents. Pak J Pharm Sci 26(2): 391-395.

Nagase H, Woessner Jr JF (1999) Matrix metalloproteinases. J Biol Chem 274: 21491-21494.

Nakamura S, Okazaki Y, Ninomiya K, Morikawa T, Matsuda H, Yoshikawa M (2008) Medicinal flowers. XXIV. Chemical structures and hepatoprotective effects of constituents from flowers of *Hedychium coronarium*. Chem Pharm Bull (Tokyo) 56(12): 1704-1709.

Nakatani N, Kikuzaki H, Yamaji H, Yoshio K, Kitora C, Okada K, Padolina WG (1994) Labdane diterpenes from rhizomes of *Hedychium coronarium*. Phytochemistry 37(5): 1383-1388.

Nam JW, Kim SJ, Han AR, Lee SK, Seo EK (2005) Cytotoxic phenylpropanoids from rhizomes of *Alpinia galanga*. J Appl Pharmacol 13: 263-266.

Ndhlala AR, Amoo SO, Ncube B, Moyo M, Nair JJ, Staden JV (2013) Antibacterial, antifungal, and antiviral activities of African medicinal plants, In: Kuete V, Eds(s), Medicinal plant research in Africa, Elsevier, Oxford, pp. 621-659.

Negi PS, Jayaprakasha GK, Jena BS (2008) Antibacterial activity of the extracts from the fruit rinds of *Garcinia cowa* and *Garcinia pedunculata* against food borne pathogens and spoilage bacteria. LWT - Food Sci Technol 41: 1857-1861.

Newman DJ and Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 75: 311-335.

Newman DJ, Cragg GM, Snader KM (2000) The influence of natural products upon drug discovery. Nat Prod Rep 17(3): 215-234.

Newman DJ, Cragg GM, Snader KM (2003) Natural products as sources of new drugs over the period 1981-2002. J Nat Prod 66: 1022-1037.

Ngo KS, Brown GD (1998) Stilbenes, monoterpenes, diarylheptanoids, labdanes and chalcones from *Alpinia katsumadai.* Phytochemistry 47(6): 1117-1123.

Nguefack J, Budde BB, Jakobsen M (2004) Five essential oils from aromatic plants of Cameroon: Their antibacterial activity and ability to permeabilize the cytoplasmic membrane of *Listeria innocua* examined by flow cytometry. Lett Appl Microbiol 39(5): 395-400.

Nichols BL, Avery S, Sen P, Swallo DM, Hahn D, Sterchi E (2003) The maltase-glucoamylase gene: Common ancestry to sucrase-isomaltase with complementary starch digestion activities. Proc Natl Acad Sci USA 100: 1432-1437.

Niyomkam P, Kaewbumrung S, Kaewnpparat S, Panichayupakaranant P (2010) Antibacterial activity of Thai herbal extracts on acne involved microorganism. Pharm Biol 48(4): 375-380.

Noble RL (1990) The discovery of the vinca alkaloids - chemotherapeutic agents against cancer. Biochem Cell Biol 68:1344-1351.

Novo DJ, Perlmutter NG, Hunt RH, Shapiro HM (2000) Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. Antimicrob Agents Chemother 44(4): 827-834.

Nuchnipa N, Apichart S (2008) Chemical constituents of the rhizomes of *Alpinia malaccensis*, Biochem Sys Ecol 36(8): 661-664.

Nyasse B, Lenta-Ndjakou B (2000) Aframodial, a labdane diterpene showing selective *in vitro* antileukemic activity. Pharmazie 55(9): 703-704.

Oberlies NH, Kroll DJ (2004) Camptothecin and Taxol: Historic achievements in natural products research. J Nat Prod 67: 129-135.

Odhav B, Kandasamy T, Khumalo N, Baijnath H (2010) Screening of African traditional vegetables for their alpha-amylase inhibitory effect. J Med Plants Res 4(14): 1502-1507.

Ogihara H, Endou F, Furukawa S, Matsufuji H, Suzuki K, Anzai H (2013) Antimicrobial activity of the carnivorous plant *Dionaea muscipula* against food-related pathogenic and putrefactive bacteria. Biocontrol Sci 18(3): 151-155.

Ogungbe IV, Setzer WN (2013) In-silico Leishmania target selectivity of antiparasitic terpenoids. Molecules 18(7): 7761-7847.

Olubomehin OO, Abo KA, Ajaiyeoba EO (2013) Alpha-amylase inhibitory activity of two *Anthocleista* species and *in vivo* rat model anti-diabetic activities of *Anthocleista djalonensis* extracts and fractions. J Ethnopharmacol 146: 811-814.

Omoregie SN, Omoruyi FO, Wright VF, Jones L, Zimba PV (2013) Antiproliferative activities of lesser galangal (*Alpinia officinarum* Hance Jam1), turmeric (*Curcuma longa* L.), and ginger (*Zingiber officinale* Rosc.) against acute monocytic leukemia. J Med Food 16(7): 647-55.

Oonmetta-aree J, Suzuki T, Gasaluck P, Eumkeb G (2006) Antimicrobial properties and action of galangal (*Alpinia galanga* Linn.) on *Staphylococcus aureus*. LWT Food Sci Technol 39: 1214-1220.

Otoguro K, Iwatsuki M, Ishiyama A, Namatame M, Nishihara-Tukashima A, Kiyohara H, Hashimoto T, Asakawa Y, Omura S, Yamada H (2011) *In vitro* antitrypanosomal activity of plant terpenes against *Trypanosoma brucei*. Phytochemistry 72(16): 2024-2030.

Paparella A, Taccogna L, Aguzzi I, Chaves-Lo'pez C, Serio A, Marsilio F, Suzzi G (2008) Flow cytometric assessment of the antimicrobial activity of essentialoils against *Listeria monocytogenes*. Food Control 19: 1174-1182.

Pape WJW, Hoppe U (1990) Standardization of an *in vitro* red blood cell test for evaluation the acute cytotoxicity potential of tensides. Drug Res 40: 498-502.

Patiño AC, María Benjumea D, Andrés Pereañez J (2013) Inhibition of venom serine proteinase and metalloproteinase activities by *Renealmia alpinia* (Zingiberaceae) extracts: Comparison of wild and *in vitro* propagated plants. J Ethnopharmacol 149(2): 590-596.

Patra JK, Gouda S, Sahoo SK, Thatoi HN (2012) Chromatography separation, ^{1}H NMR analysis and bioautography screening of methanol extract of *Excoecaria agallocha* L. from Bhitarkanika Orissa, India. Asian Pac J Trop Biomed 2: S50-S56.

Patwardhan B (2005) Ethnopharmacology and drug discovery. J Ethnopharmacol 100(1-2): 50-52.

Pertino MW, Theoduloz C, Bastías M, Schmeda-Hirschmann G (2013) Dimeric labdane diterpenes: synthesis and antiproliferative effects. Molecules 18(5): 5936-5953.

Peters RJ, Russell DG, Mann FM (2012) Diterpene modulator of macrophage phagosomal maturation. United States Patent US 8,309,322. Nov 13, 2012.

Peters RJ, Xu M (2009) Identification of syn-copalyl diphosphate synthase. United States Patent US 7,525,016. Apr 28, 2009.

Peters RJ, Xu M (2010) Identification of syn-stemodene synthase. United States Patent US 7,833,748. Nov 16, 2010.

Petersen OG (1889) Musaceae, Zingiberaceae, Cannaceae, Marantaceae. In: Engler,A., Prantl, K. (Eds.), Die Natürlichen Pflanzenfamilien. Verlag von Wilhelm Engel-mann, Leipzig, Germany, pp. 1.

Phan MAT, Wang J, Tang J, Lee YZ, Ng K (2013) Evaluation of α-glucosidase inhibition potential of some flavonoids from *Epimedium brevicornum*, LWT - Food Sci Technol 53(2): 492-498.

Phillipson JD (2001) Phytochemistry and medicinal plants. Phytochemistry 56(3): 237-243.

Phitak T, Choocheep K, Pothacharoen P, Pompimon W, Premanode B, Kongtawelert P (2009) The effects of p-hydroxycinnamaldehyde from *Alpinia galanga* extracts on human chondrocytes. Phytochemistry 70: 237-243.

Pita JC, Xavier AL, de Sousa TK, Mangueira VM, Tavares JF, de Oliveira Júnior RJ, Veras RC, Pessoa Hde L, da Silva MS, Morelli S, Ávila Vde M, da Silva TG, Diniz Mde F, Castello-Branco MV (2012) *In vitro* and *in vivo* antitumor effect of trachylobane-360, a diterpene from *Xylopia langsdorffiana*. Molecules 17(8): 9573-9589.

Pogano G, Marena S, Corgiat-Mansin L, Cravero F, Giorda C, Bozza M, Rossi CM (1995) Comparison of miglitol and glibenclamide in diet-treated type 2 diabetic patients. Diabete Metab 21: 162-167.

Pokorny J, Korczak J (2001) Preparation of natural antioxidants. In: Pokorny J, Yanishlieva N, Gordon M (Eds.), Antioxidants in Food: Practical Applications. CRC, Press, Boca Raton, pp. 311-330.

Porto TS, Furtado NAJC, Heleno VCG, Martins CHG, da Costa FB, Severiano ME, Silva AN, Veneziani RCS, Ambrosio SR (2009a) Antimicrobial ent-pimarane diterpenes from *Viguiera arenaria* against Gram-positive bacteria. Fitoterapia 80: 432-436.

Porto TS, Rangel R, Furtado N, de Carvalho TC, Martins CHG, Veneziani RCS, Da Costa FB, Vinholis AHC, Cunha WR, Heleno VCG et al (2009b) Pimarane-type diterpenes: Antimicrobial activity against oral pathogens. Molecules 14: 191-199.

Prabhakar Reddy P, Tiwari AK, Ranga Rao R, Madhusudhana K, Rama Subba Rao V, Ali AZ, Suresh Babu K, Madhusudana Rao J (2009) New Labdane diterpenes as intestinal alpha-glucosidase inhibitor from antihyperglycemic extract of *Hedychium spicatum* (Ham. Ex Smith) rhizomes. Bioorg Med Chem Lett 19(9): 2562-2565.

Prabhakaran S, Rajaram R, Balasubramanian V, Mathivanan K (2012) Antifouling potentials of extracts from seaweeds, seagrasses and mangroves against primary biofilm forming bacteria. Asian Pac J Trop Biomed 2(1): S316-S322.

Prakash V, Mehrotra BN (1995) Zingiberaceae of north-east India: Diversity and taxonomic status. In: Proceedings of the 2nd symposium on the family Zingiberaceae, pp. 262-273.

Pratt WB, Ruddon RW, Ensminger WD, Maybaum J (1994) The anticancer drugs. 2nd New York, USA: Oxford University Press.

Pushpangadan P (2005) Antioxidant approach to disease management and the role of 'Rasayana' herbs of Ayurveda. J Ethnopharmacol 99: 165-178.

Qiao CF, Quanbin H, Jingzheng S, Zhengtao W, Lu o-shan X, Hongxi X (2007) HPLC determination of two bioactive flavone glycosides and GC-MS analysis of volatile oil constituents in *Alpinia nigra*. Asian J Trad Med 2(3): 85-91.

Qiao CF, Wang ZT, Dong H, Xu LS, Hao XJ (2000) The chemical constituents of Black fruit Galangal (*Alpinia nigra*). Chinese Trad Herb Drugs 31: 404-405.

Quinn RJ, Carroll AR, Pham NB, Baron P, Palframan ME, Suraweera L, Pierens GK, Muresan S (2008) Developing a drug-like natural product library. J Nat Prod 71(3): 464-468.

Radulovic N, Denic M, Stojanovic-Radic Z (2010) Antimicrobial phenolic abietane diterpene from *Lycopus europaeus* L. (Lamiaceae). Bioorg Med Chem Lett 20: 4988-4991.

Raff JW, Glover DM (1988) Nuclear and cytoplasmic mitotic cycles continue in Drosophila embryos in which DNA synthesis is inhibited with aphidicolin. J Cell Biol 107: 2009-2019.

Raina MK (1996) Aloe in Supplement to cultivation and utilization of medicinal plants, eds. By Handa SS and Kaul MK, RRL (CSIR), Jammu-Tawi, pp. 313-322.

Raj N, Nadeem S, Jain S, Raj C, Chouhan K, Nandi P (2011) Ameliorative effects of *Alpinia calcarata* in alloxan-induced diabetic rats. Dig J Nanomater Bios 6: 991-997.

Ramiandrasoa F, Chuilon S, Moretti C, Kunesch G (1986) Labda-8(17),12-dien-15,16-dial, a component of *Renealmia guianensis* (Zingiberaceae). Plantes Medicinales et Phytotherapie 20(3): 227-230.

Rangsiruji R, Newman MF, Cronk CB (2000) Origin and relationships of *Alpinia galanga* (Zingiberaceae) based on molecular data. Edinb J Bot 57(1): 9-37.

Rao K, Ch B, Narasu LM, Giri A (2010) Antibacterial activity of *Alpinia galanga* (L.) Willd crude extracts. Appl Biochem Biotechnol 162(3): 871-884.

Rasamison VE, Ranaivo-Harimanana L, Cao S, Pan E, Ratovoson F, Randriantafika F, Rakotondrajaona R, Rakotonandrasana S, Andriantsiferana R, Kingston DG (2010) A new labdane diterpene from *Vitex cauliflora* Moldenke from the Madagascar rainforest. Fitoterapia 81(1): 55-58.

Ray A, Bharali P, Konwar BK (2013) Mode of antibacterial activity of Eclalbasaponin isolated from *Eclipta alba*. Appl Biochem Biotechnol (In press).

Reddy PP, Rao RR, Shashidhar J, Sastry BS, Rao JM, Babu KS (2009a) Phytochemical investigation of labdane diterpenes from the rhizomes of *Hedychium spicatum* and their cytotoxic activity. Bioorg Med Chem Lett 19(21): 6078-6081.

Reddy PP, Tiwari AK, Rao RR, Madhusudhana K, Rao VRS, Ali AZ, Babu KS, Rao JM (2009b) New labdane diterpenes as intestinal α-glucosidase inhibitor from antihyperglycemic extract of *Hedychium spicatum* (Ham. Ex Smith) rhizomes. Bioorg Med Chem Lett 19(9): 2562-2565.

Ren L, Qin X, Cao X, Wang L, Bai F, Shen Y (2011) Structural insight into substrate specificity of human intestinal maltase-glucoamylase. Protein Cell 2: 827-836.

Roberts SC (2007) Production and engineering of terpenoids in plant cell culture. Nat Chem Biol 3: 387-395.

Roman Junior WA, Piato AL, Conterato GMM, Wildner SM, Marcon M, Moreira S, Santo GD, Mocelin R, Emanuelli T, de Moraes Santos CA (2013) Psychopharmacological and antioxidant effects of hydroethanolic extract of *Alpinia zerumbet* leaves in mice. Pharmacogn J 5(3): 113-118.

Romero CD, Chopin SF, Buck G, Martinez E, Garcia M, Bixby L (2005) Antibacterial properties of common herbal remedies of the southwest. J Ethnopharmacol 99(2): 253-257.

Roth GN, Chandra A, Nair MG (1998) Novel bioactivities of *Curcuma longa* constituents. J Nat Prod 61(4): 542-545.

Rousseaux CG, Schachter H (2003) Regulatory issues concerning the safety, efficacy and quality of herbal remedies. Birth Defects Res B Dev Reprod Toxicol 68(6): 505-510.

Roy B, Dasgupta S, Tandon V (2009) Ultrastructural observations on Fasciolopsisbuski and its alterations caused by shoot extract of *Alpinia nigra*. Microsc Res Tech 72(2): 61-66.

Roy B, Swargiary A, Giri BR (2012) *Alpinia Nigra* (Family Zingiberaceae): An anthelmintic medicinal plant of North -East India. Adv Life Sci 2(3): 39-51.

Roy B, Tandon V (1999) Flukicidal activity of *Alpinia nigra* against the trematode, Fasciolopsis buski, in humans. Biomed Lett 60: 23-29.

Rydberg EH, Li C, Maurus R, Overall CM, Brayer GD, Withers SG (2002) Mechanistic analyses of catalysis in human pancreatic α-amylase: Detailed kinetic and structural studies of mutants of three conserved carboxylic acids. Biochemistry 41: 4492-4502.

Sahoo S, Ghosh G, Das D, Nayak S (2013) Phytochemical investigation and *in vitro* antioxidant activity of an indigenous medicinal plant *Alpinia nigra* B.L. Burtt. Asian Pacific J Trop Biomed 3(11) 87- 876.

Sakarkar DM, Deshmukh VN (2011) Ethnopharmacological review of traditional medicinal plants for anticancer activity. Int J PharmTech Res 3: 298-308.

Saklani A, Kutty SK (2008) Plant-derived compounds in clinical trials. Drug Discov Today 13: 161-171.

Sales PM, Souza PM, Simeoni LA, Silveira D (2012) α-Amylase inhibitors: A review of raw material and isolated compounds from plant source. J Pharm Pharm Sci 15(1): 141-183.

Santosh Kumar SC, Srinivas P, Negi PS, Bettadaiah BK (2013) Antibacterial and antimutagenic activities of novel zerumbone analogues. Food Chem 141(2): 1097-1103.

Sapna S, Ravi TK (2007) Approaches towards development and promotion of herbal drugs. Pharmacognosy Rev 1(1): 180-184.

Satish R, Dhananjayan R (2003) Evaluation of anti-inflammatory potential of rhizome of *Alpinia galanga* Linn. Biomedicine 23: 91-96.

Sawadogo WR, Schumacher M, Teiten MH, Dicato M, Diederich M (2012) Traditional West African pharmacopeia, plants and derived compounds for cancer therapy. Biochemical Pharmacol 84(10): 1225-1240.

Scanlon TC, Dostal SM, Griswold KE (2013) A high-throughput screen for antibiotic drug discovery. Biotechnol Bioeng. doi: 10.1002/bit.25019.

Schaefer M, Urban N, Kübler W (2013) Bicyclic labdane diterpenes for use in the treatment of TRPC6 associated diseases. European Patent EP 2556829 A1. Feb 13, 2013.

Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly *in vitro* by taxol. Nature 277(5698): 665-667.

Schlegel R, Pardee AB (1986) Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. Science 232: 1264-1266.

Schramm A, Ebrahimi SN, Raith M, Zaugg J, Rueda DC, Hering S, Hamburger M (2013) Phytochemical profiling of *Curcuma kwangsiensis* rhizome extract, and identification of labdane diterpenoids as positive GABA(A) receptor modulators. Phytochemistry. doi:pii: S0031-9422(13)00305-1.

Sears M, Caprioli J (1984) Composition and method for treatment of glaucoma. United States Patent US 4,476,140. Oct 9, 1984.

Seo S, Gomi K, Kaku H, Abe H, Seto H, Nakatsu S, Neya M, Kobayashi M, Nakaho K, Ichinose Y, Mitsuhara I, Ohashi Y (2012) Identification of natural diterpenes that inhibit bacterial wilt disease in tobacco, tomato and Arabidopsis. Plant Cell Physiol 53(8): 1432-1444.

Seth SD, Sharma B (2004) Medicinal plants of India. Indian J Med Res 120: 9-11.

Shah MB, Chauhan G (1996) In: Supplement to cultivation & utlilization of medicinal plants, (eds.) By Handa SS and Kaul MK, RRL(CSIR) Jammu-Tawi, pp. 53-96.

Shang W, Qiao J, Gu C, Yin W, Du J, Wang W, Zhu M, Han M, Lu W (2011) Anticancer activity of an extract from needles and twigs of *Taxus cuspidata* and its synergistic effect as a cocktail with 5-fluorouracil. BMC Complement Altern Med 11: 123.

Shao ZM, Wu J, Shen Z, Barsky SH (1998) Genistein exerts multiple suppressive effects on human breast carcinoma cells. Cancer Res 58: 4851-4857.

Sharma A, Shanker C, Tyagi LK, Singh M, Rao ChV (2008) Herbal medicine for market potential in India: An overview. Acad J Plant Sci 1: 26-36.

Sharma P, Sharma JD (2001) *In vitro* hemolysis of human erythrocytes by plant extracts with antiplasmodial activity. J Ethnopharmacol 74: 239-243.

Shaw P, Mead R, Higginbottom A, Barber S (2010) Therapeutics for neurological disorders. World Intellectual Property Organization WO 2010046710A1. Apr 29, 2010.

Sheeja DBA, Nair MS (2012) Phytochemical constituents of *Curcuma amada*, Biochem Sys Ecol 44: 264-266.

Sherry L, Millhouse E, Lappin DF, Murray C, Culshaw S, Nile CJ, Ramage G (2013) Investigating the biological properties of carbohydrate derived fulvic acid (CHD-FA) as a potential novel therapy for the management of oral biofilm infections. BMC Oral Health 13(1): 47.

Shi GF, An LJ, Jiang B, Guan S, Bao YM (2006) *Alpinia* protocatechuic acid protects against oxidative damage *in vitro* and reduces oxidative stress *in vivo*. Neurosci Lett 403: 206-210.

Shi H, Tan B, Ji G, Lu L, Cao A, Shi S, Xie J (2013) Zedoary oil (Ezhu You) inhibits proliferation of AGS cells. Chin Med 8(1): 13.

Shimada K, Fujikawa K, Yahara K, Nakamura T (1992) Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 40(6): 945-948.

Shin SY, Bajpai VK, Kim HR, Kang SC (2007) Antibacterial activity of eicosapentaenoic acid (EPA) against food-borne and food spoilage microorganisms. LWT- Food Sci Technol 40: 1515-1519.

Shobana S, Sreerama YN, Malleshi NG (2009) Composition and enzyme inhibitory properties of finger millet (*Eleusine coracana* L.) seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase. Food Chem 115: 1268-1273.

Shukla AK, Patra S, Dubey VK (2011) Evaluation of selected antitumor agents as subversive substrate and potential inhibitor of trypanothione reductase: An alternative approach for chemotherapy of Leishmaniasis. Mol Cell Biochem 352: 261-270.

Shutske GM (1991b) 12-halogenated forskolin derivatives. United States Patent US 5,070,209. Dec 3, 1991b.

Sielinou VT, Vardamides JC, Nkengfack AE, Laatsch H (2012) Phenolic derivatives and an antifungal and cytototoxic labdane diterpenoid from the root bark of *Turraeanthus mannii*. Phytochem Lett 5(3): 409-413.

Silva G, Jeney V, Chora A, Larsen R, Balla J, Soares MP (2009) Oxidized hemoglobin is an endogenous proinflammatory agonist that targets vascular endothelial cells. J Biol Chem 284(43): 29582-29595.

Silverstein RM, Webster FX, Kiemle DJ (2005) Proton NMR spectrometry, spectrometric identification of organic compounds. John Wiley and Sons Inc., USA, pp. 127-204.

Sim L, Jayakanthan K, Mohan S, Nasi R, Johnston BD, Pinto BM, Rose DR (2010) New glucosidase inhibitors from an ayurvedic herbal treatment for type 2 diabetes: Structures and inhibition of human intestinal maltase-glucoamylase with compounds from *Salacia reticulata*. Biochem 49: 43-451.

Sim L, Quezada-Calvillo R, Sterchi EE, Nichols BL, Rose DR (2008) Human intestinal maltase-glucoamylase: Crystal structure of the N-terminal catalytic subunit and basis of inhibition and substrate specificity. J Mol Biol 375: 782-792.

Singh HJC, Alagarsamy V, Diwan PV, Kumar SS, Nisha JC, Narsimha Reddy Y (2011a) Neuroprotective effect of *Alpinia galanga* (L.) fractions on Aβ(25–35) induced amnesia in mice. J Ethnopharmacol 138(1): 85-91.

Singh HJC, Alagarsamy V, Sathesh Kumar S, Narsimha Reddy Y (2011b) Neurotransmitter metabolic enzymes and antioxidant status on Alzheimer's disease induced mice treated with *Alpinia galanga* (L.) Willd. Phytother Res 25(7): 1061-1067.

Singh M, Tyagi S, Bhui K, Prasad S, Shukla Y (2010b) Regulation of cell growth through cell cycle arrest and apoptosis in HPV 16 positive human cervical cancer cells by tea polyphenols. Invest New Drugs 28(3): 216-224.

Singh S, Gray AI, Skelton BW, Waterman PG, White AH (1991) (+)-14β-Hydroxylabda-8(17),12-dieno-16,15-lactone [(+)-isocoronarin-D]: A new diterpene from *Hedychium coronarium* (Zingiberaceae) Aust. J Chem 44: 1789-1793.

Singh S, Kumar JK, Saikia D, Shanker K, Thakur JP, Negi AS, Banerjee S (2010a) A bioactive labdane diterpenoid from *Curcuma amada* and its semisynthetic analogues as antitubercular agents. Eur J Med Chem 45(9): 4379-4382.

Singh S, Singh R, Banerjee S, Negi AS, Shanker K (2012) Determination of anti-tubercular agent in mango ginger (*Curcuma amada* Roxb.) by reverse phase HPLC-PDA-MS. Food Chem 131(1): 375-379.

Singh SK, Rai PK, Jaiswal D, Watal G (2008) Evidence-based critical evaluation of glycemic potential of *Cynodon dactylon.* Evidence-Based Comp Alt Med 5(4): 415-420.

Singh SP, Konwarh R, Konwar BK, Karak N (2013) Molecular docking studies on analogues of quercetin with D-alanine:D-alanine ligase of *Helicobacter pylori*.Med Chem Res 22: 2139-2150.

Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Viticult 16: 144-158.

Sirat HM (1994) Study on the terpenoids of *Zingiber ottensii*. Planta Med 60(5): 497.

Sirat HM, Jamil S (1999) In: Chemical variations in the *Alpinia*, *Curcuma* and *Kaempferia* species of Malaysia (Ali AM, Shaari K, Zakaria Z eds.). FRIM (Forest Research Institute of Malaysia), Kuala Lumpur, pp. 40-45.

Sirat HM, Jani NA (2013) Chemical constituents of the leaf of *Alpinia mutica* Roxb. Nat Prod Res 27(16): 1468-1470.

Sirat HM, Masri D, Rahman AA (1994) The distribution of labdane diterpenes in the zingiberaceae of Malaysia. Phytochemistry 36(3): 699-701.

Sirat HM, Meng LL (2009) Chemical components of the rhizome oil of *Curcuma heyneana* Val. Malays J Sci 28(3): 323-328.

Sitas F, Parkin DM, Chirenje M, Stein L, Abratt R, Wabinga H (2008) PartII: Cancer in Indigenous Africans-causes and control. Lancet Oncol 9: 786-795.

Sivasothy Y, Ibrahim H, Paliany AS, Alias SA, Awang K (2013) Pahangensin A and B, two new antibacterial diterpenes from the rhizomes of *Alpinia pahangensis* Ridley. Bioorg Med Chem Lett. doi: pii: S0960-894X(13)01168-2.

Sneader W (2005) Drug discovery: A history. Chichester: John Wiley & Sons.

Solecki R, Shanidar IV (1975) A Neanderthal flower burial in northern Iraq. Science 190: 880-881.

Souza-Fagundes EM, Brumatti G, Martins-Filho OA, Corrêa-Oliveira R, Zani CL, Amarante-Mendes GP (2003b) Myriadenolide, a labdane diterpene isolated from *Alomia myriadenia* (asteraceae) induces depolarization of mitochondrial membranes and apoptosis associated with activation of caspases-8, -9, and -3 in Jurkat and THP-1 cells. Exp Cell Res 290: 420-426.

Souza-Fagundes EM, Gazzinelli G, Parreira GG, Martins-Filho OA, Amarante-Mendes GP, Corrêa-Oliveira R, Zani CL (2003a) In vitro activity of labdane diterpene from Alomia myriadenia (Asteraceae): Immunosuppression via induction of apoptosis in monocytes. Int Immunopharmacol 3(3): 383-392.

Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher H, Scheld WT, Bartlett J, Edwards J (2008) The epidemic of antibiotic-resistant infections: A call to action for the medical community from the Infectious Diseases Society of America. Clin Infect Dis 46: 155-164.

Srivastava VK, Singh BM (1996) Indian aloe in Supplement to cultivation and utilization of medicinal plants, eds. By Handa SS and Kaul MK, RRL (CSIR), Jammu-Tawi, pp. 323-332.

Srividya AR, Dhanabal SP, Misra VK, Suja G (2010a) Antioxidant and antimicrobial activity of *Alpinia officinarum*. Indian J Pharm Sci 72(1): 145-148.

Srividya AR, Dhanabal SP, Satishkumar MN, Parthkumar HB (2010b) Antioxidant and antidiabetic activity of *Alpinia galanga*. IJPPR 3: 6-12.

Stankovic N, Senerovic L, Bojic-Trbojevic Z, Vuckovic I, Vicovac L, Vasiljevic B, Nikodinovic-Runic J (2013) Didehydroroflamycoin pentaene macrolide family from *Streptomyces durmitorensis* MS405(T) : Production optimization and antimicrobial activity. J Appl Microbiol. doi: 10.1111/jam.12326.

Stavri M, Paton A, Skelton BW, Gibbons S (2009) Antibacterial diterpenes from *Plectranthus ernstii*. J Nat Prod 72: 1191-1194.

Stockwell BR, Yang WS, Sriramaratnam R (2013) Oncogenic-RAS-signal dependent lethal compounds. United States Patent US 8,546,421. Oct 1, 2013.

Stone MJ, Williams DH (1992) On the evolution of functional secondary metabolites (natural products). Mol Microbiol 6(1): 29-34.

Subbiah V (2003) Compositons comprising sclareol or scareolide for the treatment of microbial infections. European Patent EP1083895B1. Mar 12, 2003.

Subbiah V (2007) Identification of compositions, compositions, and methods of treatment of obesity and overweight conditions. United States Patent US 7,226,625. June 5, 2007.

Subbiya A, Mahalakshmi K, Pushpangadan S, Padmavathy K, Vivekanandan P, Sukumaran VG (2013) Antibacterial efficacy of *Mangifera indica* L. kernel and *Ocimum sanctum* L. leaves against Enterococcus faecalis dentinal biofilm. J Conserv Dent 16(5): 454-457.

Sukari MA, Rashid NY, Neoh BK, Abu Bakar NH, Riyanto S, Ee GCL (2010) Larvicidal activity of some Curcuma and Kaempferia rhizome extracts against dengue fever mosquito *Aedes aegypti* Linnaeus (Diptera: Culicidae). Asian J Chem 22(10): 7915-7919.

Supaphon P, Phongpaichit S, Rukachaisirikul V, Sakayaroj J (2013) Antimicrobial potential of endophytic fungi derived from three seagrass species: *Cymodocea serrulata*, *Halophila ovalis* and *Thalassia hemprichii*. PLoS One 8(8): e72520.

Suresh G, Reddy PP, Babu KS, Shaik TB, Kalivendi SV (2010) Two new cytotoxic labdane diterpenes from the rhizomes of *Hedychium coronarium*. Bioorg Med Chem Lett 20(24): 7544-7548.

Sy LK, Brown GD (1997) Labdane diterpenoids from *Alpinia chinensis*. J Nat Prod 60: 904-908.

Szallasi A, Bíró T, Modarres S, Garlaschelli L, Petersen M, Klusch A, Vidari G, Jonassohn M, De Rosa S, Sterner O, Blumberg PM, Krause JE (1998) Dialdehyde sesquiterpenes and other terpenoids as vanilloids. Eur J Pharmacol 356(1): 81-89.

Tadera K, Minami Y, Takamatsu K, Matsuoka TJ (2006) Inhibition of α-glucosidase and α-amylase by flavonoids. J Nutr Sci Vitaminol 52: 149-153.

Takahashi H, Hara S, Matsui R (1998) Purified flavonoid and diterpene 5 alpha-reductase inhibitors from *Thuja orientalis* for androgen-related diseases. United States Patent US 5,773,005. June 30, 1998.

Talebi F, Arce Garcia MA, Lee T, Chang PK, Chen H, Zaniewski TA (2011) Beverage products. United States Patent US 8,029,846. Oct 4, 2011.

Tamai E, Tsuchiya K, Nishizawa Y, Hanada M, Tokoro K (2001) Melanin inhibiting and cell growth activating compositions containing compounds having labdane structure. United States Patent US 6,313,214. Nov 6, 2001.

Tamai E, Tsuchiya K, Nishizawa Y, Hanada M, Tokoro K (2002a) Cell growth activating composition containing compound having labdane structure. United States Patent US 6,384,074. May 7, 2002a.

Tamai E, Tsuchiya K, Nishizawa Y, Hanada M, Tokoro K (2002b) Biologically active labdane or labdene derivatives from Cistus. United States Patent US 6384074 B1. May 7, 2002b.

Tanabe M, Chen YD, Saito K, Kano Y (1993) Cholesterol biosynthesis inhibitory component from *Zingiber officinale* Roscoe. Chem Pharm Bull (Tokyo) 41: 710-713.

Tanabe M, Yasuda M, Adachi Y, Ujita K, Kano Y (1991) High-performance liquid chromatography analysis of gingerols and diterpenes in Japanese gingers. Shoyakugaku Zasshi 45(4): 316-320.

Tanabe M, Yasuda M, Adachi Y, Ujita K, Kano Y (1992) Growth of Japanese Kintoki ginger and contents of pungents and diterpenes. Shoyakugaku Zasshi 46(1): 30-36.

Tatsimo SJ, Tane P, Melissa J, Sondengam BL, Okunji CO, Schuster BM, Iwu MM, Khan IA (2006) Antimicrobial principle from *Aframomum longifolius*. Planta Med 72: 132-135.

Taylor PW (2013) Alternative natural sources for a new generation of antibacterial agents. Int J Antimicrob Agents. doi: 10.1016/j.ijantimicag.2013.05.004.

Thakkar A, Sutaria D, Grandhi BK, Wang J, Prabhu S (2013) The molecular mechanism of action of aspirin, curcumin and sulforaphane combinations in the chemoprevention of pancreatic cancer. Oncol Rep 29(4): 1671-1677.

Thilagam E, Parimaladevi B, Kumarappan C, Mandal SC (2013) α-Glucosidase and α-amylase inhibitory activity of *Senna surattensis*. J Acupunct Meridian Stud 6(1): 24-30.

Thomsen R, Christensen M (2006) MolDock: A new technique for high-accuracy molecular docking. J Med Chem 49(11): 3315-3321.

Thulesius O, Christenson JT (1990) Methods for preventing thrombosis; and surgical implant having reduced platelet deposition characteristics. United States Patent US 4,909,799. Mar 20, 1990.

Trelle S, Reichenbach S, Wandel S, Hildebrand P, Tschannen B, Villiger PM, Egger M, Ju¨ni P (2011) Cardiovascular safety of non-steroidal anti-inflammatory drugs: Network meta-analysis. BMJ. doi:10.1136/bmj.c7086.

Tsopmo A, Ayimele GA, Tane P, Ayafor JF, Connolly JD, Sterner O (2002) A norbislabdane and other labdanes from *Aframomum sulcatum*. Tetrahedron 58(14): 2725-2728.

Tushar, Basak S, Sarma GC, Rangan L (2010) Ethnomedical uses of Zingiberaceous plants of Northeast India. J Ethnopharmacol 132(1): 286-296.

Ujang Z, Subramaniam T, Diah M, Wahid H, Abdullah B, Rashid A, Appleton D (2013) Bioguided fractionation and purification of natural bioactives obtained from *Alpinia conchigera* water extract with melanin inhibition activity. J Biomater Nanobiotechnol 4: 265-272.

Upadhyay A, Chompoo J, Kishimoto W, Makise T, Tawata S (2011) HIV-1 integrase and neuraminidase inhibitors from *Alpinia zerumbet*. J Agric Food Chem 59(7): 2857-2862.

Urhausen A, Torsten A, Wilfried K (2003) Reversibility of the effects on blood cells, lipids, liver function and hormones in former anabolic-androgenic steroid abusers. J Steroid Biochem Mol Biol 84(2): 369-375.

Vaidya ADB (1992) Some principles and practices of Ayurveda in selected medicinal plants of India. Bhavan's SPARC, Mumbai, pp. 365-370.

Vankar PS, Tiwari V, Singh LW, Swapana N (2006) Antioxidant properties of some exclusive species of zingiberaceae family of Manipur. J Environ Agric Food Chem 5: 1318-1322.

Verpoorte R, Choi YH, Kim HK (2005) Ethnopharmacology and systems biology: A perfect holistic match. J Ethnopharmacol 100: 53-56.

Victório CP (2011) Therapeutic value of the genus *Alpinia*, Zingiberaceae. Rev bras farmacogn 21(1): 194-201.

Vlahov G, Schiavone C, Simone N (1999) Triacylglycerols of the olive fruit (*Olea europaea* L.): Characterization of mesocarp and seed triacylglycerols in different cultivars by liquid chromatography and ^{13}C NMR spectroscopy. Eur J Lipid Sci Technol 101(4): 146-150.

Wabo HK, Tane P, Connolly JD (2006) Diterpenoids and sesquiterpenoids from *Aframomum arundinaceum*. Biochem Sys Ecol 34(7): 603-605.

Wall ME (1998) Camptothecin and Taxol: Discovery to clinic. Med Res Rev 18: 299-314.

Wallace AC, Laskowski RA, Thornton JM (1995) LIGPLOT: A program to generate schematic diagrams of protein-ligand interactions. Protein Eng 8: 127-134.

Walsh CT, Wright G (2005) Introduction: Antibiotic resistance. Chem Rev 105(2): 391-393.

Wang CC, Chang SC, Inbaraj BS, Chen BH (2010a) Isolation of carotenoids, flavonoids and polysaccharides from *Lycium barbarum* L. and evaluation of antioxidant activity. Food Chem 120: 184-192.

Wang CZ, Yuan HH, Bao XL, Lan MB (2013b) *In vitro* antioxidant and cytotoxic properties of ethanol extract of *Alpinia oxyphylla* fruits. Pharm Biol (In press).

Wang D, Chen WX, Hu YY, Peng MF (2013a) Optimization, modeling and antibacterial activity of extraction from fructus *Alpinia oxyphylla*. Adv Mat Res 781-784: 827-831.

Wang Y, Shnyra A, Africa C, Warholic C, McArthur C (2009) Activation of the extrinsic apoptotic pathway by TNF-alpha in human salivary gland (HSG) cells *in vitro*, suggests a role for the TNF receptor (TNF-R) and intercellular adhesion molecule-1 (ICAM-1) in Sjögren's syndrome-associated autoimmune sialadenitis. Arch Oral Biol 54: 986-996.

Wang Y, Yang Z, Wei X (2010b) Sugar compositions, α-glucosidase inhibitory and amylase inhibitory activities of polysaccharides from leaves and flowers of *Camellia sinensis* obtained by different extraction methods. Int J Biol Macromol 47: 534-539.

Wang YC, Huang TT (2005) Screening of anti-Helicobacter pylori herbs deriving from Taiwanese folk medicinal plants. FEMS Immunol Med Microbiol 43(2): 295-300.

Wang Z, Lu W, Li Y, Tang B (2013c) Alpinetin promotes Bax translocation, induces apoptosis through the mitochondrial pathway and arrests human gastric cancer cells at the G2/M phase. Mol Med Rep 7(3): 915-920.

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J Am Chem Soc 93: 2325-2327.

Weerakkody NS, Caffin N, Lambert LK, Turner MS, Dykes GA (2011) Synergistic antimicrobial activity of galangal (*Alpinia galanga*), rosemary (*Rosmarinus officinalis*) and lemon iron bark (*Eucalyptus staigerana*) extracts. J Sci Food Agric 91(3): 461-468.

Wheeler N, Jech K, Masters S, Brobst S, Alvarado A, Hoover A, Snader K (1992) Effects of genetic, epigenetic, and environmental factors on Taxol content in *Taxus brevifolia* and related species. J Nat Prod 13(4): 432-440.

Whitaker RJ (1993) Abienol-producing somaclonal variants of nicotiana. United States Patent US 5,177,306. Jan 5, 1993.

WHO (2007) Food safety and food-borne illness. F. s. N. 237.

WHO Expert Committee on specifications for pharmaceutical preparations (1996) World Health Organ Tech Rep Ser 863 (WHO guidelines for the assessment of herbal medicines): 178-184.

Wiart C, Au TS, Mohd Y, Hamimah H, Sulaiman M (2005) 16 Alpha hydroxy-(−)-kauran-19-oic: An antibacterial diterpene from sweet apple (*Annona squamosa* L., Annonaceae). Int J Pharmacol 1: 296-298.

Wickman GR, Julian L, Mardilovich K, Schumacher S, Munro J, Rath N, Zander SA, Mleczak A, Sumpton D, Morrice N, Bienvenut WV, Olson MF (2013) Blebs produced by actin-myosin contraction during apoptosis release damage-associated molecular pattern proteins before secondary necrosis occurs. Cell Death Differ 20(10): 1293-1305.

Williams GT, Smith CA (1993) Molecular regulation of apoptosis: Genetic controls on cell death. Cell 74: 777-779.

Williams LK, Li C, Withers SG, Brayer GD (2012) Order and disorder: Differential structural impacts of myricetin and ethyl caffeate on human amylase, an antidiabetic target. J Med Chem 55(22): 10177-10186.

Woldemichael GM, Wächter G, Singh MP, Maiese WM, Timmermann BN (2003) Antibacterial diterpenes from *Calceolaria pinifolia*. J Nat Prod 66: 242-246.

World health Organization (1999) Definition, diagnosis and classification of Diabetes Mellitus and its complications, Geneva, pp 1-49.

World Health Organization (2002) WHO Traditional Medicine Strategy 2002-2005, WHO, Geneva, Switzerland.

Wu DL (1981) Flora of China.Vol 16, Fascicle 2, Beijing: Science Press, pp. 67-106.

Wu TL, Larson K (2000) Zingiberaceae. In: Wu Raven (ed) Flora of China, vol 24. Missouri Botanical Garden Publishing, St. Louis, pp 360-363.

Wuttke W, Jarry H, Popp M, Christoffel V, Spengler B (2003) Agent for lowering prolactin. United States Patent US 6,635,672. Oct 21, 2003.

Wyllie AH, Kerr JF, Currie AR (1980) Cell death: The significance of apoptosis. Int Rev Cytol 68: 251-306.

Xu HX**,** Dong H, Sim KY (1995) The isolation of a new labdane diterpene from the seeds of *Alpinia zerumbet.* Nat Prod Lett 7(1): 29-34.

Xu HX, Dong H, Sim KY (1996) Labdane diterpenes from *Alpinia zerumbet.* Phytochemistry 42(1): 149-151.

Xu J, Liu C, Guo P, Guo Y, Jin DQ, Song X, Sun Z, Gui L, Ma Y (2011) Neuroprotective labdane diterpenes from *Fritillaria ebeiensis*. Fitoterapia 82(5): 772-776.

Xu JJ, Tan NH, Xiong J, Adebayo AH, Han HJ, Zeng GZ, Ji CJ, Zhang YM, Zhu MJ (2009a) Oxyphyllones A and B, novel sesquiterpenes with an unusual 4,5-secoeudesmane skeleton from *Alpinia oxyphylla*. Chin Chem Lett 20: 945-948.

Xu JJ, Zeng GZ, Yang SC, Shen Y, Tan NH (2013) 8-9' linked neolignans with cytotoxicity from *Alpinia conchigera*. Fitoterapia 91C: 82-86.

Xu K, Liu B, Ma Y, Du J, Li G, Gao H et al (2009b) Physicochemical properties and antioxidant activities of luteolin-phospholipid complex. Molecules 14: 3486-3493.

Yadav PN, Liu Z, Rafi MMR (2003) A diarylheptanoid from lesser galangal (*Alpinia officinarum*) inhibits proinflammatory mediators via inhibition of mitogen-activated protein kinase, p44/42, and transcription factor nuclear factor-kappa B. J Pharmacol Exp Ther 305(3): 925-931.

Yan X, Wang T, Ma Z, Zhang W, Duan J, Cai Y (2011) Crude extracts from *Andrographis paniculata*. United States Patent US RE42,718. Sep 20, 2011.

Yan X, Wang T, Ma Z, Zhang W, Duan J, Cai Y (2012) Crude extracts from *Andrographis paniculata*. United States Patent US RE43,423. May 29, 2012.

Yang HL, Chen SC, Chen CS, Wang SY, Hseu YC (2008b) *Alpinia pricei* rhizome extracts induce apoptosis of human carcinoma KB cells via a mitochondria-dependent apoptotic pathway. Food Chem Toxicol 46: 3318-3324.

Yang JM, Chen CC (2004) GEMDOCK: A generic evolutionary method for molecular docking. Proteins 5: 288-304.

Yang JS, Lin CW, Hsieh YS, Cheng HL, Lue KH, Yang SF, Lu KH (2013) *Selaginella tamariscina* (Beauv.) possesses antimetastatic effects on human osteosarcoma cells by decreasing MMP-2 and MMP-9 secretions via p38 and Akt signaling pathways. Food Chem Toxicol 59: 801-807.

Yang X, Jia Z, Li R, Jordan J (2008a) Aframodial and labdadienedial flavor enhancers. World Intellectual Property Organization WO 2008138162A1. Nov 20, 2008a.

Yang Y, Zhang S, Wu B, Ma M, Chen X, Qin X, He M, Hussain S, Jing C, Ma B, Zhu C (2012) An efficient synthesis of quinoxalinone derivatives as potent inhibitors of aldose reductase. Chem med chem 7: 823-835.

Yasuhara T, Manse Y, Morimoto T, Qilong W, Matsuda H, Yoshikawa M, Muraok O (2009) Acetoxybenzhydrols as highly active and stable analogues of 10S-10-acetoxychavicol, a potent antiallergic principal from *Alpinia galanga*. Bioorg Med Chem Lett 19: 2944-2946.

Yedjou CG, Izevbigie EB, Tchounwou PB (2013) *Vernonia amygdalina*-induced growth arrest and apoptosis of breast cancer (MCF-7) cells. Pharmacol Pharm 4: 93-99.

Yeh CB, Hsieh MJ, Hsieh YH, Chien MH, Chiou HL, Yang SF (2012) Antimetastatic effects of norcantharidin on hepatocellular carcinoma by transcriptional inhibition of MMP-9 through modulation of NF-kB activity. PLoS One 7(2): e31055. doi: 10.1371/journal.pone.0031055.

Yoshida M, Saito Y, Matsuno T (1994) Physiologically active substance. United States Patent US 5,276,177. Jan 4, 1994.

Yu X, An L, Wang Y, Zhao H, Gao C (2003) Neuroprotective effect of *Alpinia oxyphylla* Miq. fruits against glutamate-induced apoptosis in cortical neurons. Toxicol Lett 144(2): 205-212.

Yu YS, Hsu CL, Yen GC (2009) Anti-inflammatory effects of the roots of *Alpinia pricei* Hayata and its phenolic compounds. J Agric Food Chem 57(17): 7673-7680.

Yusoff MM, Ibrahim H, Hamid NA (2011) Chemical characterization and antimicrobial activity of rhizome essential oils of very closely allied Zingiberaceae species endemic to Borneo: *Alpinia ligulata* K. Schum. and *Alpinia nieuwenhuizii* Val. Chem Biodivers 8(5): 916-923.

Zani CL, Alves TM, Queiróz R, Fontes ES, Shin YG, Cordell GA (2000) A cytotoxic diterpene from *Alomia myriadenia*. Phytochemistry 53(8): 877-80.

Zhan ZJ, Wen YT, Ren FY, Rao GW, Shan WG, Li CP (2012) Diterpenoids and a diarylheptanoid from *Hedychium coronarium* with significant anti-angiogenic and cytotoxic activities. Chem Biodivers 9(12): 2754-2760.

Zhang H, Li N, Wu J, Su L, Chen X, Lin B, Luo H (2013b) Galangin inhibits proliferation of HepG2 cells by activating AMPK via increasing the AMP/TAN ratio in a LKB1-independent manner. Eur J Pharmacol. doi:pii: S0014-2999(13)00635-3. 10.1016/j.ejphar.2013.08.026.

Zhang J, Wang S, Li Y, Xu P, Chen F, Tan Y, Duan J (2013a) Anti-diarrheal constituents of *Alpinia oxyphylla*. Fitoterapia 89:149-156.

Zhang R, Li C, Williams LK, Rempel BP, Brayer GD, Withers SG (2009) Directed "*in situ*" inhibitor elongation as a strategy to structurally characterize the covalent glycosyl-enzyme intermediate of human pancreatic α-amylase. Biochemistry 48: 10752-10764.

Zhang X, Shi GF, Liu XZ, An LJ, Guan S (2011b) Anti-ageing effects of protocatechuic acid from *Alpinia* on spleen and liver antioxidative system of senescent mice. Cell Biochem Funct 29(4): 342-347.

Zhang ZJ, Cheang LC, Wang MW, Li GH, Chu IK, Lin ZX, Lee SM (2011a) Ethanolic extract of fructus *Alpinia oxyphylla* protects against 6-hydroxydopamine-induced damage of PC12 cells *in vitro* and dopaminergic neurons in zebrafish. Cell Mol Neurobiol 32(1): 27-40.

Zhou BN, Baj NJ, Glass TE, Malone S, Werkhoven MC, van Troon F, David, Wisse JH, Kingston DG (1997) Bioactive labdane diterpenoids from *Renealmia alpinia* collected in the Suriname rainforest. J Nat Prod 60(12): 1287-1293.

Zhou JH, He W (2001) Process for extracting sweet diterpene glycosides. United States Patent US 6,228,996. May 8, 2001.

Zou Y, Lu Y, Wei D (2004) Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. *in vitro*. J Agric Food Chem 52: 5032-5039.

Missed References:

Arifullah M, Namsa ND, Mandal M, Chiruvella KK, Vikrama P, Gopal GR (2013) Evaluation of anti-bacterial and anti-oxidant potential of andrographolide and echiodinin isolated from callus culture of *Andrographis paniculata* Nees. Asian Pac J Trop Biomed 3(8): 604-610.

Bora U, Sahu A, Saikia AP, Ryakala VK, Goswami P (2007) Medicinal plants used by the people of Northeast India for curing malaria. Phytother Res 21(8): 800-804.

Das A, Kasoju N, Bora U, Rangan L (2013) Chemico-biological investigation of rhizome essential oil of *Zingiber moran*-native to Northeast India. Med Chem Res 22(9): 4308-4315.

Dutta BK, Dutta PK (2005) Potential of ethnobotanical studies in North East India: An overview. Ind J Trad Knowl 4: 7-14.

Lokesh D, Amitsankar D (2012) Evaluation of mechanism for antihypertensive action of *Clerodendrum colebrookianum* Walp., used by folklore healers in north-east India. J Ethnopharmacol 143(1): 207-212.

Meetei PA, Singh P, Nongdam P, Prabhu NP, Rathore R, Vindal V (2012) NeMedPlant: A database of therapeutic applications and chemical constituents of medicinal plants from north-east region of India. Bioinformation 8(4): 209-211.

Ningthoujam SS, Das Talukdar A, Potsangbam KS, Choudhury MD (2013) Traditional uses of herbal vapour therapy in Manipur, North East India: An ethnobotanical survey. J Ethnopharmacol 147(1): 136-147.

Srivastava P, Kasoju N, Bora U, Chaturvedi R (2009) Dedifferentiation of leaf explants and cytotoxic activity of an aqueous extract of cell cultures of *Lantana camara* L. Plant Cell, Tiss Organ Cult 99: 1-7.

Tag H, Kalita P, Dwivedi P, Das AK, Namsa ND (2012) Herbal medicines used in the treatment of diabetes mellitus in Arunachal Himalaya, northeast, India. J Ethnopharmacol 141: 786-795.

Publications and Participations

P U B L I C A T I O N S

Journals:

Published:

1. **Ghosh S**, Rangan L (2013) *Alpinia*: The gold mine of future therapeutics. ***3 Biotech*** 3: 173-185.
2. **Ghosh S**, Padilla-González GF, Rangan L (2013) *Alpinia nigra* seeds: A potential source of free radical scavenger and antibacterial agent. ***Ind Crop Prod*** 49: 348-356.
3. **Ghosh S**, Indukuri K, Bondalapati S, Saikia AK, Rangan L (2013) Unveiling the mode of action of antibacterial labdane diterpenes from *Alpinia nigra* (Gaertn.) B. L. Burtt seeds. ***Eur J Med Chem*** 66: 101-105.
4. **Ghosh S**, Ozek T, Tabanca N, Ali A, Rehman JU, Khan IA, Rangan L (2014) Chemical composition and bioactivity studies of essential oils from *Alpinia nigra* (Gaertn.) B. L. Burtt. ***Ind Crop Prod*** 53: 111-119.

Manuscripts under review:

5. **Ghosh S**, Rangan L (2013) Inhibition kinetics and molecular docking of α-amylase against isolated labdane diterpenes from *Alpinia nigra*. ***Med Chem Res***.
6. **Ghosh S**, Mohan CM, Hussain M, Limaye AM, Rangan L (2013) Labdane diterpenes from *Alpinia nigra* seeds inhibit matrix metalloproteinase activity and arrest the HT 1080 fibrosarcoma cells at S-phase. ***Plos One***.
7. **Ghosh S**, Rangan L (2013) Molecular docking and inhibition kinetics of α-glucosidase activity by labdane diterpenes isolated from Tora seeds (*Alpinia nigra* B.L. Burtt). ***Plos One***.
8. **Ghosh S**, Singh RJ, Indukuri K, Dubey VK, Saikia AK, L Rangan (2013) Antileishmanial labdane type diterpene compounds from the seeds of *Alpinia nigra* (Gaertn.) B. L. Burtt. ***Ind Crop Prod***.

Patent:

Ghosh S, L Rangan, Singh RK, Dubey VK, Indukuri K, Saikia AK. Antileishmanial labdane type diterpene compounds from the seeds of *Alpinia nigra* (Gaertn.) B. L. Burtt, an ethnomedically important plant from North East India. (Submitted to DST TIFAC, New Delhi, Government of India).

P A R T I C I P A T I O N S

Training and workshops:

2012-Participated in 13th Indo-US Cytometry Workshop (IUCW), held during October 8th - 10th at **IIT Guwahati, India**.

2012-Participated in the ICS Training Course on "Extraction and modification of bioactive components and specialty chemicals from plants" held during April 23rd -27th at **Anadolu University, Eskisehir, Turkey.**

2011-Participated in short-term course on "Tools for Bioresources Conservation" held during July 11th -15th at **IIT Guwahati, India.**

Symposium:

2012-Presented research work entitled "Phytochemical analysis and anticancer property of seed extracts of *Alpinia nigra* (Gaertn.) B.L. Burtt" in 6th International Symposium on the family Zingiberaceae, September 10th -13th at **Calicut University, Kerala, India.**

Conferences:

2011-Presented poster entitled "miRNAs and Cancer Systems Biology" at conference on Cancer Biology 2011: Basic Theoretical Aspects, held during August 26th -27th at **IITG, India.**

2011-Presented poster entitled "Plant Biorepository Management in North East India- The Nature's Biorepository" in the International workshop on ISBER 2011 Annual Meeting and Exhibits, held during May 15th - 18th in **Washington DC, USA.**

2010-Presented poster entitled "Biodiversity, Biorepositories and Biobanking in India" in the International workshop on Biodiversity and Climate Change from December 19th -22nd organized by CORAL, **IIT Kharagpur**.

Biography

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Mr. Sudipta Ghosh joined as a doctoral student in the Department of Biotechnology, Indian Institute of Technology Guwahati (IITG), India in July 2010. He has carried out a multidisciplinary research during his doctoral study period under the joint supervision of Dr. Latha Rangan and Dr. Utpal Bora at IITG. His research interests mainly include natural products, medicinal chemistry, cell biology and therapeutic drug development for infectious and non-infectious diseases.

Mr. Ghosh has completed B.Sc. (Agri.) with specialization in Genetics and Biochemistry in 2006 from Uttar Banga Krishi Viswavidyalaya (UBKVV), Cooch Behar, West Bengal, India. During under graduation, he has been awarded UBKV Merit Scholarship from 2002-2005 for his academic excellence. He secured all India 33rd rank in the Junior Research Fellowship (JRF) conducted by Indian Council of Agricultural Research (ICAR) combined AIEEA -New Delhi, 2006 and joined University of Agricultural Sciences (UAS) , Dharwad, Karnataka for his post-graduation studies. He has completed M.Sc. (Agri.) in Plant Biotechnology with a UAS Merit Scholarship from 2006-2007 for his academic performance in UAS Dharwad. During post-graduation, he has identified some drought responsive microRNAs (miRNAs) in *Arabidopsis thaliana* by involving *in situ* hybridization technique and also identified the miRNA targets in ESTs of Sorghum and Cotton in 2009.

Before joining IITG for his doctoral studies, he has worked at University of Hyderabad (UoH), Department of Biochemistry, School of Life Sciences, Hyderabad, India in the Centre of Excellence project, "Identification of new therapeutic targets and immune activation markers in *Mycobacterium tuberculosis* co-infections using comparative proteomics" funded by DBT, Government of India. Later he joined Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India where he was engaged in "miRNA profiling in Colon and Breast Cancer" project using Taqman Low Density Array (TLDA).

Currently he has completed the doctoral study with a self-motivation, dedication and interest in multidisciplinary research. His doctoral research mainly focused on chemical profiling of medicinal plant extracts, isolation of bioactive natural products and its biological applications towards prospective therapeutic development. During his doctoral studies he has been awarded for best poster presentation in IIT Kharagpur, India in 2010. He has also attained overseas conference in Washington DC, USA held during May 2011. Further, he has undergone a training program related to his doctoral research at Anadolu University, Turkey in 2012 organized by ICS-UNIDO. A part of his doctoral research has been published in international peer reviewed journals and some are under review. Since 2012, he is a student member in the International Society for the Advancement of Cytometry (ISAC). He is keenly interested to continue further research in cancer therapeutics and drug development.