Investigation on the vibrational and antimicrobial potential of (E)-Labda-8(17), 12-diene-15, 16-dial from *Alpinia nigra* (Gaertn.) B.L. Burtt

A thesis submitted by

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INDIAN INSTITUTE OF TECHNOLOGY

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

STATEMENT

I hereby declare that the research embodied in this thesis entitled **"Investigation on the vibrational and antimicrobial potential of (E)-Labda-8 (17), 12-diene-15, 16-dial from *Alpinia nigra* (Gaertn.) B.L. Burtt"** is the result of experiments carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the guidance of Prof. Latha Rangan

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 research.

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January, 2019

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Investigation on the vibrational and antimicrobial potential of (E)-Labda-8 (17), 12-diene-15, 16-dial from *Alpinia nigra* (Gaertn.) B.L. Burtt"** by Ishani Chakrabartty (Roll No. 146106023) for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, IITG. The work embodied in this thesis has not been submitted elsewhere for a degree.

January, 2019

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January 2019 **Ishani Chakrabartty**

IN THE NAME OF

THE ALMIGHTY

AND

THE MOST LOVING PARENTS HE HAS GIVEN……

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List of abbreviations

ABC	ATP-binding cassette
AgNPs	Silver nanoparticles
APG	Angiosperm phylogeny group
AST	Antimicrobial susceptibility testing
ATP	Adenosine triphosphate
Au	Gold
BCS	Biopharmaceutics classification system
BOD	Biological oxygen demand
cFDA	6-Carboxyfluorescein diacetate
CLSI	Clinical and Laboratory Standards Institute
Conc.	Concentration
CoS	Co-surfactant
COSY	Correlation spectroscopy
CuNPs	Copper nanoparticles
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DFT	Density functional theory
DMSO	Dimethyl sulphoxide
DO	Dissolved oxygen
DSC	Differential scanning calorimetry
ESI	Electron spray ionization
EUCAST	European Committee on antimicrobial susceptibility testing
FC	Flow cytometry
FDA	Food and Drug Administration
FESEM	Field emission scanning electron microscopy
FETEM	Field emission transmission electron microscopy
FSC	Forward scatter

FTIR	Fourier transform infra-red
GGPP	Geranylgeranyl pyrophosphate
GMP	Goods manufacturing practices
GOD-POD	Glucose oxidase-peroxidase
HLB	Hydrophile – lipophile balance
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear quantum coherence spectroscopy
HTS	High throughput screening
IPM	Isopropyl myristate
IR	Infra-red (spectroscopy)
IV	Intravenous
KBr	Potassium bromide
LB	Luria-Bertini broth
LC-MS	Liquid chromatography mass spectrometry
ME	Microemulsions
MHB	Muller Hinton broth
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NB	Nutrient broth
NE	North East
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
o/w	Oil-in-water
OD	Optical density
PBS	Phosphate buffered saline
PEG	Polyethylene glycol

PI	Propidium iodide
PMT	Photomultiplier tube
PP	Pyrophosphate
pTLC	Preparative thin layer chromatography
QC	Quality control
QSAR/QSPR	Quantitative structure activity/property relationship
R&D	Research and Development
RBCs	Red blood cells
R_f	Retention factor
SDS	Sodium dodecyl sulphate
SERS	Surface enhanced Raman spectroscopy
SM955	Surfactant mix 9.5:0.5
Smix	Surfactant mixtures
SPR	Surface plasmon resonance
SSC	Side scatter
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
T_g	Gas transition temperature
TGA	Thermogravimetric analysis
TLC	Thin layer cheromatography
TMS	Trimethyl silane
TPF	Triphenyl formazan
TTC	2,3,5-triphenyl tetrazolium chloride
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
viz.	Namely
vs	Versus
w.r.t	With respect to
w/o	Water-in-oil

WHO	World Health Organization
YPD	1% yeast extract, 2% peptone, 2% dextrose

List of NMR units

Hz	Hertz
J	Coupling constant
M	Multiplet
MHz	Mega Hertz
Ppm	Parts per million
S	Singlet
T	Triplet
Δ	Chemical shift

List of units

%	Percentage
µl	Microliter
µM	Micro molar
µm	Micrometer
A.U.	Arbitary units
CFU/ml	Colony forming units/milliliter
cm^{-1}	Centimeter inverse
g	Gram
g/cm^3	Gram/centimeter cube
g/ml	Gram/milliliter
h^{-1}	Hour inverse
hrs	Hours
kV	Kilo volt
mg	Milligram
mg/l	Milligram/liter
mg/ml	Milligram/milliliter
mins	Minutes
mJ	Milli Joule
ml	Milliliter
ml/g	Milliliter/gram
ml/min	Milliliter/minute
mm	Millimeter
mM	Milli molar
nm	Nanometer
º	Degree
ºC	Degree centigrade
rpm	Rotations per minute

v/v	Volume/volume
w/v	Weight/volume
λ_{max}	Absorbance maxima

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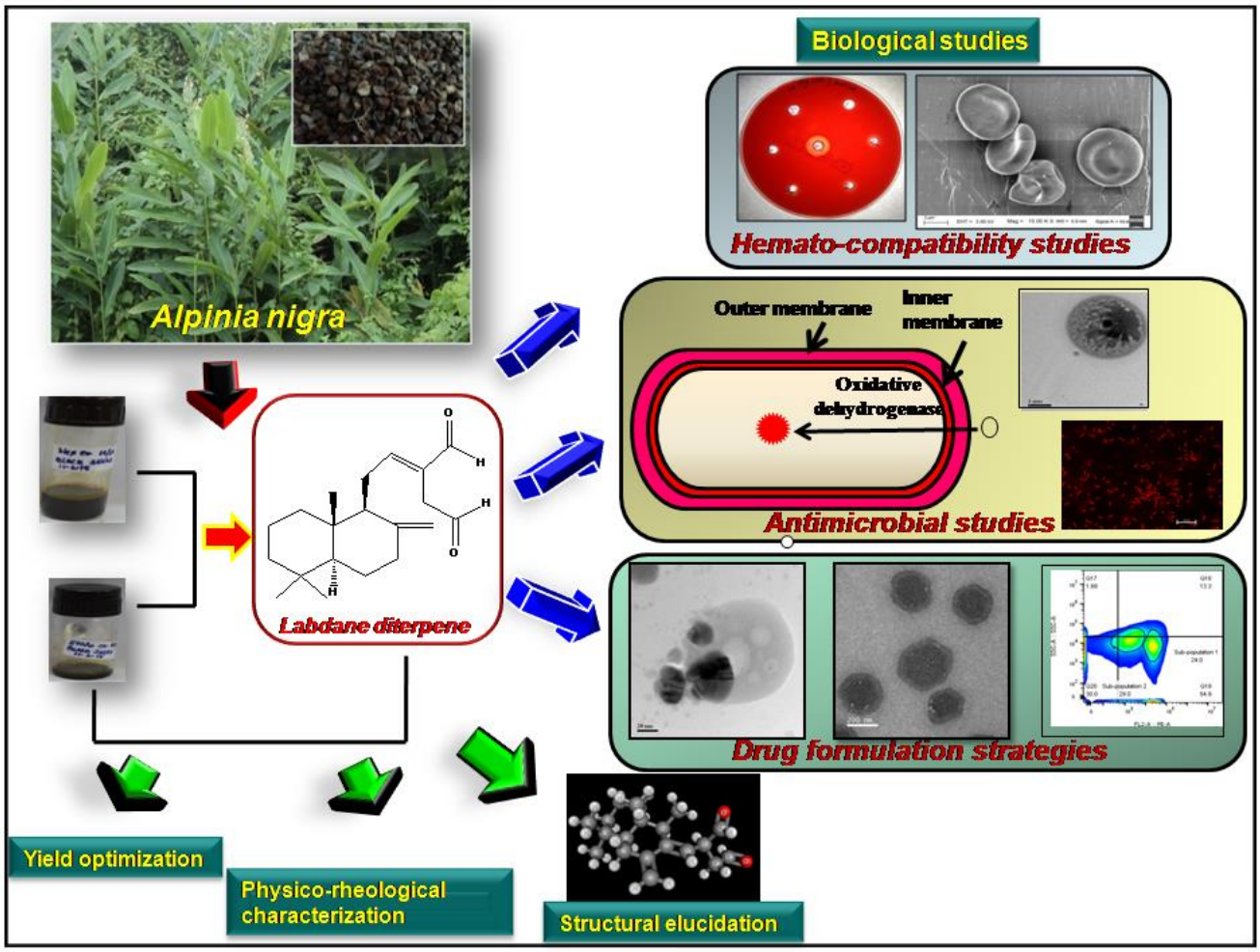
Graphical abstract

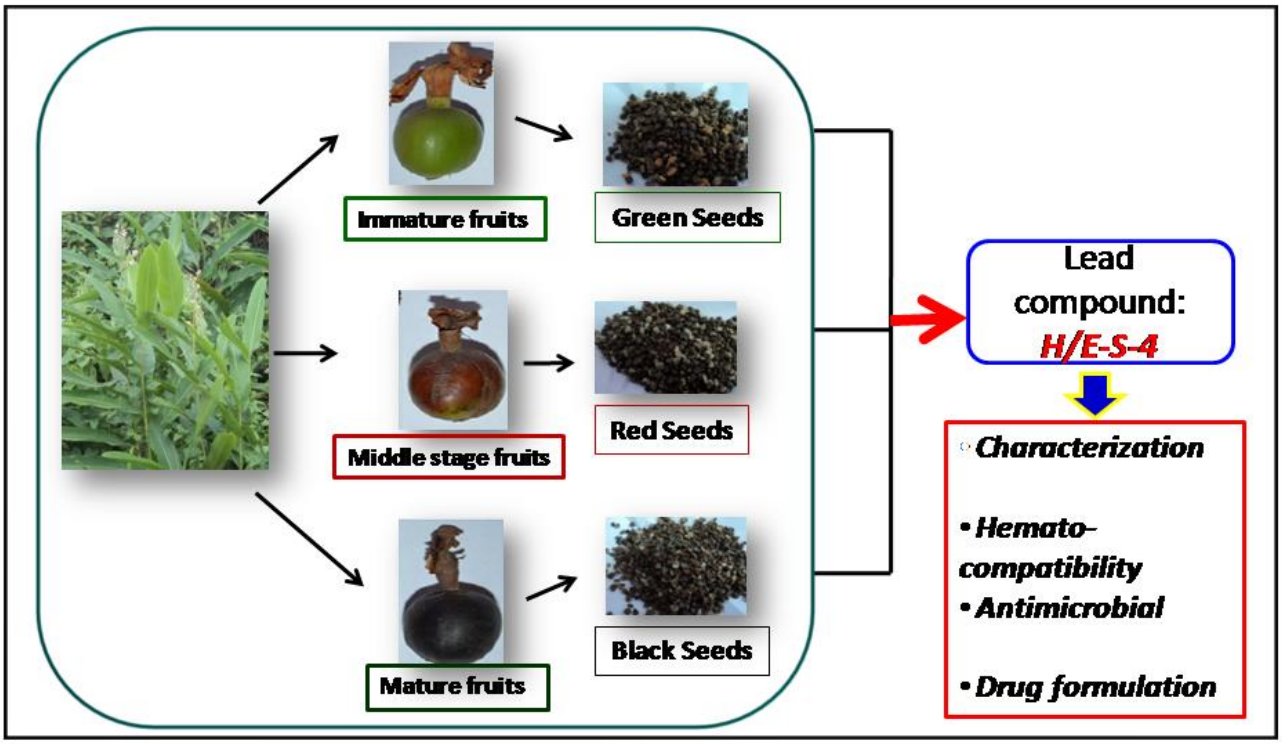


Abstract

Alpinia nigra, a rhizomatous plant belonging to the Zingiberaceae family, grows luxuriously in North-East (NE) India. Like all members of the "ginger" family, this plant too is known for its culinary properties and home remedial uses among the ethnic tribes of NE India; however, till date, this plant has not been able to draw much attention from the scientific community. Thus, the current research work was aimed at the utilization of this medicinally important plant as source bioactive compounds of pharmacological importance. A novel method was developed and the seed-to-solvent ratio was optimized for sourcing maximum yield of seed extracts, along with an active component. Characterization of this component by HRMS, FTIR, NMR and Raman spectroscopy revealed it as a labdane-type diterpene, (E)-Labda-8(17), 12-diene-15, 16-dial. Experimental vibrational spectra were correlated with the theoretical spectra obtained at DFT level. Studies on the contact angle, optical rotation, DSC, TGA and viscosity revealed important industrially important parameters of the extracts and compound. *In-silico* studies revealed this compound to be unsuitable for oral administration (theoretically); hence, hemato-compatibility studies (both qualitative and quantitative) were carried out to determine its maximum suitable dosage for IV administration. Antimicrobial studies assessed by growth kinetics study, FESEM, fluorescent microscopy, cell leakage analysis, Raman spectroscopy etc. revealed the high degree antibacterial activity. The possible mode of action of this compound against Gram negative bacteria is by cell lysis and disruption but no activity was observed against Gram positive bacteria; whereas the growth of *Candida albicans* was temporarily inhibited in a dose dependent manner possibly by cell surface interaction, preventing substrate uptake. The hydrophobic nature of the diterpene acted as a hindrance to its biological activity in an aqueous environment, hence, nanoparticles were used, which increased the efficacy of the low, yet water soluble concentrations of the compound. At the same time, the current work also provides an interesting scientific insight on the utilization of *in-house* synthesized labdane diterpene loaded oil-in-water microemulsions as a promising, futuristic antibacterial agent against a wide range of bacterial pathogens, especially the antibiotic resistant strains.

CHAPTERS

Chapter 1: Introduction



This chapter gives a brief background on the role of plant-derived natural products in the treatment of various diseases and curing ailments and finally the motivation and specific objectives of the thesis work

Chapter 1: Introduction

Nature has been an important source of drugs since time immemorial. Numerous organisms, harbored in the heart of nature, have contributed immensely to the world of modern medicine for centuries together. For a long period of time, crude extracts from natural resources were the only source of medicines available to mankind. Nature has evolved in diverse ways to synthesize a vast array of secondary metabolites, the principal component in these crude extracts. These metabolites are a mixture of various complex chemical compounds that are capable of curing various ailments. It is indeed impressive that thousands of modern drugs that are used today have their roots in natural sources due to the existence of traditional knowledge that are passed down through generations (Kumar 2009). For many years, bark and leaves of willow (containing salicyclic acid) were used for their anti-inflammatory and pain killing effects. The description of their use was found in tablets from the Sumerian period (4000 BC) and from the ancient Greek civilizations. It was only in 1763 that Reverend Edward Stone of Oxfordshire gave the 'scientific use' of willow to treat feverish patients. In 1897, Felix Hoffmann synthesized acetylsalicyclic acid (ASA), which began to be marketed under the brand name of '***aspirin***' (Chamberlain 2015). Thus, it is indeed overwhelming to know what few leaves and pieces of bark can give rise to!

Humans may boast of the tremendous advancement in medical and health science, but the inspiration for such advancements came from nature. The unfathomable wealth of nature's knowledge in medicine has provided us directly or indirectly almost 50% of FDA approved drugs at present. Tu Youyou of the Chinese National Academy of Sciences was awarded the Nobel Prize in Physiology or Medicine for the year 2015, for her pioneering work of discovering artemisinin, a novel therapeutic compound against malaria. She combed through age-old documents of the Chinese traditional medicine for her groundbreaking work and thereby, further solidifying the importance of traditional medicine.

The emergence of modern medical systems and the hype-created by them cast a shadow on the traditionally used medical system of early times. However, it is ironic that the modern system derives majority of its medicines from natural sources. With the growth of knowledge, technological advancements and breakthrough research, discovery and development of synthetic medicines has increased manifold. Though these medicines

have aided in the treatment and cure of a number of life-threatening diseases, prolonged use of such drugs are accompanied by many toxic side effects, which is major threat to human health and survival. The longstanding relationship of man with nature has again led him to explore the field to natural medicines. Increase in pollution, changing lifestyle and stress coupled with loss of biodiversity, unscientific use of medicinal plants, malpractices in traditional medicine etc. cause an alarming disturbance in the structure and function of nature (Alves and Rosa 2007). Benefits of medicinally important plants in the conventional healthcare system, owing to its lesser side effects and alternative approaches, have taken the centre stage. Plants, as the source of drugs, encourages the isolation and identification of bioactive compounds for direct use or for the production of novel bioactive compounds with improved activity (Fabricant and Farnsworth 2001). Ideally, the secondary metabolites produced by the plant that are required for its growth and development serve as the bioactive compounds; these compounds are produced by the plant as an adaptation to the environment or as a means of defense against predators (Dewick 2002; Colegate and Molyneux 2008).

Plant based products are, thus, a new, safe and affordable component in the search for effective drugs. Some promising drug candidates like celasterol and capsaicin etc. are all plant based products (Corson and Crews 2007). Today, many pharmaceutical companies have directed their attention towards the development of plant based drugs. However, in the past few years, big pharmaceutical companies had terminated their operations concerning the development of plant based drugs. This was mainly because the isolation of these compounds in pure form and their structural elucidation is very tedious and time consuming. The time required to develop a natural product from an extract hit to a pharmaceutical drug was exceptionally long. With the development of new and sophisticated spectroscopic techniques, focus has now shifted to the synthesis of small drug-like molecules to satisfy the huge demand for large library of compounds (Borman 2002). As a result, many pharmaceutical companies emphasize on natural product research with the expectation of reaping huge profits. However, expectation could not meet reality and there was no surge in the productivity of natural product based drugs. Rather, in 2001 FDA reported that they received only 16 new drug applications as opposed to 24 applications in 2000 (Newman et al. 2003). In order to improve this scenario, a lot of resources were expended by the pharmaceutical companies to combinatorial chemistry and high throughput screening. Between 1986-2006, 52% of the

total compounds reported were either natural product, a mimic or modified natural product (Newman and Cragg 2007). In between 2005-2010, 19 natural product based drugs were marketed worldwide, among which 7 were natural products, 10 semi-synthetic and 2 natural product derived drugs (Mishra and Tiwari 2011). Plants are thus, still regarded as an inexhaustible source of novel chemical entities.

From early times, India was and is still believed to be the botanical garden of the world as it is the largest producer of medicinal herbs. Not only does the country harbor thousands of herbal medicines but also caters them to the rest of the world (Seth and Sharma 2004). Existence of traditional knowledge guided by systems of Ayurveda has provided India a lead in drug discovery (Mukherjee et al. 2014). India is also taking conscious steps to preserve and protect the intellectual property surrounding herbal medicines (Jeevan 2004; Jayaraman 2009; Chakravarty 2010). The North eastern region of India has a rich diversity of medicinal plants, many of which are critically documented. The 8 states of North East (NE) India is a part of the Indo-Burma biodiversity hotspot. As such, it is no wonder that the region has a rich knowledge of traditional medicine, which developed independently and in parallel with established practices such as Ayurveda, Unani and Siddha in the rest of the country. This region harbors 130, 665 and 899 plant species under ethnomedical, food and other uses respectively. Owing to the immense importance of medicinal plants of NE India, Meetei and his co-workers created a database that contains valuable information about various natural products isolated from the medicinal plants of this region and their pharmacological applications (Meetei et al. 2012).

Zingiberaceae is the well-known "pharmacy" of the plant kingdom. Plants of Zingiberaceae are mostly rhizomatous and aromatic. Sometimes these plants are cultivated as ornamental for their showy flowers. These plants contain large amounts of essential oils in their tissues which are mainly responsible for their characteristic and stimulating odor. Due to its high medicinal and culinary value, this family has been immensely investigated by several researchers and scientists. Though Zingiberaceae has a worldwide distribution, major concentration of these plants are found in South and Southeast Asia. In India, 22 genera and 178 species of Zingiberaceae are distributed in NE and peninsular India of which around 88 different species are found in NE India alone. Tribal communities and ethnic groups of this region typically rely on the plants of

this family for treatment of illness. In addition, almost all the plants of this family have some use as a cooking ingredient. Curcumin, a well known bioactive compound, find its distribution among well known plants of Zingiberaceae like *Curcuma longa* and *Zingiber officinale*. Despite its immense pharmacological potential, curcumin was found unlikely to produce good leads in drug development due to its low solubility in aqueous medium and poor bioavailability. *Alpinia*, the largest genus of this family, has tremendous use in folk medicine, many of which are critically documented (Tushar et al. 2010). Almost all the species of this genus have pharmacological importance and hence are immensely explored for their bioactive compounds. Among the plants of *Alpinia*, till date research has been mainly concentrated on *A. galanga* and highest number of bioactive compounds has been reported from this species; most of these compounds are antimicrobial, cytotoxic, anti inflammatory, analgesic, antidiabetic etc. (Janssen and Scheffer 1985; Oonmetta-aree et al. 2006; Weerakkody et al. 2011). In addition, other species like *A. katsumadai, A. purpurata, A. zerumbet* and others are also gaining the attention of researchers for the search of bioactive compounds.

Alpinia nigra (Gaertn.) B.L. Burtt, a medium sized rhizomatous member of *Alpinia*, has however missed the attention of researchers (Roy et al. 2012). Commonly known as "Tora" or "Tara" in assamese and "Jangli ada" in Bengali, this plant is abundant in the entire NE India. Though this plant propagates vegetatively through rhizomes, some members also exhibit propagation by means of stolons and thrive abundantly in the marshy and swampy areas. It is relished as a food item by the tribal population of this region; "deragong" and "pullei eromba" are some delicacies that are made using *A. nigra* in Tripura and Manipur respectively. Ethnomedically important, this plant is used by local healers for its "magical" power. It also serves as a source of food to animals. Though limited literature is available on *A. nigra* and its phytochemicals, this plant has established itself as possible cure against helminthes, as highlighted by a leading research group of Assam (Roy and Swargiary 2009; Roy et al. 2009, 2012; Swargiary et al. 2013). A lot of research is being carried out around the other members of *Alpinia* but extensive investigation on *A. nigra* for the search of new chemical compounds for the treatment of disease is indeed lacking.

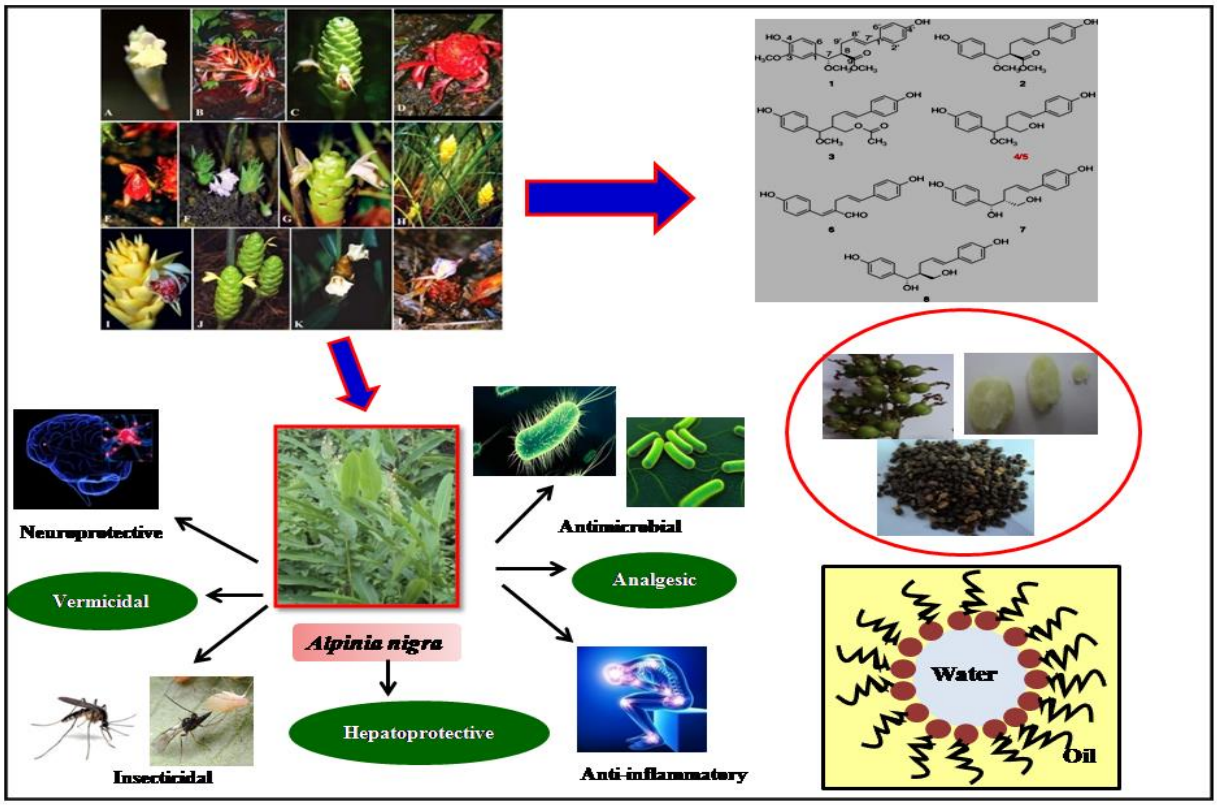
Therefore, the present research study was undertaken to isolate organic solvent extract, primarily from the seeds of *A. nigra*, optimize their yield and study the physical characteristics of the isolated extract, and finally, purification and structural elucidation of the compound isolated from the seed extract. Further, the efficacy of the isolated compound was checked against pathogenic microorganisms. Finally, the problem of poor solubility of the compound was addressed and some formulation strategies were devised in the view of prospective therapeutic application.

1.1 Objectives

Based on the background study along with the re-emergence and growing cultural acceptability of herbal medications, the research study was carried out with the broad objective of isolation and characterization of bioactive compounds from the seeds of *A. nigra*. Further, the biological potential and formulation strategies for therapeutic applications are devised. The specific objectives are outlined as follows:

1. Oil extraction and physico - rheological characterization of organic extract obtained from the seeds of *A. nigra*
2. Isolation, purification and characterization of bioactive compounds from the seeds of *A. nigra*
3. Evaluation of the bio-pharmaceutical potential of the isolated compound
   A) Studying its hemato-compatibility
   B) Screening its antimicrobial potential and deciphering its possible mode of action against:
      1. Bacteria
      2. *Candida albicans*
4. Formulating a strategic approach for the delivery of the isolated compound as a potential antibacterial agent using:
   A) Metallic nanoparticles
   B) Microemulsions

Chapter 2: Review of literature



This chapter describes the history and current status of natural products in drug discovery. Also the importance of the genus *Alpinia*, particularly *A. nigra* along with its bioactive compounds towards *in-vitro* administration for antimicrobial applications and drug formulation strategies are highlighted.

Chapter 2: Review of literature

2.1 Introduction

Natural products derived from plants, animals, bacteria, minerals have been used by man for treatment of diseases (Patwardhan et al. 2004; Lahlou 2007). Since early times, man used to wrap leaves around wounds and chew certain herbs to relieve pain and infections. Plants, in particular, are the basis of modern medicines. Paleontological studies from Kurdistan, Iraq, revealed that around 60,000 years back Neanderthals were probably aware of the medicinal properties of some plants, as evident from pollen deposits in a cave site (Solecki 1975). The earliest record of herbal medication dates back to 2600 BC, documenting around 1000 plant products from Mesopotamia (Cragg and Newman 2013). The best known documented record of plant drugs is "Ebers Papyrus" from1500 BC (Borchardt 2002). In the field of herbal medicine, Chinese medicines deserve a special mention. Centuries old practices and formulations are documented in the Chinese *Materia Medica*, with the first record dating back to 100 BC (Huang 1994). The Greeks and the Romans also had the practice of compounding drugs and storing medicinal herbs. It is reported in the *Shen Nong Ben Cao Jing* and *De Materia Medica* that Hippocrates used the extract of *Veratrum album* as an emetic (Kong et al. 2008). These Greeco-Roman methods were particularly used by the Arabs in the middle ages.The practice of privately owing pharmacies was also started by the Arabs, among which the Persian pharmacist, Avicenna, deserve special mention due his numerous contributions like *Canon Medicinae* (Cragg and Newman 2005). A review on the history of medicine can be found in the website of National Institute of Health (NIH), USA at www.nml.nih.gov/hmd/medieval.html. Traditional knowledge and folklore is in abundance in India too. Even epics, like The Ramayana highlight the importance of herbal medication in the name of "Sanjeevani booti". The documentation of the Indian Ayurvedic and Unani medicine system dates back to before 1000 BC, where we find the great works like *Charaka Samhita* (Kapoor 1990; Dev 1999).

2.2 Plants as traditional medicine

Throughout history, natural products have been used as medicines in the form of potions, essential oils, oral drugs etc. In 1985, the World Health Organization (WHO) reported that 65% of the world population relies on plant derived medicines for their health care and treatment of ailments, while the remaining population relies on chemical or

synthesized medicines inspired from plant drugs (Farnsworth et al. 1985). The main source of knowledge of medicinal plants comes from man's inquisitiveness to deal with untimely deaths and epidemics when healthcare was sparse (Hicks 1966; Kinghorn et al. 2011). The long term association of man with plants guarantees low human toxicity of any bioactive compound that may be isolated. However, not all plant derived extracts are beneficial for the health. The use of *Atropa belladonna* has been completely ruled out in folk medicine due to its poisonous nature. It is said that three berries from this plant are enough to kill a child (Allen and Hatfield 2004).

Ayurveda, Unani, Kampo and other traditional medicine have thrived on the principal plant compounds for thousands of years. These systems are still religiously followed in some of the secluded parts of India, China and other developing nations primarily because of the organizational strength in passing on the legacy to the future generation of the family (Fabricant and Farnsworth 2001). Research in medicine may raise questions on the credibility of traditional methods; however this has not decreased its use by the practitioners. The practitioners, more often than not, keep the process a secret and hence very little scientific evidence is available in this regard. Terrestrial plants like *Papaver somniferum*, *Glyccyrhiza glabra* and *Comniphora* species find mention in the clay tablets of Mesopotamia (Newman et al. 2000). The genus *Salvia* has been used by the Indian tribes of California to assist in childbirth as well as to make babies grow strong and healthy (Hicks 1966). The gummy sap from the plant *Alhagi mauroram* or Medik is reported to be used in Ayurvedic formulations to treat constipation, anorexia, leprosy, fever and obesity as well as bloody diarrhoea (Duke et al. 2008). In Northern Europe, it is believed that chewing on the plant *Ligusticum* with an empty stomach helps to protect from daily infections (Ray 1696). Among the different plant parts, the leaves are most frequently used for the treatment of diseases. The southern part of India has identified many different species of plants that have been used as traditional medicines from a long time. *Andrographis paniculata* and *Gymnema sylvestre* - leaf paste and root powder respectively, are used to treat poison bites and leaf powder is used to treat diabetes; *Ricinus communis* - leaf juice is used to increase secretion of milk and seed oil is used to treat stomach ache; *Azadirachta indica* - leaf paste is used to treat small pox, rheumatism and skin diseases; *Moringa oleifera* – boiled leaves are used to reduce body heat, to treat indigestion and eye diseases and flowers are used to cool the eyes and increase sperm production; *Zizyphus mauritiana* - leaf decoction is used to get relief from

body pain and bark powder is used to treat wounds; *Solanum torvum* - leaf juice is used to reduce body heat and unripe fruits are used to strengthen the body (Muthu et al. 2006).

2.3 Plant derived products as drugs

A survey in the countries hosting WHO-Traditional Medicinal Centers report that out of 122 bioactive compounds identified, 80% were used for ethnomedicinal purpose and derived only from 94 species of plants alone (Farnsworth et al. 1985). The primary goal behind using plants as the source of drugs is to encourage the isolation and identification of bioactive compounds for direct use (morphine, reserpin, taxol etc.) as well as production of novel bioactive compounds with higher activity or lower toxicity (metformin, nabilone etc.) (Fabricant and Farnsworth 2001). These "drugs" are ideally the secondary metabolites produced by the plant that are required for its growth and development and to combat attack from predators (Dewick 2002; Colegate and Molyneux 2008). The R&D of the pharmaceutical sector is directed to the development of plant-based drugs by means of investigation and utilization of available traditional knowledge (Patwardhan et al. 2004). Therefore, the selection of plants for isolation of bioactive compound is done by either of the following methods:

a) Chemical Screening: This method involves identification of phytochemicals such as flavonoids, isothiocyanates, alkaloids and other bioactive compounds in the preliminary screening stages (Farnsworth 1966). This is the most common method used in developing countries. However, one of its major limitations is the unpredictability of the biological effects induced by a particular compound in advance.
b) Biological Assay: This method has been used in the past by Central Drug Research Institute (CDRI) in India (Bhakuni et al. 1969, 1971). Several plant species have been tested for their biological activities like antibacterial, antifungal, anticancer, antitumor and a large number of novel bioactive compounds have been isolated by this method (Rastogi and Dhawan 1983).

In the early 20th century, it was believed that the crude and semi-pure extracts from the medicinal plants posses some "mystical power" that allows to cure illness. However, a revolutionary idea led to a new era of pharmacology. The specific interactions of drugs with biological "targets" (proteins and nucleic acids) in the human body are responsible for the effect of drug. This led to the conclusion that it is the

individual chemical compounds present in the crude extracts is "Elixir of life", causing the biological activity of a drug (Lahlou 2013). Thus, the field of "phytochemistry" or "natural product chemistry" bloomed under the umbrella of pharmacology which focused on the isolation and characterization of bioactive molecules from the crude extracts of plants that are known possess biological activity. A very classical example plant derived drug is *Morphine* that is a stimulant and is also considered important due to the mythological association of Lord Shiva with it.

Since time immemorial numerous bioactive molecules have been derived from plant sources that have successfully helped to cure a number of diseases. For example, the antimalarial drug, *Quinine* had been isolated from the bark of *Cinchona* trees in 1820 by Caventou and Pelletier (Buss and Waigh 1995). We have come a long way since then. The Nobel Prize of 2015 in Medicine awarded to a Chinese scientist for the discovery of artemisinin and avermictin highlights this. Another significant plant product is *Karanjin*, known for its insecticidal property, isolated from *Pongamia* (Prabhu et al. 2002; Vismaya et al. 2010; Katekhaye et al. 2012). Over 150 bioactive compounds have been reported from the genus *Baccharis* since 1900s (Abad and Bermejo 2006). The Amaryllidaceae alkaloids and their derivatives include novel biologically active compounds possessing anticancer, antiviral, acetylcholine esterase inhibitors and antibacterial properties (Kilgore and Kutchan 2016). Some important plant based drugs are tabulated below along with their bioactivity and plant source **(Table 2.1)**.

Table 2. 1 Common plant based drugs along with their bioactivity and sources (Fabricant and Farnsworth 2001; Lahlou 2013)

Sr.no	Drug	Action	Plant source
1.	Ajmalicine	Circulatory disorders	*Rauvolfia serpentine*
2.	Andrographolide	Dysentry	*Andrographis peniculata*
3.	Anisodine	Anticholinergic	*Anisodus tanguticus*
4.	Bergenin	Antitussive	*Ardisia japonica*
5.	Caffeine	CNS stimulant	*Camellia sinensis*
6.	Camphor	Antimicrobial agent, cough suppressant	*Cinnamonum camphora*
7.	Catechin	Haemostatic	*Potentilla fragaroides*
8.	Chymopapain	Mucolytic	*Carica papaya*
9.	Cocaine	Anaesthetic	*Erythroxylum coca*
10.	Colchicine	Anti-tumor,anti-inflammatory, antigout	*Colchicium atumnale*
11.	Curcumin	Choleretic	*Curcuma longa*
12.	Cyclosporine	Immunosuppressant	*Mentha piperita*
13.	Danthron	Laxative	*Cassia sp.*
14.	Digitalin	Cardiotonic	*Digitalis purpurea*
15.	Digoxin	Cardiotonic	*Digitalis sp.*
16.	Digoxin	Cardiotonic	*Hypercicum perforatum*
17.	Emetine	Dysentery	*Cephaelisipe cacuanha*
18.	Gossypol	Contraceptive	*Gossypium sp.*
19.	Indinavir	Anti malarial agent	*Hypericum perforatum*
20.	Kawain	Tranquilizer	*Piper methysicum*
21.	Khelin	Bronchodilator	*Ammi visnaga*
22.	Menthol	Antibacterial agent, anti perspirant	*Mentha sp.*
23.	Morphine	Analgesic	*Papaver somniferum*
24.	Noscapine	Antitussive	*Papaver somniferum*
25.	Papain	Mucolytic	*Carica papaya*
26.	Quinine	Anti malarial agent	*Colchicine ledgeriana*

Sr. No.	Drug	Action	Plant source
27.	Reserpine	Tranquilizer	*Rauvolfia sepentina*
28.	Rotenone	Piscicide	*Loncho carpusnicou*
29.	Salicin	Analgesic	*Salix alba*
30.	Stevioside	Sweetener	*Stevia rebaudiana*
31.	Theobromine	Bronchodilator	*Theobroma cacao*
32.	Tubocurrarine	Muscle relaxant	*Chondodendron tomentosum*
33.	Vicamine	Stimulant	*Vinca minor*
34.	Vinblastine	Anticancer agent	*Catharanthus roseus*
35.	Vincristine	Anticancer agent	*Catharanthus roseus*

2.4 Medicinal plants and Zingiberaceae

NE India comprises of 8 states *viz*. Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura lying between 21º34'N to 29º50'N latitude and from 87º32'E to 97º52'E longitude, comprising an area of 262060 km^2 . It has the richest diversity of plants and constitute around 50% of India's biodiversity (Mao et al. 2009). About 50% of angiosperms hail from this region, of which 40% are endemic. The dominant families present in this region are Fabaceae, Orchidaceae, Poaceae, Euphorbiaceae, Asteraceae, Cypearaceae, Rubiaceae and Zingiberaceae. All the states belong to different climatic and hilly zones which is responsible for the rich biodiversity and abundance of medicinal plants present here (Upadhaya et al. 2012).

The traditional communities living in NE India have a lot of 'traditional knowledge' handed down through generations, about the use of the plants as medicines. The local healers, known as bej or kobiraj, use various plants to cure ailments, which form an integral part of the indigenous or ethnic culture of NE India. Use of different plants (many of which are endangered or threatened) by the ethnic groups of this region for ethnomedical purposes has been documented (Sajem and Gosai 2010). Below are listed some of the widely known medicinal plants that are used locally in NE India **(Table 2.2)**.

Table 2. 2 List of some commonly found medicinal plants of NE India (Islam 2010)

Sr. No.	Name	Common name	Family	Uses
1.	*Abutilon indicum*	Indian mellow	Malvaceae	Diuretic, sedative, antipyretic, aphrodisiac, laxative, expectorant; cures urinary troubles
2.	*Acalypha indica L.*	Indian nettle	Euphorbiaceae	Emetic, laxative expectorant, antihelminthic; used in bronchitis, pneumonia, rheumatism, skin diseases, even snake bites
3.	*A. hispidum* DC.	Starbur	Asteraceae	Antimicrobial, used in cuts
4.	*Achyranthes aspera* L.	Prickly shaff	Acanthaceae	Purgative, diuretic, stomachic, depurative, astringent; useful in diarrhea, piles, dropsy, rheumatism, inflammation, gynaecological problems, pyrrhoea and gum complaints
5.	*Aconitum ferox* Wall.	Indian aconite	Ranunculaceae	Antiarthritis, diuretic, expectorant, nervine tonic, stomachic, antipyretic; locally used for leprosy
6.	*Acorus calamus* L.	Sweet flag	Araceae	Emetic, stimulant, diuretic, laxative, stomachic, expectorant; snake repellent, insecticide; relieves bronchial and chest infections
7.	*Ageratum conyzoides* L.	Goat weed	Asteraceae	Coagulant, anti-tetanic, styptic; used in leprosy, piles, edema and uterine infections
8.	*Alpinia galanga* (L.) Wild	Greater galanga	Zingiberaceae	Carminative, stomachic, antipyretic; used medically in rheumatism and catarrhal infection; locally used in diarrhea
9.	*Andrographis paniculata (Burm.f)* Nees	The king of bitter	Acanthaceae	Antihelminthic, febrifuge, stomachic, tonic; useful in diarrhea, dysentery, dyspepsia, influenza, bronchitis; decoction is useful in jaundice
10.	*Argemone mexicana* L.	Prickly poppy	Papaveraceae	Laxative, emetic, expectorant, demulcent;useful during gonorrhea, blenorrhagia, cutaneous diseases, jaundice, dropsy, herpes, scabies, rheumatism, asthma, whooping cough, pulmonary diseases and catarrhal infections
11.	*Artemisia vulgaris* L.	Motherwort	Asteraceae	Antihelminthic, expectorant, antilithic, stomachic, febrifuge, insect repellent; locally used for burns, nose bleeding, skin diseases, headache; snake bites

Sr. No.	Name	Common name	Family	Uses
12.	*Boerhavia diffusa* L.	Spreading hogweed	Nyctaginaceae	Expectorant, diuretic, laxative, emetic, ophthalmic; good for asthma, jaundice and gonorrhea
13.	*Cannabis sativa* L.	Indian hemp	Cannabaceae	Appetizer, antispasmodic, digestive, sedative, diuretic, stimulant, stomachic; source of narcotics; locally used during indigestion, melancholy and impotence
14.	*Capsella bursa-pastoris*	Shepherd's purse	Brassicaceae	Coagulant, diuretic, astringent; useful in blennorhagia and chest trouble
14.	*Cassia sophera* L.	Sickle cassia	Caesalpiniaceae	Depurative, expectorant, antipyretic, cathartic; useful in eczema, rheumatism and skin diseases
15.	*Centella asiatica* (L.)Urban	Indian pennywort	Apiaceae	Diuretic, brain and cardiac tonic, carminative, expectorant, febrifuge; useful in diseases of nerve, blood, constipation, cough; given as memory booster to children
16.	*Centratherum anthelminticum*	Kinka oil plant	Asteraceae	Antihelmintic, antiserptic, diuretic, stimulant
17.	*Chenopodium album*	Lamb's quarters	Amaranthaceae	Antihelminthic and laxative; recommended in leucoderma, liver enlargement, piles, bolis and sores
18.	*Litsea cubeba* Pers.	Mountain pepper	Lauraceae	Used locally to cure foot and mouth disease
19.	*Lysimachia racemose* Lam.	Swamp candles	Primulceae	Emetic, effective antihelmithic
20.	*Poulzolzia hirta* Hassk.	Nettle	Urticaceae	Fabrifuge, depurative; used in skin infections, warts, boils, leucorrhoea; fractured joints

Zingiberaceae is considered to be the "blessing of nature" as it a resource for many useful products like spices, medicines, perfumes, dyes, aesthetics etc. Members of this rhizomatous family are recognized by their characteristic odor, which is due to the presence of volatile, essential oils and oleoresins. India is one of the richest abodes of Zingiberaceae. The NE region of India houses the greatest abundance, comprising of 19 genera and about 88 species. The ethnomedical use of members of Zingiberaceae has been studied critically and documented (Tushar et al. 2010). Members of this family are traditionally used by tribal populations in NE India as a home remedy for a wide range of ailments. This implies that these plants synthesize a wealth of chemicals or secondary metabolites that increases their survival ability and help to overcome challenges.

Extraction of these active components from the plants coupled with chemical synthesis, are helpful in the development of highly effective drugs. A large number of nations of the world are investing a major share of their budget towards developing "herbal medicine", and India, a country so rich in ethnic and traditional knowledge, is taking an active part in this aspect.

The largest, most complex and diverse genus of Zingiberaceae is *Alpinia*, with 230 species occurring in tropical and subtropical Asia alone, spreading from Sri Lanka to Western Ghats of India to China, Japan, southeast Asia, the Pacific and Australia. *Alpinia* belongs to the group of flowering plants (angiosperms) and as per the Angiosperm Phylogeny Group II (APG II) system, it comes under monocotyledonous plants. *Alpinia* is a member of the tribe Alpinieae. These herbaceous plants lack true stem; rather they have pseudostem which are well developed. They generally grow from thick, underground, creeping rhizomes (Wu and Larsen 2000). Members of this genus are generally aromatic due to their high content of essential oils; hence various parts of these plants are subjected to fractionation process to yield volatile oils, extracts and bioactive components. A review on the pharmaceutical potential of this genus has been previously reported (Ghosh and Rangan 2013). Extensive research is being carried out on some members of this genus *viz*. *Alpinia galangal, A. purpurata, A. katsumadai, A. officinarum*, etc.

2.5 *Alpinia nigra*: The plant under study

A lesser explored member of the genus *Alpinia* is *Alpinia nigra*, a medium sized rhizomatous, herbaceous plant, endemic to South-east Asia including Bhutan, China, India, Thailand, Bangladesh, Burma and Sri Lanka. This plant is consumed by the indigenous natives of Tripura, India, as a vegetable mixed with rice. It is considered as a plant related to magico- religious beliefs (Dobur Uie) in some parts of the world (Sharma and Pegu 2011). This herb is the only member of Zingiberaceae which serves as a fodder to rhinoceros (Ghosh and Das 2007). Few research and review papers outline the importance of the different extracts obtained from this plant and further research continues to isolate the various bioactive compounds from its different parts and enumerate their mechanism of action in controlling diseases.

2.5.1 Botanical description

A. nigra is an herbaceous plant that grows commonly in shady places, forest margin and wasteland (Tag and Das 2004). It grows from an underground fleshy rhizome and has

well developed pseudo stems above the ground. Leaves are simple, alternate, oblong, sessile or sub-sessile, pointed at the ends with a very short petiole and very long leaf sheath. The plant has panicle type of inflorescence and racemose or inderminate growth. Flowers are solitary, perfect, terminal, protected by bracts. They are white in color, have irregular symmetry (3 sepals and 3 lobes, 3 petals and 3 lobes) and are monoecious. One stamen is fertile, 5 are sterile, showy, and are white to pinkish in color. Ovary is inferior with a single pistil, style between anthers; axile placentation; ovary is sectioned by radial spokes into locules and is trilocular. Fruit is berry with many seeds; green when young (**Fig.2.1**) and changes from red to black with maturity (Roy and Swargiary 2009).

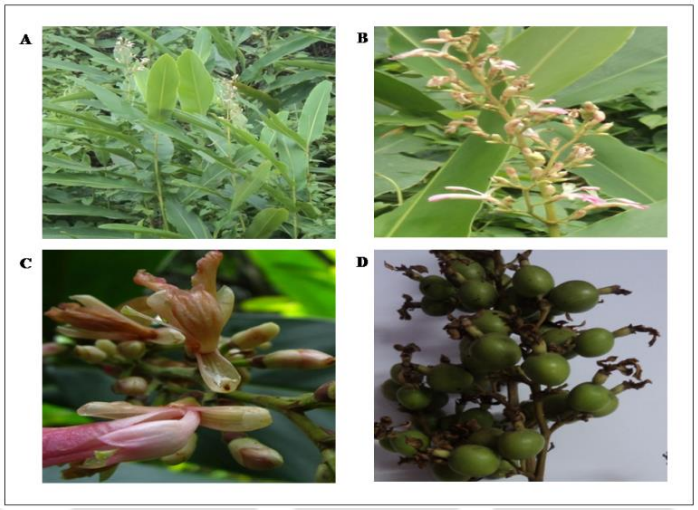


Fig 2. 1 Different parts of *A. nigra* (**A**) Oblong, sessile leaves having long sheath; (**B**) Racemose inflorescence of flowers; (**C**) Pinkish flowers with irregular symmetry; (**D**) Fruit cluster with immature fruits

2.5.2 Chemical description

2.5.2.1 Chemical and mineral content

Minerals and trace elements are very important in regulating growth and metabolic function of the human body even though they do not provide any calorie (Ismail et al. 2011). Trace elements like potassium, zinc, calcium, manganese etc. are known to play indirect role in the management of the non-communicable disease, Type II diabetes (Kar et al. 1999). The proximate composition and mineral content of the rhizome of *A. nigra* is shown in **Table 2.3**.

Table 2. 3 Proximate composition (%) and mineral content (mg/100) in rhizomes of *A. nigra*

Sr. no.	Proximate Composition (%)		Mineral Content (mg/100g)	
	Constituent	**Content**	**Constituent**	**Content**
1.	Ash	1.86±0.05	Copper	7.50±0.87
2.	Moisture	9.87±0.15	Zinc	12.00±0.28
3.	Crude fiber	76.53±1.06	Iron	32.75±0.43
4.	Crude protein	5.64±0.06	Manganese	6.25±0.14
5.	Crude fat	6.1±0.23	Calcium	0.37±1.3
6.	Ascorbic acid	1.23±0.03	Nickel	0.17±0.07
7.			Magnesium	1.2±1.55

However, this plant is a poor source of minerals. These values indicate that this plant is a rich source of carbohydrates and iron and hence is ideal to be used as medicinal and food supplement. The plant has approximately 10% lower content of crude protein and fat than *Zingiber officinale* (Shahid and Hussain 2012).

2.5.2.2 Oil content

Alpinia, which have a high content of essential oils, show a high amount of aromaticity. It means that the chemical compounds, rather than the percentage yield, control the aromaticity of the plant (Roy 1998). *A. nigra* is also no exception to it. A lot of research has been done to estimate the oil content in various parts of this plant **(Table 2.4)**.

Table 2. 4 Oil content estimated from different parts of *Alpinia nigra* (Ghosh et al. 2014; Islam et al. 2014)

Sr. No.	Parts used	Oil content (%)
1.	Rhizomes	0.15
2.	Dried fruits	1.25
3.	Leaves	0.02-0.15
4.	Flowers	0.06
5.	Leaves	0.23
6.	Seeds	0.76
7.	Rhizomes	0.18

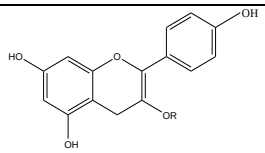
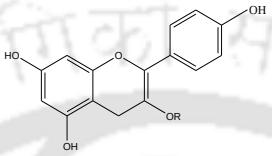
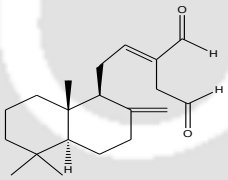
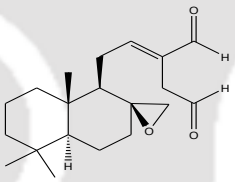
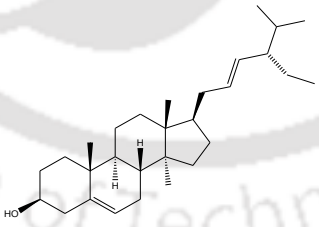
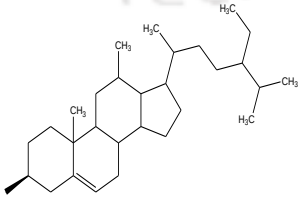
The oil content from the leaves is slightly higher in Assam (Ghosh et al. 2014) than Bangladesh (Islam et al. 2014). The climate, temperature, soil, harvest time and other environmental factors along with the mode of oil extraction used influence the oil yield (Meier and Mediavilla 1998; Mejdoub and Katsiotis 1998).

2.5.3 Phytochemistry

Phenolic compounds from medicinal herbs and dietary plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others that have a wide range of bio-pharmaceutical properties such as anti-inflammatory, anti-oxidant, anti-cancerous etc. (Huang et al. 2010). The diverse range of bioactive substances like flavonoids, tannins, terpenes and alkaloids harbored in the aqueous and organic solvent extracts obtained from various parts of *A. nigra* is the key to its therapeutic efficacy. A wide range of bioactive compounds have been isolated and studied from the genus *Alpinia* (Ghosh and Rangan 2013), however, limited literature is available on *A. nigra* and its phytochemicals (Roy et al. 2012). Naturally occurring bioactive compounds have been characterized extensively using a number of analytical techniques like preparative thin layer chromatography (pTLC), high performance liquid chromatography (HPLC), ultraviolet (UV) spectral analysis, fourier transform infrared spectroscopy (FTIR), ^{13}C and ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry (MS). The few, yet notable compounds isolated from *A. nigra* are enlisted in **Table 2.5**.

In addition, 3 hydroxypropyl esters *viz*. 3 dihydroxypropyl ester (III), 3 dihydroxypropyl ester (IV) and 3 dihydroxypropyl ester (V) have also been isolated from the seed clusters of *A. nigra* (Qiao et al. 2000). The pinene compound class is pre-dominant in the oil and the major constituents are β-pinene and α-pinene constituting 20.02% (w/w) and 6.65% (w/w) respectively in fruits (Qiao et al. 2007) and 55.3% (w/w) and 9.7% (w/w) respectively in rhizomes (Prakash et al. 2007). There is a notable high content of Kaempferol-3-*O* glucuronide in the seeds cluster (around 0.926 to 2.012%) than astraglin. These two compounds are not detected in the rhizomes. Kaempferol-3-*O*-glucuronide has been found to be primarily distributed in the pulp with a high content (3.043% w/w) which is approximately six times to that found in the seeds (Qiao et al. 2007).

Table 2. 5 Bioactive compounds isolated from *A.nigra* (Qiao et al. 2000; Ghosh et al. 2013a, 2017; Ghosh and Rangan 2014a; Sethi et al. 2015)

Sr. no.	Compound Name	Structure	Class of compound	Bioactivities
1.	Astraglin	 R= Glucose	Flavone glycoside	Antibacterial, antiprotozoal, antioxidant, hepato-protective
2.	Kaempferol-3-*O*-glucuronide	 R= Glucouronic acid	Flavone glycoside	Antibacterial, antiprotozoal, antioxidant, hepato-protective
3.	(E)-labda-8(17), 12-diene-15, 16-dial		Diterpene	Antibacterial, Antidiabetic, Antileishmanial
4.	(E)-8β,17-Epoxylabd-12-ene-15, 16-dial		Diterpene (epoxy derivative)	Antibacterial, Antidiabetic, Antileishmanial
5.	Stigmasterol		Sterol	-
6.	γ-Sitosterol		Sterol	-

2.5.4 Bio-pharmaceutical potential

The genus *Alpinia* has immense bio-pharmaceutical potential which is evident from earlier published reports. In addition, the different spices obtained are in use as food flavoring agents (Qureshi et al. 1992; Parthasarathy and Kandiannan 2007). *A. nigra* is consumed as a vegetable diet with rice in Tripura and also as a culinary spice. The aqueous shoot extract is consumed by the natives of Tripura against intestinal infections (Roy et al. 2009); the rhizomes are used in gout and colic diseases (Shahid and Hussain 2012). A brief account of the biological efficacy towards therapeutic uses of this plant **(Fig 2.2)** is described below:

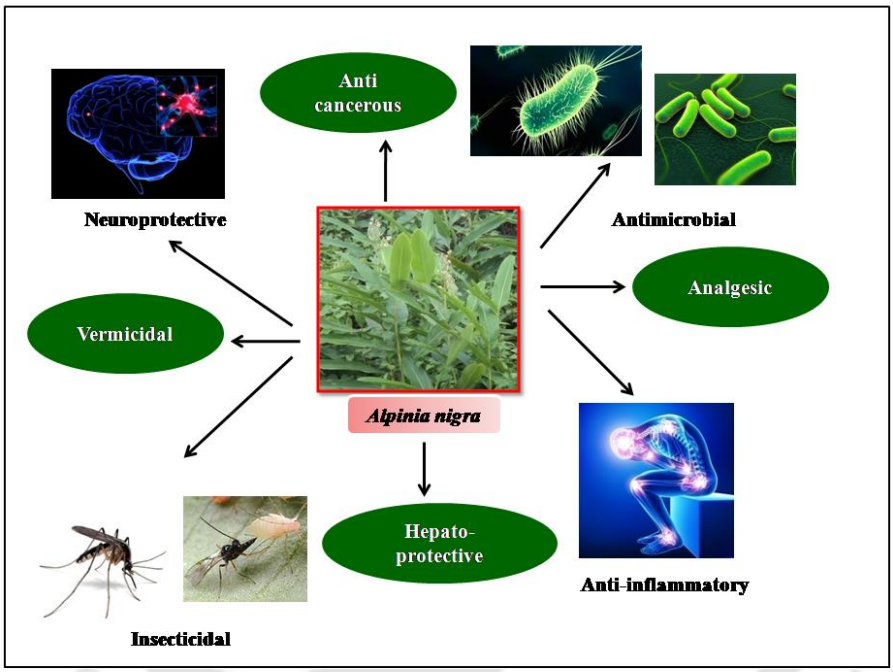


Fig 2. 2 Schematic representation of the biopharmaceutical potential of *A. nigra*

2.5.4.1 Antimicrobial activity

Infectious diseases account largely for ill health. A number of plants are used as traditional medicines against microbial infections. Organic solvent extracts from different parts of *A. nigra* are highly effective antibacterial agents. Methanolic extracts of the rhizome of *A. nigra* show moderate antibacterial activity against *S. aureus* and *B. cereus* (Bhunia and Mondal 2012) and the aqueous extract of the rhizome show antibacterial activity against *S. aureus, B. cereus, S. paratyphi* and *E. coli*. In addition the aqueous extracts of the flowers and seeds also show moderate antibacterial activity (Ghosh et al. 2014). The antibacterial efficacies of the different extracts obtained from the different parts of *A. nigra* are listed in **Table 2.6**. While the aqueous leaf extract of this plant show

low antibacterial activity, the methanolic extract has a moderate activity against a wide range of both Gram positive and Gram negative bacteria (Abu Ahmed et al. 2015).

Table 2. 6 Antibacterial activity of the various organic crude extracts of *A. nigra* (Bhunia and Mondal 2012; Ghosh et al. 2014; Abu Ahmed et al. 2015)

Sr. no.	Tested strains	Parts of the plant used	Bioactive fractions	Zone of inhibition (mm)
1.	*S. aureus*	Rhizome	Methnolic extract	13
			Essential oil by hydrodistillation	8
		Leaf	Methanolic extract	9
			Essential oil by hydrodistillation	7
		Flower	Essential oil by hydrodistillation	6
		Seed		6
2.	*B. cereus*	Rhizome	Methanolic extract	12
			Essential oil by hydrodistillation	7
		Leaf	Methanolic extract	8
			Essential oil by hydrodistillation	6
		Flower	Essential oil by hydrodistillation	6
		Seed		6
3.	*S. paratyphi*	Rhizome	Essential oil by hydrodistilation	6
		Leaf	Methanolic extract	7
			Essential oil by hydrodistilation	6
		Flower	Essential oil by hydrodistilation	6
		Seed		6

Sr. no.	Tested strains	Parts of the plant used	Bioactive fractions	Zone of inhibition (mm)
		Rhizome		7
4.	*E. coli*	Leaf	Essential oil by hydrodistilation	8
		Flower		8
		Seed		7

2.5.4.2 Antiparasitic and insecticidal activity

A large number of parasites pose great threat to the human health. Crude alcoholic shoot extract of *A. nigra* possesses efficient vermicidal activity against the intestinal parasite, tapeworms (Roy et al. 2009) by inhibiting the activity of alkaline phosphatase, an enzyme associated with the digestion and absorption of food materials in helminth parasites (Swargiary et al. 2013). The essential oils of leaf, rhizome and seed of this plant show 100% mortality at 125 ppm against *Aedes aegypti* larvae. The essential oils of *A. nigra* also show bitting deterrent activity against *A. aegypti*; the essential oil of the seed was found to be most effective while flower oil was least effective (Ghosh et al. 2014).

2.5.4.3 Antioxidant and cytototoxic activity

Plants contain phytochemicals with various bioactivities including antioxidant, anti-inflammatory and anticancer. Natural antioxidants found in foods increase the antioxidant capacity of the plasma and reduce the risk of diseases (Shekhar et al. 2011). The leaves of *A. nigra* possess high phenolic and flavonoid content that imparts potential antioxidant activity (Sahoo et al. 2013). The methanolic extract of rhizome has the highest antioxidant activity followed by hexane extract whereas ethanolic extract has the least (Sattar et al. 2013).

Bioactive compounds are almost always toxic at higher doses. The petroleum ether, ethyl acetate and chloroform fractions of methanolic extract of the rhizomes of *A. nigra* are found to be considerably cytotoxic. In addition, these fractions may have mild

anti-tumor and pesticidal activity that can be confirmed from further assays (Paul et al. 2015). The crude leaf extract of this plant has an LC50 value of 57.12 µg/ml, which indicates significant pharmacological effects of this extract (Abu Ahmed et al. 2015).

2.5.4.4 Anti-inflammatory and analgesic activity

Inflammation is a protective response by the body to eliminate the cause of injury and initiate healing. This complex biological response involves a large number of immune cells to eliminate detrimental stimuli (Ferrero-Miliani et al. 2007). A large number of steroidal and non-steroidal drugs have been used as medication for inflammation but they are accompanied with many side effects including liver dysfunction (Urhausen et al. 2003). Hence, herbal medicines are an ideal choice to treat inflammation and acute pain. Several bioactive compounds isolated from *Alpinia* species show anti-inflammatory and analgesic activity (Ghosh and Rangan 2013).

An equal proportion mixture of petroleum ether, ethyl acetate and methanolic bark extract of *A. nigra* exhibits significant anti-inflammatory effect (Das and Biswas 2012). In addition, there is significant and dose-dependent anti-inflammatory activity of ethanolic fruit extract (Das et al. 2014) and methanolic extract of shoot-bark and leaves of *A. nigra* (Ameen et al. 2014). The methanolic leaf extract of *A. nigra* exhibits slightly weaker analgesic activity in comparison to standard drug nalbuphine HCl (Abu Ahmed et al. 2015) while the ethanolic fruit extracts of this plant possess profound analgesic activity (Das et al. 2014). The presence of numerous phytochemicals from moderate to high in these extracts is responsible for the analgesic and anti-inflammatory activities as observed previously (Ramesh et al. 1998; Küpeli and Yesilada 2007; Fawole et al. 2010).

2.5.4.5 Neuroprotective activity

Anxiety and depression are the most common psychiatric disorders that affect about 20% of adult population in their lifetime (Ghosh and Rangan 2013). Most of the antidepressants prescribed have a high risk to benefit ratio with prominent long term side effects (Newman et al. 2004). Hence, there is an urgent need to search for highly efficient drugs, that are without any side effects, to treat anxiety and depression. Recent studies reveal that the methanolic leaf extract of *A. nigra* has anxiolytic and antidepressant activities (Sharmen et al. 2014). The plant extract is known to have a number of flavonoids and neuro-active steroids that act as ligands to GABAA receptors in central

nervous system (CNS), thus imparting antidepressant like properties, as previously described (Fernández et al. 2006; Jäger and Saaby 2011).

2.5.4.6 Other activities

In general, non-steroidal anti-inflammatory, analgesic drugs possess antipyretic effect by acting on the hypothalamus (Clark and Cumby 1975). The methanolic extract of *A.nigra* also shows strong antipyretic activity (Ameen et al. 2014). In addition, *A. nigra* also shows moderate to good clot lysis activity in synergy with streptokinase as well as synergistic antithrombotic action (Mannan et al. 2011). Also, this plant can be used as a dietary adjunct for the treatment of diabetes as the ethanolic extract of *A. nigra* shows significant decrease in the digestion and absorption of carbohydrate, thus, showing a pronounced hypoglycemic effect (Ghosh et al. 2013a).

The above consolidated data reveals that *A. nigra*, like other members of this genus, possess a huge wealth of phytochemicals which are responsible for its bio-efficacy. Rhizome is mostly investigated for pharmacological efficacy followed by leaf while other vegetative and reproductive parts are used moderately. In addition, different solvent systems are utilized in bioactivity studies and methanol is the most preferred solvent system for initial crude oil extraction and extraction of bioactive compounds. The crude fractions of *A. nigra* are found to be promising therapeutic agents and can overcome a large number of biomedical challenges.

2.6 Labdane diterpene: The compound under investigation

Terpenes are the largest class of secondary metabolites present in plants that possess defined physiological functions. The basic chemical unit of any plant secondary metabolite is a hydrocarbon chain known as isoprene unit **(Fig 2.3 A)**. Diterpenes constitute a large family of secondary metabolites having 4 isoprene units (C20). They are synthesized *via* geranylgeranyl pyrophosphate (GGPP) through mevalonate pathway. Protonation of GGPP yields coplyl PP owing to a cyclization process and subsequently produces labdadienyl PP by an alternative process (Dewick 2002). The most well-known member is taxane or taxol, commercially available under names such as paclitaxel, docetaxel and cabazitaxel and used for chemotherapy. Other standard diterpenes include clerodane, kaurane, gibberellane and so on (Morita and Itokawa 1988; Gonzalez-Burgos and Gomez-Serranillos 2012). Terpene and terpene derivatives, obtained from plant sources, have a wide range of biological activities like effects against pathogenic bacteria,

diabetes, soreness, rheumatism etc. (Daniewski et al. 1989; Landau et al. 1994; Fortuna et al. 2001; Cabral et al. 2008a; Steinbach et al. 2008). It has been shown that the sesquiterpene, cnicin, can induce death of myeloma cells even in presence of cytokines (Jöhrer et al. 2012). Sawamura et.al have reported the antiviral potential of terpenoids isolated from *Alpinia officinarum* (Sawamura et al. 2010) against influenza virus.

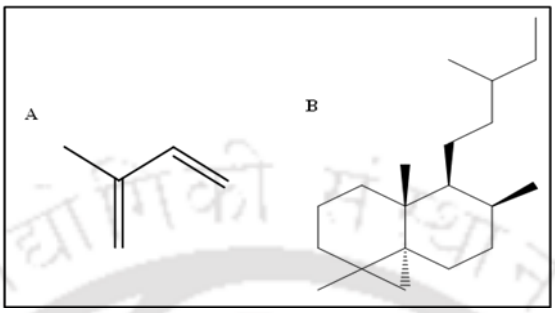


Fig 2. 3 Structure of (**A)** Isoprene unit (**B)** Labdane

Labdane diterpenes are basically bicyclic diterpenes that form its central core **(Fig 2.3 B)** (https://pubchem.ncbi.mlm.nih.gov/search/compound/labdane#section=2D-Structure). "Labdane", the family of diterpenes, derived its name from "Labdanum", a gum obtained from a group of plants in 1956, which is considered to be the very first member described for this group of compounds (Cocker and Halsal 1956; Cocker et al. 1956). Various labdane diterpenes and their derivatives, isolated from different plants of Zingiberaceae family, possess a wide range of biological activities (Garbarino and Molinari 1992; Demetzos and Dimas 2001; Rodrigues-Filho et al. 2002; Tomla et al. 2002; Sob et al. 2007; van Wyk and Davies-Coleman 2007; Kunnumakkara et al. 2008; Prabhakar Reddy et al. 2009; González et al. 2010; Suresh et al. 2010). The anti-oxidative potential of various terpenes including labdane-type diterpenes has been thoroughly highlighted by González-Burgos E. et al. (Gonzalez-Burgos and Gomez-Serranillos 2012). Apart from these, a comprehensive review on the biological activities of labdane-type diterpenes by Demetzos, C. et al. gives a bird's eye view of the antibacterial, antifungal, antiprotozoal, anti-inflammatory, immunomodulatory, anti-cancer and other therapeutic potential of the compounds (Demetzos and Dimas 2001).

The labdane diterpene, (E)-labda-8(17), 12-diene-15, 16-dial (Fig 3 C), was isolated from the seeds of *Alpinia galanga* for the very first time (Morita and Itokawa 1988). This particular compound is also very widely distributed in Zingiberaceae (Kimbu et al. 1979, 1987; Firman et al. 1988; Sirat and Hadi 1993; Sirat et al. 1994; Sy and Brown 1997; Abreu and Noronha 1997; Abe et al. 2002; Abas et al. 2005; Kenmogne et

al. 2005; Tatsimo et al. 2006; Singh et al. 2010; Sheeja and Nair 2014). Not only in *Alpinia galanga*, this compound is also reported to be found in other species of *Alpinia viz*. *Alpinia chinensis* (Sy and Brown 1997), *Alpinia katsumadai* (Ngo and Brown 1998; Hua et al. 2009), *Alpinia calcarata* (Kong et al. 2000, 2004; Hema and Nair 2009), *Alpinia mutica* (Sirat and Jani 2013) and *Alpinia zerumbet* (Chompoo et al. 2011; Upadhyay et al. 2011) etc. In addition, four species of *Alpinia viz*. *A. javanica*, *A. purpurata*, *A. speciosa* and *A. oxymitra*, are reported to contain this compound in major amounts (Sirat et al. 1994). Ghosh S. et al isolated the labdane diterpene, (E)-labda-8(17), 12-diene-15, 16-dial from the seeds of *Alpinia nigra* and found good anti-bacterial, anti-diabetic and anti-leishmanial properties of the compound (Ghosh et al. 2013a, 2017; Ghosh and Rangan 2014b). It was found to be antifungal against various fungal species and cytotoxic against KB cell line by Morita H. et al. (Morita and Itokawa 1988). This particular labdane diterpene also has a number of other biological attributes like antimicrobial (Tatsimo et al. 2006), antiviral (González et al. 2010; Grienke et al. 2010), antiplasmodial (Duker-Eshun et al. 2002) cancer chemoprevention (Endringer et al. 2014) and others (Sukari et al. 2010; Upadhyay et al. 2011; Awang et al. 2012; Martins et al. 2014).

2.7 Antimicrobial potential of plant-derived compounds: The inspiration for development of modern antimicrobials

Even before mankind became aware of the existence of microbes, they accepted the concept that plants had natural 'healing properties', which, in today's age is termed as 'antimicrobial principles'. It is a well known fact that all plants produce certain chemicals as their "defense mechanism" that are naturally toxic to bacteria and fungi. In simple land plants like bryophytes, where 'anatomical barriers' are less effective, synthesis of 'secondary metabolites' serve as 'chemical barriers' and as the most effective defense system (Harborne 1988) against microorganisms, insects and herbivores. Apart from their defensive role, secondary metabolites have certain other roles; for example, terpenes and terpenoids are mostly responsible for odor and flavor of plants (eg. capsaicin from chilli pepper) whereas quinones and tannins are responsible for plant pigment.

Hippocrates had quoted "Let food be thy medicine and medicine be thy food". In the literal sense, this is a very significant statement for natural product chemists and pharmacologists. Most microbial infections occur due to contaminated food and hence,

are a major concern for consumers, food industry and food safety authorities. Many consumers have become aware of the deleterious side effects of synthetic preservatives. Hence, there is a growing demand for natural antimicrobials that can inhibit bacterial and fungal growth in order to improve the quality and shelf-life of food (Tajkarimi et al. 2010). Rios and Recio had proposed, back in 2005, that plants with known antimicrobial activity, that are consumed as foods, should be subjected to fractionation and isolation of pure, active compounds, rather than phytochemical screening (Ríos and Recio 2005). These active compounds mostly include a large number of phenolic compounds that disrupt the membrane of microorganisms (Cueva et al. 2010; Xue et al. 2013). A lot of food items from plants possess some active components that possess antimicrobial properties, some of which are listed in **Table 2.7**.

Table 2. 7 Common plant derived food items with their active components possessing antimicrobial properties (Cowan 2006; Tajkarimi et al. 2010; Gyawali and Ibrahim 2014)

Sr. No.	Plant	Category	Active component
1.	Allspice	Spice	Eugenol
2.	Apple	Fruit	Phloretin
3.	Basil	Herb	Linalool
4.	Black pepper	Spice	Piperine
5.	Chamomile	Tea	Anthemic acid
6.	Cinnamon	Spice	Cinnamic acid/ Eugenol
7.	Clove	Spice	Eugenol
8.	Coca	Fruit	Cocaine
9.	Eucalyptus	Herb	Tannins
10.	Fava bean	Vegetable	Fabatin
11.	Garlic	Spice	Allicin/ Ajoene
12.	Ginger	Spice	Gingerol
13.	Green tea	Tea	Catechin
14.	Mustard	Spice	Allyl isothiocyannate
15.	Nutmeg	Spice	Myristicin/ Sabinene
16.	Oregano	Herb	Carvacrol/ Thymol
17.	Papaya	Fruit	Latex

Sr. No.	Plant	Category	Active component
18.	Poppy	Spice	Opium
19.	Rosemary	Herb	Camphor/ Borneol
20.	Sage	Herb	Camphor/ Borneol
21.	Thyme	Herb	Thymol/ Carvacol

2.8 Solubility of natural bioactive compounds: The issue that is usually ignored

Solubility is the property of a solid, liquid or gaseous substance (called solute) dissolvable in a solid, liquid or gaseous solvent to form a homogenous solution. The extent of solubility ranges from fully soluble (such as ethanol in water) to insoluble or poorly soluble (such as gum in water) (Clugston and Flemming 2000).

For the action of any drug or molecule, it has to be soluble enough to enter and get absorbed into the biological system. The FDA provides a Biopharmaceutics Classification System (BCS) that acts as a guide for predicting intestinal drug absorption using the parameters of solubility and intestinal permeability. As per Amidon et.al, a drug is considered soluble when the highest dosage of the drug is soluble in 250 ml of water or aqueous media over a pH range of 1-7.5; intestinal permeability is generally considered for drugs that are generally administered by the intravenous (IV) route (Amidon et al. 1995). Based on these two parameters, drugs are generally divided into four classes as given in **Table 2.8**.

Table 2. 8 Classification of drugs as given by FDA (Savjani et al. 2012)

Sr. No.	Drug Class	Characteristic
1.	Class I	High soluble and high permeable
2.	Class II	High soluble and low permeable
3.	Class III	Low soluble and high permeable
4.	Class IV	Low soluble and low permeable

2.8.1 Importance of solubility and solubility limitations

The most common and convenient route of drug administration is oral route due to ease of administration, cost effectiveness, flexibility in design of dosage etc. The major challenge with oral dosage designs is posed by the poor bio-availability, which in turn, in most cases is attributed to poor solubility and low permeability. Not only oral

administration, solubility of drugs also plays a critical role in other routes of drug administration like intravenous, parenteral etc. (Krishnaiah 2010).

In the recent years, there has been a growing awareness regarding the issue of solubility in the field of drug discovery. Lipinski et.al was probably the first group to raise awareness in the matter of limitations associated with orally administered drugs that have low solubility (Lipinski et al. 1997). Despite this awareness, it is seen that a number of compounds for drug discovery have low solubility. The structural features of these compounds that produce potent biological activity (like lipophilic structures) are responsible for reducing solubility. Ideally, bioactive compounds are dissolved in dimethyl sulphoxide (DMSO) for bioassays but this never guarantees their good aqueous solubility. It is reported that 30% of bioactive compounds have aqueous solubility of less than 10 µM (Lipinski 2001; Di and Kerns 2007). As a result, a number of discrepancies and erroneous results occur in bioassays, some of which are summed up in **Table 2.9**. This can actually lead to an "artificial low potency" for the compound as the actual concentration in solution is much less than the predicted/reported target concentration.

Table 2. 9 Problems associated with solubility (Di et al. 2009)

Sr. No.	*In vitro* impairment	*In vivo* impairment
1.	Erratic assay results	Poor oral bioavailability
2.	Erroneous structure – activity relationship (SAR)	Lack of efficacy
3.	Discrepancies between assays	Abnormal pharmokinetic (PK) profile
4.	Low high throughput screening (HTS) hit rate	Problematic formulation
5.	Artificially low potency	Inter – subject, inter - species variation
6.	Underestimated toxicity	Expensive and prolong development
7.	Cannot be measured	Burden to patients

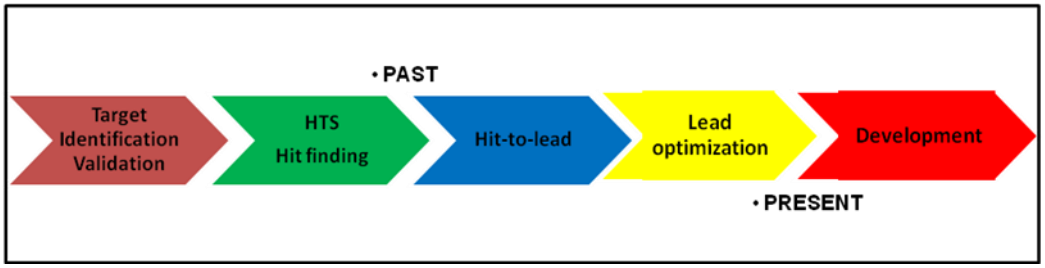


Fig 2. 4 Timing of profiling of drug-like properties; adapted from (Di et al. 2009)

The profiling of drug like properties along with their biological activity **(Fig 2.4)** is very critical, so that issues and challenges are recognized and resolved early. Matter of solubility is overlooked during the hit finding and hit-to-lead phases of drug development, which later leads to many highly potential leads being dropped from the race to drug development (Di et al. 2009). Therefore, the solubility of compounds for drug development should be optimized by structural modifications, development of formulation strategies etc. to increase the success rate in drug production, particularly in case of natural bioactive compounds.

2.8.2 Strategies to determine and improve solubility

Solubility of compounds receives the utmost importance in the new drug development strategy, adopted by pharmaceutical companies, because improved solubility can eventually lead to the development of a successful drug candidate. Thus, early solubility profiling leads to earlier formulation development for drug delivery so that this does not remain as a "rate-limiting step" in the current drug development scenario. **Table 2.10** lists the methods currently used to determine solubility of organic compounds and other small molecules.

Table 2. 10 Methods to determine solubility

Sr. No.	Methods	References
1.	Shake flask assay	(Kerns 2001; Kerns and Di 2004; Glomme et al. 2005; Alsenz and Kansy 2007; Dai et al. 2008; Kerns et al. 2008)
2.	Nephelometric assay	(Bevan and Lloyd 2000; Kerns 2001; Kerns and Di 2004; Glomme et al. 2005; Alsenz and Kansy 2007; Dai et al. 2008; Kerns et al. 2008)
3.	Direct Ultra-Violet (UV) assay	(Kerns 2001; Teng-Man Chen et al. 2002; Kerns and Di 2004; Glomme et al. 2005; Alsenz and Kansy 2007; Dai et al. 2008)
4.	High Pressure Liquid Chromatography (HPLC)	(Kerns 2001; Pan et al. 2001; Dai et al. 2008; Kerns et al. 2008)
5.	Roche in-house Parallel Incremental Solubility Assay (PISA)	(Alsenz and Kansy 2007)
6.	Roche in-house Lyophilization Solubility Assay (LYSA)	(Alsenz and Kansy 2007)
7.	pH-metric assay (commercial "pSol" instrument; pION, Woburn, MA)	(Kerns 2001)
8.	Computational approach	(Lipinski et al. 1997; Lipinski 2000; Jorgensen and Duffy 2002; Balakin et al. 2004, 2006; Delaney 2005; Faller and Ertl 2007)

2.8.2.1 Microemulsion

A well known method to deal with the low solubility problem of bioactive, natural compounds in aqueous medium is by the preparation of microemulsions (ME). The definition of ME cannot be made further precise or apt than the one provided over three decades ago by Danielsson and Lindman (Danielsson and Lindman 1981), Microemulsion is defined as "the system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution".

A thorough review of ME as a novel drug delivery system has been done by Lawrence, M. J. et al (Lawrence and Rees 2000) and Karasulu, H. Y. (Karasulu 2008), ranging from formation to advantages and disadvantages and the applications of the system. **Fig 2.5** shows the different types of ME that are generally formed or used in drug delivery applications. The major advantages of the ME are that they are thermodynamically stable, easy to make and have a high solubilization capacity. The system is slowly gathering popularity and there has been an increase in the number of

studies especially regarding the delivery of hydrophobic drugs or leads (Al-Adham et al. 2000; Garti et al. 2001; Zhang et al. 2010; Prajapati et al. 2013; Xiao et al. 2013; Guimarães et al. 2014). However, the main disadvantage associated with ME is choosing a biocompatible surfactant and co-surfactant. Studies on possible permutations and combinations of various surfactants and co-surfactants have come up but the question of toxicity still remains (Constantinides 1995; Papadimitriou et al. 2008; Mahdi et al. 2011; Syed and Peh 2014).

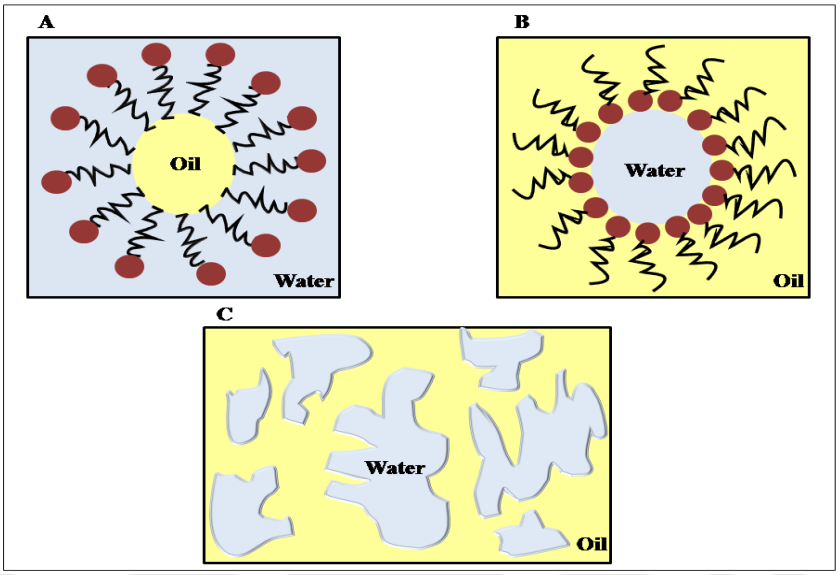
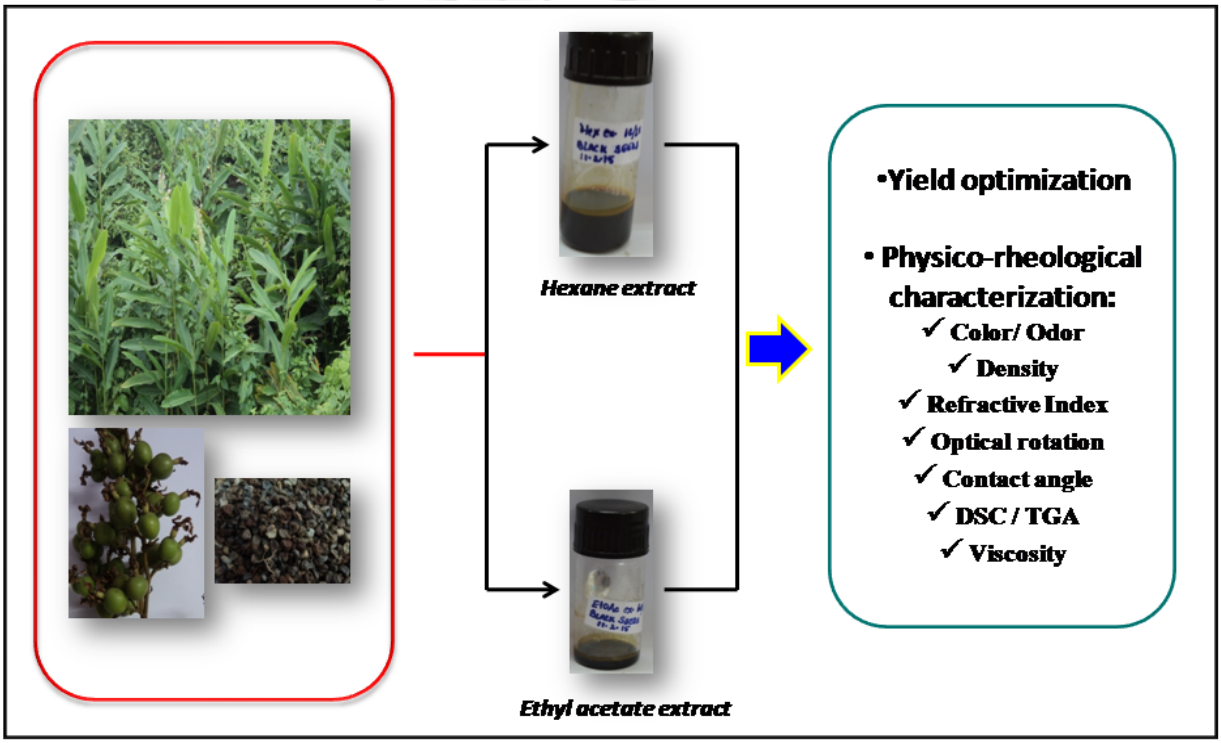


Fig 2. 5 Schematic representation of different types of microemulsions: **(A)** Oil in water **(B)** Water in oil and **(C)** Bi-continuous microemulsion; adapted from (Karasulu 2008)

Since limited information is available on the phytoconstituents of *A. nigra*, detailed ethno medical information along with scientific approach is required to establish this plant as a potential candidate in the field of herbal and complementary medicine. This plant needs to be explored thoroughly for its available compounds that impart bioactivity to the plant extracts, particularly against pathogenic microbes. In order to develop such compounds as potential drugs, their aqueous solubility needs to be taken into consideration, along with devising suitable means for drug targeting and delivery.

Chapter 3: Oil extraction and physico-rheological characterization of organic extracts obtained from the seeds of *Alpinia nigra*



This chapter describes the various extraction methods using different organic solvents from the different stages of the seeds of *A. nigra* and optimization of the best possible yield, along with their physical and rheological profiling

Chapter 3: Oil extraction and physico – rheological characterization of organic extracts obtained from the seeds of *A. nigra*

3.1 Introduction

Nature houses an inexhaustible wealth of resources in its core, commonly called as bio-resources. Amongst all its resources, medicinal plants deserve a special mention. An economy based on biological resources provides hope to the developing countries for a better sustainability. There is a worldwide awareness for the improvement of bioresource preservation and their sustainable utilization. Plants are one of most important and beneficial natural resources. They have always attracted the attention of the scientific community due to their uncountable benefits to mankind in various areas like medicines, cosmetics, food etc. In addition, plants play the most important role of environmental clean-up and add enormously to the beauty and aesthetic appeal of nature. The current century observes a paradigm shift of the bioprocess and pharmaceutical industry towards the use of plants as they open new avenues for product development, less processing cost, drug development and environmental impact. Thus, the advancement of green chemistry and biological studies provide a new network of inter-disciplinary research to ensure maximum benefit with the lowest possible risk to mankind (Harborne 1998).

Like all other countries of Asia, the Indian subcontinent is endowed with natural beauty and resources. The luxurious forests of the country are rich in "green" wealth that house plants containing many bioactive compounds. The NE India has different climatic zones like tropical, sub-tropical, temperate and alpine; hence, this region flourishes in its flora and fauna, particularly in the growth of valuable medicinal plants (Shankar et al. 2017). In the plant kingdom, the Zingiberaceae family is well known for its medicinal properties. NE India houses its greatest abundance, comprising of 19 genera and about 88 species; the ethno medical use of its members has been studied critically and documented (Tushar et al. 2010). A survey carried out in 2007-2011 in the Dibrugarh region of Assam lists 22 different species belonging to 9 different genera of this monocotyledonous family *viz*. *Alpinia*, *Amomum, Boesenbergia, Curcuma, Globba, Hedychium, Kaempferia, Larsenianthus* and *Zingiber* (Borah and Sharma 2012). *Alpinia nigra*, a lesser explored member of this family, is considered to be related to magico- religious beliefs (Dobur Uie) (Sharma and Pegu 2011) and is the only member of Zingiberaceae which serves as a fodder to rhinoceros (Ghosh and Das 2007). *A. nigra*, also known as 'Tora', is consumed

by the indigenous natives of Tripura, India, as a vegetable mixed with rice. A large number of reports are available on the bio-pharmaceutical potential of the different extracts obtained from the different parts of this plant; seeds being the most extensively studied (Ghosh et al. 2013b, 2014; Sharmen et al. 2014; Abu Ahmed et al. 2015). However, no reports are available on yield optimization of the extracts from the seeds of *A. nigra*.

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 the 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 preparation (Rousseaux and Schachter 2003). Most of the plant organic extracts (except oil yielding plants like *Jatropa curcas* or *Pongamia pinnata*) are usually non – flowing, sticky gum or resins. According to Balogun and Fetuga, there is a lack of knowledge, systemic collection and utilization of the gum like resinous extracts and bioactive molecules obtained from plant parts (Balogun and Fetuga 1986). As a result, the industrial potential of large number of such valuable natural products remains unexplored and unexploited. A comprehensive study on the physico-chemical and rheological characterization of plant extracts and products are thus, of utmost importance. Although most plants of the genus *Alpinia* (including *A. nigra*) have been thoroughly investigated for their medicinal properties, their physico-rheological characterization for industrial application have not been investigated yet.

Therefore, the present study of this chapter focuses on the preparation of various organic extracts from the seeds of *A. nigra*, to optimize the yield and characterize them. Further, the physico-chemical and rheological characteristics of the organic seed extracts were carefully determined and analyzed.

3.2 Materials and methods

3.2.1 Collection of fruits of *A. nigra*

The fruits of *A. nigra* grow in 3 stages *viz*. immature (green), partially mature (red) and mature (black). Sufficient quantity of healthy fruits of each stage were collected in an autoclave bag during June - August and from November – January every year between 2014 – 2016 from the wildly growing plants in and around the Indian Institute of Technology Guwahati (IITG) campus (26◦12.476' N to 91◦41.965'E). They were shade dried and stored at room temperature (25°C) till further processing.

3.2.2 Processing of dried fruits

The ectocarp and the pericarp portions of the dried fruits were removed manually and the seeds from the trilocular chambers of the fruits were collected and stored in an air tight bag at 25°C until further usage.

3.2.3 Preparation of organic extracts

Various extraction methods *viz*. conventional maceration, soxhlet extraction, microwave assisted extraction, ultrasound assisted extraction and many other methods are available for the extraction of chemicals components from plant materials in the form of crude extracts. In the current study, 2 different methods were used for preparation of crude extracts from the seeds of *A. nigra.*

3.2.3.1 Soxhlet extraction

Among all the methods, hot solvent extraction using soxhlet extractor is reported to give significantly higher yield in terms of phenolic content and other secondary metabolites in terms of economic investment (Aspé and Fernández 2011). Seeds from the 3 different stages of fruits were washed, cleaned and shade dried for a few days. The seeds were then powdered and subjected to hot solvent extraction in soxhlet apparatus using organic solvents (n-hexane, ethyl acetate, methanol), with increasing polarity. After each extraction, the same samples were dried properly to remove any traces of previous solvent so as to reduce the carry over effect and subjected to the next polar solvent. The parameters of each extraction were carefully recorded. The extract obtained, was concentrated by rotary evaporator (Cole parmer, USA), and stored for further analysis. All the solvents were of HPLC grade (Ranken, India).

3.2.3.2 Room temperature extraction

The dried seeds were macerated into a fine powder using a mortar and pestle, with continuous addition of organic solvent (n-hexane and ethyl acetate), with increasing polarity. After maceration, the mixture was sealed tightly and kept overnight at 25ºC. The extract obtained was filtered and then, concentrated using rotary evaporator. The seed-to-solvent ratio was optimized as per the parameters given in **Table 3.1**.

Table 3. 1 Parameters for optimization of seed-to-solvent ratio

Sr. No.	Seed-to-solvent ratio (w/v)	Seeds (g)	Solvent (ml)
1.	1:4	32.05	128.2
2.	1:6		192.3
3.	1:9		288.45

3.2.4 Physico – rheological characterization

3.2.4.1 Physical properties

Color, odor and state of the extracts were noted at room temperature by visual inspection (Onyeike and Acheru 2002). Determination of refractive index was done using refractometer (Atago DR-A1, India). Density was calculated by measuring the weight of known volume of samples on a balance (Sartorius; max. 220 g ± 0.1 mg) and then changing the calculation units.

3.2.4.2 Fourier transform infrared (FTIR) spectroscopy

A drop of each sample was cast on a small amount of potassium bromide (KBr; HiMedia, India) and made into a pellet in preparation for FTIR analysis. The spectrum was recorded in the spectrophotometer (Perkin Elmer, Germany) in the range of 700-4000 cm^{-1}.

3.2.4.3 Contact angle

Water contact angle was measured using a drop shape analyzer (Kruss, Germany), having a needle diameter of 0.5 mm, droplet size 4 µm, droplet rate of 0.16 per min and having a frame rate of 60. Samples were spread over a glass slide and the contact angle was measured.

3.2.4.4 Optical rotation

Optical polarimeter (AUTOPOL II, Rudolph Research Laboratory, USA) was used to calculate the specific rotation and optical purity of the test samples, measured at a wavelength of 589 nm.

3.2.4.5 Differential scanning calorimetry (DSC)

DSC was performed on a Netzsch DSC instrument. The analysis was completed in two thermal scans; the first cycle was from -30 to 240ºC at a heating rate of 5ºC per min followed by 2 mins of isothermal condition at 240ºC and then cooling from 200 to 30ºC with a cooling rate of 5ºC per min.

3.2.4.6 Thermogravimetric analysis (TGA)

TGA was executed by thermogravimetric analyzer (TGA-4000, Perkin Elmer, USA) under a nitrogen atmosphere with a flow rate of 50 ml/min. The test samples were placed in a alumina crucible and the analyses were performed at a temperature range of 30 to 650ºC at a heating rate of 10ºC per min.

3.2.4.7 Viscosity

Ubbelohde type capillary viscometer with a temperature controlled system was used for the measurement of viscosity of the 2 plant extracts. The concentrations of the samples used for measurement were 0.0001 g/ml, 0.0002 g/ml, 0.0008 g/ml, 0.0014 g/ml and 0.0018 g/ml in chloroform. The measurements were taken at 15ºC, 25ºC and 50ºC to see the effect of temperature on the intrinsic viscosity of the samples. The relative viscosity (η_r) of the samples was calculated from the flow time of the pure solvent and the sample solution and the specific viscosity (η_{sp}) was calculated by subtracting 1 from the relative viscosity obtained as shown in equation (1) and (2) respectively.

$$\eta_r = t/to \quad \text{.....(1)}$$

$$\eta_{sp} = 1 - \eta_r \quad \text{.....(2)}$$

where, t = flow time of samples in secs

to = flow time of pure solvent in secs

3.2.5 Statistical analysis

All the experiments were set up in a completely randomized manner, performed in triplicate and mean of the three was used for the plots. All graphs were plotted using Origin 8.5; $R^2 \geq 0.97$.

3.3 Results and discussion

3.3.1 Preparation of organic extracts

Organic solvents are frequently used for the extraction of secondary metabolites from plant samples, particularly 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 polarity of the chemical compounds in the extracts. Hence, selection of a suitable solvent system is a determinant factor on the extract properties, along with the diverse composition of the samples (Al-Farsi and Lee 2008).

3.3.1.1 Soxhlet extraction

In the current study, soxhlet extraction was used initially to prepare the organic seed extracts. For seed oil extraction from biofuel plants like *Pongamia pinnata,* a seed-to-solvent ratio of 1:6 was found to be ideal (Kesari et al. 2010); hence the same ratio was used for *A. nigra*. The different parameters for the extract yield of all the 3 stages of seeds are given in **Table 3.2** to **3.4**. .Among all the 3 stages of seeds, red seeds or the partially mature seeds yielded the maximum extracts, hence this stage maybe considered the best stage for oil extraction **(Table 3.3)**. However, due to the low yield of polar extracts (methanol extract), this extract was not used for further studies.

Table 3. 2 Parameters for organic extraction from green seeds of *A. nigra*

Sr. No.	Seed type	Parameters	Hexane extract	Ethyl acetate extract	Methanol extract
1.	Green or immature	Weight of seeds (g)	50	50	50
2.		Volume of solvent (ml)	300	300	300
3.		Time for 1st cycle (mins)	19	23	51
4.		Total time required (mins)	104	139	140
5.		Total no. of cycles	12	7	4
6.		Yield of extract (g)	3.84±0.5	0.37±0.02	0.20±0.011
7.		Volume of recovered solvent (ml)	260±1	250±5	234±2.5

Table 3. 3 Parameters for organic extraction from reds seeds of *A. nigra*

Sr. No.	Seed type	Parameters	Hexane extract	Ethyl acetate extract	Methanol extract
1.	Red or partially mature	Weight of seeds (g)	50	50	50
2.		Volume of solvent (ml)	300	300	300
3.		Time for 1st cycle (mins)	17	22	42
4.		Total time required (mins)	67	72	132
5.		Total no. of cycles	6	5	4
6.		Yield of extract (g)	9.28±0.8	0.45±0.5	0.31±0.031
7.		Volume of recovered solvent (ml)	264±1.3	252±2.1	236±1.2

Table 3. 4 Parameters for organic extraction from black seeds of *A. nigra*

Sr. No.	Seed type	Parameters	Hexane extract	Ethyl acetate extract	Methanol extract
1.	Black or mature	Weight of seeds (g)	50	50	50
2.		Volume of solvent (ml)	300	300	300
3.		Time for 1st cycle (mins)	14	24	51
4.		Total time required (mins)	67	112	160
5.		Total no. of cycles	6	6	4
6.		Yield of extract (g)	2.72±0.2	0.37±0.34	0.19±0.32
7.		Volume of recovered solvent (ml)	241±1.8	235±3	232±2.1

3.3.1.2 Room temperature extraction

Room temperature extraction or mechanical maceration is an age old method for the extraction of phytochemicals from plants. The traditional methods of Ayurveda, Unani and Sidhha medicines all use this method for the extraction of phytochemicals (Thangaraj 2015). Selection of a right extraction technique, along with optimum seed-to-solvent ratio, is of utmost importance in order to source maximum secondary metabolite containing extracts, particularly if the seeds are seasonal and hence, limiting. It was found that the yield of crude extracts from red seeds was more by this method than by soxhlet extraction; hence for subsequent studies, room temperature extraction was considered to be ideal for the seeds of *A. nigra*. The average yield of the hexane and ethyl acetate seed extracts at various seed-to-solvent ratios using room temperature extraction are listed in **Table 3.5**. The maximum yield was obtained at a ratio of 1:6 for both the solvent extracts and ethyl acetate extract had more yield than the hexane extract. This indicated that the secondary metabolites present in the seeds of *A. nigra* have a better solubility in ethyl acetate than hexane at room temperature. Though soxhlet extraction is an ideal method for oil extraction from biofuel plants like *Pongamia pinnata* (Kesari et al. 2010), as demonstrated previously by our group, medicinal plants like *A. nigra* give a better yield when extracted at room temperature as observed here.

Table 3. 5 Yield of extracts at different seed-to-solvent ratios at room temperature from *A. nigra*

Sr. No.	Seed-to-solvent ratio (w/v)	Average yield of crude extracts[a] (g)	
		Hexane extract	Ethyl acetate extract
1.	1:4	2.153 ± 0.013	7.337 ± 0.016
2.	1:6	9.842 ± 0.143	11.920 ± 0.532
3.	1:9	7.424 ± 0.120	4.294 ± 0.048

[a]Optimized yields are means of triplicate determination with standard deviation

3.3.2 Physico – rheological characterization

3.3.2.1 Physical properties

The physical properties of the 2 seed extracts of *A. nigra* are given in **Table 3.6**. All the samples are initially oily at room temperature and have a sticky consistency but tend to solidify with the increase in shelf life or change in storage temperature. This indicated that the extracts are rich in saturated fatty acids which solidify at low temperature. This behavior also indicated low degree of unsaturation in the components of the extracts. The hexane extract has a characteristic, distinguishable spicy odor, whereas the ethyl acetate extract was odorless. The observed density of the samples are within the range of seed oil as previously reported (Onyeike and Acheru 2002).

Table 3. 6 Physical properties[a] of seed extracts from *A. nigra*

Sr. No.	Sample	Color	State[b]	Odor	Refractive index[b]	Density (g/cm^3)
1.	Hexane extract	Reddish brown	Thick liquid	Characteristic (Spicy)	1.427±0.025	0.929±0.03
2.	Ethyl acetate extract	Blackish	Thick liquid	Odorless	1.458±0.01	1.04±0.23

[a]Values are mean of triplicate determination with standard deviation
[b]Measured at 25ºC

3.3.2.2 FTIR

The IR spectrum of the hexane and ethyl acetate extracts of the seeds of *A. nigra* is shown in **Fig 3.1**. It was observed that the IR spectra are relatively simple and "less crowded", indicating that not much variety of bonds are present in the extracts. Both the extracts exhibited almost identical vibrational patterns with slight differences. Two sharp peaks at 2872 cm^{-1} and 2925 cm^{-1} indicated the C-H bond stretch of CH_3 and CH_2 groups respectively. Intensity of these peaks was higher in the ethyl acetate extract in comparison to the hexane extract. Moderately strong peaks at 1731 cm^{-1}, 1683 cm^{-1} and 1638 cm^{-1}

were the signature vibrations of C=O stretch of –CHO group, α, β unsaturated C=O stretch of –CHO or –C=O group and stretch of conjugated C=C groups respectively. Peaks between 1300 cm^{-1} – 1500 cm^{-1} were attributed to the skeletal vibrations of –C-C groups. Hexane extract exhibited 2 sharp and distinct peaks at ~ 960 cm^{-1} and 1022 cm^{-1} respectively, which were merged together in the ethyl acetate extract. This indicated that hexane extract contained large number of compounds with cyclohexane groups, which were present in small amounts in the ethyl acetate extracts. This might be in concordance to the fact that compounds possessing such non – polar groups had poor solubility in semi – polar solvent like ethyl acetate and hence, were predominantly present in the hexane (non polar) solvent extract. The wavenumbers of the 2 extracts and their corresponding vibrational modes are tabulated below **(Table 3.7)**.

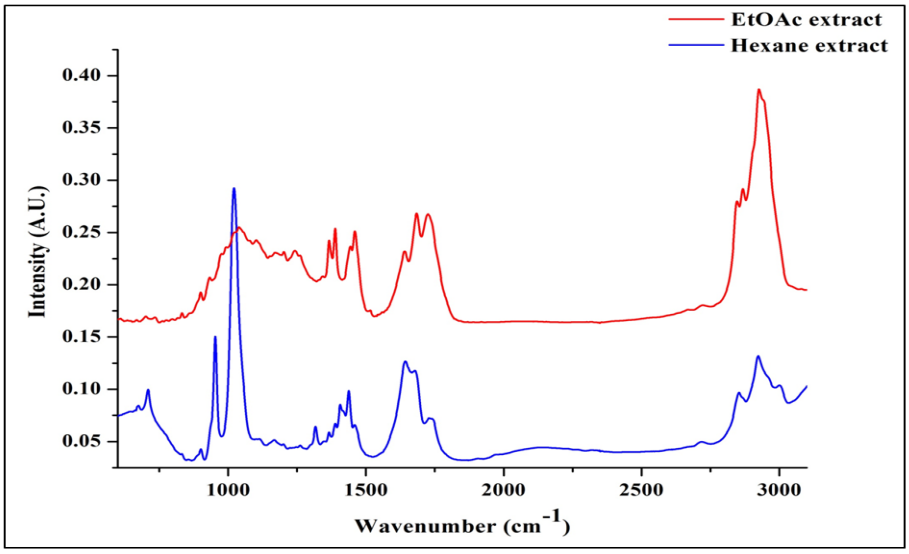


Fig 3. 1 IR spectra of hexane and ethyl acetate extracts of the seeds of *A. nigra*

Table 3. 7 Experimental wavenumbers of the 2 seed extracts of *A. nigra* and their corresponding vibrational modes

Sr. No.	Exp. Wavenumber (cm^{-1})		Corresponding modes
	Hexane extract	Ethyl acetate extract	
1.	960.755	1045.639 (merged)	Skeletal C-C vibrations
2.	1022.295		Cyclohexane ring vibrations
3.	1300 – 1500		Skeletal C-C vibrations
4.	1638.961	1635.966	Stretch of conjugated C=C groups
5.	1683.791	1683.786	C=O stretch of α, β unsaturated –CHO or –C=O groups
6.	1736.577	1731.873	C=O stretch of –CHO groups
7.	2857.997	2872.371	Asymmetric C-H stretch of CH_3
8.	2925.162	2925.162	C-H stretch of CH_2 group

3.3.2.3 Contact angle

Contact angle is a parameter to determine the hydrophobic and hydrophilic nature of any material. In order to check the surface wettability, the contact angle measurements of hexane extract and ethyl acetate extract were calculated and found to be 93.8º and 89.9º respectively **(Fig 3.2)**. The water droplet on the compound was stable for a long duration and remained as a round droplet, with minimal contact to the sample film. It is evident that the hexane extract was clearly more hydrophobic than the ethyl acetate extract. Polarity index of ethyl acetate (4.4) is higher than hexane (0.1). Thus, the hexane extract of the seeds of *A. nigra* will be more rich in hydrophobic groups like $–(C-H)_n$, C-C, C=C than the corresponding ethyl acetate extract, which was congruent to its contact angle value. However, the small difference of their contact angle values suggested that both the seed extracts are rich in non-polar, hydrophobic compounds like terpenes and terpenoids.

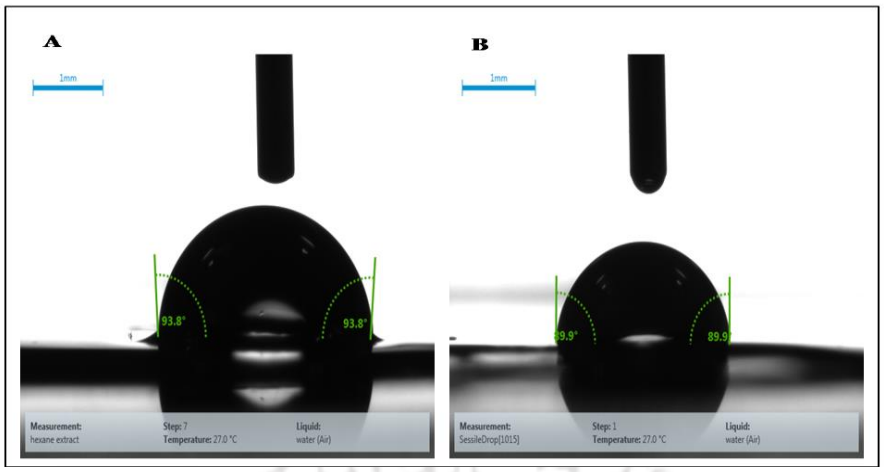


Fig 3. 2 Contact angle of water on the surface of **(A)** hexane and **(B)** ethyl acetate extracts of seeds of *A. nigra*

3.3.2.4 Optical rotation

Optical rotation or optical activity is the rotation of the plane of polarization of linearly polarized light as it travels through materials. Optical activity occurs only in chiral materials, the ones lacking microscopic mirror symmetry. In chemistry, specific rotation (α) is a property of a chiral chemical compound. It is defined as the change in orientation of monochromatic plane polarized light per unit distance – concentration product, as light passes through a sample of compound in solution.

The specific rotation of the two plant extracts are given in **Table 3.8**. Both the extracts displayed some amount of optical activity but the activity of hexane extract is much higher than that of the ethyl acetate one. This indicated that hexane extract of the seeds of *A. nigra* must have more chiral compounds than the ethyl acetate extract. Ideally the optical activity of plant organic extracts are not usually studied but some mention about this property has been made by Willow J.H. Liu in his book (Liu 2011).

Table 3. 8 Specific rotations of the seeds extracts from *A. nigra*

Sr. No.	Sample	Specific rotation[a] (α)
1.	Hexane extract	19.19
2.	Ethyl acetate extract	5.59

3.3.2.5 DSC

The DSC thermogram of the extracts is shown in **Fig 3.3**. From the first heating cycle of DSC, it was observed that the extracts had very low gas transition temperatures (T_g). For

both the samples, a broad melting or crystalline peak was observed. This suggested the non – crystalline or amorphous nature of the samples. No exothermic behavior was exhibited by the samples which further confirmed the absence of crystallization effect or solid phase. This broad endothermic behavior exhibited by the plant extracts are characteristic of oils (Aboul-Gheit et al. 1997; Tan and Che Man 2000). The DSC result is in coherence with the physical appearance/property of both the extracts as highlighted above in **Table 3.6**.

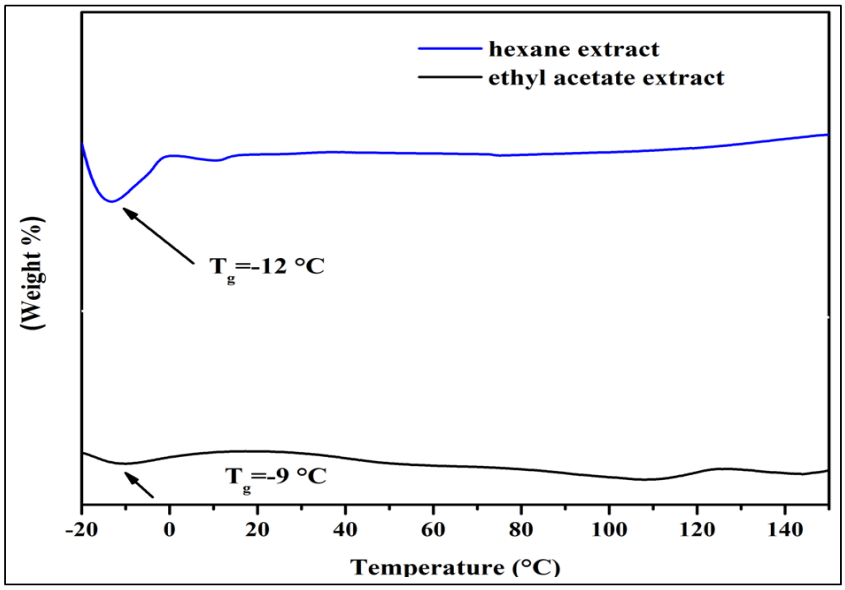


Fig 3. 3 DSC thermogram of hexane and ethyl acetate extracts of *A. nigra* seeds

3.3.2.6 TGA

The TGA thermograms of the 2 seed extracts are shown in **Fig 3.4**. From the thermograms, it is clear that the thermal degradation of all the samples passed through a two-stage degradation. Initial 5% weight loss of all the samples maybe attributed to the loss of adsorbed moisture from the surface. Among the 2 extracts, hexane extract was found to be more thermally stable than the ethyl acetate extract; its T_{90} is 461ºC while that of ethyl acetate extract is 420ºC. Hexane extracts of plants are usually rich in fatty acids like palmitic acid, linoleic acid etc. and hence, are thermally stable (Shao et al. 2015).

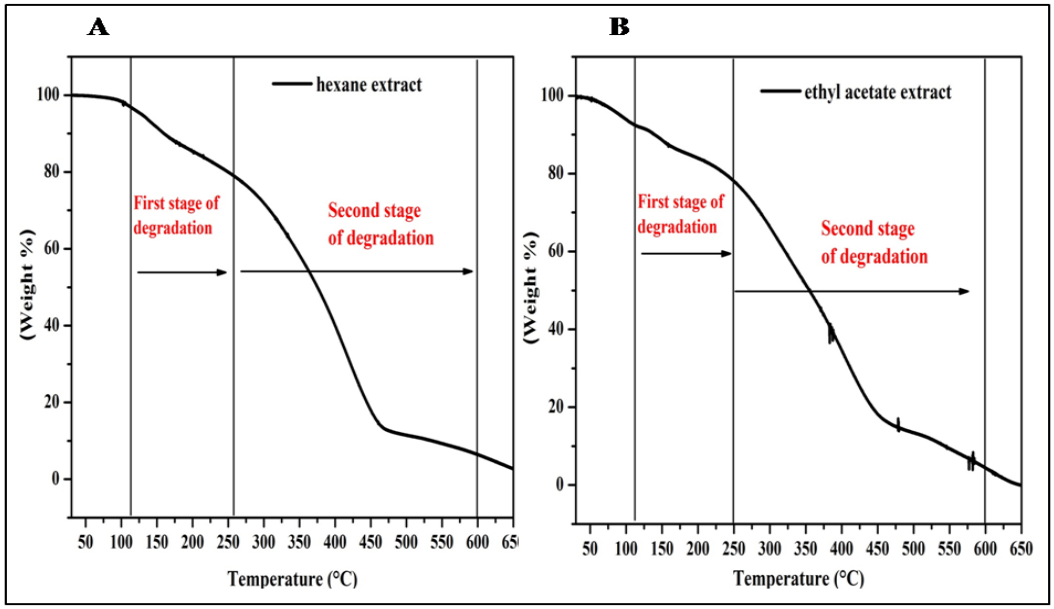


Fig 3. 4 TGA thermogram of **(A)** hexane and **(B)** ethyl acetate extract of seeds of *A. nigra*

3.3.2.7 Viscosity

Reduced and intrinsic viscosity of the extracts as a function of concentration and temperature respectively, is shown in **Fig 3.5**. For all temperature tested, the reduced viscosity seemed to increase with the increase in concentration of the samples. Among the 2 extracts, hexane extract showed the highest reduced viscosity. There was an increasing trend of reduced viscosity of approximately 35 to 80 ml/g and 30 to 70 ml/g for the hexane and ethyl acetate extract respectively, when their concentrations increased from 0.0001 g/ml to 0.0018 g/ml. The viscosity values are higher than free flowing oils and are comparable to that of *Lepidium staivum* seed extract (Karazhiyan et al. 2009). Intrinsic viscosity of all the samples decreased with the increase in temperature hence, temperature dependency of viscosity is highly evident. Intrinsic viscosity showed the same trend as reduced viscosity. It is evident that both the extracts, particularly the hexane extract of the seeds of *A. nigra* are rich in diterpenes, which are usually resinous, and hence viscous in nature (Keeling and Bohlmann 2006). However, the decrease in intrinsic viscosity is not very significant, which showed the stability of the samples at different temperatures.

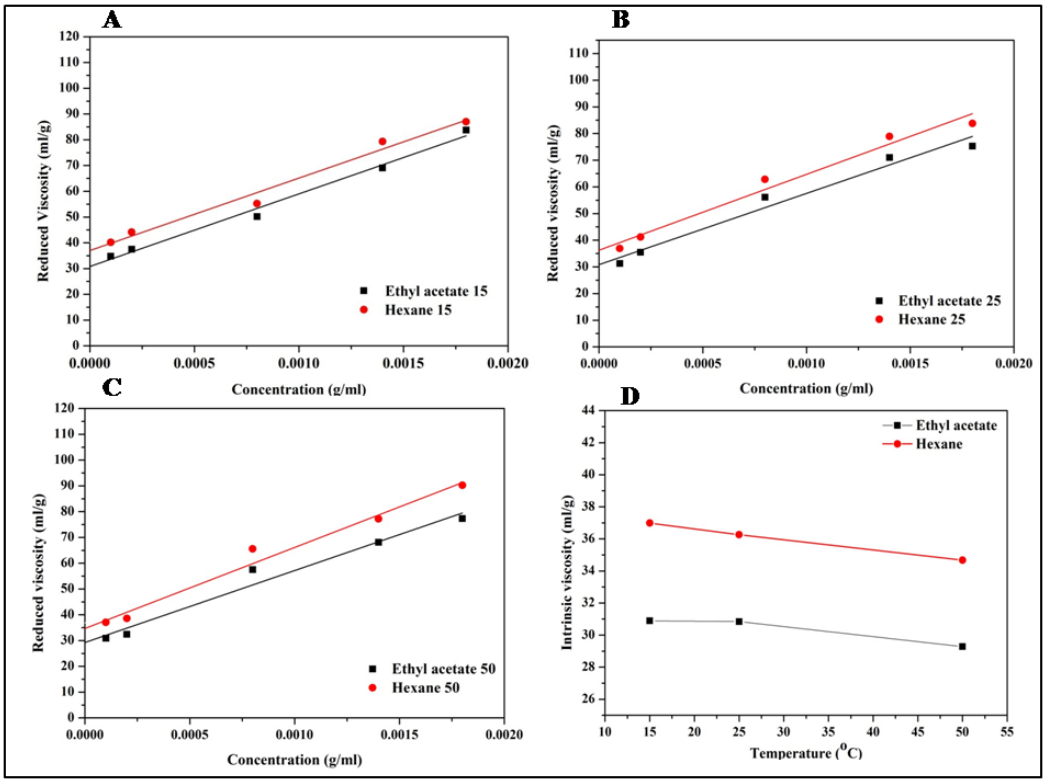


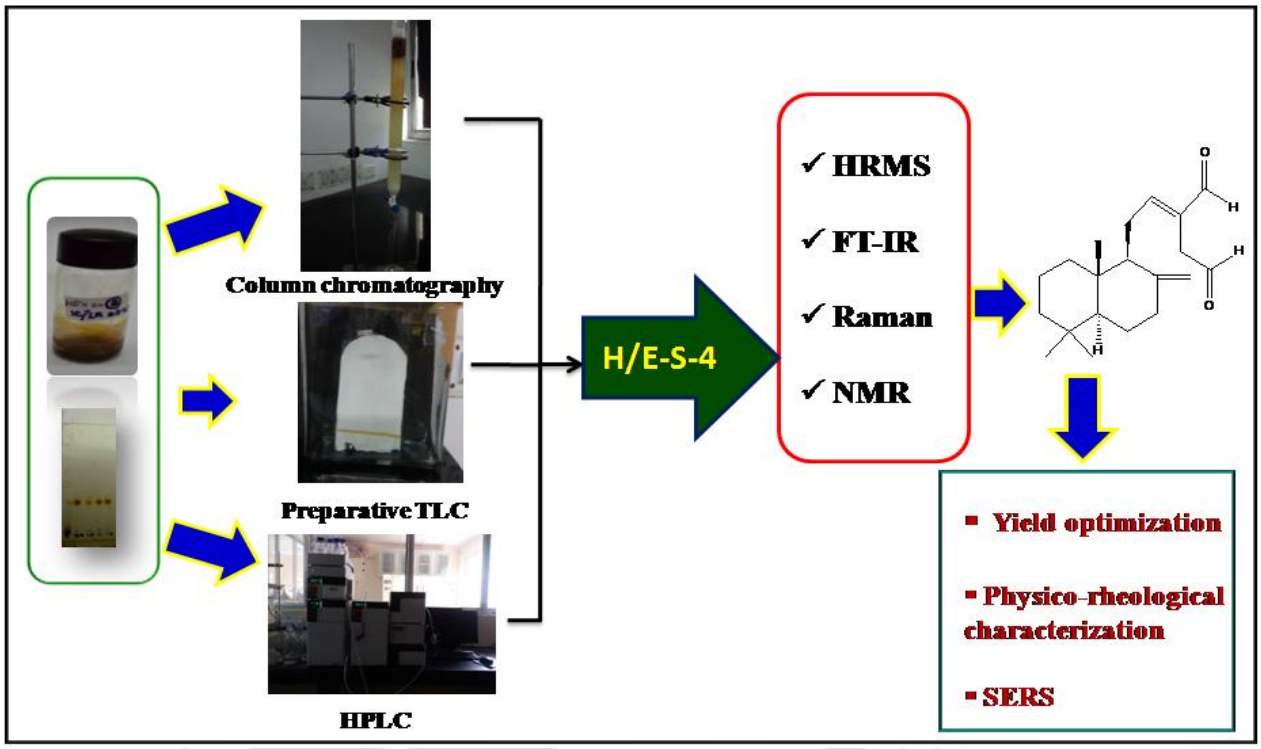
Fig 3. 5 Reduced viscosity of extract as a function of concentration at **(A)** 15ºC **(B)** 25ºC and **(C)** 50ºC; **(D)** Intrinsic viscosity of extracts as a function of temperature

3.4 Conclusion

In this chapter, it was demonstrated that mechanical maceration or room temperature extraction is the most economically viable and effective extraction method from the seeds of *A. nigra*. By this extraction methodology, a seed-to-solvent ratio of 1:6 (w/v) proved to be adequate for maximum yield of extracts from the seeds, presenting a yield of ~ 13 g. This work also encompasses the physico – rheological characterization of the extracts obtained from the seeds of *A. nigra*. The physical properties exhibited by the extracts are in close agreement with various other plant seed oils. In agreement with the other species of *Alpinia*, *A. nigra* seed extracts were found to be particularly rich in non – polar compounds like terpenes and terpenoids. The low T_g value of these plant derived samples are suggestive of their amorphous behavior. Oral consumption of *A. nigra* seeds cannot lead to any health hazards, which is indicated by the rapid degradation of the samples studied. These extracts possess immense potential to act in food systems and as excipient. Because of the presence of a large number of secondary metabolites, these extracts can serve as a viable resource for bioprocess and biomedical applications. Characterization of *A. nigra*- derived extracts for its added attributes also has other important implications

and may establish a case study for the medicinal property profiling of *A. nigra* varieties of NE India. These results therefore, offer a scientific basis for the use of these seed extracts in food, pharmaceutical and other commercial product industries. However, further work is required on the extensive rheological characterization of these extracts because they can significantly contribute to a better understanding of the use this medicinal crop and its applications in pharmaceutical industries. Also, these *A. nigra*-derived extracts are known for their antimicrobial properties; hence, the principal chemical entities responsible for this important property need to be isolated and identified.

Chapter 4: Isolation, purification and characterization of bioactive compound obtained from the seeds of *A. nigra*



This chapter describes the isolation, structural elucidation and physico-rheological characterization of a labdane diterpene from the seeds of *A. nigra* along with SERS studies using nanoparticles

Chapter 4: Isolation, purification and characterization of bioactive compounds from the seeds of *A. nigra*

4.1 Introduction

The NE region of India carries a rich diversity of medicinal plants in its womb. The various ethnic groups and tribal populations of this region are aptly trained in using the natural wealth around them as home remedies for curing a number of ailments. These medicinal plants possess unique and diverse chemical compounds, mostly organic, which are commonly called as natural compounds. Throughout the course of evolution, these compounds have also evolved enormously to give rise to various analogues for better sustainability and survival. However, from the past two decades the use of natural products as drugs has diminished because of technical barriers in high-throughput screening of these compounds against molecular targets. As John A. Beutler had rightly expressed that when it comes to natural products for drug development, the general notion is that "The pond has all been fished out", but the truth is that many "fish" still remain in the pond (Beutler 2009). With the development of analytical techniques and combinatorial chemistry, along with growing socio-cultural acceptability, a re-emergence of natural products is observed in the drug discovery scenario (Harvey et al. 2015). Thus, herbal therapy as an alternative medicine is becoming an attractive approach for the treatment many disorders and diseases. Natural products in the form of crude extracts (both organic and aqueous), pastes or decoctions usually contain large amounts of flavanoids, terpenoids, phenolics etc. that are known to possess tremendous biological potential (Ramesh et al. 1998; Al-saraireh et al. 2015). The NE India, though a small area on the world map, still rely on medicinal plants and other natural sources for home remedies due to the existence of a huge "folk" population that believe in traditional knowledge that are handed down through generations. In the plant kingdom, Zingiberaceae is well known as a repository for spices, condiments, medicines, dyes, perfumes etc. (Jantan et al. 2008). The largest genus, *Alpinia*, of the Zingiberaceae family has also attracted the attention of researchers for several years due to its wide range of bio-pharmaceutical potential (Ghosh and Rangan 2013). Many of the bioactive compounds from this genus has been isolated and thoroughly investigated.

However, limited researchers have focused their attention on the bioactive compounds of *A. nigra*, the plant under study. Qiao et.al had isolated astraglin and

kaempferol-3-*O*-glucuronide way back in 2000 from the seeds of this plant; content of kaempferol was manifold higher than astraglin (Qiao et al. 2000, 2007). In 2013, two diterpenes were also isolated from its seeds (Ghosh et al. 2013a). Very recently, two sterols had been reported from the rhizomes of *A. nigra* (Sethi et al. 2015). However, the vibrational spectroscopic studies and the biological efficacy of most of these compounds remain unexplored.

Secondary metabolites like terpene derivatives that are sourced from plants display a spectrum of biological activity, ranging from the anti pathogenic to anti diabetic and analgesic (Nowak et al. 1989; Christensen and Lam 1990; Landau et al. 1994; Fortuna et al. 2001; Cabral et al. 2008b; Steinbach et al. 2008). Studies on the structural and vibrational properties of analogous terpene derivatives could yield vital chemical clues to accentuate their biological potential (Chain et al. 2015). IR spectroscopy is an excellent non invasive, label-free and vibrational analytical method that elucidates the general biochemical profile and specific functional groups of the compound under investigation. In addition, Raman spectroscopy has the added advantage of requiring a very small amount of sample for analysis with nearly no interference from water molecules (De Gelder et al. 2007; Baker et al. 2014). In fact, Raman spectroscopy has been used to study molecular associations and thermal vibrations in gases (Vigasin and Denisov 1992; Komyak et al. 1993). Yet, the widespread use of Raman scattering has been curtailed by its inherently small signal intensity (Haynes et al. 2009). Subsequently, the intensity of Raman scattering has been greatly enhanced by positioning a Raman scatterer on or near a roughened metal surface (Jeanmaire and Van Duyne 1977). This enhancement, known as Surface Enhanced Raman Scattering (SERS), can be used to increase signal strength in any system handicapped by an intrinsically weak Raman signal (Sharma et al. 2012).

Copper nanoparticles (CuNPs) can be employed as SERS substrates since they exhibit Surface Plasmon Resonance (SPR). The SERS is, in turn, responsible for significant enhancement in electromagnetic field, due to oscillations of incident light and the free movement of electrons in the NPs. The SPR property of CuNPs is adopted in various optical enhancement applications; with the SERS function, being one of its better known/accepted applications (Nath and Khare 2011). Further, the widespread utility of CuNPs for SERS is accelerated by its low procurement cost which has offset its inherent

disadvantage of metal reactivity. These factors have encouraged researchers to study CuNPs and optimize its applicability as a SERS substrate, despite the associated disadvantages. SERS is known to exhibit a high sensitivity for a wide range of bioactive compounds (Beljebbar et al. 1995, 1997). It is a powerful tool to probe chemical interactions between the adsorbing molecules and the surface of metals like Cu, Au, Ag, etc. SERS is, therefore, capable of stimulating even a 10 fold amplification of the Raman signal from the adsorbed molecule (Moskovits 1985; Campion and Kambhampati 1998; Kneipp et al. 2002). However, it must be noted that SERS is a viable approach to adopt, when only one or two biomolecules are of interest (De Gelder et al. 2007).

Therefore, the present chapter was focused on the isolation and characterization of major bioactive compound(s) from the organic seed extracts of *A. nigra*. The vibrational spectroscopic studies were given more importance and SERS studies were conducted using CuNPs. In addition, the physico – rheological characterization and drug likeliness studies were carried out for the possible industrial and biopharmaceutical application of the isolated compound(s) in the future.

4.2 Materials and methods

4.2.1 Isolation of the compound

4.2.1.1 Column chromatography

A glass column (4×55 cm; Borosil, India) was packed using silica gel (60-120 mesh; Merck, India), dissolved in hexane, which served as the stationary phase. To isolate bioactive compounds, 2 g of crude extract (dissolved in hexane) was loaded on the column. The fractions were gradually eluted with increasing polarity of hexane-ethyl mobile phase. The purity of the eluted compounds was subsequently checked on silica gel TLC plates (TLC Silica gel 60 F_{254}; Merck, Germany) using hexane-ethyl acetate mobile phase.

4.2.2 Purification of the compound

4.2.2.1 Preparative thin layer chromatography (pTLC)

Slurry was prepared from 60 g of TLC silica gel G (SRL, India) containing 13% $CaSO_4.1/2H_2O$ as binder, with methanol in ethyl acetate (10%). Thin glass plates were coated uniformly with the slurry. These plates were first allowed to dry for an hour at room temperature; subsequently, drying was completed in an oven at 80°C. The fractions obtained above was diluted in dichloromethane (DCM) and spotted/applied on the

activated silica gel plates with a capillary tube. The major components of the fraction collected were separated on the plates using 10% ethyl acetate-hexane mobile phase system. Thick bands relating to the individual compound were visualized/detected by placing the TLC plates in an iodine chamber. The bands were scraped out, washed with DCM and the individual compound was filtered out and collected. The solvent was evaporated using rotary evaporator and stored at room temperature for further analysis.

4.2.2.2 High performance liquid chromatography (HPLC)

The purity of the isolated compound and optimization of its yield with respect to (w.r.t) seed-to-solvent ratio was assessed by using reversed phase HPLC (RP-HPLC; Shimadzu, Japan) in the following manner:

4.2.2.2.1 Chemical reagents

1. Trifluoroacetic acid (TFA)
2. Solvent A: Acetonitrile
3. Solvent B: High purity grade water (with 0.3% TFA)

All the solvents used above are HPLC grade (Ranken, India).

4.2.2.2.2 Column

Reversed phase octadecylsilica (C18; Shimadzu, Japan) column was used.

4.2.2.2.3 Sample preparation

4.2.2.2.3.1 Solvent preparation

All the solvents were first filtered through 0.20 µm filter (Sartorius, India) and then degassed at 40ºC.

4.2.2.2.3.2 Test sample preparation

For purity assessment, 1 mg of the isolated compound was dissolved in 1 ml of acetonitrile. For yield optimization of the compound w.r.t seed-to-solvent ratio, 50 µl of each extract was dissolved in 1950 µl of acetonitrile. All the sample solutions were filtered through 0.20 µm filter and degassed at 40 ºC.

4.2.2.2.4 Running of column

The procedure for running the RP-HPLC was as follows:

4.2.2.2.4.1 Column equilibration

The system was set up as per the requirements and equilibrated under the following initial conditions **(Table 4.1)** till a flat baseline was obtained.

Table 4. 1 Parameters for initial washing and set up of RP-HPLC

Sr. No.	Parameters	First wash	Second wash	Third wash
1.	Solvent (%)	100 (Solvent A)	50:50 (Solvent A:Solvent B)	100 (Solvent B)
2.	Flow rate (ml/min)	1	1	1
3.	Detection wavelength (nm)	235	235	235
4.	Temperature (ºC)	30	30	30
5.	Duration (mins)	15	15	15

4.2.3 Characterization of the compound

4.2.3.1 Spectroscopic characterization

4.2.3.1.1 High resolution mass spectrometry (HRMS) and Liquid chromatography-mass spectrometry (LC-MS)

For mass spectral analysis, 1 mg of the pure isolated compound was dissolved in 2 ml of methanol (HPLC grade). The spectrum for mass spectrometry was recorded on Agilent Technologies 650 Accurate Mass Q-TOF LC/MS instrument with electron spray ionization (ESI) technique.

4.2.3.1.2 Vibrational spectroscopy

4.2.3.1.2.1 FTIR

Sample preparation for FTIR analysis was done as given in the previous chapter **(section 3.2.4.2)**.

4.2.3.1.2.2 Raman spectroscopy

The compound was dissolved in DCM and drop cast on a coverslip. The thin film obtained, was placed in Raman spectrophotometer (Horiba LabRAM HR) at excitation wavelengths of 488 nm and 514 nm of Argon-ion laser.

4.2.3.1.2.3 DFT calculations

The vibrational modes of the isolated compound recorded experimentally by FTIR and Raman, were correlated with theoretical results, obtained by DFT calculation. DFT calculations were performed at B3LYP/6-311++G (d, p) level, using the Gaussian 09 software package (Frisch, M. J.; Trucks, G.W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.;Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenber 2009). Vibrational frequencies were scaled by 0.97 for comparative evaluation against the experimental results. All the vibrational spectra were plotted and analyzed using software Origin 8.5.

4.2.3.1.3 Nuclear magnetic resonance (NMR)

NMR was carried out by dissolving the compound in 800 µl of deprotonated solvent, $CDCl_3$, and transferring it to 5 mm NMR tubes. ^1H NMR was recorded on a Bruker 600 MHz instrument. The singlet for $CDCl_3$ appears at $\delta = 7.260$. ^{13}C NMR was recorded at 150 MHz, with the triplet for internal standard at $\delta = 77.019$-77.443, along with trimethylsilane (TMS) as internal reference. For distortionless enhancement by polarization transfer (DEPT) experiment, decoupler pulse angle of 135º was used. The Top Spin 2.1 and Mestronova 8.0 software were used for NMR acquisition and NMR spectra analysis respectively. ^1H and ^{13}C spectral chemical shifts and coupling constants were expressed as δ and Hz respectively. The structure was drawn and peaks were assigned using Chemdraw 8.0.3.

4.2.3.1.4 Vibrational spectroscopy based interaction studies

4.2.3.1.4.1 Synthesis of CuNPs

CuNPs, employed as SERS substrates, were synthesized by the pulse laser ablation in distilled water. The 2nd harmonic of a high power Q-switched Nd:YAG laser (Litron LPY7864-10G) was focused using a lens of 25 cm focal length, on a copper target immersed in distilled water. The incident laser energy was maintained at 30 mJ, and the ablation of the target was continued for duration of 60 mins. The repetition rate of the laser was fixed at 10 Hz. The target was continuously moved during the ablation, to avoid the formation of a crater, and hence facilitating the uniform ablation, on a shot to shot basis.

4.2.3.1.4.2 Sample preparation and analysis

4.2.3.1.4.2.1 FTIR

The adsorption of the compounds on CuNPs was also assessed by IR spectroscopy and the sample was prepared as given in the previous chapter **(section 3.2.4.2)**.

4.2.3.1.4.2.2 SERS

A small amount of the compound, dissolved in DCM, was added to 100 µl of the solution containing CuNPs. A thin layer of the mixture was drop cast on a coverslip, allowed to dry and exposed to excitation wavelengths of 488 nm and 514 nm respectively, using Argon-ion laser.

The enhancement factor is calculated as:

$$E = \frac{I_{SERS} \times C_{SOL}}{I_{SOL} \times C_{SERS}}$$

Where, I_{SERS}= Intensity of SERS spectra

C_{SOL} = Concentration of the compound

I_{SOL} = Intensity of the normal Raman spectra of the compound (without CuNPs)

C_{SERS} = Concentration of the compound in the SERS mixture

4.2.3.2 Physico-rheological characterization

The physical and rheological properties of the isolated compound *viz*. color, odor, density, refractive index, contact angle, optical rotation, DSC, TGA and viscosity were studied as described in the previous chapter **(section 3.2.4)**.

4.2.4 *In silico* studies

4.2.4.1 Lipinski's rule of five

To understand the applicability of the isolated compound as a drug, the descriptors for bioavailability were examined on the basis of Lipinski's rule of five using the Molinspiration server (http://www.molinspiration.com/).

4.2.4.2 Drug likeliness and toxicity predictions

The drug likeliness of the isolated compound was checked using "Advanced Chemistry Development" (ACD/I-lab). This online property explorer was used to estimate the drug relevant properties like Log P (lipophilicity), Log S (solubility), Log D (distribution), Log

$K^{(OC)}$ (absorption) and molecular mass. In addition, this was also used to estimate the risks of side effects of the compound like mutagenic, irritant, toxicity categories and other health hazards (http://ilab.acdlabs.com/) (Szumilak et al. 2017).

4.3 Results and Discussion

4.3.1 Isolation of the compound

The schematic representation of column chromatography is shown in **Fig 4.1**. Six different fractions were eluted using column chromatography at different polarities of gradient mobile phase (0 – 10% ethyl acetate in hexane) as shown in **Table 4.2**.

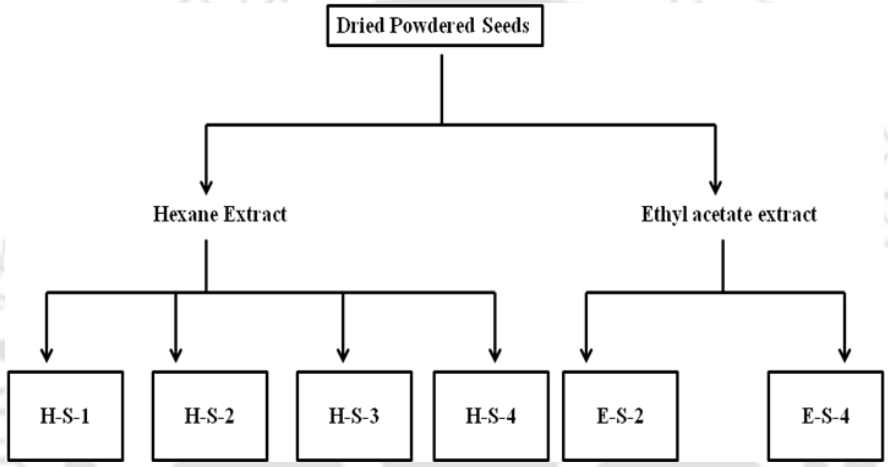


Fig 4. 1 Schematic representation of elution of fractions using column chromatography

Table 4. 2 Elution of different fractions from column chromatography

Sr. No.	Extract	Fraction name
1.	Hexane	H-S-1
2.		H-S-2
3.		H-S-3
4.		H-S-4
5.	Ethyl acetate	E-S-2
6.		E-S-4

Due to their extremely low yield, the fractions H-S-1 and H-S-3 were not considered suitable and further purification process was continued using the remaining four fractions: H-S-2, H-S-4, E-S-2 and E-S-4. By general perception and visualization on

TLC plates, the fractions 2 and 4 respectively from each extract were found to be same; the fractions would, thus, be referred as H/E-S-2 and H/E-S-4.

4.3.2 Purification of the compound

4.3.2.1 pTLC

The fraction, H/E-S-2 could not be properly resolved on the pTLC plate and hence, was dropped from the race. However, a distinct band relating to the individual compound, H/E-S-4, having a retention factor (Rf) value of 0.364, was separated/purified by pTLC and collected by washing. After solvent evaporation, thick, dark golden colored sticky oil was obtained, which was subjected to further purification and characterization process.

4.3.2.2 HPLC

The compound, H/E-S-4 was found to have absorbance maxima (λ_{max}) at 235 nm. Using the given parameters of HPLC, the compound was eluted at a retention time of 46.578±0.049 min. The optimum yield of the compound from hexane extract w.r.t seed-to-solvent ratio was found to be 1:4 as shown in **Fig 4.2**. The yield of the compound from ethyl acetate extract was found to be negligible in all the ratios (**Fig 4.3**). The retention time of H/E-S-4 from all the extracts are given in **Table 4.3**.

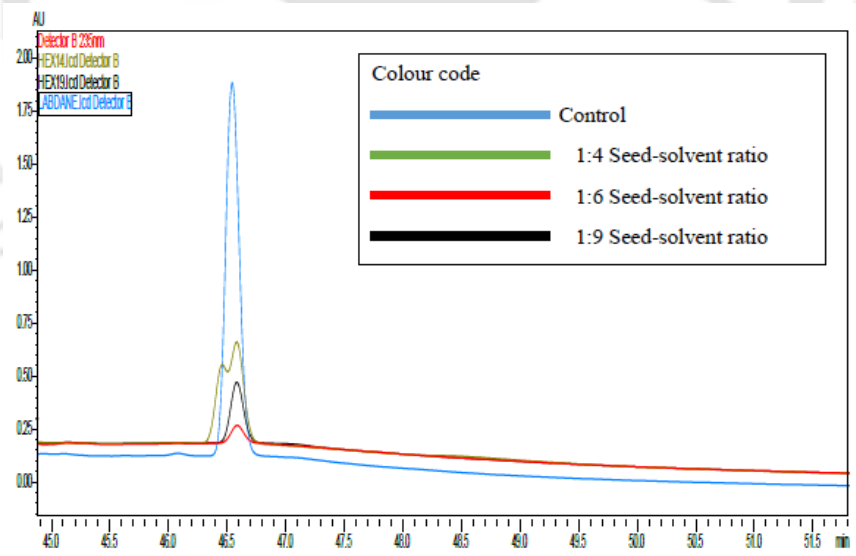


Fig 4. 2 HPLC chromatogram of hexane extracts

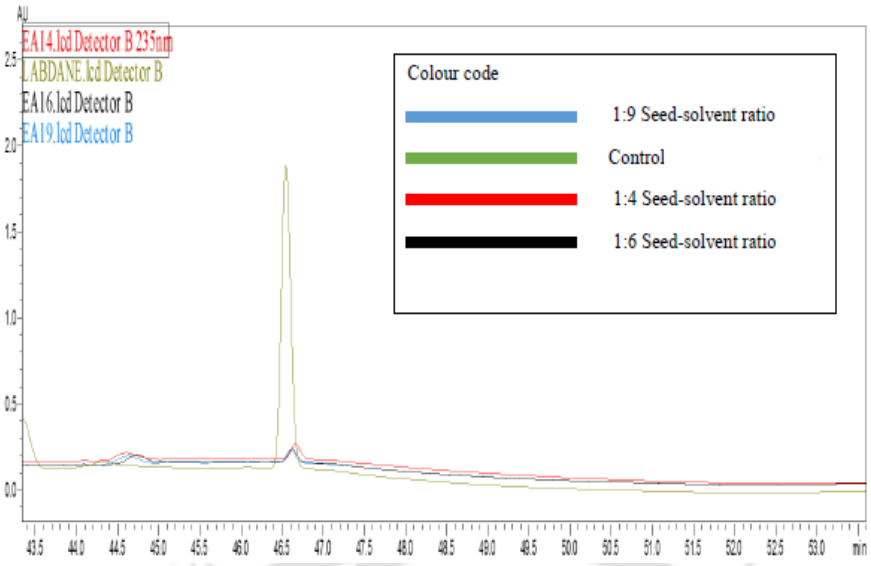


Fig 4. 3 HPLC chromatogram of ethyl acetate extracts

Table 4. 3 Retention time of the compound from different extracts

Sr. No.	Seed-to-solvent ratio	Retention time of H/E-S-4 (min)	
		Hexane extract	Ethyl acetate extract
1.	1:4	46.562±0.027	46.603±0.085
2.	1:6	46.564±0.030	46.582±0.055
3.	1:9	46.563±0.028	46.574±0.043
4.	**Retention time of H/E-S-4 (min)**		
	46.578±0.049		

4.3.3 Characterization of the compound

The isolated compound H/E-S-4 turned out to be a labdane diterpene, (E)-labda-8(17), 12-diene-15, 16-dial. The structure of the compound **(Fig 4.4)** has been deduced using a number of analytical techniques given below.

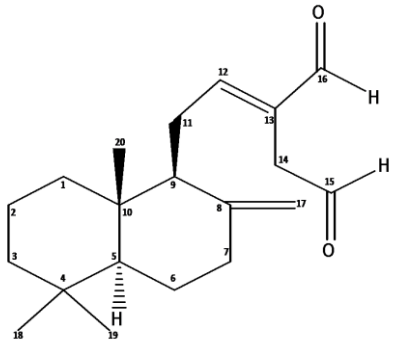


Fig 4. 4 Structure of labdane diterpene, (E)-labda-8(17), 12-diene-15, 16-dial

4.3.3.1 Spectroscopic characterization

4.3.3.1.1 HRMS/ LC-MS analysis

The mass of the isolated compound was confirmed by HRMS and LC-MS **(Fig 4.5 and 4.6)**.

$[M-H]^+$ was 303.2092

$[M-H]^-$ was 302.3200

The molecular mass matched well with the previously published reports (302, 303.2330) (Sirat et al. 1994; Ghosh and Rangan 2014b).

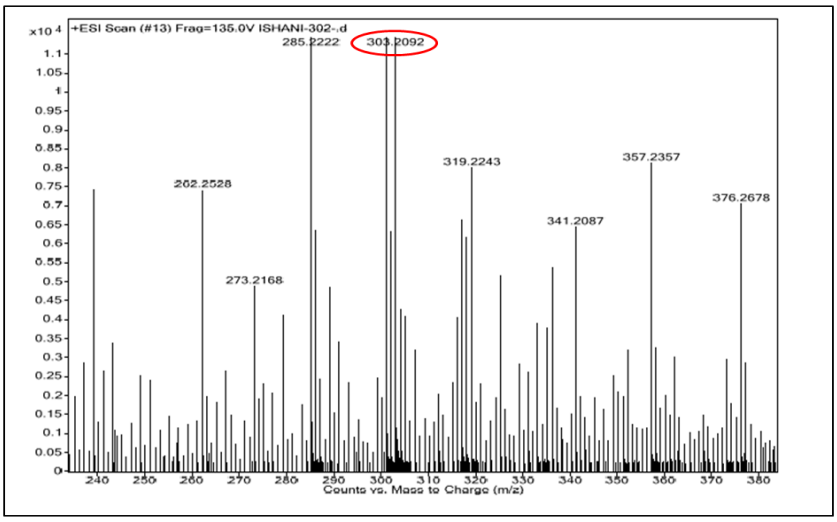


Fig 4. 5 HRMS mass spectrum of labdane diterpene in ESI positive mode

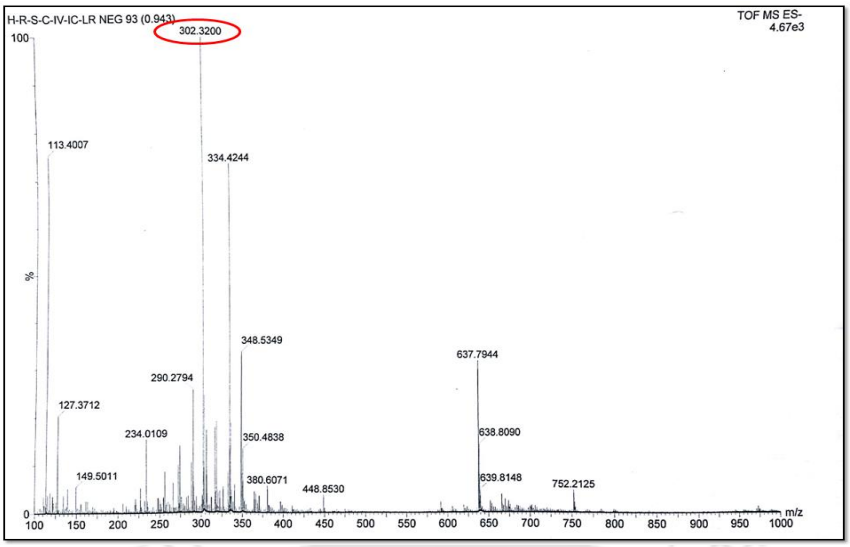


Fig 4. 6 LC-MS mass spectrum of labdane diterpene in negative mode

4.3.3.1.2 Vibrational spectroscopy

4.3.3.1.2.1 FTIR

The IR spectra of labdane diterpene with the correlated DFT spectra are shown in **Fig 4.7**. The characteristic C=O stretching vibration of aldehyde appeared as a prominent band at ~1735 $cm^{-1,}$ which is congruent with previous literature (Ghosh and Rangan 2014b). However, this bond for C=O stretch is not very strong as opposed to that ideally observed. It may happen that in the molecular environment, the isolated -CHO (carbon 16) can form an enolate (known as conjugation - alpha proton migration to the aldehydic/ketone oxygen). Therefore, it no longer remains as pure CHO group. This C=O seemed to get a comparable characteristic bond character of the other CHO group (carbon 15) which already has a double bond next to it; thus same frequency gets as a summed up. For this reason, a clear and sharp peak was observed at 1646 cm^{-1} (for conjugated aldehyde) and a low intensity peak at 1735 cm^{-1} (pure aldehyde) was observed as it lost its true character. This peak matched well with the calculated DFT value. Experimental peak at ~1646 cm^{-1} displayed the presence of alkenyl C=C stretch in the compound, in conjugation with –CHO group, and exhibited a clear agreement with the calculated DFT value at ~1641 cm^{-1}. A strong peak in the experimental spectrum at ~892 cm^{-1} suggested the presence of vinylidene C-H group; the out of plane geometry of this group matched well with the calculated value at ~918 cm^{-1}. Presence of the asymmetric C-H stretch of CH_3 group was confirmed by a distinct peak at ~2851 cm^{-1} in the experimental spectrum and ~2893 cm^{-1} in the DFT spectrum. The peak at ~976 cm^{-1} in the experimental spectrum was due to skeletal C-C vibrations. An intense peak at 2929.505 cm^{-1} in the

experimental spectrum was attributed to C-H stretch of CH_2 group, which was congruent with the calculated value at ~2961 cm^{-1}. The experimental wavenumbers and their corresponding vibrational modes are tabulated below **(Table 4.4)**. Nearly all the peaks matched well with calculated values.

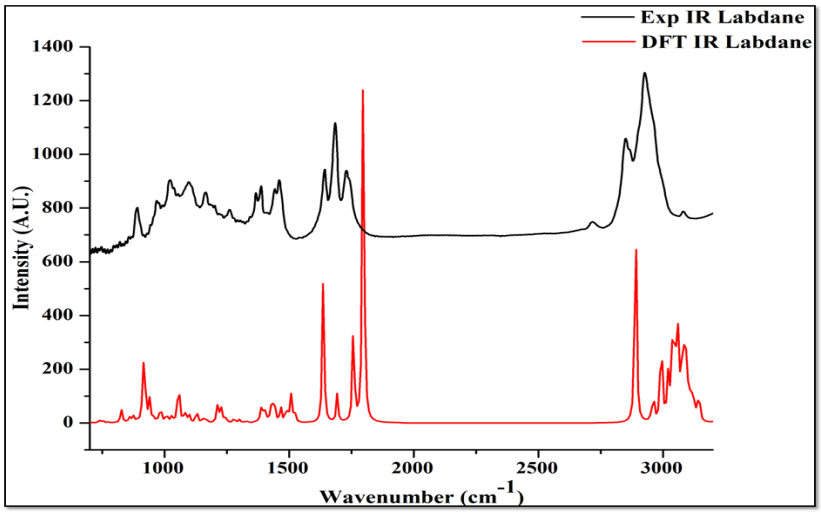


Fig 4. 7 Comparison between experimental IR spectra of pure labdane diterpene and calculated DFT

Table 4. 4 Calculated and experimental wavenumbers of FTIR spectra of pure labdane diterpene and their corresponding vibrational modes

Sr. No.	DFT wavenumber (cm^{-1})	Exp. wavenumber of compound (cm^{-1})	Corresponding modes
1.	918.843	892.795	Vinylidene C-H group
2.	992.157	976.049	Skeletal C-C vibrations
3.		1023.328	Vibration of the cyclohexane ring
4.	1641.457	1646.456	Alkyl C=C stretch in conjugation with –CHO
5.	1743.090	1735.325	C=O stretch of –CHO group
6.	2961.164	2929.505	C-H stretch of CH_2 group
7.	2892.846	2851.189	Asymmetric C-H stretch of CH_3
8.	1501.375	1463.167	
9.	3086	3081	

4.3.3.1.2.2 Raman spectroscopy

The Raman spectrum of the isolated compound, recorded at excitation wavelengths of 488 nm and 514 nm, is shown in **Fig. 4.8 A and B** respectively. However, the relative intensities of the Raman bands showed drastic reduction with increasing wavelength (decreasing energy) of the incident laser. Also included in the figure are the predicted Raman spectra obtained by DFT calculation. The DFT calculations have been adjusted by a factor of 0.97 to obtain the best fit with the experimental spectra. The calculated and experimental wavenumbers validated the unambiguous confidence in the assignments. At 488 nm excitation wavelength, the most intense bands were ~999 cm^{-1}, ~1003 cm^{-1}, ~1031 $cm^{-1}$and ~1602 cm^{-1} as shown in **Fig. 4.8 A**. For 514nm the bands were ~984 cm^{-1}, ~1005 cm^{-1}, ~1032 and ~1641 cm^{-1} as shown in **Fig. 4.8 B**. The first 3 peaks were attributed to the bending vibrations of C-H bonds in the entire molecule, while the fourth peak was an outcome of the stretching of C=O bond within the CHO group. The theoretical DFT calculations were in good agreement with the experimental spectrum and their corresponding modes are tabulate below **(Table 4.5)**.

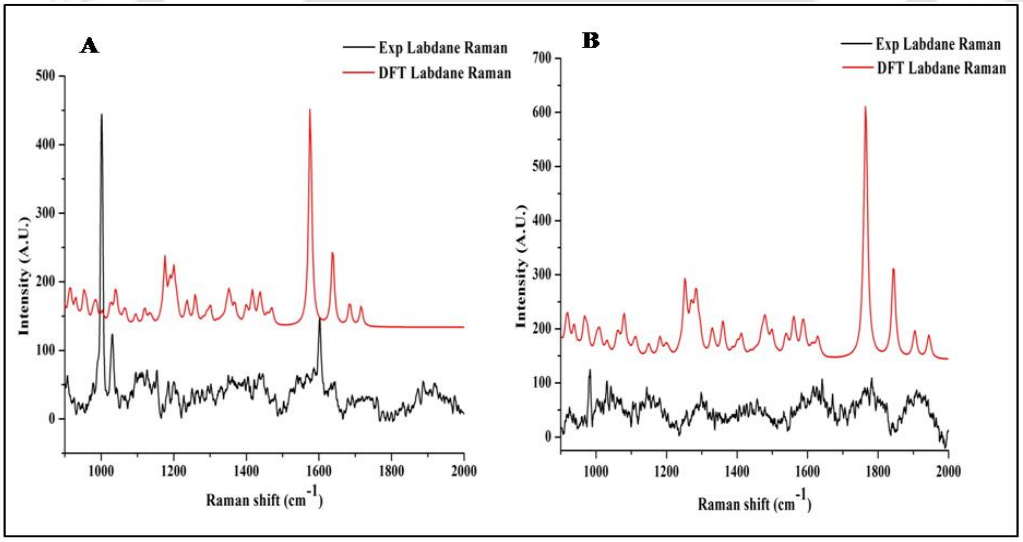


Fig 4. 8 Comparison of experimental Raman spectra with calculated DFT spectra of pure labdane diterpene at **(A)** 488nm **(B)** 514nm

Table 4. 5 Calculated and experimental wavenumbers of Raman spectra of pure labdane diterpene and their corresponding vibrational modes

Sr. No.	DFT Wavenumber (cm^{-1})	Exp Wavenumber (cm^{-1})	Corresponding modes
1.	986.7751	999.0668	Bending vibration of C-H group in the entire molecule
2.	1007.8649	1003.0668	Bending vibration of C-H group in the entire molecule
3.	1041.0122	1031.3263	Bending vibration of C-H group in the entire molecule
4.	1579.7348	1602.7105	Stretching of C=O bond of –CHO group

4.3.3.1.3 NMR

^{1}H NMR spectrum of the isolated compound **(Fig. 4.9)**, showed three singlets at δ = 0.726 (3H), 0.748 (3H) and 0.818 (3H), for the three individual –CH_3 groups, associated with quarternary carbons. A doublet of protons at δ = 3.376-3.468 (2H) denoted allylic methylene. Two singlets at δ = 4.364 (^{1}H) and 4.859 (1H), along with one triplet at δ = 6.750 signified the olefinic proton. Finally, the 2 singlets at δ = 9.401 and 9.631 represented the 2 –CHO groups. The ^{13}C NMR spectrum **(Fig. 4.10)** showed the presence of 20 carbons, indicating the presence of –CH_3, -CH_2, -CH and quartenary carbons. Two carbons at δ = 193.78 and 197.52 indicated carbons linked to an oxygen atom in -CHO group. A –C=C was confirmed by the signals δ = 160.17 (1C) and δ = 148.26 (1C). The presence of a number of methylene groups was ascertained by the signals at 29.91, 33.79, 38.06 39.46, 39.57, 39.82, 42.81, 55.59 and 56.68. Three signals from 14.62-24.88 indicated the presence of –CH_3 group. The ^{1}H and ^{13}C NMR data tabulated below **(Table 4.6)** correlated well with previously published reports (Sirat et al. 1994; Ghosh and Rangan 2014b). DEPT experiments were used for CH_n multiplicity determination by variation of the selection angle parameter (the tip angle of the final ^{1}H pulse). Angle 135° displayed all CH and CH_3 in a phase opposite to CH_2. Consequently, the DEPT 135 spectra **(Fig. 4.11)** demonstrated the presence of 6 –CH_2 and 9 –CH_3 and –CH groups. The two-dimensional correlation spectrum (COSY) between the protons that are separated by a single bond is shown in **Fig 4.12**. The HSQC spectrum **(Fig. 4.13)** exhibited the interactions between the protons and carbons.

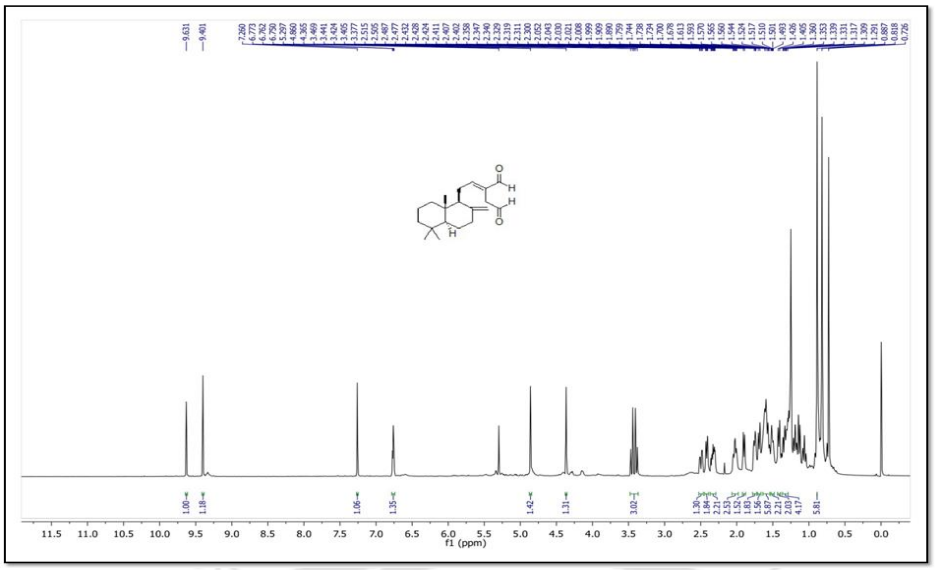


Fig 4. 9 ^{1}H NMR spectrum of labdane diterpene

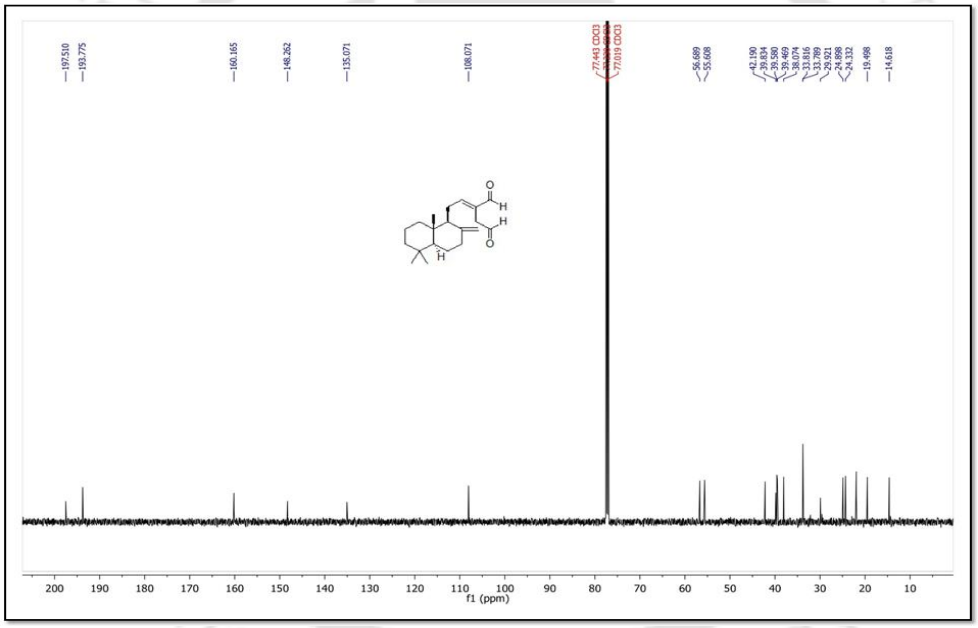


Fig 4. 10 ^{13}C NMR spectrum of labdane diterpene

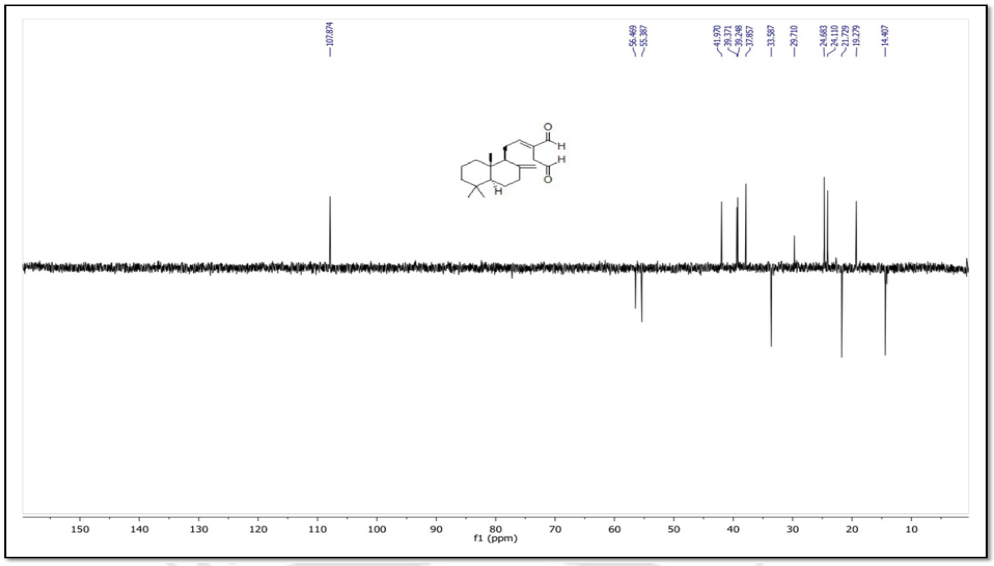


Fig 4. 11 DEPT 135 NMR spectrum of labdane diterpene

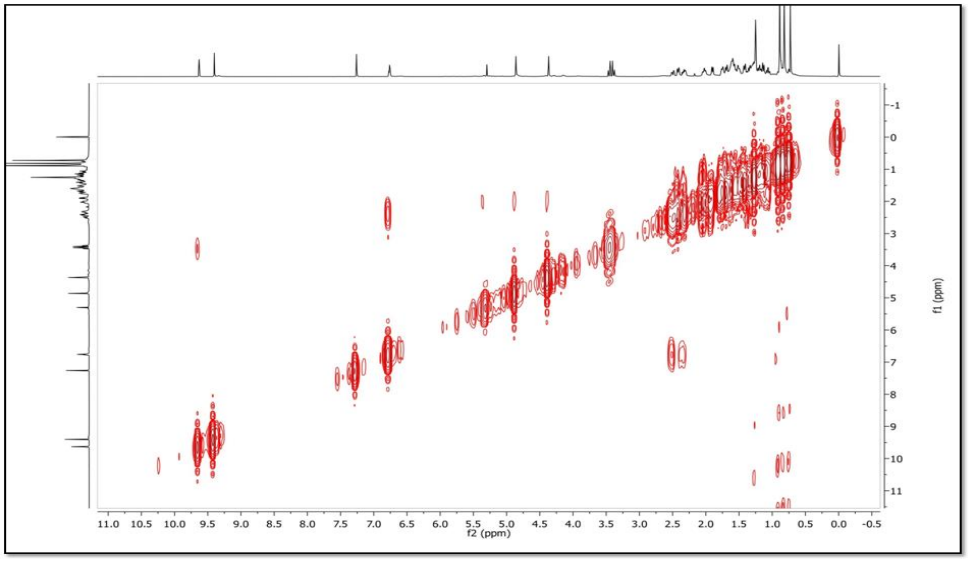


Fig 4. 12 COSY spectrum of labdane diterpene showing proton-proton interaction

Table 4. 6 ^{1}H and ^{13}CNMR data of labdane diterpene dialdehyde (δ in ppm, J in Hz)

Atom No.	1H (δ, J)	13C (δ)
1		39.580
2		19.498
3	2.338 – 1.041 (m)	42.190
4		33.789
5		56.689
6		39.834
7	2.432 – 2.402 (m)	38.074
8		148.262
9	2.515 – 2.477 (m)	55.608
10		39.489
11		29.921
12	6.761 (t, J = 4056.6)	160.166
13		135.071
14	3.423 (q, J = 2053.8)	33.816
15	9.631 (s)	197.510
16	9.401 (s)	193.775
17	4.860 (s) 4.365 (s)	108.071
18	0.897 (s)	24.898
19	0.818 (s)	24.332
20	0.778 (s)	14.618

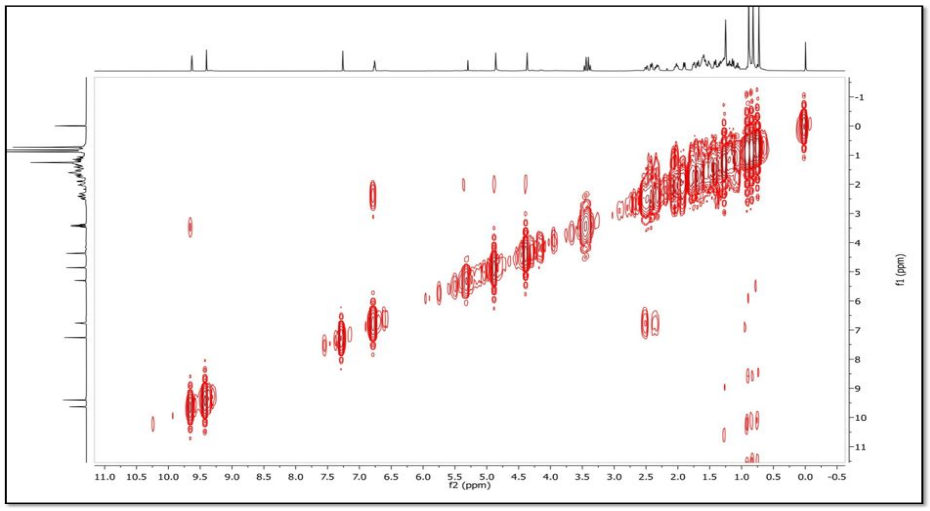


Fig 4. 13 HSQC NMR spectrum of labdane diterpene that shows the interaction between the protons and carbons

4.3.3.1.4 Vibrational spectroscopy based interaction studies

4.3.3.1.4.1 FTIR

The adsorption of labdane diterpene on CuNPs was studied by IR spectroscopy. The IR spectrum of CuNPs in conjugation with labdane diterpene was congruent with that of the individual compound **(Fig. 4.14)**. This confirmed compound adsorption on the metallic surface. The IR spectrum of the untreated CuNPs with a distinct peak at ~3435 cm^{-1} was displayed in figure given below; the presence of the characteristic –OH group corroborated the aqueous preparation of NPs. This peak was also present in the mixture sample at ~3435 cm^{-1}, which indicated that the compound was adsorbed on the Cu surface, when dissolved in water. However, the peaks between 900-1000 cm^{-1} were either suppressed or merged together. The peaks characteristic of C=O and C=C were also merged together, indicating their individual red and blue shift respectively. This broadening of individual peaks may be due to the interaction of the O^- ion of already suppressed C=O group with the Cu^{2+} groups of the CuNPs. As a result, these bonds lose their individual behavior and exhibit the vibrations of conjugated groups. All the peaks of the mixture sample attested to the excellent agreement of the CuNPs with the compound. **Table 4.7** given below draws a comparison of the peaks and their corresponding vibrational modes between the individual compound and that adsorbed on the CuNPs.

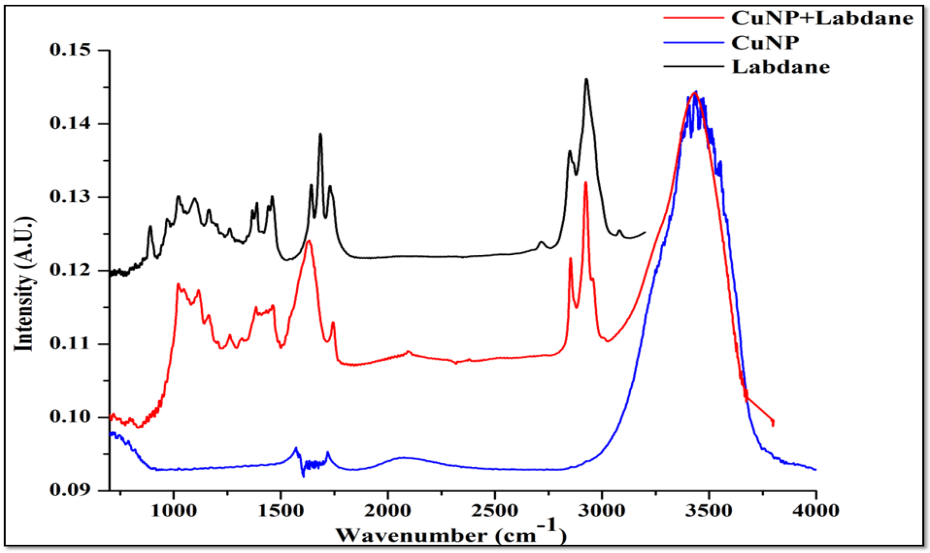


Fig 4. 14 Comparison between IR spectra of labdane diterpene (with and without CuNPs); spectrum of CuNPs also shown

Table 4. 7 Experimental wavenumbers of IR spectra of labdane diterpene (with and without CuNPs) and their corresponding vibrational modes

Sr. No.	Exp Wavenumber of compound (cm^{-1})	Exp Wavenumber of mixture (cm^{-1})	Corresponding modes
1.	892.795	-	Vinylidene C-H group
2.	976.049	-	Skeletal C-C vibrations
3.	1023.328	1029.927	Vibration of cyclohexane ring
4.	1646.456	1638.458	Alkyl C=C stretch in conjugation with –CHO
5.	1735.325	1741.835	C=O stretch of –CHO group
6.	2929.505	2924.439 (merged)	C-H stretch of CH_2 group
7.	2851.189	2854.788	Asymmetric C-H stretch of CH_3
8.	1463.167	1458.831	
9.	3081	-	
10.	-	3435.458	-O-H group from water

4.3.3.1.4.2 SERS

Use of metallic nanoparticles for the enhancement of the intensity of Raman spectrum is a widely used phenomenon. The SERS spectra recorded at 488 nm and 514 nm are shown in **Fig. 4.15 A and B** respectively.

Several prominent bands were apparent in both spectra of different excitation wavelengths; however, they displayed severely altered relative intensities. The most intense bands in the SERS spectra were observed at ~983 cm^{-1} and ~1004 cm^{-1} at 488nm **(Fig. 4.15 A)**, and ~982 cm^{-1} and ~1005 cm^{-1} at 514nm **(Fig. 4.15 B)** respectively. Both these bands were greatly enhanced in the SERS spectra. This enhancement was accredited to generation of a strong electromagnetic field, following the excitation of localized SPR on colloidal nanoparticle surfaces (Haynes et al. 2009). Moreover, some bands in the range of 1080-1200 cm^{-1} were visible in the SERS spectra, but were absent in the normal Raman spectra. The bands in the region between 400-750 cm^{-1}, assigned to the ring C-C in-plane deformation modes, were also enhanced. The fact, that the most enhanced bands corresponded to in-plane modes in the SERS spectra, indicated that labdane must be in a perpendicular orientation with respect to the metal surface (Albrecht 1961). However, the intense peak at ~1031 cm^{-1}, which was attributed to the stretching vibration of the C-H bonds in the molecule, and the prominent peak observed at ~1602 cm^{-1}, from the stretching of C=O bond of the CHO group, were severely reduced in the SERS spectra. Such behavior may be attributed to the interaction of these groups with the Cu^{2+} ions as described in the previous section **(section 4.3.3.1.4.1)**. These observations lead us to also believe that the molecule was attached perpendicular to the Cu surface, through a chelated structure of the C=O. Such reduced intensities of the C=O bond, in proximity to colloidal surface, are commonly observed in flavones (Teslova et al. 2007; Corredor et al. 2009). Enhancement factor at 488 nm is 10^2 fold and at 514 nm is 10^3 fold.

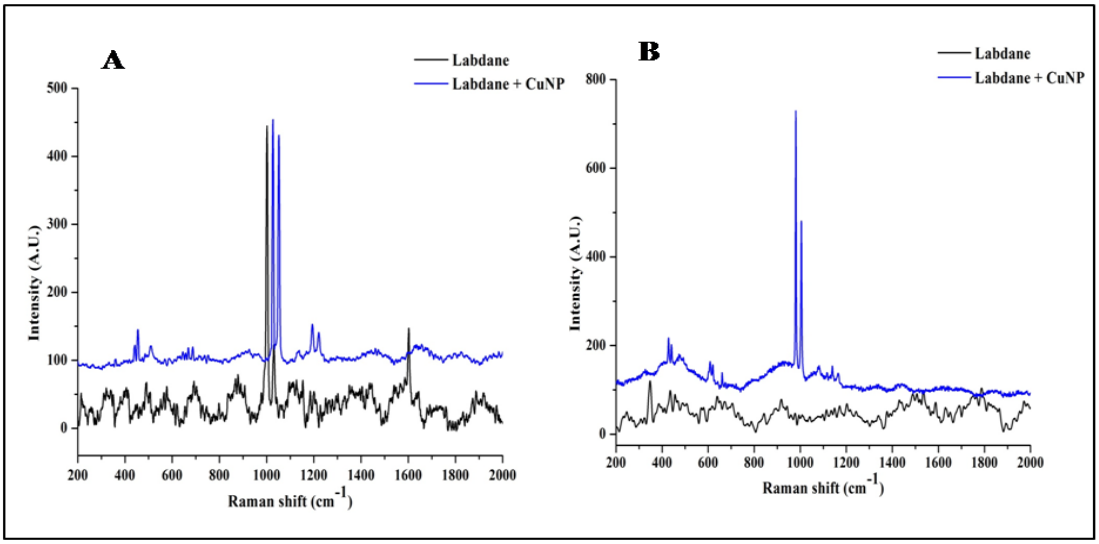


Fig 4. 15 Comparison of SERS spectra of labdane diterpene with and without CuNPs with FT-Raman of labdane diterpene at different excitation wavelengths at **(A)** 488 nm and **(B)** 514 nm

4.3.3.2 Physico – rheological characterization

4.3.3.2.1 Physical properties

The compound, labdane diterpene, is thick, dark golden colored, sticky oil. It has a characteristic odor and a refractive index of 1.505±0.03 at room temperature. Like the seed extracts, the compound was also oily initially but tends to harden with the change of temperature and increased shelf life, which is concordant with its low degree of unsaturation. The measured density of compound was found to be 0.99±0.1 g/cm^3, which is in the range of seed oil as previously reported (Onyeike and Acheru 2002).

4.3.3.2.2 Contact angle

The contact angle of water droplet on the surface of labdane diterpene was found to be 108.5º **(Fig. 4.16)**. The water droplet on the compound was stable for a long duration and remained as a round droplet, with minimal contact to the compound film. It is evident that labdane diterpene is clearly more hydrophobic than the 2 crude extracts themselves. This hydrophobic nature of the compound is characteristic of diterpenes that facilitates its interaction and crossing of plasmatic membranes (Mateo et al. 2000; Urzúa et al. 2008; González et al. 2013).

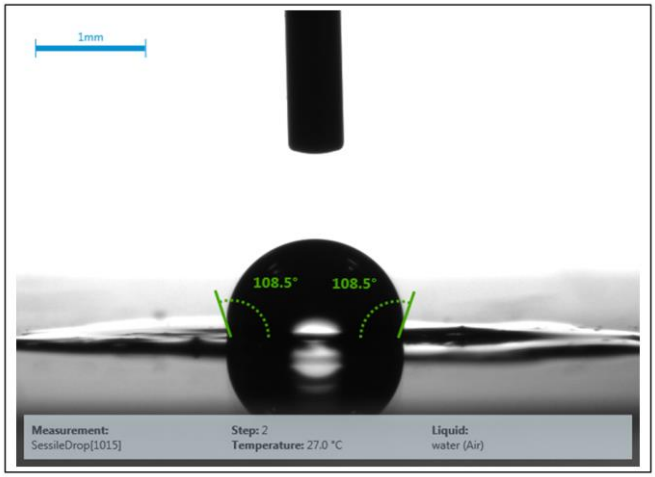


Fig 4. 16 Contact angle of water on the surface of labdane diterpene

4.3.3.2.3 Optical rotation

The specific rotation of labdane diterpene was found to be 10.19, which indicated some amount of optical activity. This isolated molecule, like all other diterpenes of the labdane series, also showed the presence of asymmetric center; its α value is comparable to that of 8α- Drimenol (Carman 1966; Carman and Sutherland 1979).

4.3.3.2.4 DSC

The DSC thermogram of the isolated compound is shown in **Fig. 4.17.** From the first heating cycle of DSC, it was observed that the T_g of the extracted compound was higher than the seed extracts obtained, given in the previous chapter **(section 3.3.2.5)**. Like the 2 extracts, the compound exhibited a broad melting or crystalline peak, which suggested its amorphous nature. Absence of exothermic behavior further confirmed the absence of crystallization effect or solid phase. This broad endothermic behavior exhibited by this plant – derived compound is characteristic of oils (Aboul-Gheit et al. 1997; Tan and Che Man 2000) and is concordant with its physical appearance/ property as highlighted above. The hydrophobic labdane diterpene exhibited characteristic endothermic behavior of diterpenes (Matsingou et al. 2005), which increases upon structural modification like acetylation, hydration etc.

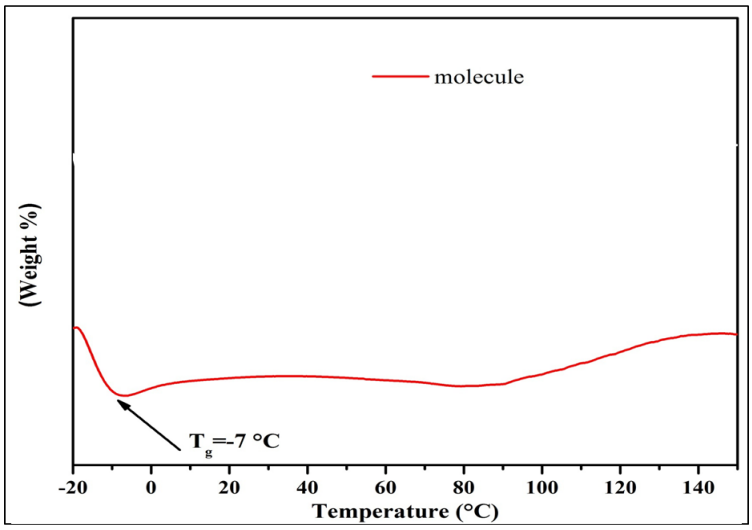


Fig 4. 17 DSC thermogram of labdane diterpene

4.3.3.2.5 TGA

The TGA thermogram of labdane diterpene is shown in **Fig. 4.18**. It is clear that like the extracts, the compound molecule also passed through a two-stage degradation process. The compound was found to be more thermally stable than the two extracts. Though the onset degradation temperature of labdane was 117ºC, the rate of degradation was slow with T_{90} taking place at 470ºC. The early degradation of the molecule maybe attributed to the release of the volatile aldehyde groups (Kebelmann et al. 2013). Major thermal degradation of the compound occured between 250-450ºC, which is quite similar to naturally occurring resins that comprise of a mixture of diterpenes and sequiterpenes (Tsanaktsidis et al. 2013).

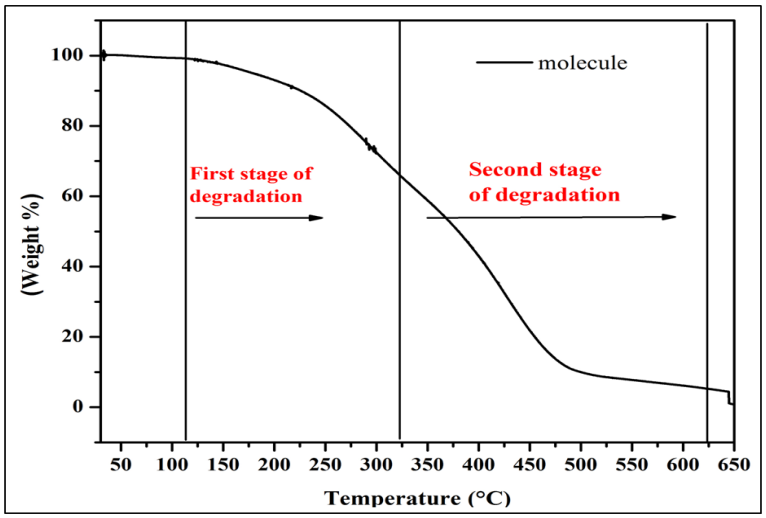


Fig 4. 18 TGA thermogram of labdane diterpene

4.3.3.2.6 Viscosity

Reduced and intrinsic viscosity of the compound as a function of concentration and temperature respectively is shown in **Fig. 4.19**.The compound shows a similar trend of reduced viscosity for all temperatures as shown by the extracts, that has been discussed in the previous chapter **(section 3.3.2.7)**; the compound molecule showed the highest reduced viscosity when compared to the 2 extracts. There was an increasing trend of reduced viscosity of approximately 40 to 110 ml/g for the extracted molecule when its concentrations increased from 0.0001 g/ml to 0.0018 g/ml. Intrinsic viscosity decreased with the increase in temperature hence, temperature dependency of viscosity is highly evident. The molecule exhibited the highest intrinsic viscosity when compared to extracts described in the previous chapter **(Fig 3.5)**. Diterpenes from plant sources are usually resinous, and hence viscous in nature (Rojatkar and Nagasampagi 1994; Keeling and Bohlmann 2006); they are abundantly found in bee propolis (Aminimoghadamfarouj and Nematollahi 2017). Thus, the isolated molecule exhibited the characteristic viscous nature of diterpenes. However, the decrease in intrinsic viscosity is not very significant, which showed the stability of the molecule at different temperatures.

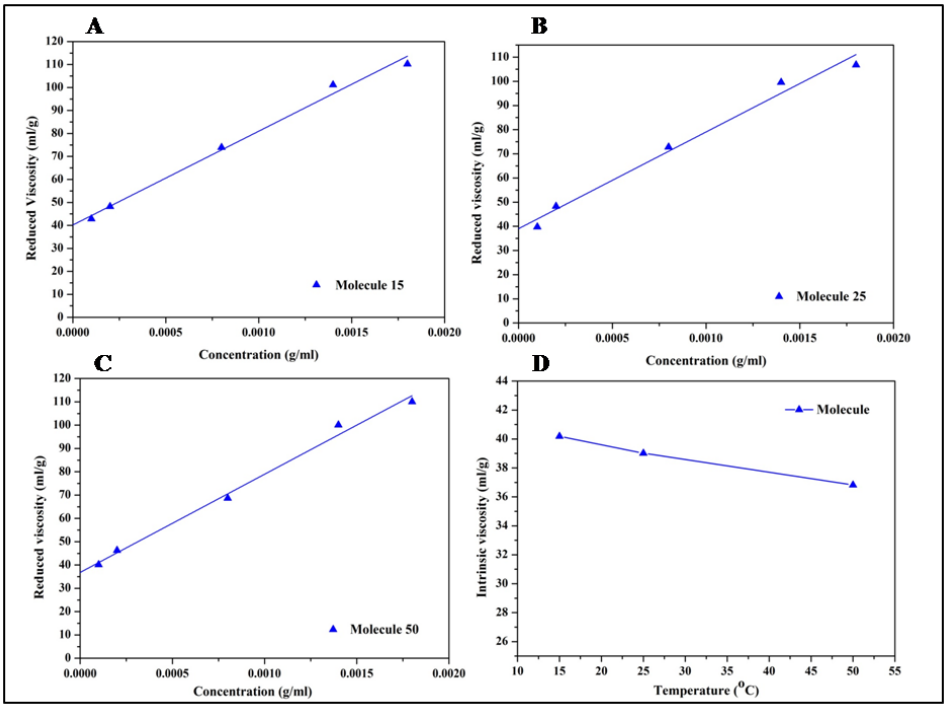


Fig 4. 19 Reduced viscosity of molecule, labdane diterpene as a function of concentration at **(A)** 15ºC **(B)** 25ºC and **(C)** 50ºC; **(D)** Intrinsic viscosity of molecule as a function of temperature

4.3.4 *In silico* studies

4.3.4.1 Lipinski's rule of five

Lipinski's rule of five states that a drug is orally active if it fulfils all of the following criteria: not more than 10 hydrogen bond acceptors, not more than 5 hydrogen bond donors, molecular weight to be below 300 Dalton, and the partition coefficient (log P) should be less than 5 (Lipinski et al. 1997). Though the 'rule of five' is the most widely used predictor for any drug molecule, in reality, it doesnot include natural products that have major therapeutic applications (Zhang and Wilkinson 2007). The calculation obtained shows that labdane diterpene satisfied all but one clause of the Lipinski's rule, suggesting that this compound is not suitable for oral administration **(Table 4.8)**. The log P value of this compound exceeded 5 indicating its higher lipophilicity. However, the log P value of labdane diterpene satisfied the claims of the 'modified Lipinski's rule of five' (Ghose et al. 1999); hence it becomes a potential "oral" therapeutic drug molecule.

Table 4. 8 Lipinski parameters for isolated labdane diterpene

Sr. No.	Parameters	Predicted Values
1.	H-bond acceptor	2
2.	H-bond donor	0
3.	Molecular weight	302.45
5.	Log P[#]	5.36
6.	Violations	1

[#]log P is the logarithm of partition coefficient between water and 1-octanol, indicates lipophilicity, should be < 5

4.3.4.2 Drug likeliness and toxicity predictions

According to the known structure and activity relationships, it is considered that certain small heterocyclic molecules act as highly functionalized scaffolds and are known pharmacophores of a number of biologically active and medicinally useful molecules (Thompson and Ellman 1996). The logarithm of this coefficient, log Po/w, has been shown to be one of the key parameters in quantitative structure activity/property relationship (QSAR/QSPR) studies (Bayat and Nassab 2010).

Table 4.9 shows some important predicted parameters of a potential drug molecule and its toxicity. The higher Log D value (5.37) indicated that the compound is more lipophilic; such compounds are more easily absorbed by the cells and the

elimination of these drugs from the body is less. Labdane diterpene is highly insoluble in water (log S = -4.95) which significantly affects its absorption and distribution properties. The structure of this compound correlates with its bioavailability properties such as intestinal absorption and blood-brain barrier penetration (Zhao et al. 2002).

Table 4. 9 Bioavailability and toxicity parameters of labdane diterpene

Sr. No.	Parameters	Predicted values
1.	Log S	-4.95
2.	Log D	5.37 (From pH 1-8)
3.	Log $K^{(OC)}$	4.9±1.0
4.	TPSA	34.14
5.	Toxicity category[#]	Class 2 (Oral) Cass 4 (IV)
6.	Ames test[a]	+ 0.09
7.	Bioavailability	≤ 30% (Oral) ≥ 70% (Intestinal)

[#]Indicates toxicity category of chemical compounds; class 2 indicates toxic if swallowed and class 4 indicates no hazard statement

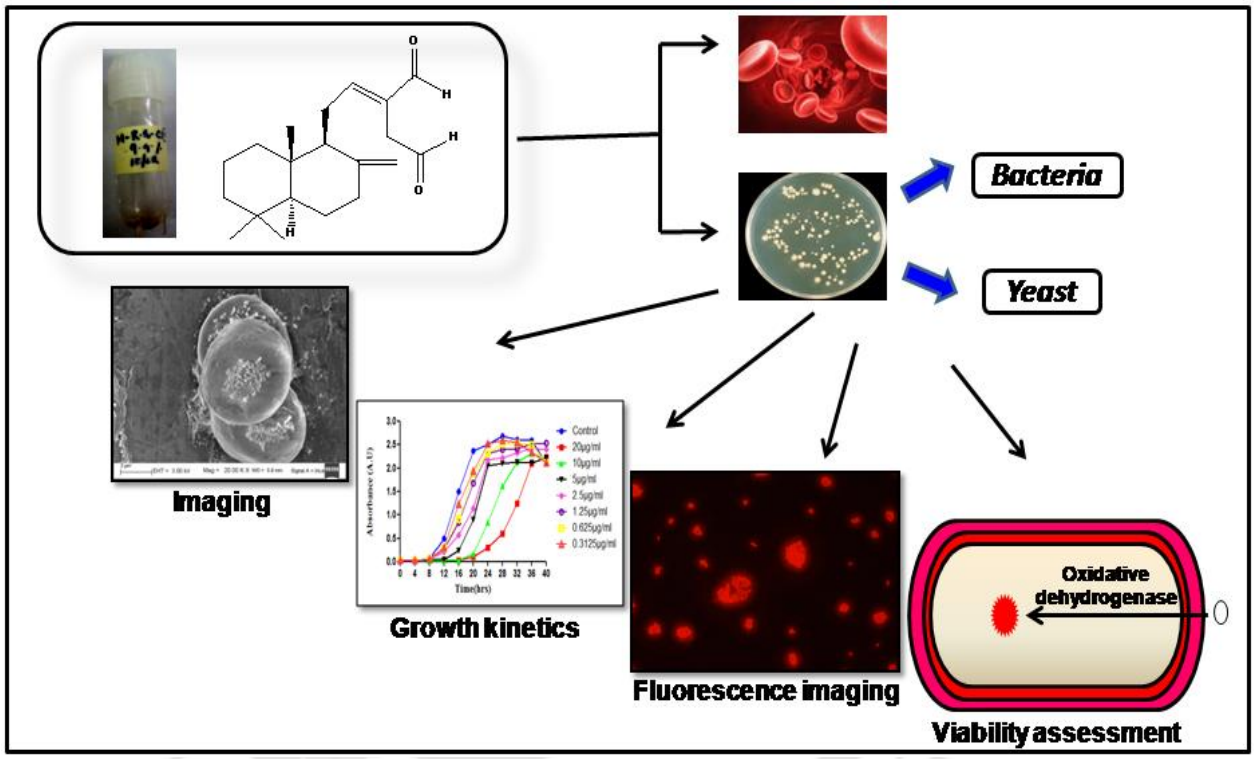
[a]Determines the mutagenic activity of chemical compounds on bacteria; value indicates a moderate activity

4.4 Conclusion

In this chapter, it was demonstrated that the seeds of *A. nigra* contain four non – polar compounds, of which two are found in major amounts. One of these was found to be a principle labdane diterpene and characterized as (E)-labda-8(17), 12-diene-15, 16-dial. Using 1:4 as the seed-to-solvent ratio of hexane extract, maximum yield of this compound was sourced. The vibrational spectroscopy (FT-IR and Raman) study reveal that the experimental spectra of the compound are in good agreement with the theoretical wavenumbers calculated by DFT. The SERS technique sustained intense enhancement in the Raman spectra along with the suppression of fluorescence. Spectral analysis indicated the perpendicular orientation of the compound on the metal surface; hence the importance of the orientation of the molecule on the metallic surface and the interaction of some ionic groups of the compound and metal is highly significant. The SERS spectra, thus, provide a signature of vibrational spectra for labdane diterpene that would help in its easy discrimination and hence, identification from a mixture of compounds. Analysis of the

physico – rheological properties of the compound suggests its highly hydrophobic, viscous and amorphous nature. The compound has high thermal stability and some optical behavior. *In silico* predictions suggest that labdane diterpene is a potential drug candidate and has a high intestinal absorption. However, *in silico* predictors or softwares have also indicated, theoretically, the compound to be unsuitable for oral administration. But diterpenes are known to be effective antibacterial agents; hence it is of utmost necessity to evaluate the biomedical application of this compound, especially for intra – venous application.

Chapter 5: Evaluation of the bio-pharmaceutical potential of the isolated compound



This chapter describes the hemato-compatibility of the isolated labdane diterpene for *in-vitro* administration. Also, it describes the effectiveness and identifies the possible mode of action of the compound against pathogenic bacteria and yeast

Chapter 5: Evaluation of the biopharmaceutical potential of the isolated compound

A) Studying its hemato - compatibility

5A.1 Introduction

Due to the rapidly changing environment and lifestyle of people as well as emergence of wide range of resistant microbes, a number of new and lethal diseases are emerging. In this regard, sustainable compounds for therapeutic applications are in much demand. Focus is being narrowed down towards plant based drugs due to their competent efficacy, rare chances of side-effects, society faith and sustained availability (Dias et al. 2012). Owing to the easy and cultural acceptability, herbal treatment using plant derived drug/bioactive compounds are preferred by most people in developing countries (Ghasi et al. 2000). Indian, Chinese and African civilizations have documentations dating back to 1000 BC that highlights the use of plants for the treatment of various diseases (Taylor et al. 2001; Roy et al. 2007; Cragg and Newman 2013). India has a rich heritage of traditional medicine systems such as Ayurveda, Siddha and Unani (Dev 1999) that exploit plant derived formulations to heal ailments. The individual chemical, bioactive compounds isolated from the crude extracts of different plant parts (called as "Elixir of life"), when formulated as drugs, are responsible for their biological activity (Lahlou 2013). However, in the past decade difficulty in the synthesis of natural products coupled with the lack of resupply and the perceived notion of natural product based drug discovery being a slow process are all valid critiques.

Damage to biological cells during infection form the basis of a number of pathological conditions. Emergence of a number of side effects upon administration of many drugs maybe an added detriment to such condition. Often, infusions and hypersensitivity reactions are frequent side effects of *in – vitro* administered medicines, which may lead to fatal allergic reactions due to the activation of the complement system. These reactions have remarkable similarities with the clinical symptoms but marked differences from IgE – mediated allergy. Hence, the effect of any molecule (intended for therapeutic application) on the cells of the biological system exposed to the drug molecule need to be evaluated, along with assessment of its effect on the specific drug target. Hemato - compatibility, a major component of biocompatibility, is a critical evaluation of the interactions of any foreign material with blood to explore the possible

adverse effects as a result of this exposure (Szebeni 2012). Ideally, erythrocytes or red blood cells (RBCs) have been used as the convenient model for drug – host cell interaction studies (D'Aquino et al. 1983). In case of intended intra-venous administration of drugs, study on blood cells is mandatory and as such erythrocytes constitute the major part of cells in the blood stream. Compounds possessing potent biological activity may not be useful in pharmacological formulations when they possess hemolytic effect (Zohra and Fawzia 2014). Lysis of erythrocytes, commonly known as hemolysis, induced by such molecules may lead to hemolytic anemia, in addition to causing cell cyto-toxicity.

Zingiberaceae is a family of medicinal plants that grows abundantly in the Indian subcontinent. Plants of Zingiberaceae are not only used in various aspects such as food, spice and condiments, perfumes and aesthetics to mankind, but also form vital species in ethnomedicine (Devi et al. 2014). Well known medicine books of ancient times like 'Charaka Samhita', 'Susmta Samhita' and 'Materia Indica' describes the medicinal, curative and aromatic properties of plants belonging to this family (Borchardt 2002; Kasarkar and Kulkarni 2016). A wide range of bioactive compounds have been reported in the genus *Alpinia* (Ghosh and Rangan 2013), the largest genus of Zingiberaceae, but the species *Alpinia nigra* remains an unexplored goldmine in this regard (Roy et al. 2012). Seeds were found to be most effective for the isolation of bioactive compounds (Qiao et al. 2007; Ghosh et al. 2013a). (E)-Labda-8(17), 12-diene-15, 16-dial, the compound under study, is known to possess anti-bacterial (Ghosh et al. 2013a), antidiabetic, anticancer (Ghosh and Rangan 2014b) and anti-leishmanial (Ghosh et al. 2017) properties which makes it a potent therapeutic molecule. However, no reports are currently available on the hemato – compability of this compound.

Therefore, this part of the present chapter focuses on studying the compatibility of labdane with human erythrocytes and a safe dosage for *in – vitro* administration has been determined. In addition, the effect of a higher dosage of hydrophobic diterpene on RBCs has also been studied.

5A.2 Materials and methods

5A.2.1 Hemolytic assay

Qualitative and quantitative hemolytic activity assays was performed for different concentrations of labdane diterpene. Approximately 2% ethanol in 1X PBS (pH 7.4) was used as carrier solvent for all the sample preparations as pure water is hemolytic,

something which was reported way back in 1914 (Krumbhaar 1914). Serial dilution of labdane was done and a concentration range of 0.2 mg/ml - 1 mg/ml was used for the hemolytic assay.

5A.2.1.1 Qualitative hemolytic activity assay

Qualitative hemolytic assay was performed on sheep blood agar plates (Hi-media, India) by agar well diffusion method. Around 50 µl of the different concentrations of the compound were loaded into the wells and incubated at 37ºC for 4 hrs. 1X PBS and 5% Triton X were used as vehicle and positive controls respectively as previously reported (Santhana akshmi et al. 2014). After incubation, the plates were visually analyzed for the clearance zones.

5A.2.1.2 Quantitative hemolytic assay

5A.2.1.2.1 Erythrocyte / RBC sample preparation

The RBCs were isolated from whole blood as per protocol previously given (Zohra and Fawzia 2014) with some modifications. Briefly, fresh blood [O group] was drawn from healthy volunteers after obtaining informed consent. The whole blood was centrifuged at 2000 rpm for 10 mins and 20% erythrocyte suspension was prepared in 1X PBS (pH 7.4 prepared from its constituent salts procured from HiMedia, India).

5A.2.1.2.2 Assay

Hemolytic activity of labdane was tested *in-vitro* using spectrometry. The different concentrations of the compound were mixed with erythrocyte suspension in 1:1 (v/v) ratio and incubated at 37ºC for 40 mins. 1X PBS was used as negative control and 0.5% Triton X as positive control. After incubation, the cells were spun down and the supernatant was collected. Absorbance reading taken at 540 nm using multimode microplate reader (Tecan, Infinite M – 200 pro, Switzerland) and the results were presented as percentage hemolysis or hemolytic ratio that was calculated using the following formula (Shrestha et al. 2016):

$$\%\, Lysis = \frac{[As - An]}{[Ap - An]} \times 100$$

where, As: Absorbance of test sample

An: Absorbance of negative control

Ap: Absorbance of positive control

All the experiments were carried out in triplicate and the mean of the three and standard deviation was used for plotting the graph. The graphs were plotted using Origin 8.5 software.

5A.2.2 Light - microscopic analysis

After quantitative hemolytic assay, the pellets of treated erythrocytes for each sample were collected, washed with 1X PBS and a smear was made on a clean grease free slide. The smear was air dried and visualized under light microscope (Olympus) with a 40X magnification lens. At least 10 images were captured at different places and the morphological modifications in cell structure were noted.

5A.2.3 Field Emission Scanning Electron Microscopic (FESEM) analysis

In order to confirm our speculations from the light microscopic analysis, higher magnification imaging – FESEM was used. Erythrocyte suspension (20%) was treated with the different concentrations of labdane for 40 mins at 37°C and samples were prepared as previously given with minor modification (Hortolà 1992). In brief, treated and untreated cells were washed with 1X PBS (pH 7.4) and fixed in 2.5% glutaraldehyde solution for 24 hrs at 4°C. Cells were washed twice with 1X PBS and were dehydrated with increasing concentration of ethanol (Molecular biology grade; Merck, India) (10% - 100%). Samples were coated with gold film in a Polaron sputter coater. The images were acquired with FESEM (Carl zeiss) at 3.0 kV power.

5A.3 Results and discussion

5A.3.1 Qualitative hemolytic assay

Qualitative hemolytic activity assay was performed on sheep blood agar plates. The hemolytic potential of labdane was determined by the zone of clearance observed on the plates after incubation. The zones of clearance were created by loss of opacity of media at the places where RBCs are disrupted by the used compound. Size of clearance zones is directly proportional to the hemolytic potential of the compound. Image of the assay are presented in **Fig 5A.1**. It was seen that none of the concentrations of labdane showed distinct clearance zones. Clear and prominent zone of clearance formed by Triton X (positive control) validated the efficacy of the assay. Though zones were visible for the

higher concentrations of labdane, they were not measurable which indicated that none of the samples possess appreciable hemolytic potential.

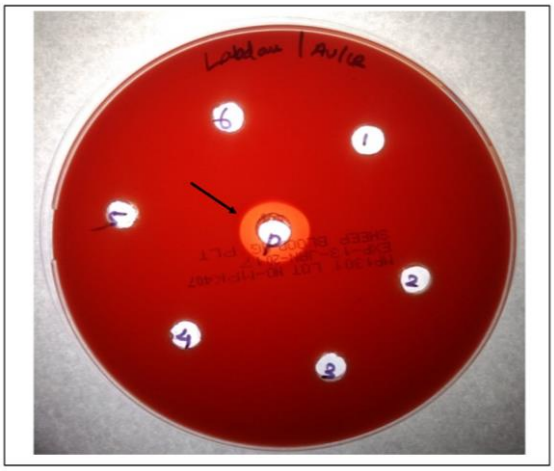


Fig 5A. 1 Sheep blood agar plates treated with labdane; **1 – 5** corresponds to 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of labdane

5A.3.2 Quantitative hemolytic assay

The amount of hemoglobin released upon treatment due to damage of RBC membrane is the indicator of hemolytic potential of the samples. Total hemolysis was obtained using Triton X and no hemolysis was obtained using 1X PBS. The results are depicted as percentage lysis. It is reported that any drug causing 0.5% damage to RBCs is suitable for medicinal purpose (Nguyen and Ness 2014). Hemolytic potential of labdane is shown in **Fig 5A.2.**

From **Fig. 5A.2 A**, it was clear that concentration < 0.6 mg/ml is suitable to be used in blood stream (IV administration). However, there was a drastic increase in percentage lysis between 0.4 mg/ml and 0.6 mg/ml (0.33% to 1.62%). Hence the concentration range in between these two were analyzed (**Fig. 5A.3 B)**. It was seen that concentrations $\leq$ 0.44 mg/ml were suitable for administration in blood stream. The % lyses of positive and solvent controls are also shown in **Fig. 5A.2 B inset**. These results are comparable with the previous literature; copalic acid is a labdane diterpene from *Copaifera* spp. which showed significant hemolysis of 38.4% at 100 mM while other labdane diterpenes such as 3-hydroxy-copalic and 3-acetoxy-copalic from same plant showed no significant hemolysis (Vargas et al. 2015). Thus, the hemolytic potential or rather the hemato – compatibility of labdane diterpenes maybe related to the type of substituent groups attached to the labdane backbone.

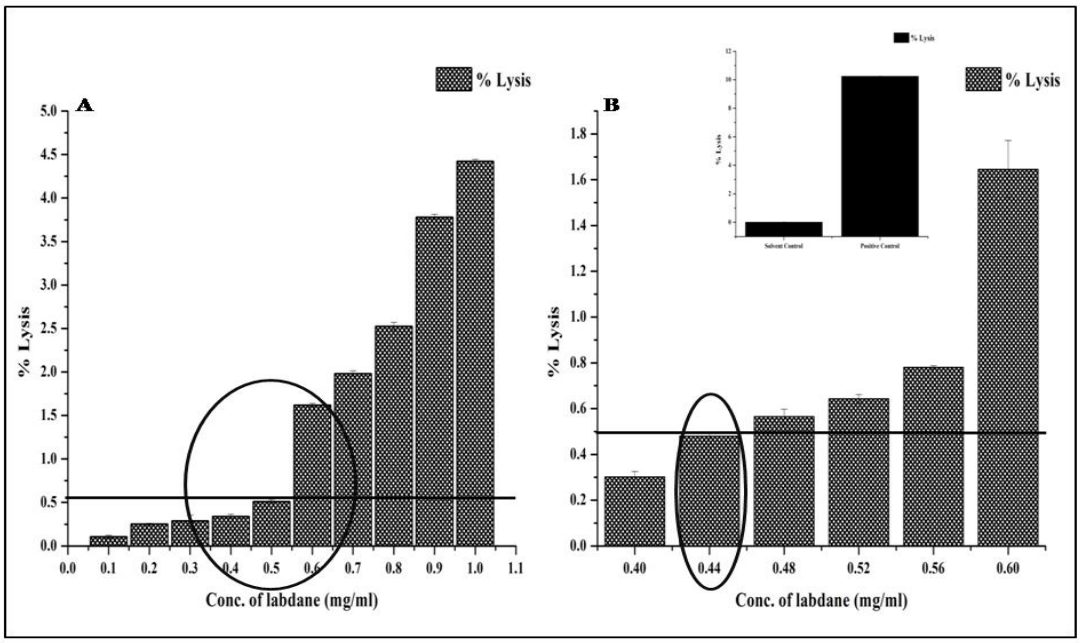


Fig 5A. 2 Percentage lysis of erythrocytes caused by different concentrations labdane **(A)** 0.1 mg/ml to 1.0 mg/ ml **(B)** 0.4 mg/ml to 0.6 mg/ ml; inset shows the percentage lysis of solvent and positive control

5A.3.3 Light – microscopic analysis

The release of hemoglobin obtained during quantitative hemolytic assays indicated certain degree of damage (maybe partial or complete) to RBC membrane upon treatment. The type of damage caused by labdane was studied using light microscopy (**Fig 5A.3**). Upon treatment with Triton X (positive control), the RBCs showed total lysis **(Fig 5A.3 A)** whereas all the RBCs appeared intact when treated with IX PBS (solvent control; **Fig 5A.3 B).** It was observed that the membranes of the RBCs remain intact upon labdane treatment with a concentration of $\leq$ 0.4 mg/ml and no cell cleavage or lysis was seen (**Fig. 5A.3 C)**. However, the release of hemoglobin was observed. This might be because of pores created on the erythrocyte membrane which was observed when treated at a concentration of $\geq$ 0.6 mg/ml of labdane (**Fig 5A.3 D; clear on inset**).

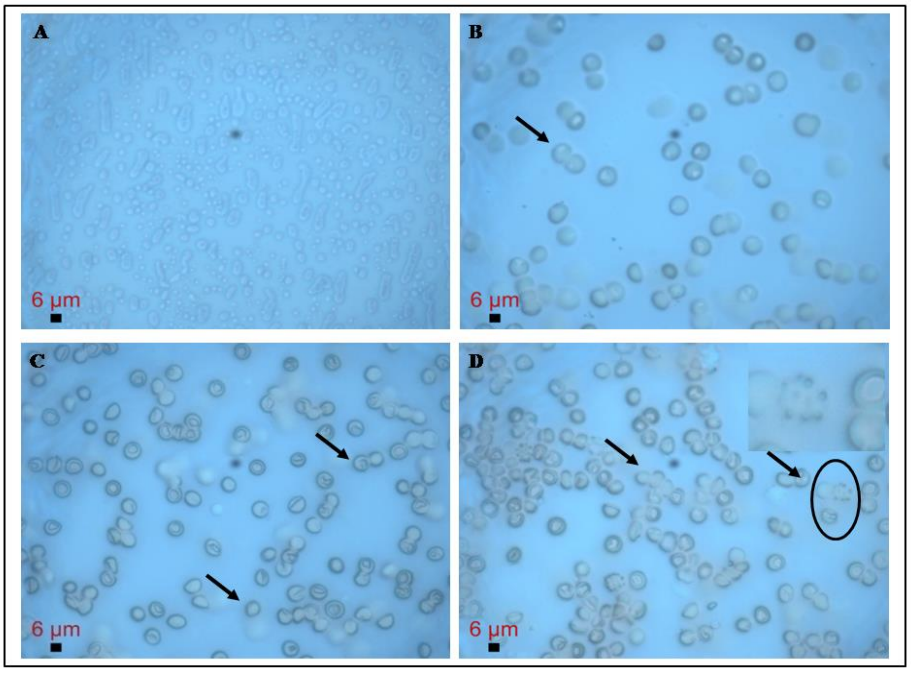


Fig 5A. 3 Light microscopic images of RBCs treated with **(A)** Positive control (Triton X 100), **(B)** Solvent control (1X PBS), **(C)** Labdane (0.4 mg/ml), **(D)** Labdane (0.8 mg/ml); inset shows porated RBC

5A.3.4 FESEM analysis

The results obtained by light microscopic analysis were confirmed by using FESEM. These high resolution and magnified images further provide a conclusive idea about the effect of labdane on RBCs. The images of FESEM analysis are shown in **Fig. 5A.4.** Most of the RBCs appeared healthy and intact after treatment with the compound. **Fig. 5A.4 A** showed healthy RBCs that were unaffected by PBS (negative control). From **Fig 5A.4 B (inset)**, it became evident that indeed pores were created on the surface of the erythrocytes after treatment with the compound. The morphology of the cells were slightly distorted and such aberrations were not observed in the healthy RBCs. Leakage of hemoglobin occured by these pores upon treatment with labdane. Appearance of both damaged as well as healthy RBCs from a treated sample clearly indicated the low degree of damage caused to the blood cells upon treatment.

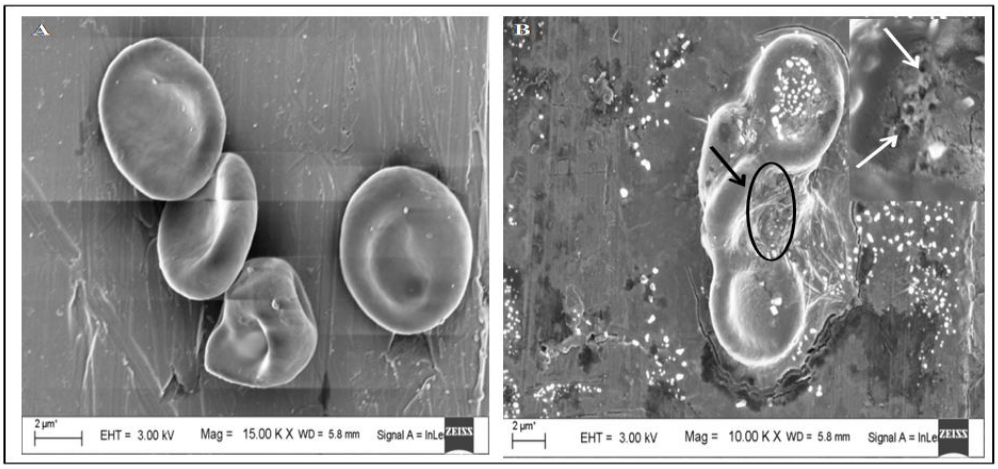


Fig 5A. 4 FESEM images of RBCs treated with **(A)** Solvent control (1X PBS), **(B)** Labdane (0.8 mg/ml); white arrows on the inset shows pores on RBC membrane

5A.4 Conclusion

For the administration of any plant based drugs or formulations *per os* or by any other methods, evaluating the hemolytic potential is of utmost importance. The effect of (E)-labda-8(17), 12-diene-15, 16-dial was studied. The maximum dosage of labdane for IV administration was 0.40 – 0.44 mg/ml. Results from light microscopy and FESEM revealed that labdane created pores in erythrocyte membrane leading to leakage of hemoglobin; at this concentration the rupture of erythrocytes was not observed. Thus, this plant derived bioactive compound manifests low hemolytic activity and hence, can be administered in the bloodstream without any serious risk of hemolytic anemia. Hemato - compatibility of drugs is, thus, a major and important aspect of drug delivery and administration. In case of prolonged administration of labdane, particularly *in vivo* models, various hematological parameters should be checked and followed up to prevent any possible risk of anemia.

B) Screening the antimicrobial potential of the compound and deciphering its possible mode of action against:

1. Bacteria

5B 1.1 Introduction

A major healthcare concern worldwide in the 21st century is the emergence of serious infections caused by microorganisms that are resistant to the commonly used antimicrobials of today. The seriousness of this problem is such that the WHO had used "Antimicrobial resistance: No action today no cure tomorrow" as the theme for World Health Day in the year 2011 (Rai et al. 2013). There has been overuse or abuse of these antimicrobials, which further worsen the situation. Thus, it is necessary to limit but preserve the use of effective antimicrobials for human use (Chang et al. 2015) and at the same time find suitable alternatives which do not have any indirect adverse effects (Cheng et al. 2014). From time immemorial, nature has been exploited as the source of medicines. Natural products, including plants, animals, bacteria, minerals have been used by man for treatment of microbial infections (Lahlou 2007). Plants, in particular, are the basis of modern medicines. The traditional Welsh rhyme says "*Eat leeks in March and wild garlic in May , and all the year after the physicians may play*" (Tyler 1982). Though the practice of finding healing powers from plants is an ancient idea, the use of natural antimicrobials had become non-existent with the advent of antibiotics in the 1950s. However, due the easy availability of over-the-counter herbal medicines from local suppliers and self medication with them, use plant extracts as antimicrobials started gaining popularity from the late 1990s (Eisenberg et al. 1993). The past decade has witnessed a tremendous surge in the acceptance and use of herbal medicines by the public both in developed and developing countries (Ekor 2014).

The extensive traditional use of many members of the Zingiberaceae family for curing ailments has been handed down as legacy in India, China and Southeast Asian countries from thousands of years (Kunnumakkara et al. 2008). Members of *Alpinia*, the largest genus of this family, possess attractive inflorescence and aromatic aerial parts that are usually subjected to fractionation for essential oils, extracts and bioactive compounds (Ghosh and Rangan 2013). Till date, *Alpinia galanga* has been shown to possess the highest number of bioactive compounds possessing antimicrobial properties (Weerakkody

et al. 2011), whereas *Alpinia nigra* still remains an unexplored hub, particularly with respect to its antimicrobial potential (Roy and Swargiary 2009).

Among the different phytoconstituents of plants, diterpenes are known to display a wide spectrum of biological activities, including antibacterial activity (Kuźma et al. 2007; Porto et al. 2009). Previous studies have demonstrated that various classes of diterpenes like pimarane, clerodane, labdane, isopimarane etc. have been extensively used as antibacterial agents (Kalpoutzakis et al. 1998; Wiart et al. 2005; Radulović et al. 2010). However, till date no studies have been done to study the mode of action of these diterpenes against the pathogenic bacteria.

Over the past 50 years, microbiological methods were available only to determine whether the bacterial cells were dead or alive. A large number of current reports mention that bacteria may exist in an eclipsed or dormant state, avoiding detection by standard methods. The existence of such states for microbes, particularly the pathogenic ones, can have very serious and life – threatening implications for public health and welfare. It is very important to answer whether such physiological states of bacteria are closer to dead or alive bacterial states in matters relating to food safety, drinking water purity, sterility of pharmaceuticals etc.(Bogosian and Bourneuf 2001).

Therefore, this part of the present chapter focuses on studying the inhibitory potential of the labdane diterpene, isolated from the seeds of *A. nigra*, against pathogenic gut bacteria. In addition, investigation has also been carried out to understand the possible mode of action of these plant derived diterpene against the tested bacteria.

5B 1.2 Materials and methods

5B 1.2.1 Preparation for assay

5B 1.2.1.1 Microbial cultures

The antibacterial activity of labdane diterpene was evaluated against 2 Gram negative bacteria, *Escherichia coli* enterotoxic (MTCC 723) and *Salmonella paratyphi* (MTCC 735) and 2 Gram positive bacteria, *Staphylococcus aureus* (ATCC 6538) and *Listeria monocytogens* (ATCC 19115). The bacterial strains were grown and maintained as previously described (Kesari et al. 2010) on nutrient agar plates. Before each experiment, the bacteria were sub-cultured twice in nutrient broth to ensure their active growth.

5B 1.2.1.2 Test sample preparation

A stock of concentration 1 mg/ml of labdane diterpene was prepared in 5% DMSO (SRL, India) in 1X PBS (pH 7.2; prepared from its constituent salts procured from HiMedia, India) and further diluted in 1X PBS. The dilution of labdane was done within a hemato-compatible range (0.006 mg/ml - 0.4 mg/ml), using the results from the previous part of the present chapter **(section 5A.3.2)**.

5B 1.2.2 Antibacterial assay

5B 1.2.2.1 Growth curve analysis

Growth curve analysis was carried out in 96-well micro-titre plates (Eppendorf) as per previous protocol (Sekse et al. 2012) with some modifications. Briefly, around 100 µl of bacterial culture (adjusted to 10^6 cells) was added to corresponding well and to this, different volumes of diluted labdane were added so that the final concentrations, mentioned above, were obtained. Antibiotic kanamycin (Sigma, India) (25 µg/ml) was used as positive control and 1X PBS was used as negative control. Plates were incubated at 37^{o}C in a shaking incubator (Orbitek, India) and absorbance values were recorded at regular intervals of 2 hrs at 600 nm for 24 hrs using multimode microplate reader (Tecan, Infinite M – 200 pro, Switzerland). The observed optical density values were plotted against time to obtain the growth pattern.

5B 1.2.2.2 Viability assessment by TTC assay

2,3,5-triphenyl tetrazolium chloride (TTC) assay is the simple method to assess the cell viability. In presence of live cells, TTC is reduced to triphenyl formazan (TPF) by the respiratory linked dehydrogenase enzymes that require NAD or NADP (Alkisnson et al.

1950). The development of red color due to formation of formazan salt is the indicator for presence of live cells and its intensity is directly proportional to number of live cells.

For cell viability analysis after treatment with compound, around 100 µl of bacterial culture adjusted to 10^6 cells was added to corresponding microfuge tubes (Eppendorf) and to this the compound was added so that the final concentrations mentioned above was obtained. Tubes were incubated at 37ºC in shaking incubator (Orbitek, India) for 12 hrs. Kanamycin (0.025 mg/ml) was used as positive control and 1X PBS was used as negative control. After incubation, the cells were spun down and washed with 1X PBS twice. Then the 1% TTC solution (SRL, India), having a final concentration of 2.5 mM, was added to the tubes and incubated in dark for 8 hours at 25ºC. The formazan salts that resulted were solubilized using 1% SDS (SRL, India; dissolved in 50% methanol) for 30 mins at 60ºC. The cells were again spun down and supernatant was collected. The absorbance values were recorded at 485 nm and the results were represented as relative viability percentage (Castro-concha et al. 2012). Relative viability percentage is calculated using the formula:

$$\text{Relative Viability} = \frac{[A_s - A_n]}{[A_p - A_n]} \times 100$$

where, A_s - Absorbance of test sample

A_n - Absorbance of negative control

A_p - Absorbance of positive control

5B 1.2.2.3 FESEM analysis

FESEM was used to visualize the changes in the morphology of the bacteria after treatment with the compound (0.006 mg/ml and 0.4 mg/ml). The bacterial cells were washed with freshly prepared 50 mM phosphate buffer (pH 7.2), fixed with 2.5% (v/v) glutarldehyde (HiMedia, India) in PBS and rinsed again with the same buffer. The specimen was dehydrated with increasing grades of ethanol from 10% to 100%. The specimens were then, coated with gold and analyzed through FESEM (Carl Zeiss Ultra 55), as previously described (Ghosh et al. 2013a).

5B 1.2.2.4 Fluorescence microscopy

Extent of bacterial cell damage and lysis after treatment with the compound was also determined using fluorescence microscopy. Freshly inoculated bacterial cells were treated

with labdane (0.006 mg/ml and 0.4 mg/ml) and allowed to grow for 6 hrs at 37ºC. Post incubation, the cells were collected and washed with 1X PBS twice. Cells were later fixed with glutaraldehyde (2.5% v/v) for 3 hrs, followed by washing with 1X PBS twice. Fixed cells were then, stained with propidium iodide (PI; Sigma, India) for 10 mins. Cells were visualized with inverted fluorescent microscope (Olympus) with 100X lens and at least 10 images were captured at different places to observe the average number of stained cells. Cells were counted using Image J software.

5B 1.2.3 Statistical analysis

All experiments were set up in a completely randomized design and with a minimum of three replicates. All the graphs were plotted as mean of the observed values using Origin 8.5. The statistical analysis was carried out using SPSS Statistics 17.0 ($R^2 \geq 0.96$). Significance of analysis for mean values of each experiment was compared to the means of corresponding vehicle control at respective time points using Ttest ($p < 0.05$).

5B 1.3 Results and discussion

5B 1.3.1 Growth curve analysis

The OD values are directly proportional to the turbidity of microorganism in suspension and thus, represent relative quantity of microorganism under a certain experimental condition (Ma et al. 2014). It was seen that labdane diterpene was highly effective in inhibiting the growth of Gram negative bacteria upto a period of 26 hrs **(Fig. 5B 1 A and B)**. The inhibition of labdane at higher concentrations was comparable to that of the positive control. However, low concentrations of the compound (0.012 mg/ml and 0.006 mg/ml) were not able to inhibit the growth of *E. coli*. In case of *S. paratyphi* at the above concentrations, the lag phase of the bacteria was extended upto 16 hrs of treatment, after which it resumed its normal growth pattern. Whether the compound kills the bacteria or merely prevents its growth and extends its lag phase need to be investigated further.

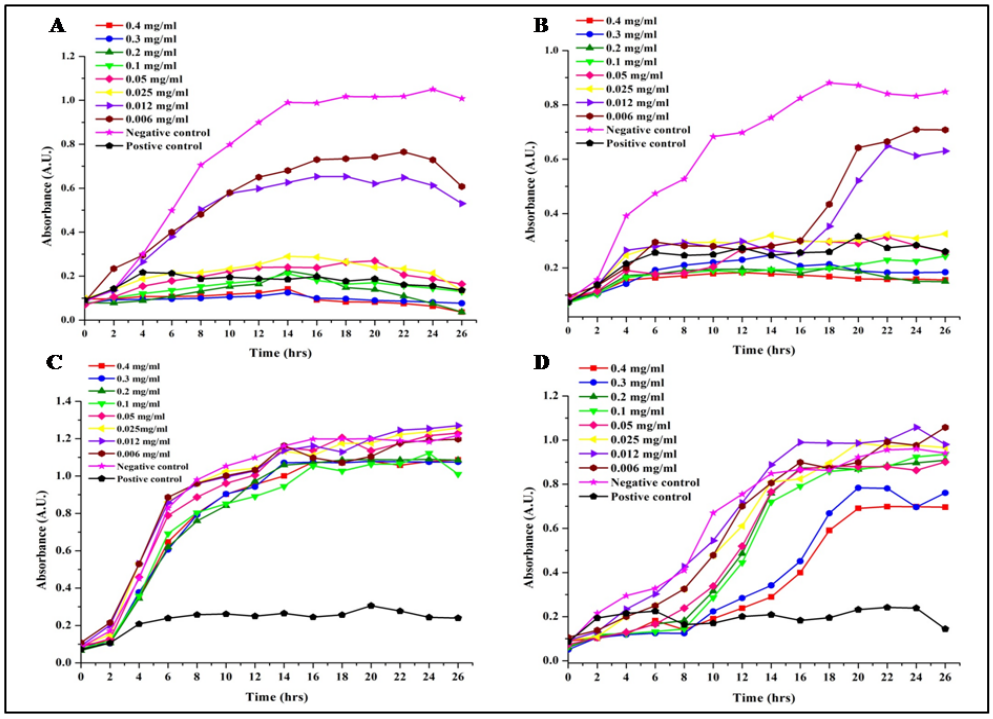


Fig 5B 1. 1 Growth curve of **(A)** *E. coli* **(B)** *S. paratyphi* **(C)** *S. aureus* **(D)** *L. monocytogens* after treatment with labdane diterpene

However, the compound showed no effectiveness against Gram positive bacteria **(Fig 5B 1.1 C and D)**. This may be due to the complex cell wall composition of these bacteria and the hydrophobic nature of the compound. Even though a higher concentration of the compound was able to restrict the growth of *L. monocytogenes* up to 12 hrs, there was no pronounced inhibitory effect.

5B 1.3.2 Viability assessment by TTC assay

TTC is a metabolic dye and hence growth conditions of bacteria have to be optimum while using this dye. Insufficient atmospheric and suboptimal growth conditions by mishandling may lead to inefficient reduction of TTC. The viability assessment of the 4 bacteria after treatment with labdane diterpene is shown in **Fig 5B 1.2**.

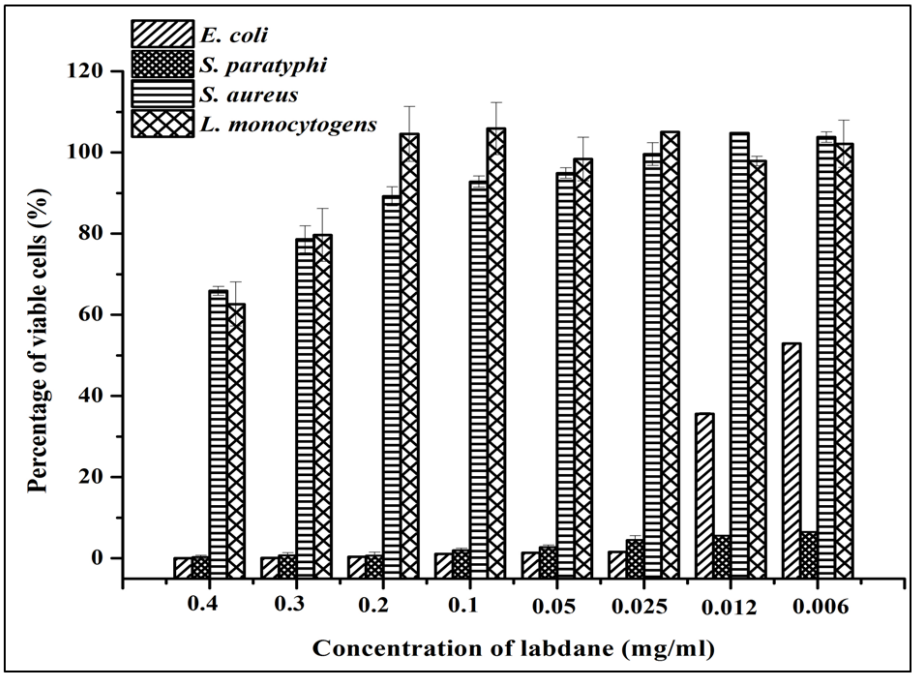


Fig 5B 1. 2 Percentage of viable bacterial cells after treatment with labdane diterpene

From the above figure, it was observed that negligible percentage of viable cells of *E. coli* were present after treatment with labdane diterpene (≥ 0.025 mg/ml). At lower concentrations of 0.012 mg/ml and 0.006 mg/ml, high amount of viable cells were observed, which was in coherence with the growth curve of the bacteria. The percentage of viable cells in *E. coli* increased with decrease in labdane concentration. However, in case of *S. paratyphi*, an almost similar percentage of viable cells (higher than that of *E. coli*) were observed when treated with all the concentrations of the compound. In presence of a minute amount of compound (0.012 mg/ml and 0.006 mg/ml), the viability of the cells were almost equal to that in presence of 0.025 mg/ml. This indicated that with the passage of time, these viable cells were able to override the inhibitory effect of the low concentrations of the compound and were able to grow properly **(Fig. 5B 1.1 B)**. This was similar to that of previously published reports on the bacteriostatic and fungistatic properties of secondary metabolites, particularly terpenes and terpenoids (Schafer et al. 2004; Cushnie and Lamb 2005; Ma et al. 2014).

Also, it was clear that labdane was not effective against Gram positive bacteria. The percentage of viable cells is equivalent to negative control. However, at high concentration (0.4 mg/ml), a very mild inhibition was observed in both the Gram positive pathogens.

5B 1.3.3 FESEM analysis

Membrane damage is found to be the key mechanism by which plant extracts and plant based compounds exert their antimicrobial activities (Hammer and Heel 2012). Since, it was observed from the previous section that labdane diterpene has a profound effect on Gram negative bacteria but not on Gram positive bacteria, the effect of the compound was checked on *E. coli* and *S. paratyphi* (Gram negative bacteria) and *S. aureus* (Gram positive bacteria) by FESEM to unveil the changes in bacterial cell morphology after compound treatment.

FESEM study of bacteria treated with 0.006 mg/ml of the compound revealed undamaged bacterial cell morphology, which was similar to that of untreated bacteria **(Fig 5B 1.3 A-C)**. Whereas, membrane disintegration, cell shrinkage and cell lysis was observed in the Gram negative bacteria treated with 0.4 mg/ml of the compound **(Fig 5B 1.3 D and E)**. This indicated that the labdane diterpene, isolated from the seeds of *A. nigra*, caused destruction of Gram negative bacteria by degrading their cells walls and hence, the cells lost their viability. In case of the Gram positive bacteria, *S. aureus*, some morphological distortions were observed on their cell surface upon treatment with a higher concentration of the compound **(Fig 5B 1.3 F)**, but there was no lysis and cell morphology appeared intact; hence their viability was not disrupted.

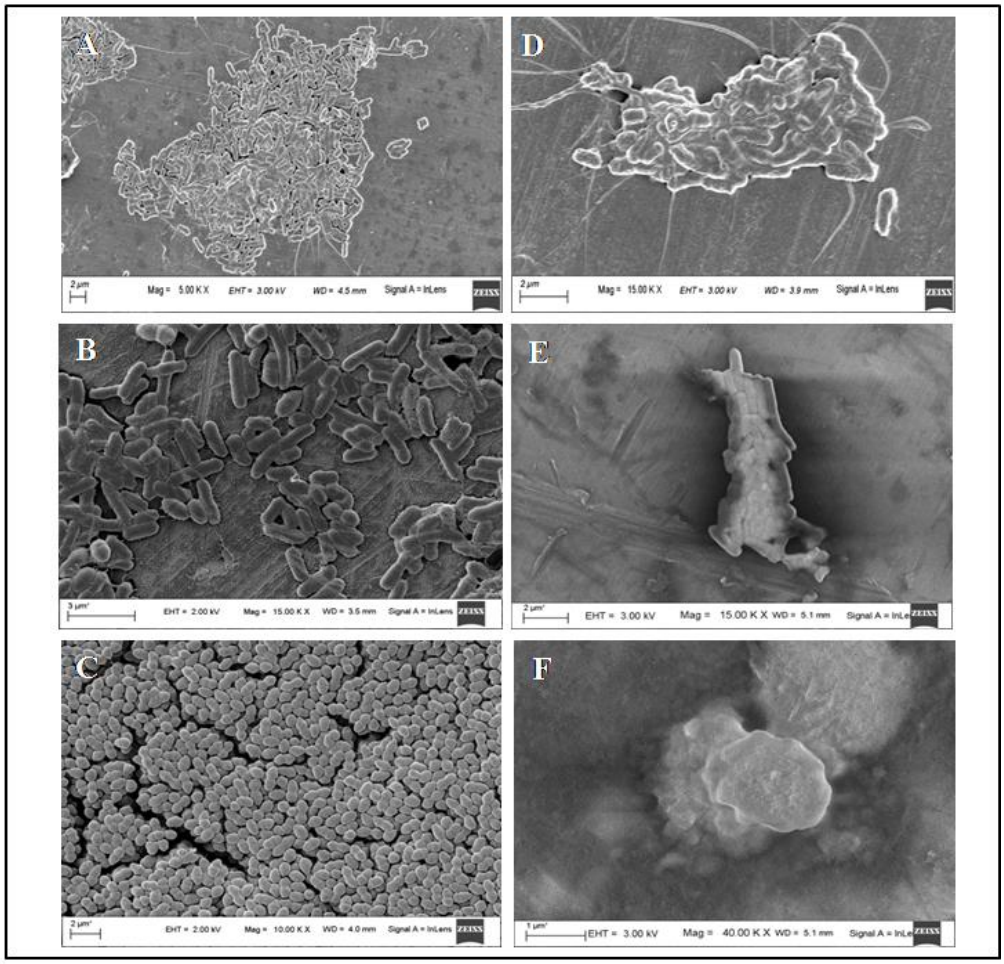


Fig 5B 1. 3 FESEM images of *E. coli* **(A** and **D)**, *S. paratyphi* **(B** and **E)** and *S. aureus* **(C** and **F)**; bacterial cells after treatment with 0.006 mg/ml of labdane **(A-C)**; bacterial cells after treatment with 0.4 mg/ml labdane **(D-F)**

Such abnormalities of bacterial cell morphology are evidential of membrane disruption, as reported previously (Koyama et al. 1997; Shin et al. 2007). Previous study of purified bee venom revealed their antimicrobial activity on the skin bacterium, *Propionibacterium acnes*, where clear damage in the bacterial cell damage was observed in the electron microscopic images (Han et al. 2013). The current study of bacterial cell damage caused by plant derived antibacterial agent is congruent with previously published reports (Kairyte et al. 2013; Kamonwannasit et al. 2013; Sherry et al. 2013; Supaphon et al. 2013).

5B 1.3.4 Fluorescence microscopy

Viability assessment of bacteria using PI uptake as a measure for dead (PI stained; membrane permeable) and live (PI unstained; membrane impermeable) cells are applied in various scopes of research and industry (Shi et al. 2007). Cells stained with PI

fluoresce bright red under green excitation (Williams et al. 1998). From **Fig 5B 1.4 (A and B)**, it was seen that *E. coli* and *S. paratyphi* cells treated with 0.006 mg/ml of labdane exhibit around 10% of stained cells; whereas bacterial cells treated with 0.4 mg/ml of labdane show around 90 - 95% of stained cells **(Fig 5B 1.4 D and E)**. It had been reported that PI only enters cells with "compromised" membranes and stains their nucleic acids, thus, PI is thought to stain "dead" cells (Sgorbati et al. 1996). The stoichiometric binding of PI with DNA has also been previously reported (Schutte et al. 1985). Thus, it was clearly evident that treatment of bacteria with higher concentration of labdane diterpene "killed" the bacteria by poration or lysis. The membrane of both the Gram negative bacteria were compromised or destroyed after treatment with the compound, facilitating the entry of PI into the cells, and may ultimately undergo complete destruction and lysis. *S. aureus* exhibited no red fluorescence upon PI staining when treated with both high and low concentrations of labdane **(Fig 5B 1.4 C and F)**. Since it is known that PI stains membrane compromised cells, it was confirmed that *S. aureus* had no effect of labdane treatment on it. The peptidoglycan cell wall of Gram positive bacteria, which imparts antibiotic resistance to this group of bacteria (Utili 2001), was probably responsible to its inertness, in comparison to the susceptibility of Gram negative bacteria as previously observed (Dahl et al. 1989), towards labdane treatment. The corresponding bright field microscopic images are shown in **Fig 5B 1.5**.

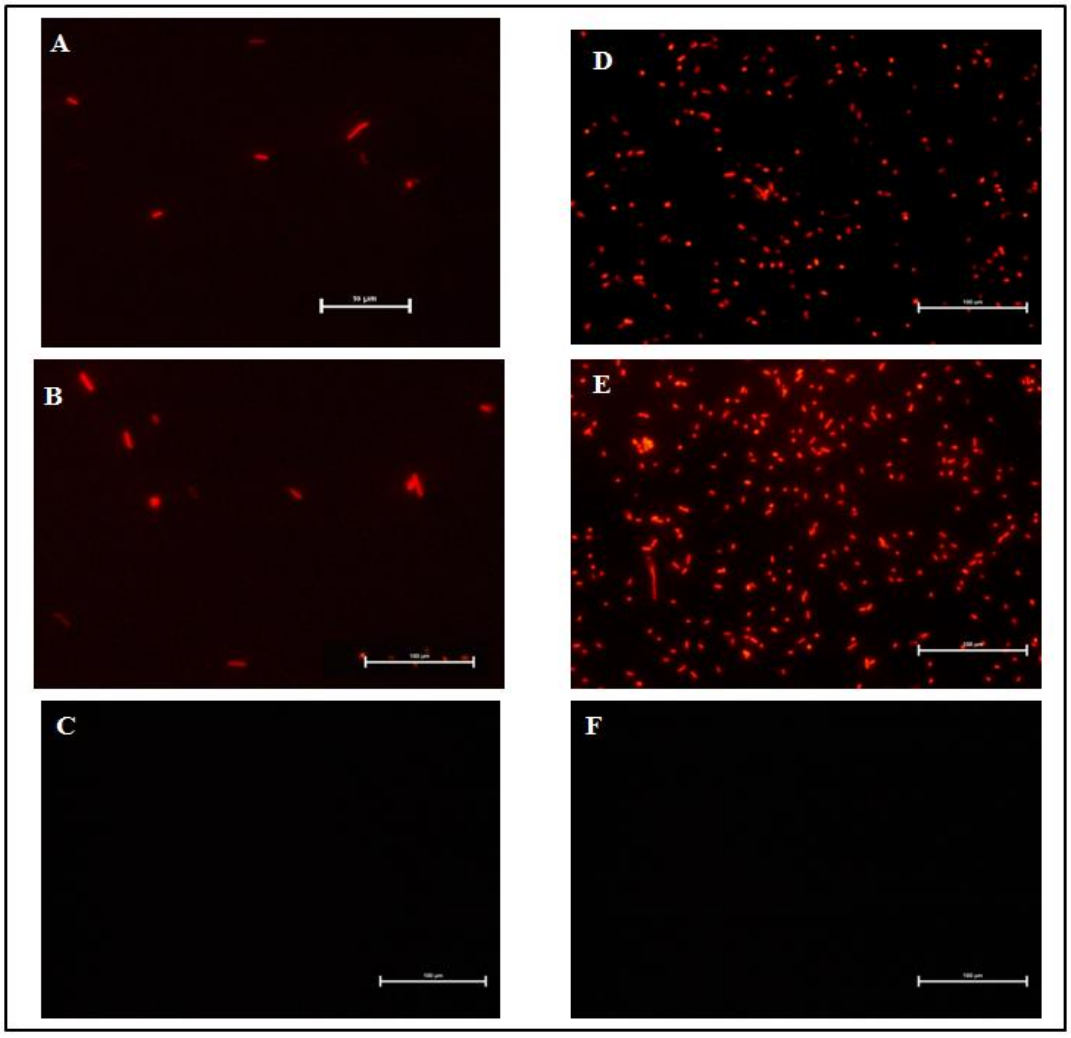


Fig 5B 1. 4 Fluorescent microscopic images of *E. coli* **(A** and **D)**, *S. paratyphi* **(B** and **E)** and *S. aureus* **(C** and **F)**; bacterial cells after treatment with 0.006 mg/ml of labdane **(A-C)**, bacterial cells after treatment with 0.4 mg/ml labdane **(D-F)**

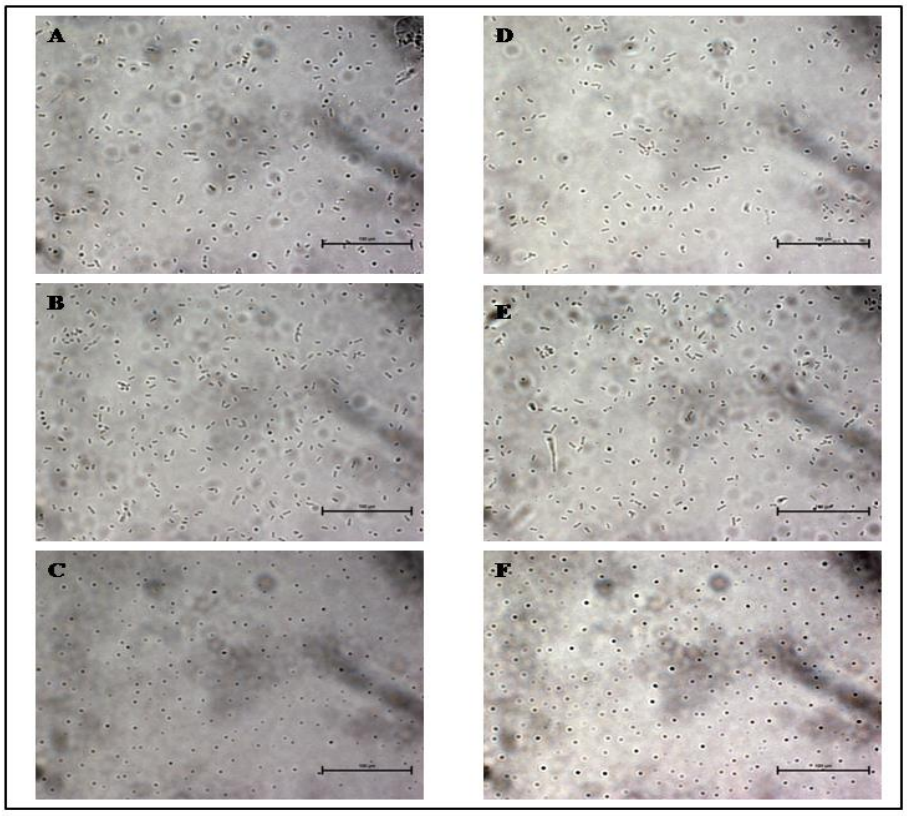


Fig 5B 1. 5 Bright field microscopic images of *E. coli* **(A** and **D)**, *S. paratyphi* **(B** and **E)** and *S. aureus* **(C** and **F)**; bacterial cells after treatment with 0.006 mg/ml of labdane **(A-C)**, bacterial cells after treatment with 0.4 mg/ml labdane **(D-F)**

5B 1.4 Conclusion

In the current study, the antibacterial potential of labdane diterpene, isolated from the seeds of *A. nigra*, has been reported against bacteria. The compound was found to have pronounced antibacterial effect on Gram negative bacteria but has no effect on Gram positive bacteria. Bacterial cell membrane damage and cell poration, accompanied by lysis was found to be the key mechanism underlying the efficacy of the compound. Thus, labdane diterpene can be considered an effective antibacterial agent against Gram negative bacteria, though deeper investigation in this matter is required in order to correctly understand the active nature of the compound. Therefore, the application of this crop and its isolated compound in the future by the pharmaceutical companies is an urgent need. However, at higher concentrations, the compound tends to form turbidity in the aqueous medium, which may be attributed to its innate hydrophobic nature. Recognition of the low solubility of labdane diterpene in the aqueous medium can lead to development of useful strategies to improve the efficacy and performance of the compound for bioassays.

B) Screening the antimicrobial potential of the compound and deciphering its possible mode of action against:

2. *Candida albicans*

5B 2.1 Introduction

From time immemorial, natural resources have been exploited for useful medicines for the treatment of various ailments and disorders. A survey in the countries hosting WHO-Traditional Medicinal Centers report that out of 122 compounds identified, 80% were used for ethno-medicinal purposes and derived only from 94 species of plants alone (Farnsworth et al. 1985). The primary goal behind using plants as the source of drugs is to encourage the isolation and identification of bioactive compounds for direct use as well as production of novel bioactive compounds with higher activity or lower toxicity. A large portion of this constitute secondary metabolites produced by the plant that are not required for the growth and development of the plant but are produced as a means of defense against predators (Colegate and Molyneux 2008).

India is one of the richest abodes of Zingiberaceae. Members of this family are traditionally used by tribal populations of NE India as a home remedy for a wide range of ailments. This implies that these plants synthesize a wealth of chemicals or secondary metabolites that increase their survival ability and to overcome challenges (Tushar et al. 2010). The labdane diterpene, (E)-labda-8(17), 12-diene-15, 16-dial, isolated from the seeds of *A. nigra*, is widely distributed in the Zingiberaceae family (Sirat et al. 1994; Abas et al. 2005). This compound is found to possess antidiabetic, anticancer (Ghosh and Rangan 2014b) and anti-leishmanial (Ghosh et al. 2017) properties.

Fungal pathogens are known to cause severe systemic and local infections in human beings. Very few antifungal agents are currently available in the market and most of them are not completely effective and safe for human use. Indigenous people have used several plant species belonging to algae, bryophytes, pteridophytes and angiosperms for the treatment of fungal infections, with effective outcomes (Vidyasagar 2016). Secondary metabolites, such as terpene derivatives, have a wide range of biological activities (Fortuna et al. 2001; Cabral et al. 2008a). Terpenes are the chemicals mainly responsible for the culinary, medicinal and fragrant properties of aromatic and medicinal plants. Volatile oils obtained from plants are variable mixture of terpenoids mainly

monoterpenes and sesquiterpenes, occasionally diterpenes and a variety of hydrocarbons. Terpenes are known to possess the ability of skin penetration that has a immense potential in transdermal drug delivery (Lim et al. 2009). It is reported that the terpenes and terpenoids possess bacteriostatic and fungistatic properties that increase upon carbonylation (Dorman and Deans 2000). The major components of essential oils *viz.* cineole, thymol, carvacrol, geranial etc. are known to possess antifungal activity. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic nature. However, this antimicrobial activity gets decreased drastically upon addition of hydrophilic groups to diterpenoids (Arif et al. 2009). Due to their lipophilicity, terpenes interfere with cell membrane system of organisms and decrease their activity (Schafer et al. 2004). Studies hypothesize that these secondary metabolites may not be killing microbial cells, as previously believed, but merely causing their aggregation that reduces the viable counts (Cushnie and Lamb 2005).

Candida albicans is a polymorphic fungus that grows either as ovoid shaped budding yeast, as elongated ellipsoid cells with pseudohyphae or as parallel walled true hyphae. The yeast and true hyphal forms are observed during infections and have distinct functions but the role of pseudohyphae is still not clear. It is a remarkable pathogen that adopts 2 different mechanisms to penetrate the host cells: induced endocytosis and active penetration. The fungus occurs in the warm and moist parts of the body like mouth, rectum, vagina and parts of skin causing candidiasis. The ability of *C. albicans* to form biofilms as a defense mechanism against biotic and abiotic stresses is considered an important virulence factor (Mayer et al. 2013). Not only that, the fungus is only known to possess some genetic mechanism that confer inherent resistance to certain antibiotics to this organism (Wu et al. 2017). Thus there is a need to constantly look for better, safer and more effective therapeutic agents against *Candida* species. Naturally occurring substances like honey and secondary metabolites like flavonoids are known to possess anti-*Candida* activity (Ahmed et al. 2013).

Therefore, this part of the present chapter focuses on the *in vitro* aspect of the anti-*Candida* activity of labdane diterpene. Further, investigation has been made on the growth kinetics and substrate uptake of this pathogenic fungus in the presence of the compound to understand its mode of action.

5B 2.2 Materials and methods

5B 2.2.1 Microbial strain

The biological potential of labdane diterpene was tested against *C. albicans* (NCCPF no. 400035), which was obtained as a gift from Fungal Biotechnology lab, Dept. of Biosciences and Bioengineering, IIT Guwahati. The fungal strain was grown and maintained as previously described (Kesari et al. 2010) on YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates. Before each experiment the organism was sub-cultured twice in YPD broth to ensure their active growth at 28ºC for 24 hrs.

5B 2.2.2 Candicidal activity

5B 2.2.2.1 Minimum effective concentration determination

The isolated compound was screened to evaluate the minimum effective concentration required to inhibit the growth of *C. albicans* using agar well diffusion method as previously described (Magaldi et al. 2004). Different dilutions of the compound were prepared in 1% DMSO (0.020 mg/ml – 0.000312 mg/ml). Flucanozole (> 98% purity; Sigma) at a concentration of 0.020 mg/ml and 1% DMSO were used as positive and vehicle controls respectively.

The minimum effective concentration was also determined by broth micro-dilution method in 96 – well micro-titre plate as per previous protocol (Nagao et al. 2017) with some modifications. Two fold serial dilution of the compound was prepared in 1% DMSO ranging from 0.020 – 0.000156 mg/ml and added to each individual well. Around 50 µl of *Candida* suspension (adjusted to 10^6 cells/ml) was added and the plates were incubated at 28ºC for 18 hrs. Results were analyzed with multimode microplate reader (Tecan, Infinite M – 200, Switzerland) at 660 nm and the lowest concentration at which fungal growth was inhibited was considered as the minimum effective concentration.

5B 2.2.2.2 FESEM analysis

FESEM was used to visualize the changes in the morphology of *C. albicans* before and after treatment with the compound. Untreated fungus was used as a negative control. The sample for FESEM was prepared as described in the previous part of this chapter **(section 5B.1.2.2.3)**.

5B 2.2.2.3 Raman spectroscopy

Fungal cells were characterized before and after treatment with the compound using Raman spectroscopy. Untreated *Candida* cells were used as negative control. About 3 ml of fungal cultures at exponential growth phase (10^8 CFU/ml) were centrifuged at 8000 rpm for 5 mins. Cells were washed and suspended in sterile distilled water. The sample was put on clean coverslip and allowed to dry at 37 ºC. The decrease in the intensities of Raman shift indicates fall in the number of microorganism, which may be due to inhibition of growth upon treatment (Das et al. 2013).

5B 2.2.2.4 Cell leakage analysis

In order to confirm the membrane damaging efficacy of the compound, cell leakage analysis was performed by monitoring the absorbance at 260 nm of the cell supernatants. Overnight culture of *C. albicans* grown in YPD broth was harvested by centrifugation at 10,000 rpm for 5 mins. Culture was then freshly inoculated in YPD broth and treated with the different concentrations of the compound. The cultures were incubated for 4, 8, 12, 16, 20 and 24 hrs respectively. After incubation, the samples were centrifuged at 5000 rpm for 5 mins in order to separate the fungal cells from low molecular weight metabolites such as nucleotides, amino acids and inorganic ions which are known to "leak" from the cells after membrane damage. Finally, the level of released material from the fungal cells was determined by measuring the optical density of the supernatant at 260 nm using UV-Vis spectrophotometer (Gene Quant 1300, India).

5B 2.2.2.5 Dissolved oxygen (DO) analysis

Dissolved oxygen (DO) in media is a direct measurement of the bacterial respiration and reflects the growth state of microorganism (Krishnaraj et al. 2010). A 1% (v/v) inoculum of overnight grown fungal culture was added to 50 ml YPD media in 250 ml Erlenmeyer flasks, treated with the compound (0.020 mg/ml) and incubated in a shaker incubator at 28 ºC and 180 rpm (Orbiteck, India). Oxygen uptake for growth of the *C. albicans* cells was performed using Biological oxygen demand (BOD) monitor (Hach, India). Untreated *C. albicans* culture was used as control. Level of oxygen was measured by dipping the probe of the BOD monitor in the culture media after 0, 3, 6, 12 and 24 hrs of incubation.

5B 2.2.3 Kinetic experiment

5B 2.2.3.1 Growth curve analysis

A 1% (v/v) inoculum of active fungal culture was added to 50 ml YPD media in 250 ml Erlenmeyer flasks. *C. albicans* was treated with different concentrations of labdane (0.000312 mg/ml – 0.020 mg/ml). The flasks were incubated in a shaker incubator at 28ºC and 180 rpm (Orbiteck, India). The measurement of growth and glucose uptake was done as previously described (Dutta et al. 2015). Briefly, 1 ml of culture was taken out from the flasks every 2 hrs in pre-autoclaved micro-centrifuge tubes (Eppendorf, India). The samples were centrifuged at 10,000 rpm for 5 mins to separate the cells from the media. The supernatant was collected to measure the substrate uptake (given below) and the cells were re-suspended in IX PBS. Growth of cells was determined by measuring the optical density of the re-suspended cells at 600 nm in a UV-Vis spectrophotometer (AnTech, India). Growth of the samples was measured till stationary phase of growth was obtained for all the samples.

5B 2.2.3.2 Substrate uptake

The uptake of glucose was obtained by colorimetric assay using GOD-POD kit as described previously by Krishnaveni (Krishnaveni et al. 1984).

5B 2.2.4 Statistical analysis

All experiments were set up in a completely randomized design and with a minimum of three replicates. All the graphs were plotted as mean of the observed values using Origin 8.5. The statistical analysis was carried out using SPSS Statistics 17.0 ($R^2 \geq 0.96$). Significance of analysis for mean values of each experiment were compared to the means of corresponding vehicle control at respective time points using one-way ANOVA followed by Tukey's test ($p < 0.05$).

5B 2.3 Results and discussion

5B 2.3.1 Candicidal activity

5B 2.3.1.1 Minimum effective concentration determination

The minimum effective concentration of the compound, inhibiting the growth of *C. albicans* was found to be 0.000312 mg/ml, by agar well diffusion assay, **(Fig. 5B 2.1)**. The zone of inhibitions are given in **Table 5B 2.1**. However, the minimum effective concentration of the compound by broth micro-dilution method was found to be 0.000625

mg/ml. For all the further assays, the concentration range and result obtained from the agar well diffusion was used.

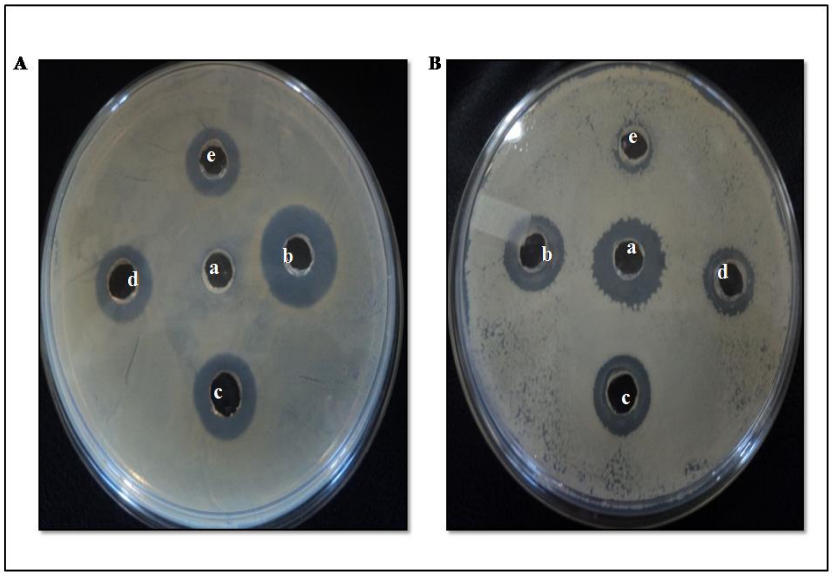


Fig 5B 2. 1 Plates showing agar well diffusion assay; **Plate A**: **a**. 1%DMSO **b.** Flucanozole (0.020 mg/ml) **c-e** Labdane diterpene (0.020 mg/ml, 0.010 mg/ml, 0.005 mg/ml)' **Plate B**: **a**. Flucanozole (0.020 mg/ml) **b-e** Labdane diterpene (0.0025 mg/ml, 0.0125 mg/ml, 0.000625 mg/ml, 0.0003125 mg/ml)

Table 5B 2. 1 Inhibitory efficacy of different concentrations of labdane diterpene dialdehyde against *C. albicans*

Sr. No.	Concentration of labdane diterpene (mg/ml)	Zone of inhibition (mm)
1	0.020	15.95±0.5
2	0.010	14.35±1.85
3	0.005	12.1±1.1
4	0.0025	8.25±1.4
5	0.00125	6.11±0.55
6	0.000625	3.07±1.25
7	0.0003125	2.0±1.02

Zone of inhibition of flucanozole (0.020 mg/ml) is 21.25±1.25 mm

5B 2.3.1.2 FESEM analysis

FESEM was used to observe the morphological changes in *C. albicans* caused by the treatment of labdane diterpene. FESEM images of the untreated fungus showed a well defined and smooth cell surface **(Fig. 5B 2.2 A)**, whereas cell shrinkage and membrane disintegration was observed in the fungal cells treated with the compound **(Fig. 5B 2.2 B-C)**. However, no sign of poration or lysis was seen. These findings indicated that labdane diterpene caused some amount of cell distortion but no pore formation in the cell wall of the fungus. Whether the fungus is killed or damaged completely by the membrane damage needs to be investigated further.

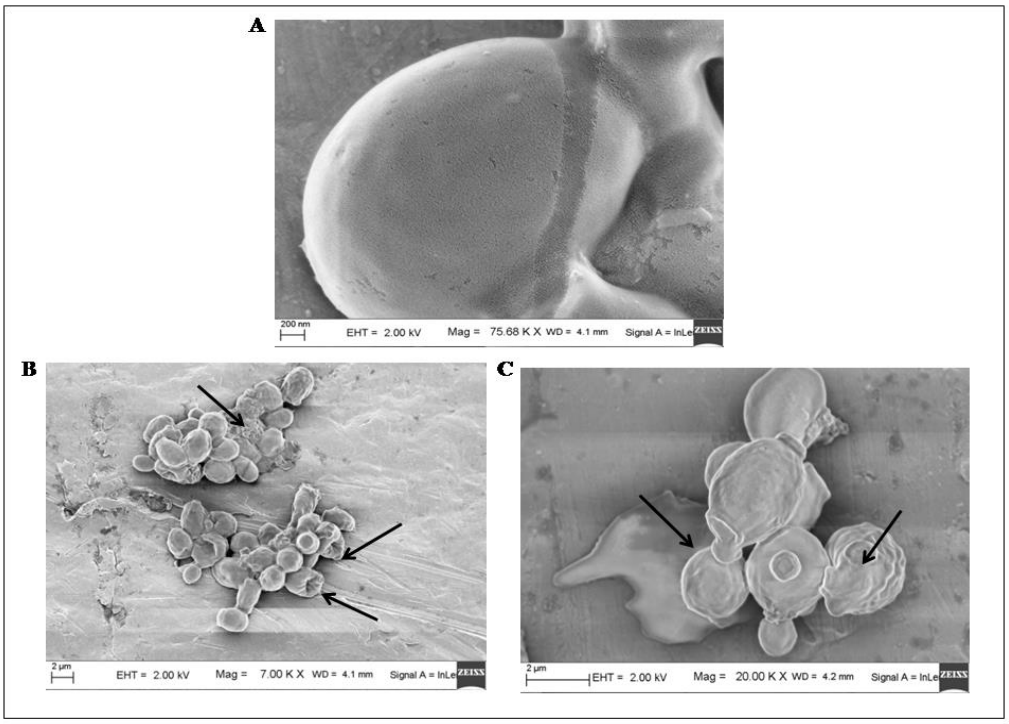


Fig 5B 2. 2 FESEM images of **(A)** untreated and **(B-C)** treated *C. albicans* with labdane (0.020 mg/ml); arrows indicate distorted morphology of cells upon treatment

5B 2.3.1.3 Raman spectroscopy

Raman spectroscopy gives detailed information about the composition of the fungal cell and provides a fingerprint region below 1800cm^{-1} (Movasaghi et al. 2007). **Fig 5B 2.3** shows the Raman spectrum of the *C. albicans* with and without treatment of labdane diterpene. Sharp peaks at 1289 cm^{-1} and 1342 cm^{-1} corresponds to amino acids. Very low intensity peaks from 792- 852 cm^{-1} were attributed to the nucleotides: nitrogenous bases and O-P-O stretch of DNA (Chouthai et al. 2015). Peak at 1003 cm^{-1} corresponds to glucose. The sharp peak at 1378 cm^{-1} corresponds to nucleic acid and amide III band of

tryptophan. The peak at 1437 cm^{-1} was attributed to the presence of δCH_3 group. It was observed that the treated *Candida* cells also exhibited the above mentioned peaks but there was a sharp decline in the prominent peak intensities. This decrease in intensity after treatment with the compound might be due to due to lack of cell growth as cell damage or destruction was not observed.

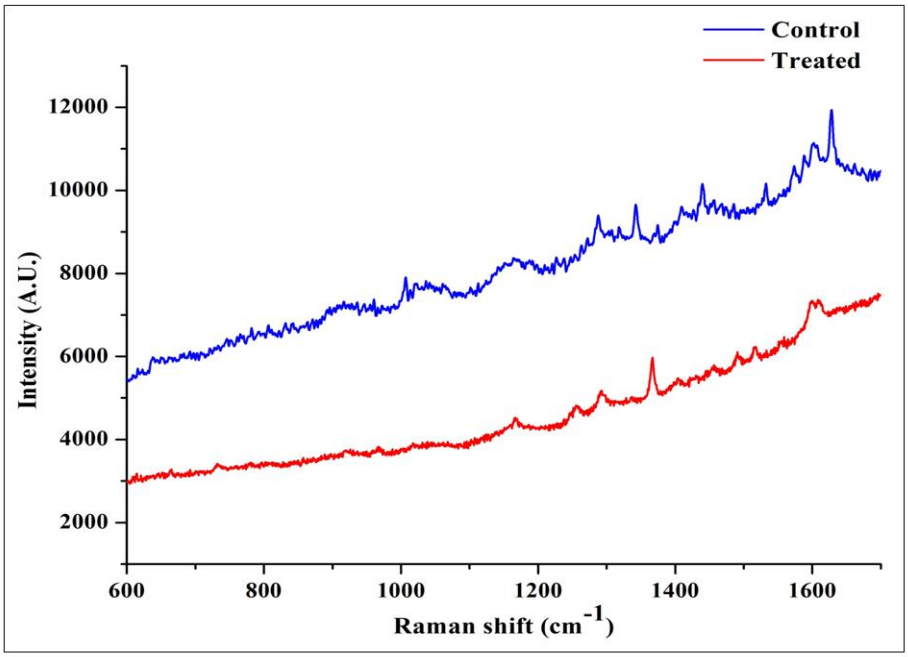


Fig 5B 2. 3 Raman spectra of *C. albicans* showing antagonistic activity of labdane diterpene

5B 2.3.1.4 Cell leakage analysis

It is known that nucleotides, purines, pyrimidines "leak" from compromised fungal cells and the release of such moieties are determined by measuring optical density at 260 nm. It was observed that there was no change in the amount of low molecular weight metabolites and they remained constant with increasing time of treatment **(Fig 5B 2.4)**. This indicated that there was no release of metabolites from the yeast cells; the amount of released metabolites being comparable to the untreated cells. This further added to the observation that no cell lysis or surface pore formation occurred upon compound treatment; hence cell materials were not released.

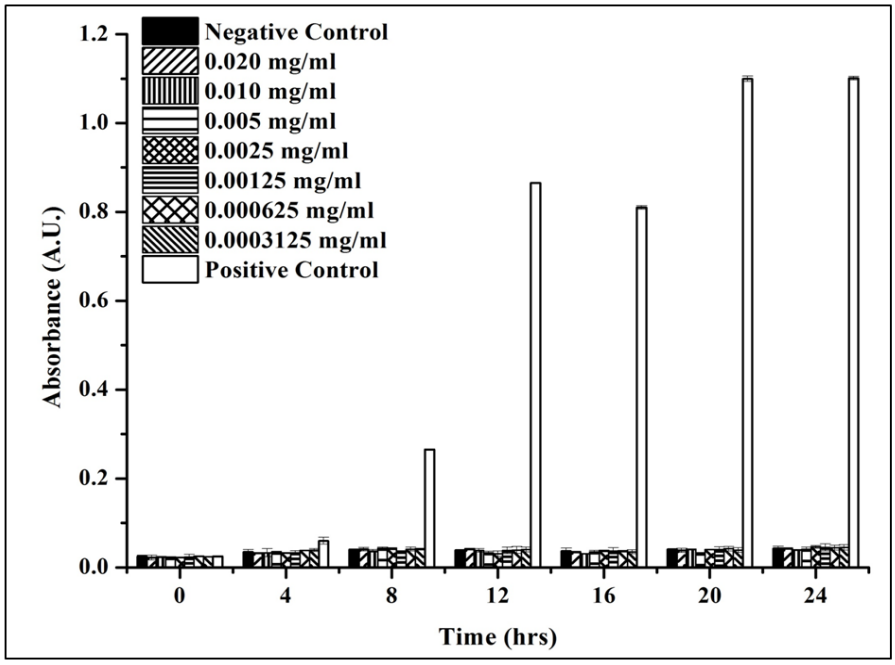


Fig 5B 2. 4 Absorbance of cell materials at 260 nm released from *C. albicans* after treatment with labdane diterpene at 0, 4, 8, 12, 16, 20 and 24 hrs

5B 2.3.1.5 DO analysis

Uptake of oxygen by *C. albicans* was studied to elucidate the possible mode of action of the labdane diterpene. It was observed that the amount of dissolved oxygen remained constant in the treated cells when compared with untreated fungal culture. Amount of dissolved oxygen in the media reduced < 0.7 mg/l in the untreated cells whereas in the treated cells, it remained almost equal to its initial concentration (> 7 mg/l). This suggested that the respiration rate and, as a result the growth of the *Candida* cells, was inhibited under the effect of labdane diterpene **(Fig 5B 2.5)**. It can be speculated that labdane diterpene, like all plant derived diterpenes, might interact with certain membrane proteins or enzymes like ATP- binding cassette (ABC) transporters, acetylcholine receptor and protein transferases which are crucial for the fungal growth (Eterović et al. 1993; Seo et al. 2012).

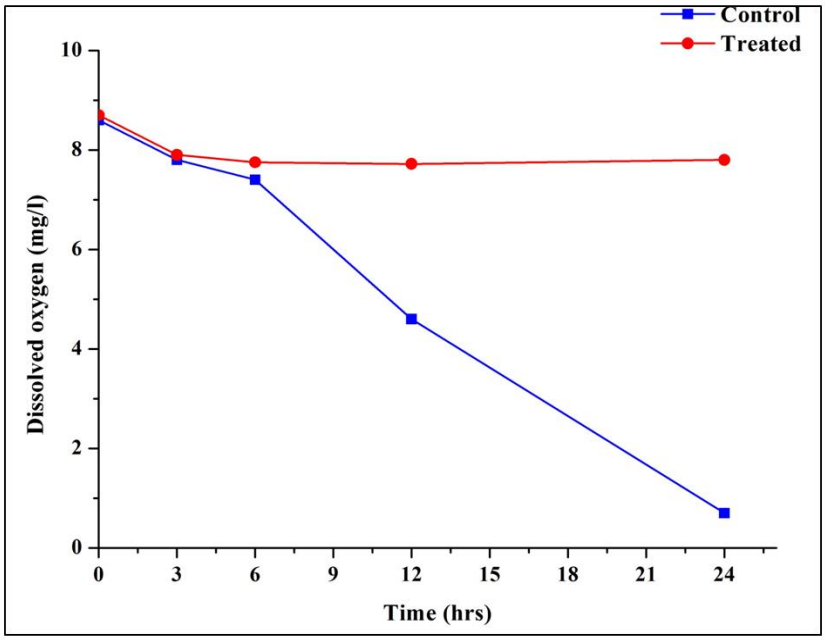


Fig 5B 2. 5 Changes in the respiratory activity of *C. albicans* with respect to time after treatment with labdane diterpene

5B 2.3.2 Kinetic experiment

5B 2.3.2.1 Growth curve analysis

The optical density (OD) values are directly proportional to the turbidity of microorganism in suspension and thus, represent relative quantity of microorganism present under certain experimental condition (Ma et al. 2014). The results showed that the lag phase of *C. albicans* was prolonged in a dose dependent manner after treatment, in comparison to the untreated fungus. The fungus entered into log phase of growth within 8-12 hrs of treatment with low concentrations of the compound (0.000312 mg/ml – 0.0025 mg/ml); whereas in case of treatment with higher concentrations (0.005 mg/ml – 0.020 mg/ml), log phase was attained only after 18-24 hrs of treatment **(Fig. 5B 2.6 A)**. This indicated that the growth of *C. albicans* was inhibited in a dose dependent manner and the inhibitory effect was immediate. **Fig. 5B 2.6 B** showed the effect of a fixed concentration of compound (0.020 mg/ml) on the growth of fungus under different concentrations of substrate. *C. albicans* treated with the antibiotic flucanozole at a concentration of 0.020 mg/ml and 1% DMSO were used as positive and vehicle controls respectively.

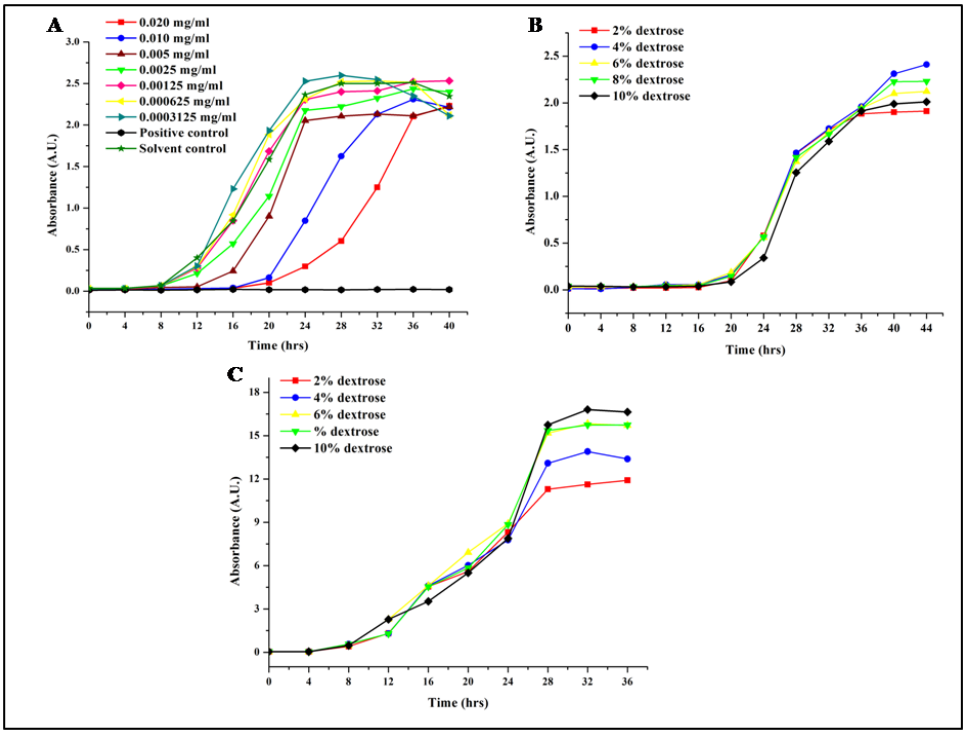


Fig 5B 2. 6 Growth profile of *C. albicans* **(A)** at different concentrations of labdane diterpene in the presence of fixed initial substrate concentration (2% dextrose); **(B)** in the presence of labdane and **(C)** in the absence of labdane at different initial substrate concentration

It was seen that varying the substrate concentration had no affect on the inhibition of fungus growth by the compound. At all the substrate concentrations, *C. albicans* entered log phase within 20-24hrs. A control experiment was also set up at varying substrate concentrations without treatment **(Fig. 5B 2.6 C)**. It was observed that in the absence of compound, *Candida* entered log phase within 8 hrs of inoculation; hence no substrate dependent inhibition was observed. This further confirmed that labdane diterpene retarded or stalled the growth of the fungus irrespective of the substrate concentration. These results lead to a hypothesis that, like many *Candida* species, the pathogen might possess the ability to produce some surfactants (Sobrinho et al. 2008) which may be responsible for the removal of the oily labdane diterpene from its vicinity and override its inhibitory effect.

5B 2.3.2.2 Specific growth rate

The specific growth rate of the organism dropped from 0.297 h^{-1} to 0.13 h^{-1} when treated with the compound at a concentration of 0.020 mg/ml **(Fig 5B 2.7 A)**. This decrease in specific growth rate (< 0.2 h^{-1}), when treated with a concentration of ≥ 5µg/ml, was

attributed to the retarding or temporary fungistatic effect of labdane diterpene. When checked for substrate inhibition in the presence of a high concentration of compound (0.020 mg/ml), it was seen that at the highest substrate concentration (10% dextrose), the specific growth rate was 0.604 h^{-1} whereas it was 1.115 h^{-1} at the lowest concentration (2% dextrose) **(Fig 5B 2.7 B)**. However, in the absence of the compound, there was no substrate-dependent inhibition on the growth kinetics of the fungus **(Fig 5B 2.7 C)**; the specific growth rate increased from 0.312 h^{-1} at 2% dextrose to 0.484 h^{-1} at 10% dextrose. This behavior exhibited by the pathogen was in contradiction to "substrate dependent growth inhibition" observed in such organisms (Dutta et al. 2015). Thus, it can be inferred that along with the inhibitory effect labdane diterpene, substrate inhibition (which is otherwise absent) became evident on the growth pattern of the organism.

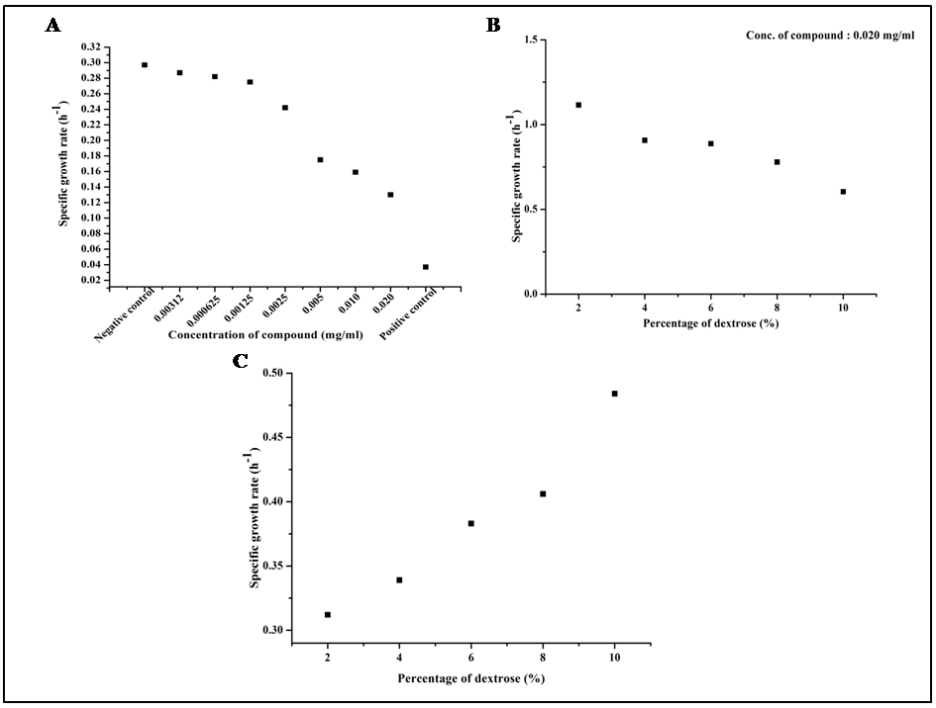


Fig 5B 2. 7 Specific growth rate **(A)** as a function of labdane concentration; **(B)** in the presence fixed concentration of labdane and **(C)** in the absence of labdane diterpene as a function of substrate concentration; ($R^2 \geq 0.96$)

5B 2.3.2.3 Substrate uptake

The concentration of substrate in the medium at any given time can be taken as a measure of the growth of the organism. The concentration of substrate decreased as the organism multiplied in the presence of different concentrations of the compound **(Fig 5B 2.8 A)**. Concentration of substrate in the medium was comparable to the growth stage of the organism. Substrate was completely utilized by the treated cells (0.005-0.020 mg/ml)

within 28-36 hrs, which was in contrast to untreated cells where substrate concentration became negligible within 20 hrs itself. The same trend was observed when different concentrations of substrate were used in the presence of fixed concentration of compound (0.020 mg/ml) **(Fig 5B 2.8 B)**. In the absence of labdane diterpene, the organism started utilizing the substrate immediately for its growth irrespective of its initial concentration, further confirming the absence of substrate inhibition kinetics **(Fig 5B 2.8 C)**.Thus, it was seen that the labdane diterpene delayed the growth of *C. albicans* in a dose dependent manner by preventing its substrate uptake.

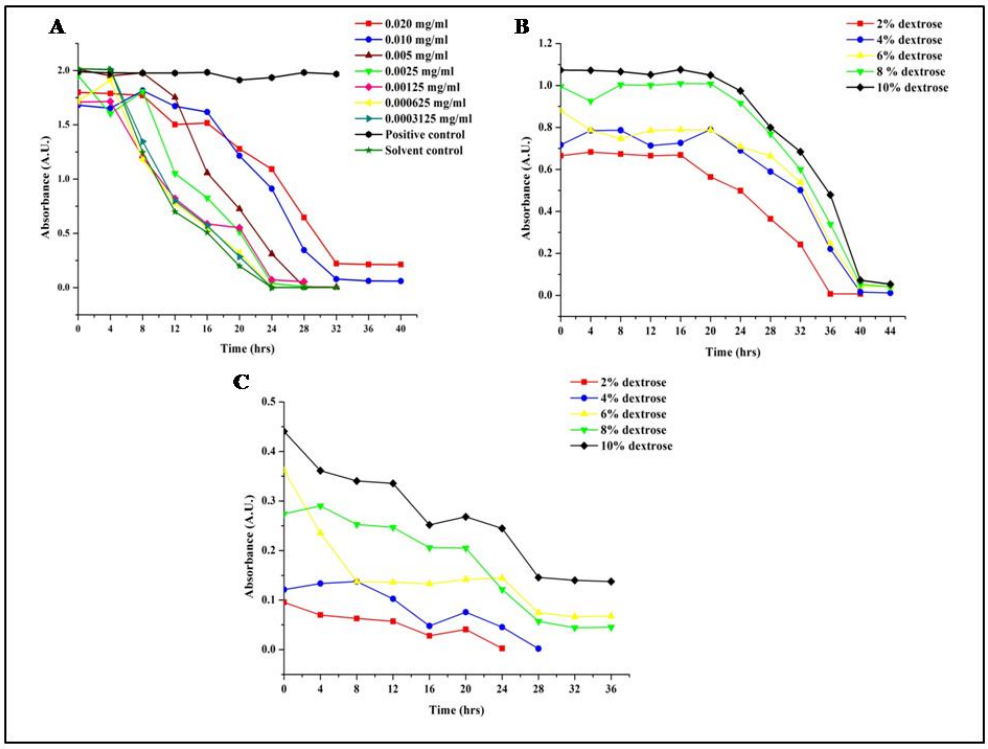


Fig 5B 2. 8 Substrate uptake for growth by *C. albicans* **(A)** at different initial labdane concentration; **(B)** in the presence of fixed concentration of labdane and **(C)** in the absence of labdane at different initial substrate concentration

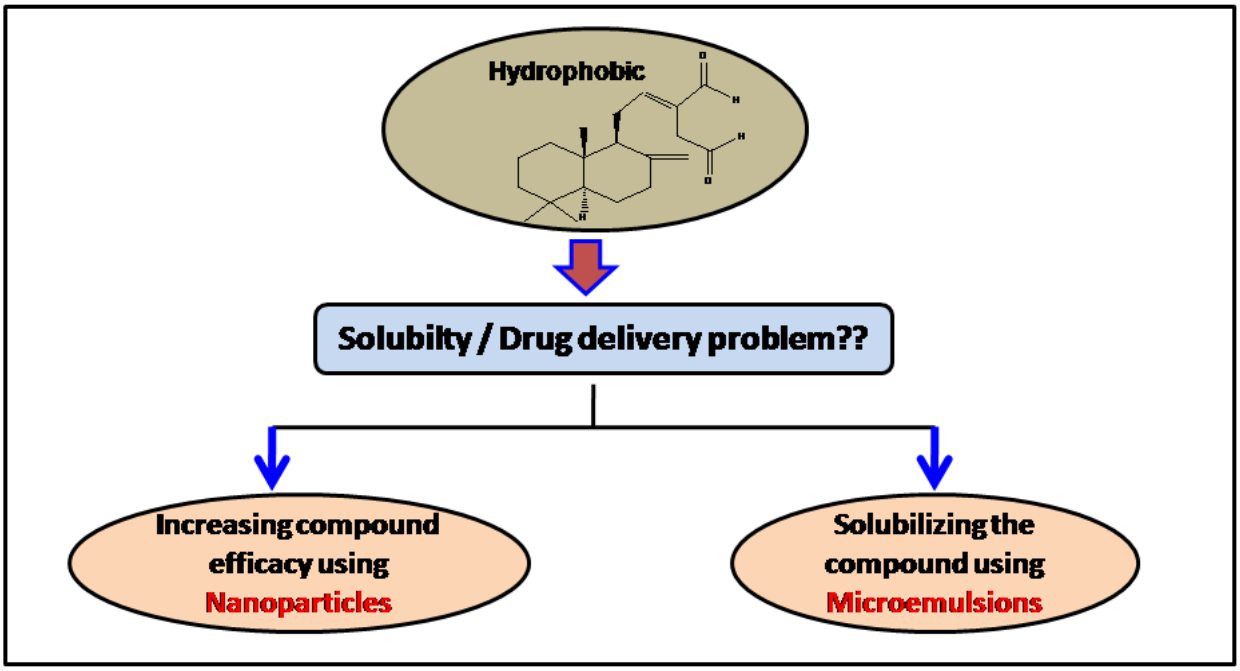
5B 2.4 Conclusion

(E)-labda-8(17), 12-diene-15, 16-dial, isolated from seeds of *A. nigra* in good quantity, has an antagonistic effect on the growth of *C. albicans*. The minimum effective concentration of the compound against *C. albicans* was found to be 0.000312 mg/ml by agar well diffusion. Depressions and distortions on the cell surface of the fungus was observed upon treatment. Growth curve analysis indicated that the compound prolonged the lag phase of *C. albicans* in a dose dependent manner and this effect ceased after ~24hrs. Different analyses to understand its mechanism of action led to the conclusion

that the labdane diterpene not only caused cell surface changes of *C. albicans* but also prevented substrate uptake by the organism leading to its growth inhibition.

These findings provide a new and interesting insight in understanding the biological efficacy of this naturally occurring diterpene towards the development of herbal medicines for the treatment of candidiasis in the future.

Chapter 6: Formulating a strategic approach for the delivery of the isolated compound as a potential antibacterial agent



This chapter addresses the problem of aqueous solubility associated with the hydrophobic labdane diterpene and provides possible solution to this by using nanoparticles and microemulsions

Chapter 6: Formulating a strategic approach for the delivery of the isolated compound as a potential antibacterial agent using:

A) Metallic nanoparticles

6A.1 Introduction

Discovery and development of new drugs is a matter of great challenge for the pharmaceutical industry. Not only the regulatory mechanisms have become more stringent but the growing concern about safety has also increased manifold. The pharmaceutical industry has opted for and implemented new and useful strategies for increasing the R&D productivity (Ruffolo 2006). One of the most important strategies is to incorporate the screening of drug-like properties early in the drug discovery process.

Traditional design of pharmaceuticals was governed solely by the potency of the new lead molecule/compound against therapeutic targets based on the pharmacological screening. Thus, very little information was available on the drug-like properties during the drug development process (Di et al. 2009). As a result, many compounds failed to exhibit efficacy *in vivo*, although they demonstrate good potency *in vitro*. This was mainly because of inadequate drug-like properties (which are usually ignored and note taken in to consideration) like low solubility, poor permeability, high metabolism and strong reflux by transporters (Prentis et al. 1988).

Solubility is one of the most challenging properties in drug discovery. Solubility is the amount of substance that dissolves in a given volume of solvent at a specified temperature. Drug like properties such as solubility are very rarely considered during the process of drug development which leads to many potential drugs being dropped from the race. Various issues like poor oral bioavailability, lack of efficacy, problematic formulations, discrepancies between assays etc. may be associated with the solubility problem of a compound (Di et al. 2009). The therapeutic potential of labdane diterpene has been reported widely but sadly no mention has been made regarding its solubility profile in any aqueous or culture medium. Ignorance of the solubility profile shows how low solubility leads to errors in bioassay such as underestimated activity (Di and Kerns 2006).

Nanotechnology comes as an aid for solving this solubility issue. Nanoparticles (NPs) have a number of advantageous properties like small particle size, increased surface area to volume ratio, improved solubility along with their ability to cross bold-brain barrier (Singh and Lillard Jr 2009). Ideally metallic NPs such as Au, Ag, Pt, Pd etc. are used as a therapeutic agent as they have a very prominent effect against microorganisms (Schrand et al. 2010; Wernicki et al. 2014). Metallic NPs possess inherent toxicity and high electrostatic properties, hence, the concentration of the NPs along with the stabilizers used for their synthesis have to be maintained such that they are biocompatible and non toxic (Buzea et al. 2007). In the recent times, the use of plant materials, microbes, phytochemicals etc. as reducing agents for the synthesis of nanoparticles has been gaining much importance (Krishnaraj et al. 2010; Lee et al. 2014). The use of NPs in targeted drug delivery system as a conjugate to a drug and as a vector to ligate to specific targets has a tremendous overall effect of it as an effective drug (J 2015). Nanotechnology is a rapidly emerging field and pharmaceutical sciences are using NPs to reduce the toxicity and side effects of potential drugs (De Jong and Borm 2008). Novel properties of NPs like small size, customized surface, improved solubility and their multi-functionality along with its ability to cross blood brain barrier has opened avenues to target, diagnose and treat devastating diseases like cancer (Singh and Lillard Jr 2009). A number of metals like Au, Ag, Cu etc. are used in the synthesis of NPs and used as imaging probes and delivery vehicles, along with polymers and semiconductors (Prabhu et al. 2011).

Thus, this part of the present chapter focuses on using metallic NPs for targeted *in-vivo* delivery of the hydrophobic labdane diterpene and thus, solving its problem of aqueous solubility. In addition, the compatibility of the diterpene, combined with metal oxide NPs with human erythrocytes and the antibacterial potential of this nano-composite has also been studied.

6A.2 Materials and methods

6A.2.1 Solubility assay: % transmittance method

6A.2.1.1 Preparation of labdane (stock 1)

An initial stock solution of concentration 10000 µg/ml was prepared by dissolving 10 mg of labdane diterpene in 1 ml of DMSO.

6A.2.1.2 Assay

The various dilutions required for the solubility assay for the labdane diterpene in water and nutrient broth (NB) were made as given in **Table 6A.1**. The corresponding blanks and 200 µl of each dilution were loaded into separate wells of a flat-bottomed 96-well microtiter plate. The absorbance of the samples was measured at 620 nm and later converted to % transmittance. A plot of labdane diterpene concentration *vs* % transmittance was made to determine the solubility of the compound in the aqueous and NB culture media used.

Table 6A. 1 Sample preparation for solubility test of labdane diterpene

Stock 1 conc. (µg/ml)	Dilutions from Stock 1		Stock 2 conc. (µg/ml)	Dilutions for solubility assay in								Final labdane diterpene conc. (µg/ml)
				Water				NB medium				
				With 2% DMSO		With 5% DMSO		With 2% DMSO		With 5% DMSO		
	Stock 1 (µl)	DMSO (µl)		Stock 2 (µl)	Water (µl)	Stock 2 (µl)	Water (µl)	Stock 2 (µl)	Medium (µl)	Stock 2 (µl)	Medium (µl)	
10000	2	38	500	4	196	10	190	4	196	10	190	10
	4	36	1000									20
	6	34	1500									30
	8	32	2000									40
	10	30	2500									50
	12	28	3000									60
	14	26	3500									70
	16	24	4000									80
	18	22	4500									90
	20	20	5000									100
	22	18	5500									110
	24	16	6000									120
	26	14	6500									130
	28	12	7000									140
	30	10	7500									150
	32	8	8000									160
	34	6	8500									170
	36	4	9000									180
	38	2	9500									190
	40	0	10000									200

6A.2.2 Synthesis of NPs

Copper and silver NPs (CuNPs and AgNps) were synthesized by the pulse laser ablation in distilled water. The 2nd harmonic of a high power Q-switched Nd:YAG laser (Litron LPY7864-10G) was focused using a lens of 25 cm focal length, on a copper target immersed in distilled water. The incident laser energy was maintained at 30 mJ, and the ablation of the target was continued for duration of 60 mins. The repetition rate of the laser was fixed at 10 Hz. The target was continuously moved during the ablation, to avoid the formation of a crater, and hence facilitating the uniform ablation, on a shot to shot basis. The initial concentration of the CuNPs and AgNPs were 0.34 mg/ml and 0.2 mg/ml respectively.

6A.2.3 Characterization of NPs

6A.2.3.1 Transmission electron microscopy (TEM)

To evaluate the association of the copper and silver NPs with the compound, TEM analysis of the samples (NPs with and without treatment) were carried out. The sample was diluted with de-ionized (MilliQ) water and sonicated for about 2 hrs. About 10 µl of this sonicated sample was drop cast on carbon-coated copper grids, and the solvent was allowed to evaporate under visible light. The residue was viewed using an FETEM (JEOL JEM-2100F), at an accelerating voltage of 200 kV.

6A.2.3.2 FESEM

The size and morphology of the NPs along with their association with labdane were determined by FESEM analysis. Around 10 µl of the sonicated mixture of CuNPs/AgNPs and labdane was drop cast on a clean glass slide (covered with foil) and the solvent was allowed to evaporate under visible light. The materials were then coated with gold and analyzed through FESEM (Carl Zeiss Ultra 55) at 3.0 kV.

6A.2.4 Biological studies

6A.2.4.1 Hemato-compatibility studies

6A.2.4.1.1 Preparation of test samples

Qualitative and quantitative hemolytic activity assays was performed for different concentrations of NPs and their combinations with labdane diterpene. Approximately 2% ethanol in 1X PBS (pH 7.4) was used as carrier solvent for all the sample preparations as pure water is hemolytic (Krumbhaar 1914). Serial dilutions of NPs were done and a

concentration range of 0.1 mg/ml – 0.02 mg/ml was used for the hemolytic assay. Around 4 different, hemato-compatible concentrations of labdane (0.1 mg/ml – 0.4 mg/ml) as obtained from the previous chapter **(section 5A.3.2)** were used in combinations with the NPs for the hemolytic assay.

6A.2.4.1.2 Hemolytic assay

The qualitative and quantitative hemolytic assays were performed for the different concentrations of NPs and their combinations with labdane as described in the previous chapter **(section 5A.2.1.1** and **5A.2.1.2)**.

6A.2.4.1.3 Microscopic imaging

In order to visually observe the effect of the above test samples on the erythrocytes, the treated RBCs were subjected to light microscopy and FESEM imaging. The RBCs were treated by both the NPs, individually, at a concentration of 0.1 mg/ml in order to observe the kind of damage caused to the erythrocytes. The samples for these microscopic analyses were then prepared and the analysis was performed as described in the previous chapter **(section 5A.2.2** and **5A.2.3)**.

6A.2.4.2 Antibacterial studies

6A.2.4.2.1 Preparation for assay

6A.2.4.2.1.1 Microbial cultures

The antibacterial activity of labdane diterpene was evaluated against 2 Gram negative bacteria, *Escherichia coli* enterotoxic (MTCC 723) and *Salmonella paratyphi* (MTCC 735) and 2 Gram positive bacteria, *Staphylococcus aureus* (ATCC 6538) and *Listeria monocytogens* (ATCC 19115). The bacterial strains were grown and maintained as previously described (Kesari et al. 2010) on nutrient agar plates. Before each experiment the bacteria were sub-cultured twice in NB to ensure their active growth.

6A.2.4.2.1.2 Test sample preparation

A stock of concentration of labdane diterpene was prepared in 5% DMSO (SRL, India) in 1X PBS (pH 7.2; prepared from its constituent salts procured from HiMedia, India and further diluted in 1X PBS. The dilution of labdane was done within a hemato-compatible range (0.006 mg/ml - 0.1 mg/ml), using the results from the previous chapter **(section 5A.3.2)**. Armed with the result of hemolytic assay, CuNPs and AgNPs were diluted in 1X

PBS, within a hemato-compatible range (0.005 mg/ml - 0.1 mg/ml). The above concentrations were used in different combinations for the antibacterial assays.

6A.2.4.2.2 Assay

6A.2.4.2.2.1 Growth curve analysis

The growth curve analysis of the 4 bacteria was performed after treatment with the NPs as per the protocol given in the previous chapter **(section 5B 1.2.2.1)**. This analysis was performed in order to determine a low concentration of the CuNPs and AgNPs, respectively, which does not have any detrimental effect and cause no damage to the bacterial cells.

Having determined the low concentrations of NPs which is compatible/tolerable to the bacterial cells, another growth curve analysis was performed, in a similar fashion, by using different concentrations of labdane in combination with the CuNPs and AgNPs respectively. Using NPs to target the labdane may help to increase the efficacy of the low concentrations of the compound, which were otherwise ineffective, and may result in the killing of the bacterial cells.

6A.2.4.2.2.2 Viability assessment by TTC assay

It was very necessary to determine the viability of the bacterial cells after treatment with the NPs in order to determine the suitable concentration of NPs to be used in combination with labdane diterpene. Metallic NPs are toxic to microorganisms at higher concentrations (Niazi and Gu 2009). Hence, any concentration that is killing or inhibiting the growth of the bacteria would not be suitable for targeting labdane against microbial cells.

Viability assessment of the bacterial cells after treatment with the NPs and a combinations of NPs and labdane was done as per the protocol given in the previous chapter **(section 5B 1.2.2.2).**

6A.2.4.2.2.3 FESEM analysis

FESEM was used to visualize the changes in the morphology of the Gram negative bacteria after treatment with the NPs and the combination of compound and NPs. Actively growing Gram negative bacteria (*E. coli* and *S. paratyphi*) cells were inoculated in NB. Both the bacteria were treated, individually, with low concentrations of CuNPs (0.005 mg/ml), AgNPs (0.002 mg/ml) and a combination of these concentrations with

0.006 mg/ml of labdane. The bacterial cultures were allowed to grow in a shaking incubator (37ºC, 180 rpm; Orbitek, India) for 6 hrs. The cells were, then, centrifuged at 10,000 rpm for 5 mins to separate the cells from the other components. The different samples of the treated Gram negative bacteria were prepared for FESEM as per the protocol given in the previous chapter **(section 5B 1.2.2.3)**.

6A.2.4.2.2.4 Fluorescence microscopy

Extent of bacterial cell damage and lysis after treatment with the different samples (CuNPs, AgNPs, CuNPs + labdane and AgNPs + labdane), at the concentrations mentioned above **(section 6A.2.4.2.2.3)**, was also determined using fluorescence microscopy. Freshly grown Gram negative bacteria were treated with different samples and allowed to grow for 6 hrs at 37ºC and 180 rpm. Post incubation, the bacterial cells were prepared for fluorescence microscopic analysis as given in the previous chapter **(section 5B 1.2.2.3)**.

6A.2.4.3 Candicidal activity

6A.2.4.3.1 Microbial strain

The biological potential of CuNPs and AgNPs were tested against *Candida albicans* (NCCPF no. 400035), which was obtained as a gift from Fungal Biotechnology lab, Dept. of Biosciences and Bioengineering, IIT Guwahati. The fungal strain was grown and maintained as previously described (Kesari et al. 2010) on YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates. Before each experiment the organism was sub-cultured twice in YPD broth to ensure their active growth at 28ºC for 24 hrs.

6A.2.4.3.2 Growth curve analysis

The growth curve analysis of *Candida albicans* (NCCPF no. 400035) was performed after treatment with the NPs as per the protocol given in the previous chapter **(section 5B 2.2.2.6)**. This analysis was performed in order to determine a low concentration of the CuNPs and AgNPs, respectively, which does not have any detrimental effect and cause no damage to the fungal cells.

6A.3 Results and discussion

6A.3.1 Solubility assay: % transmittance method

The solubility assay was carried out using the % transmittance assay. It is based on the principle of scattering of light by insoluble precipitated particles of the compound.

Although its sensitivity is less compared to other high throughput methods like direct UV absorption and chromatographic methods, it is fast, cheap and gives a satisfactory approximate estimate of solubility for the early stages of potential drug hits screening and *in vitro* bioassays (Kerns and Di 2004; Glomme et al. 2005; Dai et al. 2008; Kerns et al. 2008). The permissible concentration of DMSO in a culture medium has always been a topic of controversy, with different types of cells showing varying levels of susceptibility to DMSO. But it is best to keep it at a minimum.

From **Fig 6A.1 (A and B),** it was found that the solubility of the labdane diterpene was ranging from 10 µg/ml at 2% DMSO to 20 µg/ml at 5% DMSO respectively in both water and NB media. Labdane is, therefore, poorly soluble in water or a highly hydrophobic compound.

Higher percentage of DMSO is required to prevent labdane from getting precipitated in order to check its bioactivity at concentrations > 20 µg/ml.

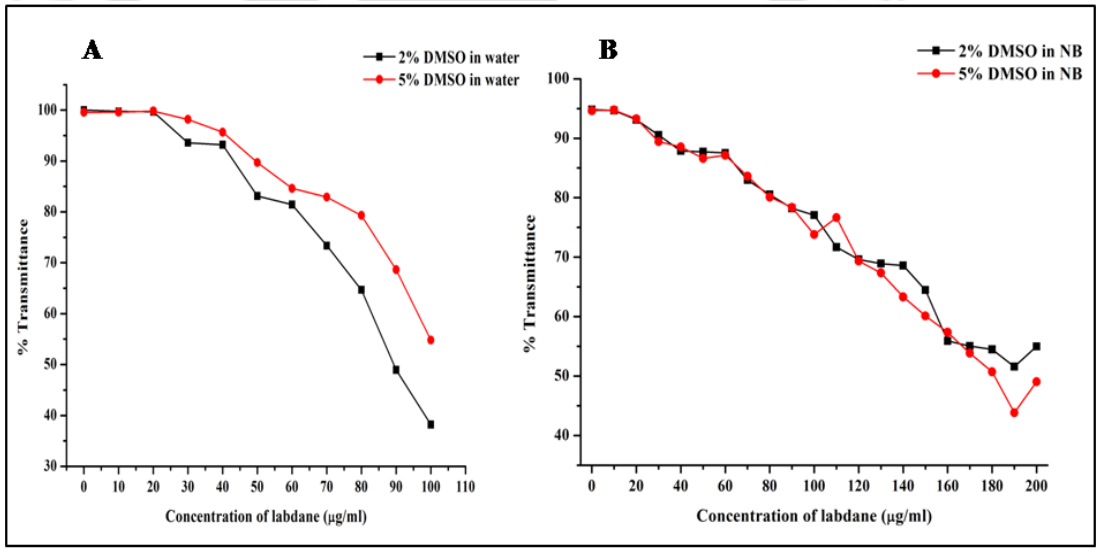


Fig 6A. 1 Concentration vs % transmittance plot for labdane in **(A)** water and **(B)** NB culture media

6A.3.2 Characterization of NPs

6A.3.2.1 TEM

TEM images of the NPs (with and without NPs) are shown in **Fig 6A.2**. **Fig 6A.2.A and C** showed high concentration of small CuNPs and AgNPs respectively that were mono-dispersed in the area of focus. Some AgNPs showed high degree of agglomeration. The NPs treated with compound **(Fig. 6A.2 B and D)** displayed sparse volume of Cu and Ag NPs that were lodged in a droplet of labdane oil. This interaction was mainly a physical

adsorption of the NPs on the labdane oil. This region of interaction gave rise to enhanced Raman spectra (SERS) of the compound in case of the CuNPs, described in Chapter 4 **(section 4.33.1.4.2)**.

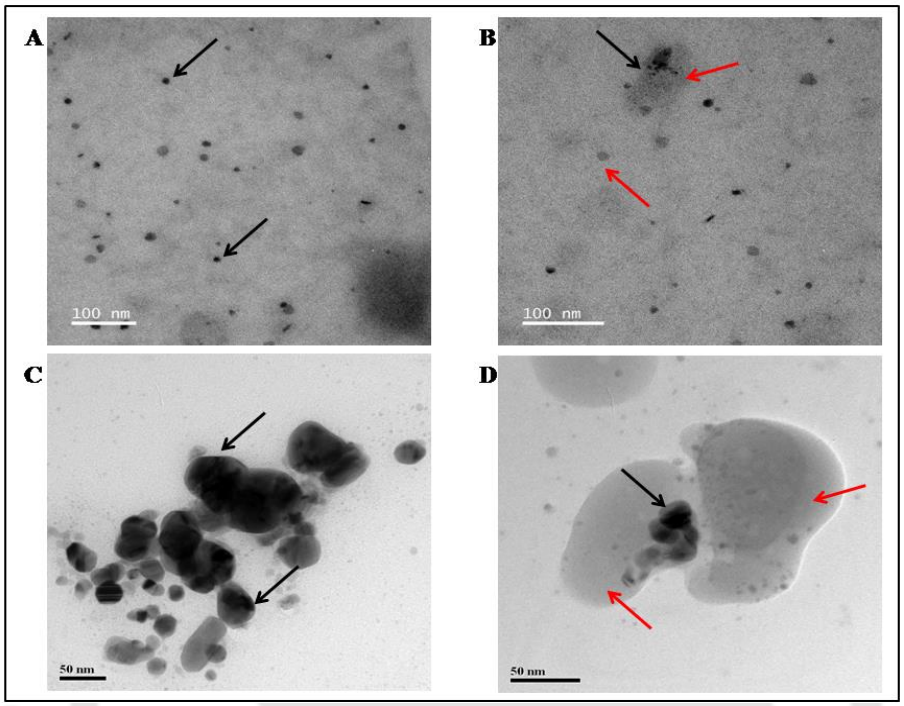


Fig 6A. 2 TEM images of untreated **(A** and **C)** and labdane treated **(B** and **D)** CuNPs and AgNPs; black arrows indicate NPs and red arrows indicate labdane oil droplet

6A.3.2.2 FESEM

FESEM was done to determine the size and morphology of the CuNPs and AgNPs and to visualize their interaction with the compound. FESEM images indicating the size of the NPs are shown below in **Fig 6A. 3**. Images showed round CuNPs and AgNPs, having sizes of 14±0.31 nm and 17.48±0.24 nm respectively **(Fig 6A.3 A and C)**. Smaller the particle size, greater is the surface area-to-volume ratio. Greater surface area makes the drug molecules readily available at surface of particle and enables faster release (Redhead et al. 2001). Hence these synthesized CuNPs and AgNPs are well suited for drug delivery applications. **Fig 6A.3 B and D** displayed NPs that were homogenously distributed with the compound and physically adsorbed on the scattered droplets of labdane oil.

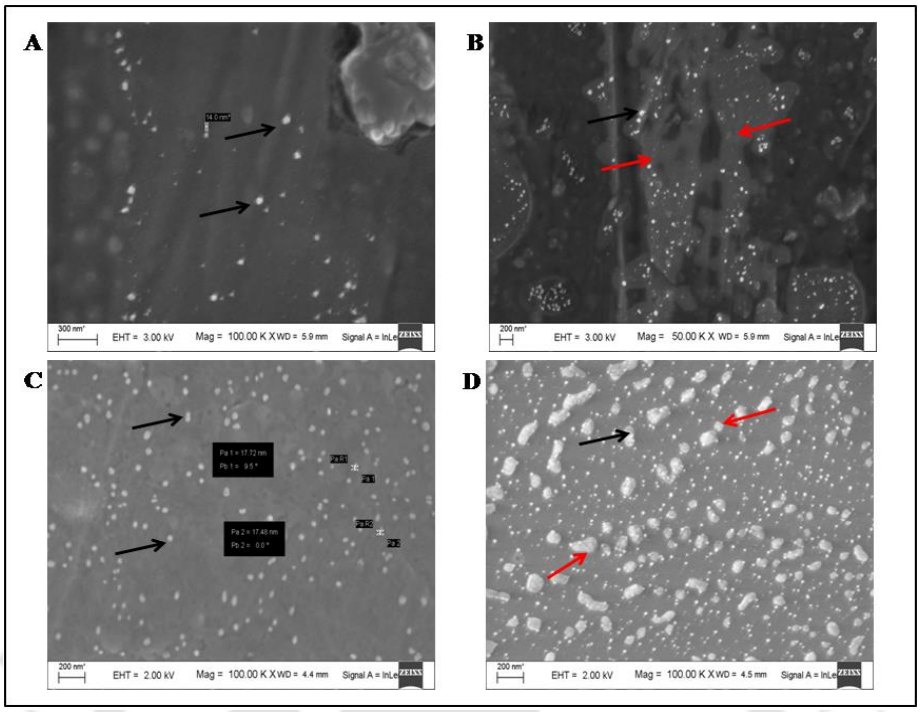


Fig 6A. 3 FESEM images of untreated **(A and C)** and labdane treated **(B and D)** CuNPs and AgNPs; black arrows indicate NPs and red arrows indicate labdane oil droplets

6A.3.3 Biological studies

6A.3.3.1 Hemato-compatibility studies

6A.3.3.1.1 Hemolytic assay

Qualitative hemolytic activity assay was performed on sheep blood agar plates. The hemolytic potential of the NPs and their combination with labdane was determined by the zone of clearance observed on the plates after incubation. The zones of clearance were created by loss of opacity of media at the places where RBCs are disrupted by the samples. Size of clearance zones is directly proportional to the hemolytic potential of the samples. Images of the assay are presented in **Fig 6A.4**. It was observed that neither the NPs nor their combinations used with labdane diterpene showed distinct clearance zones. Clear and prominent zone of clearance formed by Triton X (positive control) validated the efficacy of the assay. Though zones were visible for some samples, they were not measurable which indicated none of the samples possess appreciable hemolytic potential.

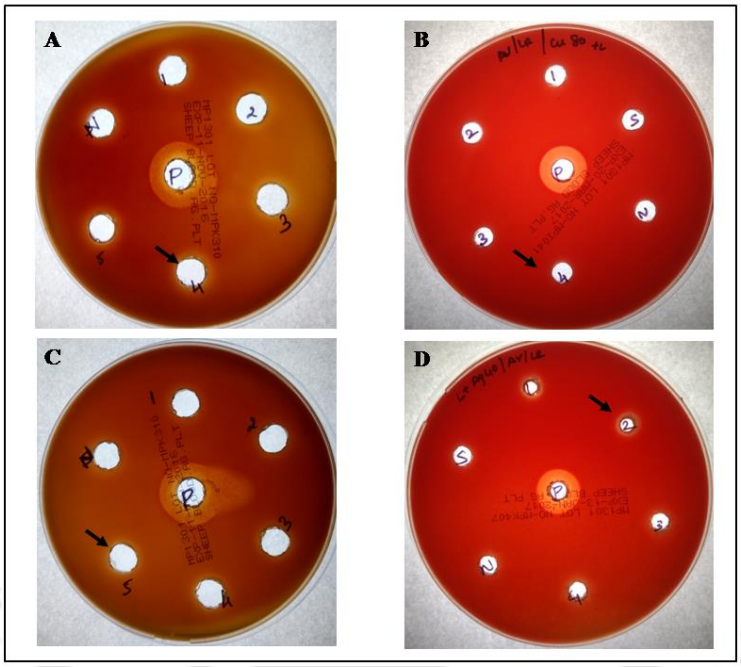


Fig 6A. 4 Sheep blood agar plates treated with CuNPs and AgNPs **(A** and **C)** respectively; 1 – 4 corresponds to 0.02, 0.04, 0.06 and 0.08 mg/ml of corresponding NPs. Plates treated with mixture of labdane and 0.06 mg/ml and CuNPs and AgNPs **(B** and **D)** respectively; 1 – 4 corresponds to 0.1, 0.2, 0.3 and 0.4 mg/ml of labdane. P represents the positive control, N represents the negative control and S represents solvent control

Quantitative hemolytic assay was performed using absorbance method. The amount of hemoglobin released upon treatment due to damage of RBC membrane is the indicator of hemolytic potential of the samples. Total hemolysis was obtained using Triton X and no hemolysis was obtained using 1X PBS. The results are depicted as percentage lysis. As reported previously, any drug causing 0.5% damage to RBCs is suitable for medicinal purpose (Nguyen and Ness 2014). Hemolytic potential of NPs is shown in **Fig 6A. 5**. From the figure **(Fig 6A. 5 A)**, it was observed that the individual NPs i.e. CuNPs and AgNPs were compatible with blood cells at a concentration of $\leq$ 0.08 mg/ml and $\leq$ 0.06 mg/ml respectively. This was in coherence with previous reports that copper oxide NPs showed 100 % hemolysis at a concentration of > 0.08 mg/ml (Chen et al. 2013) while the hemolytic effect of Al_2O_3 NPs increased when their size decreased to 13 nm (Vinardell et al. 2015). Thus, it was evident that NPs used here had less toxicity, were relatively bio-compatible and hence, could be used as carrier for intravenous administration. Equipped with the individual hemolytic potential of labdane and the NPs, study was now carried out to understand effect of labdane along with its carrier NPs on erythrocytes and results are shown in **Fig 6A.5 (B** and **C)**. The optimum

concentration of both the NPs for the different concentrations of labdane was obtained and is tabulated below (**Table 6A.2**). Thus, targeted delivery of labdane into the bloodstream using the suitable concentrations of CuNPs and AgNPs would account for a negligible hemolysis.

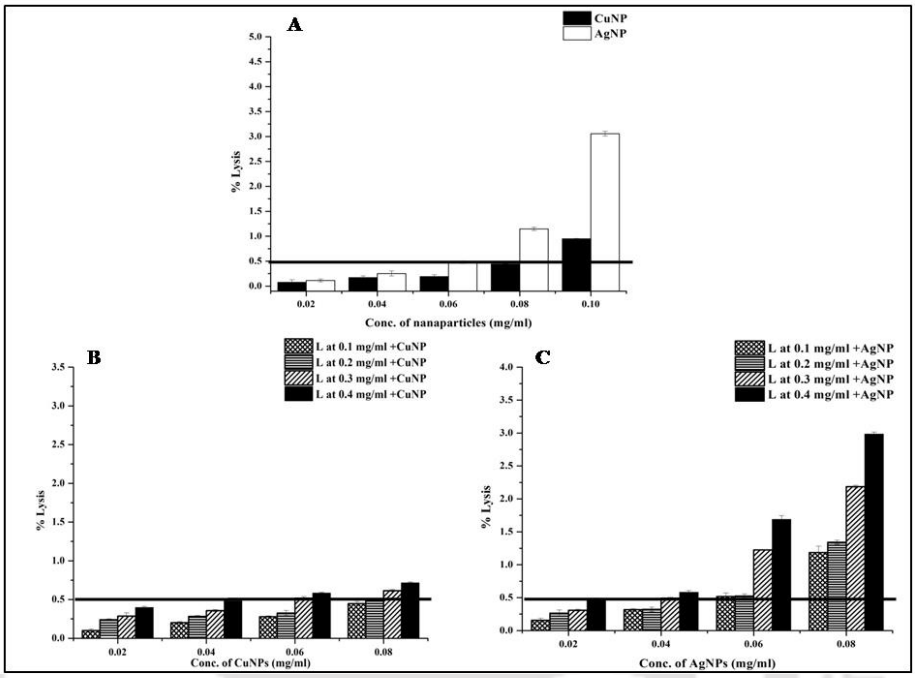


Fig 6A. 5 Percentage lysis of erythrocytes caused by **(A)** CuNPs and AgNPs (0.02 mg/ml to 0.1mg/ ml) **(B)** CuNPs (0.02 mg/ml to 0.08 mg/ml) + labdane (0.1 mg/ml to 0.4 mg/ml) **(C)** AgNPs (0.02 mg/ml to 0.08 mg/ml) + labdane (0.1 mg/ml to 0.4 mg/ml)

Table 6A. 2 Hemato- compatible concentrations of labdane in combination with CuNPs and AgNPs (≤ 0.5% hemolysis)

Sr. No.	Conc. of labdane (mg/ml)	Conc. of CuNPs (mg/ml)	Conc. of AgNPs (mg/ml)
1.	0.1	0.08	0.06
2.	0.2	0.08	0.06
3.	0.3	0.06	0.04
4.	0.4	0.06	0.04

6A.3.3.1.2 Microscopic imaging

The release of hemoglobin obtained during hemolytic assays indicates certain degree of damage (maybe partial or complete) to RBC membrane upon treatment. The type of damage caused by the NPs and their combination with labdane was studied using light

microscopy **(Fig 6A.6)**. Unlike labdane diterpene which caused minute pores on the cell surface of RBCs at higher concentrations, both CuNPs and AgNPs caused cleavage and total lysis of erythrocytes as prominent damage to the cell membrane was observed **(Fig 6A.6 A** and **B** respectively).

The results obtained by light microscopic analysis were confirmed by using FESEM. These high resolution and magnified images further provide a conclusive idea about the effect of NPs and NPs + labdane on RBCs. The images of FESEM analysis are shown in **Fig 6A.6**. Most of the RBCs appeared healthy and intact after treatment. **Fig 6A.6 C** and **D** showed the lysis of erythrocytes upon treatment with CuNPs and AgNPs respectively, indicating that the released hemoglobin was due to rupture of erythrocyte membrane. Appearance of both damaged as well as healthy RBCs from a treated sample clearly indicated the low degree of damage caused to the blood cells upon treatment.

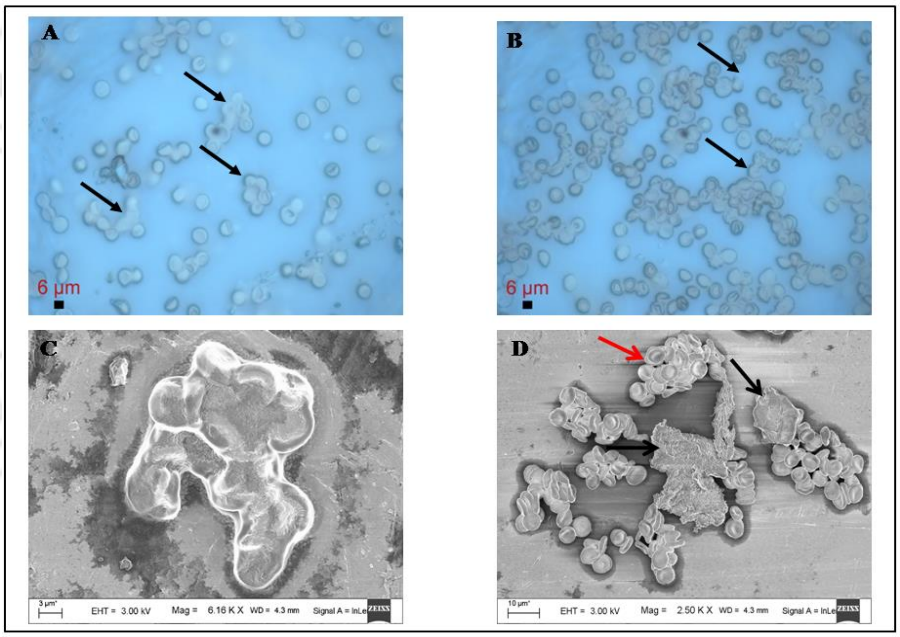


Fig 6A. 6 Light microscopic and FESEM images of RBCs treated with CuNPs (0.1 mg/ml) **(A** and **C)** and AgNPs (0.1 mg/ml) **(B** and **D)** respectively

6A.3.3.2 Antibacterial studies

6A.3.3.2.1 Growth curve analysis

The solubility of labdane in the aqueous media, as observed above **(section 6A.3.1)**, was a problem especially at higher concentrations and tends to get precipitated out from the solution. Hence, it was required to increase the efficacy of lower concentrations of labdane in some way in order to resolve the problem of compound solubility at higher concentrations. Nanotechnology comes as an aid to this. In this case, CuNPs and AgNPs

were used at suitable concentrations to enhance the efficacy of the compound at lower concentrations against bacteria. From **Fig 6A.7** and **6A.8**, it was seen that the metallic NPs were highly effective against Gram negative bacteria; this was is congruence with previous reports (Lara et al. 2010; Schrand et al. 2010). Growth of both the Gram negative bacteria was observed in presence of CuNPs at a concentration of $\leq$ 0.025 mg/ml **(Fig 6A.7 A** and **B)**, while the bacteria exhibited some growth in presence of AgNPs at a concentration of $\leq$ 0.005 mg/ml **(Fig 6A.8 A** and **B)**. However, both the CuNPs and AgNPs were unable to exhibit any inhibition on the growth of the Gram positive bacteria; their growth was equivalent to that of negative control.

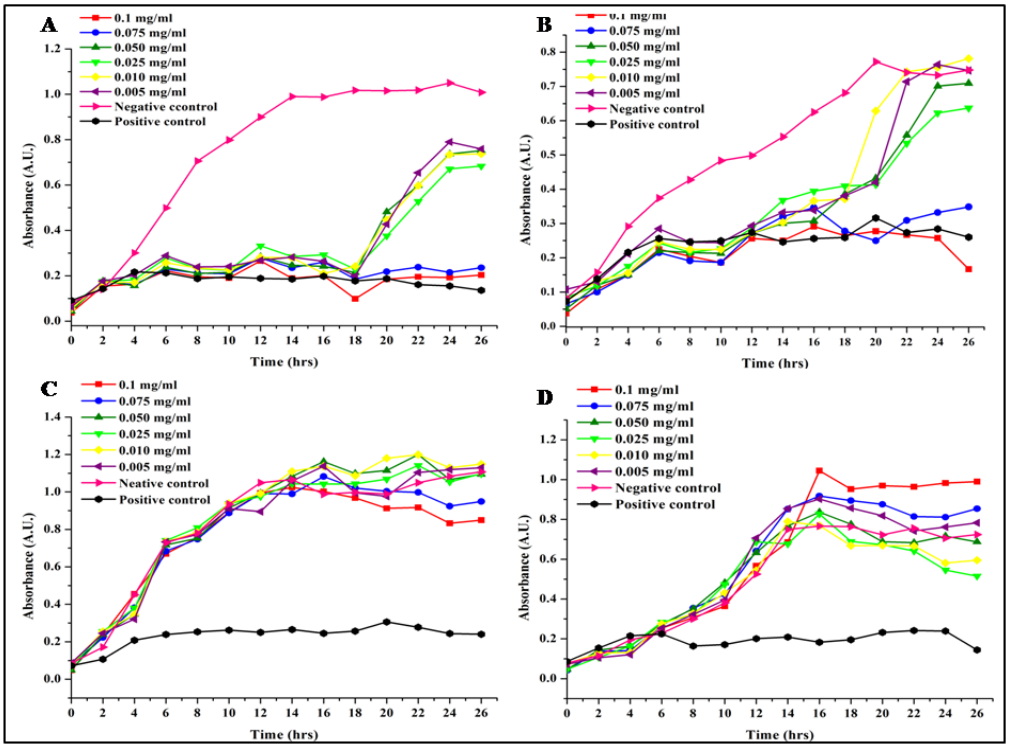


Fig 6A. 7 Growth curve of **(A)** *E. coli* **(B)** *S. paratyphi* **(C)** *S. aureus* **(D)** *L. monocytogens* after treatment with CuNPs

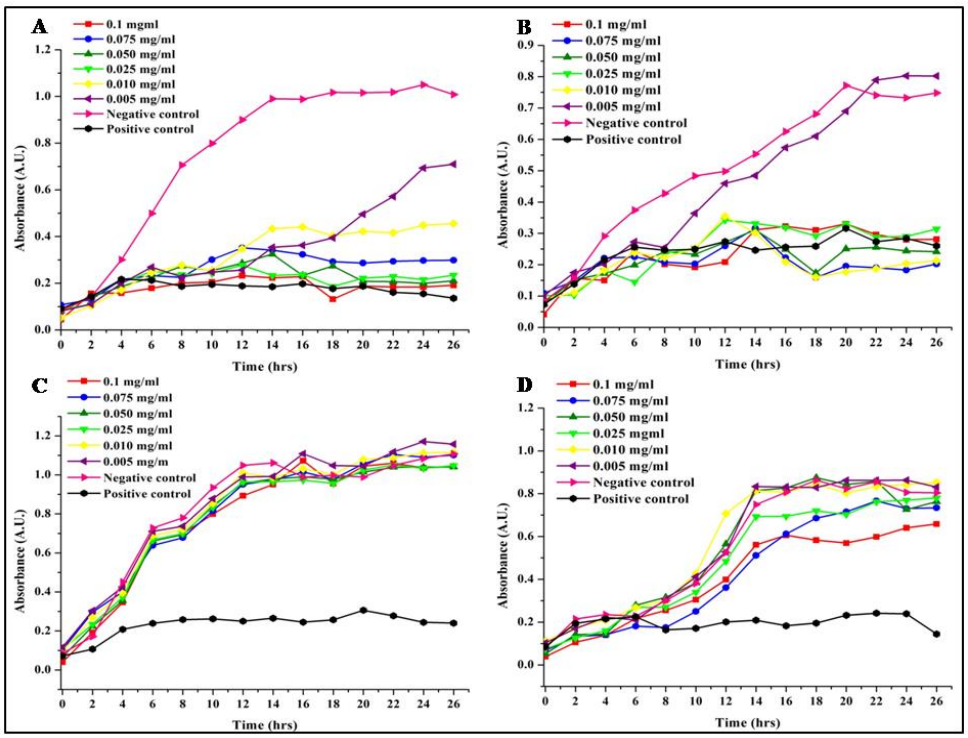


Fig 6A. 8 Growth curve of **(A)** *E. coli* **(B)** *S. paratyphi* **(C)** *S. aureus* **(D)** *L. monocytogens* after treatment with AgNPs

Therefore, various concentrations of labdane were combined with CuNPs and AgNPs (0.005 mg/ml and 0.002 mg/ml) respectively and targeted against the 4 bacteria. From **Fig 6A.9** and **6A.10**, it was observed that the efficacy of labdane increased greatly in combination with the NPs. In combination with CuNPs and AgNPs respectively, labdane was able to inhibit the growth of both *E. coli* **(Fig 6A.9 A** and **Fig 6A.10 A)** and *S. paratyphi* **(Fig 6A.9 B** and **Fig 6A.10 B)** at low concentrations ($\leq$ 0.012 mg/ml), which were not effective when used alone, as observed in the previous chapter **(section 5B 1.3.1)**. Thus, use of NPs helped to solve the issue of solubility of the compound in the media by increasing its efficacy at concentrations which were not previously effective against these bacteria but were soluble in aqueous media. However, even this combination of labdane with the NPs was not able to show any inhibitory effect on the growth of Gram positive bacteria. Since the 2 Gram positive bacteria were not inhibited or killed by the NPs and their combination with labdane, all the further antibacterial assays were carried out for the 2 Gram negative bacteria.

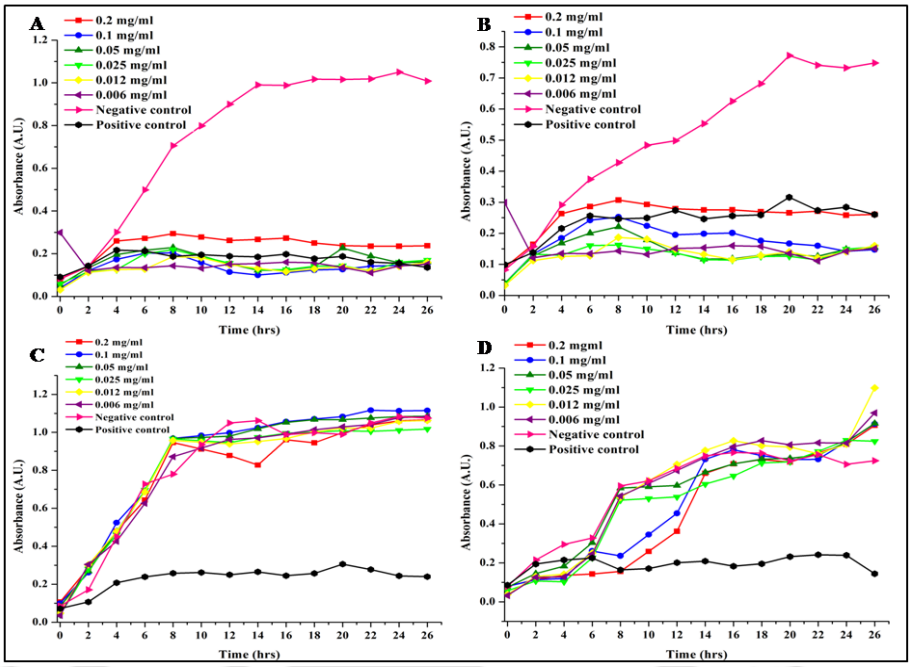


Fig 6A. 9 Growth curve of **(A)** *E. coli* **(B)** *S. paratyphi* **(C)** *S. aureus* **(D)** *L. monocytogens* after treatment with labdane + CuNPs

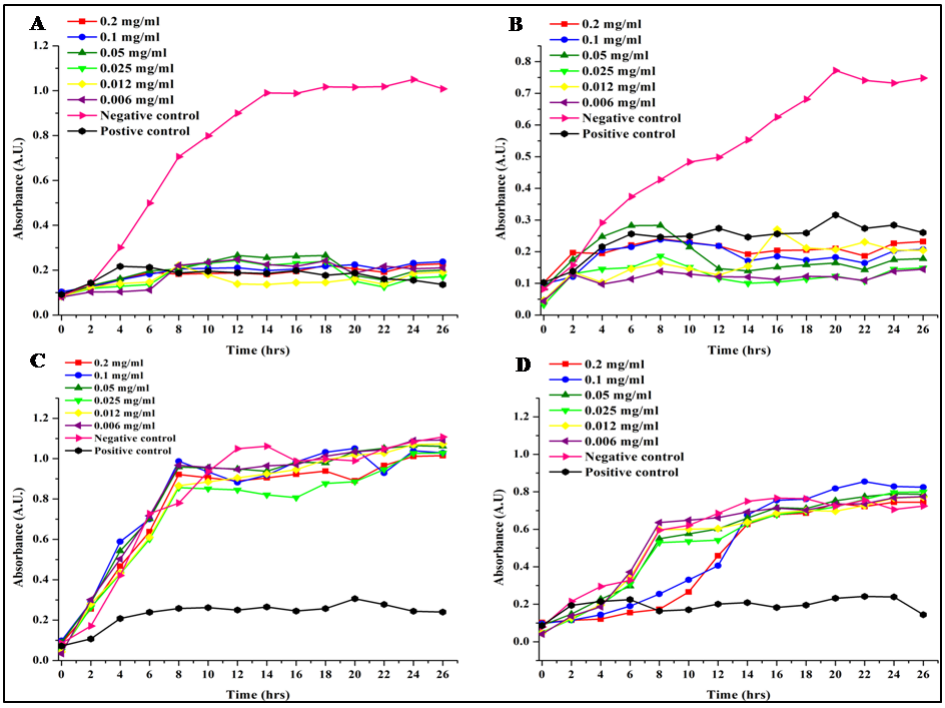


Fig 6A. 10 Growth curve of **(A)** *E. coli* **(B)** *S. paratyphi* **(C)** *S. aureus* **(D)** *L. monocytogens* after treatment with labdane + AgNPs

6A.3.3.2.2 Viability assessment by TTC assay

TTC is a metabolic dye and hence growth conditions of bacteria have to be optimum while using this dye. Insufficient atmospheric and suboptimal growth conditions by

mishandling may lead to inefficient reduction of TTC. Since Gram positive bacteria showed no inhibition of growth upon treatment by NPs, the underlying assays are reported for Gram negative bacteria alone. The viability assessment of the 2 Gram negative bacteria after treatment with NPs and NPs + labdane is shown below in **Fig 6A.11**.

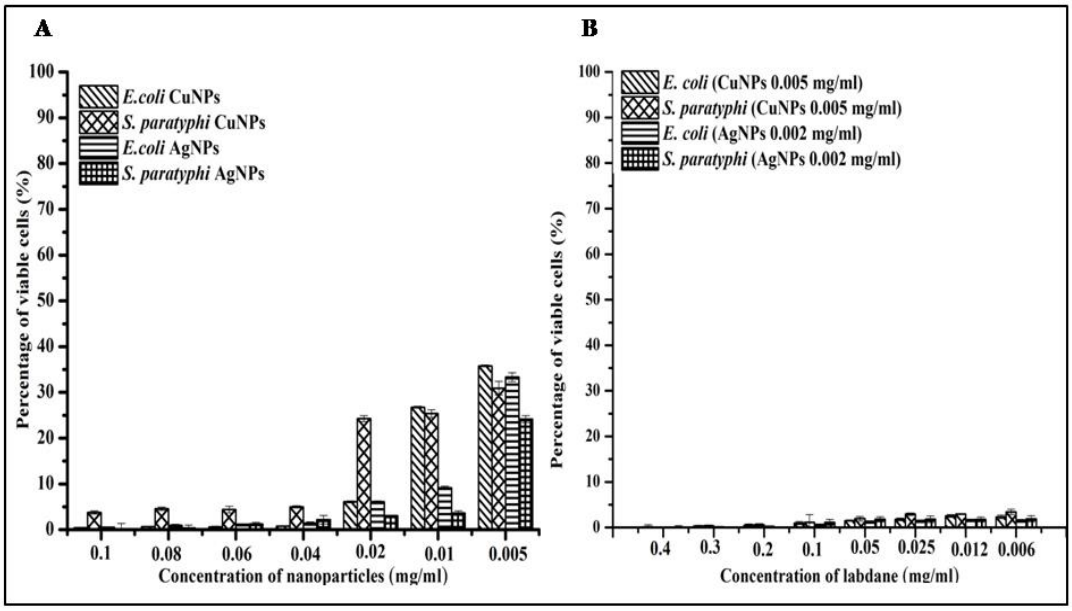


Fig 6A. 11 Percentage of viable Gram negative bacteria after treatment with **(A)** NPs and **(B)** NPs + labdane diterpene

Viability of *E. coli* was affected more than *S. paratyphi* in the presence of NPs **(Fig 6A.11 A)** at higher concentrations. However, viability of both bacteria was less in the presence of AgNPs than CuNPs. This can be very well correlated with the inherent antibacterial property of silver (Lei 2007; Prabhu and Poulose 2012). Both bacteria exhibited dose dependent loss of viability; though loss of viability was more gradual in *S. paratyphi* whereas it was more drastic in case of *E. coli* especially at higher concentrations. But when low concentrations of NPs (0.005 mg/ml and 0.002 mg/ml for CuNPs and AgNPs respectively) were combined with different concentrations of labdane diterpene, a sharp decline in the relative viability was observed **(Fig 6A.11 B)**. Using these combinations, even low concentrations of labdane killed the bacteria which were otherwise in-effective. Thus, not only targeted delivery of labdane was achieved by using the NPs but increased efficacy at water soluble concentrations was obtained.

6A.3.3.2.3 FESEM analysis

The morphological changes of bacterial cells (*E. coli* and *S. paratyphi*) were observed by FESEM upon treatment with NPs and NPs + labdane **(Fig 6A.12)**. The bacterial cells treated with the NPs (0.005 mg/ml for CuNPs and 0.002 mg/ml for AgNPs) were typically rod shaped. Each cell size was almost same and damage on cell surface was not detected **(Fig 6A.11 A-B** and **C-D** respectively**)**. This indicated that the bacterial ells were not damaged by these low concentrations of NPs and they showed intact cell surface morphology. However, in the cells treated with a combination of labdane and NPs (0.006 mg/ml labdane with 0.005 mg/ml of CuNPs and 0.002 mg/ml of AgNPs), irregular fragments and wrinkled cells were seen. This indicated that not only morphological changes of cell surface but also cell fragmentation occurred due to damage of cell membranes **(Fig 6A.12 E-H).** The cells underwent complete lysis and stuck together, forming a gooey mass. Morphological destruction of the Gram negative bacterial cells treated with labdane + CuNPs was feeble **(Fig 6A.12 E** and **G)** than those with labdane + AgNPs **(Fig 6A.12 F** and **H)**. This difference is possibly attributed to the extensive and inherent antibacterial effect of silver over copper metal, which is also in coherence with the previous published reports (Trentin et al. 2011; Nabipour and Rostamzad 2015).

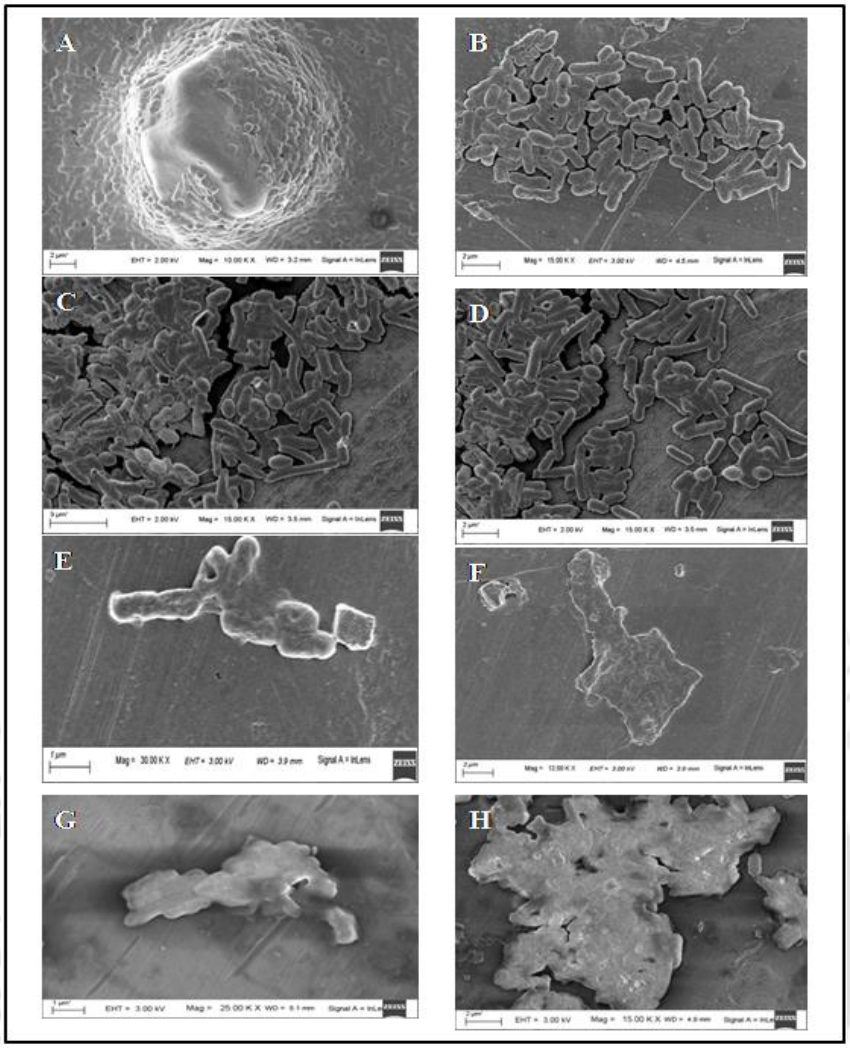


Fig 6A. 12 FESEM images of *E. coli* **(A, B, E** and **F)** and *S. paratyphi* **(C, D, G** and **H)**; bacterial cells after treatment with 0.005 mg/ml of CuNPs **(A** and **C)**; bacterial cells after treatment with 0.002 mg/ml of AgNPs **(B** and **D)**; bacterial cells treated with CuNPs + labdane **(E** and **G)** and bacterial cells treated with AgNPs + labdane **(F** and **H)**; labdane concentration is 0.006 mg/ml

6A.3.3.2.4 Fluorescence microscopy

Viability assessment of bacteria using PI uptake as a measure for dead (PI permeable) and live (PI impermeable) cells are applied in various scopes of research and industry (Shi et al. 2007). Cells stained with PI fluoresce bright red under green excitation (Williams et al. 1998). The fluorescent microscopic images of *E. coli* and *S. paratyphi* upon treatment with NPs and NPs + labdane are shown in **Fig 6A.13**; the corresponding bright field images are show in **Fig 6A.14**.

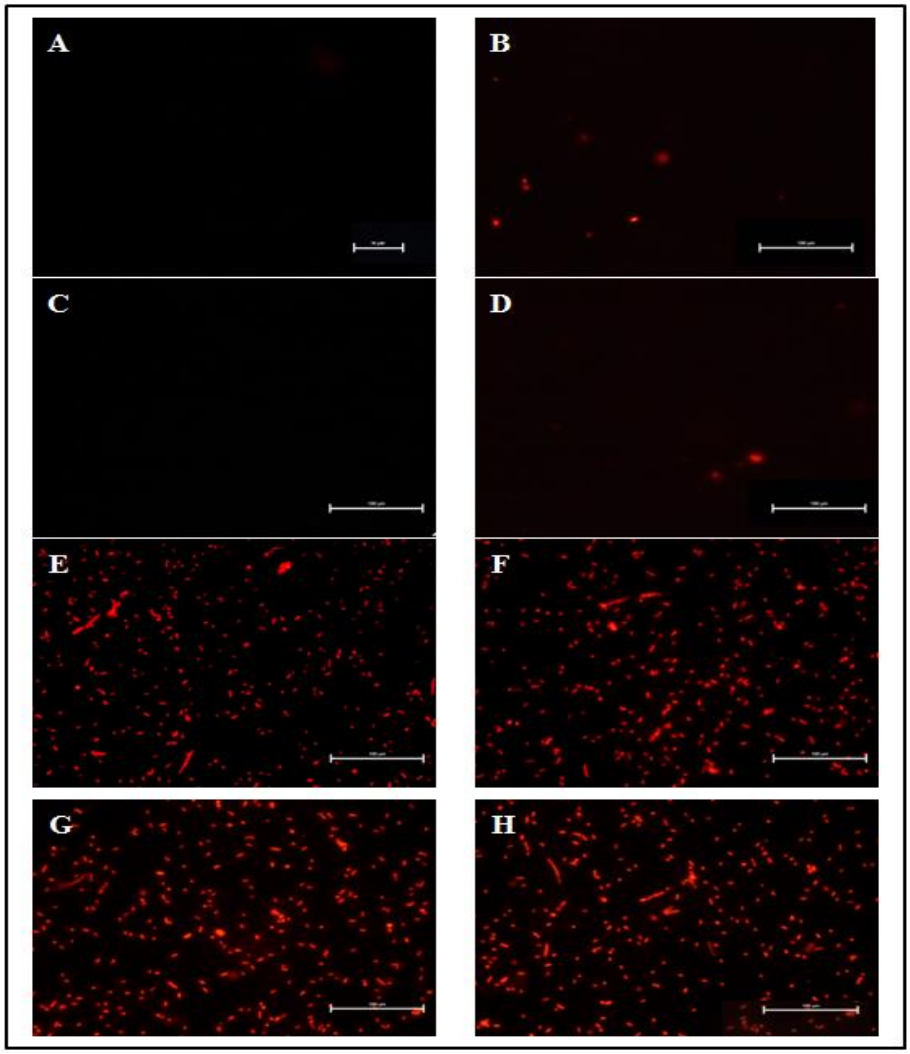


Fig 6A. 13 Fluorescent microscopic images of *E. coli* **(A, B, E** and **F)** and *S. paratyphi* **(C, D, G** and **H)**; bacterial cells after treatment with 0.005 mg/ml of CuNPs **(A** and **C)**; bacterial cells after treatment with 0.002 mg/ml of AgNPs **(B** and **D)**; bacterial cells treated with CuNPs + labdane **(E** and **G)** and bacterial cells treated with AgNPs + labdane **(F** and **H)**; labdane concentration is 0.006 mg/ml

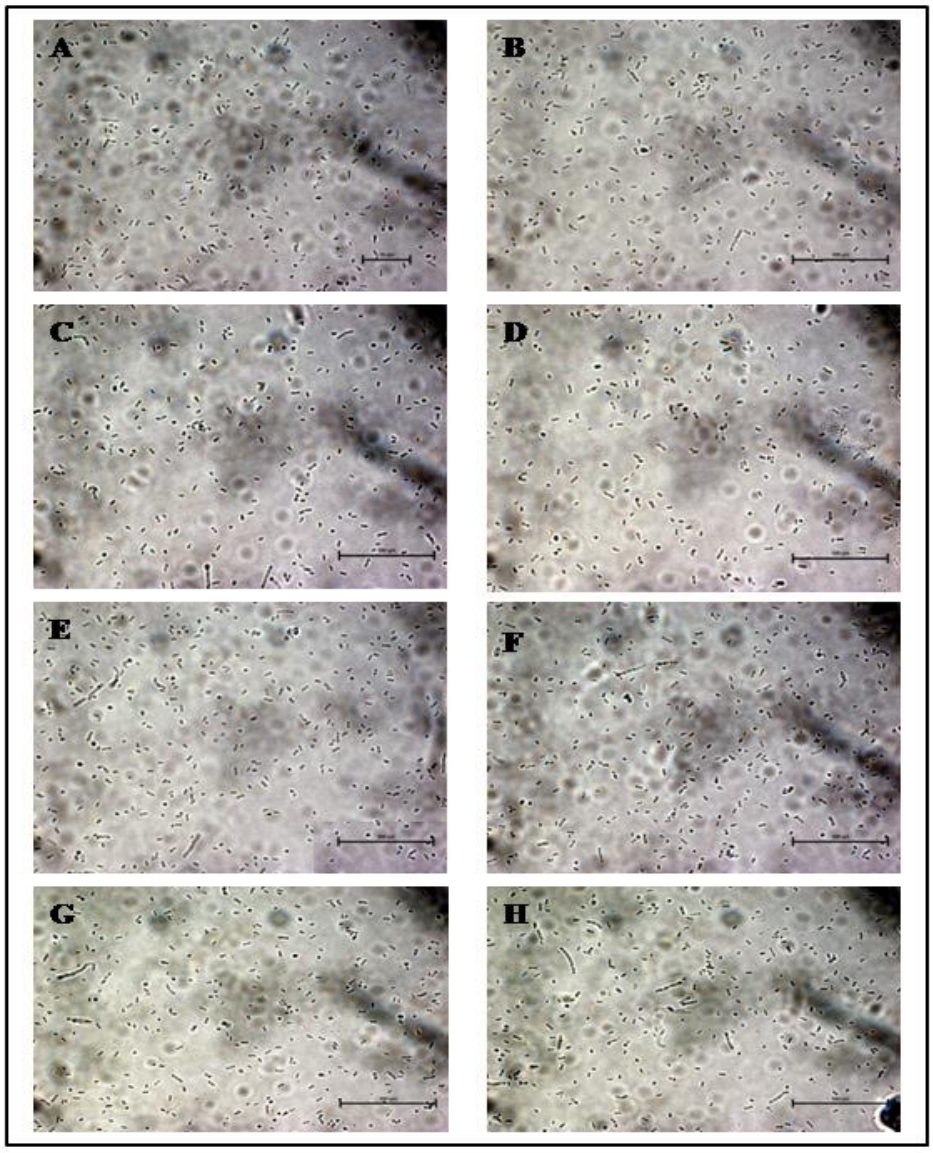


Fig 6A. 14 Bright field microscopic images of *E. coli* **(A, B, E** and **F)** and *S. paratyphi* **(C, D, G** and **H)**; bacterial cells after treatment with 0.005 mg/ml of CuNPs **(A** and **C)**; bacterial cells after treatment with 0.002 mg/ml of AgNPs **(B** and **D)**; bacterial cells treated with CuNPs + labdane **(E** and **G)** and bacterial cells treated with AgNPs + labdane **(F** and **H)**; labdane concentration is 0.006 mg/ml

Approximately 2% of PI stained Gram negative bacterial cells are observed when treated with 0.005 mg/ml of CuNPs **(Fig 6A.13 and 6A.14 A** and **C)** whereas around 5% of bacterial cells were stained when treated with 0.002 mg/ml of AgNPs **(Fig 6A.13 and 6A.14 B and D)**. Interestingly, when bacterial cells were treated with a combination of 0.006 mg/ml of labdane with 0.005 mg/ml of CuNPs **(Fig 6A.13 and 6A.14 E** and **G)** and with 0.002 mg/ml of AgNPs **(Fig 6A.13 and 6A.14 F** and **H)** respectively, 90 – 95% of bacterial cells exhibited red fluorescence. It had been reported way back that PI only

enters cells with "compromised" membranes and stains their nucleic acids, thus, PI is thought to stain "dead" cells (Sgorbati et al. 1996). The stoichiometric binding of PI with DNA has also been previously reported (Schutte et al. 1985). Thus, it was clearly evident that the use of NPs increased the efficacy of labdane diterpene immensely (approximately 20 fold) at a very low concentration of 0.006 mg/ml. The membrane of both the Gram negative bacteria were compromised or destroyed after treatment with this combination, facilitating the entry of PI into the cells, which ultimately undergo complete destruction and lysis.

6A.3.3.3 Candicidal activity

6A.3.3.3.1 Growth curve analysis

C. albicans, a well known human pathogen, has the ability to grow in various amino acid and dextrose mixtures at 28ºC, 30ºC and 37ºC **(**Brennan et al. 1971). The growth of this pathogen can be inhibited or killed by a number of azo compounds like flucanozole, pyrazole etc. The growth kinetics of *C. albicans* after treatment with high concentrations of CuNPs and AgNPs is shown below in **Fig 6A.14**.

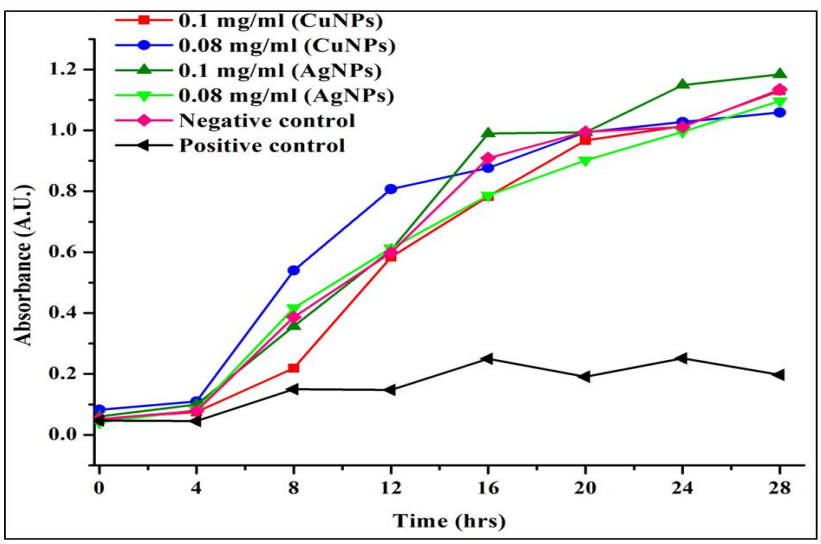


Fig 6A. 15 Growth curve of *C. albicans* after treatment with 0.1 mg/ml and 0.08 mg/ml of CuNPs and AgNPs

It was observed that both CuNPs and AgNPs were not effective in killing or inhibiting the growth of *C. albicans*. It has been previously reported that the toxicity of metal oxide NPs are less in comparison to metallic NPs (Dizaj et al. 2014). In 2015, it was reported that MgO and SiO_2 NPs have a minimum inhibitory concentration (MIC) of > 3.2 mg/ml against *C. albicans* whereas the MIC of CuO and ZnO was $\geq$ 0.4 mg/ml

(Karimiyan et al. 2015). The concentration of the NPs used in the present study was in the hemato-compatible range (≤ 0.1 mg/ml). Thus, the in-affectivity of the CuNPs and AgNPs against *C. albicans* was in concordance with the previous reports. Hence, it can be hypothesized that a combination of NPs and labdane diterpene would not be able to kill the fungal cells.

6A.4 Conclusion

Labdane diterpene has very low solubility of 10-20 µg/ml in aqueous medium and hence, it belongs to either class II or IV of Biopharmaceutics Classification System (BCS). The effect of the combination of the compound with CuNPs and AgNPs on erythrocytes was also studied. For targeted delivery of labdane (0.4 mg/ml) in the bloodstream, AgNPs (≤ 0.04 mg/ml) and CuNPs (≤ 0.06 mg/ml) was found to be optimum. Both the NPs caused the rupture and lysis of erythrocytes.

Though the antibacterial potential of the compound has previously been reported, an attempt has been made to address a core problem associated with this hydrophobic compound – its solubility. The increase in efficacy of the compound at lower concentrations was achieved by using CuNPs and AgNPs. Thus, through the use of NPs, targeted drug delivery and increase in solubility of the compound has been achieved, which was probably responsible for its higher efficacy. FESEM and fluorescent microscopic images revealed a comparative picture of the degree of damage and DNA leakage caused when treated with the NPs alone and their combination with the compound. Recognition of the low solubility of labdane diterpene in aqueous medium can lead to the development of useful strategies to improve the efficacy and performance of this compound for bioassays. The use of NPs, highlighted here, is one such strategy that can be adopted. However, no affect of NPs was observed on *C. albicans*. For ensuring validation of bioassays, performance of compound can be checked across wide solubility range. Deep insight and food for thought is provided for drug developers/scientists regarding the solubility of potential, futuristic drug molecules.

Chapter 6: Formulating a strategic approach for the delivery of the isolated compound as a potential antibacterial agent using:

B) Microemulsions

6B.1 Introduction

High throughput screening (HTS) has revolutionized the process of drug discovery. Solubility and pK_a are among the most important features to be considered for drugs to be administered and absorbed via oral route. Development of low solubility/low bioavailability drugs is a very time consuming and expensive process; as such they prove difficult to get to the market and repay their development costs. Therefore, it is very necessary to determine the solubility profile of potential drug compounds and solve any issue of low solubility associated with them; HTS serves as a very valuable tool in this regard (Bevan and Lloyd 2000).

A well known method to deal with the low solubility problem of bioactive, natural compounds in aqueous medium is by the preparation of microemulsions. The IUPAC definition of miroemulsion (ME) is "a dispersion made of water, oil, and surfactant(s) that is an isotropic and thermodynamically stable system with dispersed domain diameter varying approximately from 1 to 100 nm, usually 10 to 50 nm." It is a transparent or slightly opalescent formulation which has several advantages for pharmaceutical use like ease of preparation, long-term stability and high drug solubilization capacity (Xiao et al. 2013). It helps in incorporating poor water-soluble drugs and increasing their bioavailability, thus, solving the issues of solubility and stability associated with them (Kim et al. 1997; Gao et al. 1998; Xiao et al. 2013).

One of the most common converging points of research in natural products is the *in vitro* antimicrobial susceptibility test (AST). There are various protocols for antimicrobial susceptibility testing including standardized protocols from the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Nonetheless, the root of all these protocols lies in the *in vitro* assessment of the effectiveness of a pure compound(s) or a formulation of the compound against various pathogens. In a broad overview, a standard AST can be performed using the pioneering Kirby-Bauer disk diffusion assay and

modified versions of it, macro- and micro- broth dilution assays and TLC based bio-autography (Balouiri et al. 2016).

The conventional diffusion based techniques are qualitative in nature whereby the appearance of the zone of inhibition indicates the susceptibility of the microbes to the antimicrobial compound(s) or their formulation under study. The MIC of these compounds cannot be accurately determined using these techniques, however, E-test method, which utilizes commercialized antibiotic concentration gradient strips, can give an approximate MIC value via the intersection point between the strip and the elliptical zone of inhibition (Balouiri et al. 2016). The broth dilution technique has been the go-to technique for most laboratories to determine the MIC values. The principles behind the broth macrodilution and microdilution techniques remain the same - the lack of a threshold turbidity or cell growth in the broth. The MIC value (or the inhibition zone diameter) provides us with the breakpoint values to declare the microbe or the pathogen as susceptible, intermediate or resistant (CLSI 2015; Balouiri et al. 2016; Syal et al. 2017). However, the major drawback of the above techniques is time consumption owing to the incubation periods ranging from 16 hours to 48 hours or more (Van Belkum and Dunne 2013; Balouiri et al. 2016; Syal et al. 2017); hence, there is a constant quest for a quick, reliable and reproducible techniques to study antimicrobial susceptibility.

One such technique is the flow cytometry based AST. The use of flow cytometry (FC) for AST had been suggested back in the 1990's (Pore 1990). FC can be used to determine microbial subpopulations of healthy, injured and non-viable cells, which is not possible in the conventional techniques. It is reproducible and can be completed in 2-4 hrs. However, the high cost of the instrumentation and the reagents involved are main reasons behind the lack of widespread use (Pore 1990; Alvarez-Barrientos et al. 2000; Vale-Silva and Buchta 2006; Assunção et al. 2007).

Thus, this part of the present chapter focuses on solving the issues of solubility associated with the isolated compound by the synthesis and characterization of labdane diterpene-loaded ME. Further, the antibacterial assessment of the ME treated bacteria has been done to understand its physiological state after treatment with the compound.

6B.2 Materials and methods

6B.2.1 Preparation and characterization of ME

6B.2.1.1 Determination of surfactant mix (Smix) ratio

Oil phase used – Castor oil, Ethyl oleate, Eucalyptus oil, Isopropyl myristate (IPM).

Co-surfactants used – Butanol, Iso-butanol, Ethanol, Iso-propanol, PEG-400, 1-octanol

For ME preparation, an important phenomenon to be taken into consideration is the HLB or the Hydrophile - lipophile balance value. HLB is an empirical expression for the relationship of the hydrophilic ("water-loving") and hydrophobic ("water-hating") groups of a surfactant. The table below lists HLB values along with typical performance properties. The higher the HLB value, the more water-soluble is the surfactant (Chemmunique 1980). The HLB system is particularly useful to identify surfactants for oil and water emulsification.

The various surfactant mixtures (Smix) of different HLB values were prepared according to **Table 6B.1** with constant stirring on a magnetic stirrer. With constant stirring, 400 µl of milliQ water was added drop by drop to each sample. The visual characteristics of the samples were checked for transparency/translucence and viscosity. Those samples satisfying the desired criteria were selected for further studies.

Table 6B. 1 Composition of samples for determination of suitable Smix ratio

Smix		Volume (ml)		Weight (g)		Mass fraction		HLB value	Oil (ml)	Co-surfactant (ml)
Tween80: Span 80 (v/v)	Sample code	Tween 80	Span 80	Tween 80	Span 80	Tween 80	Span 80			
5:5	SM55	0.5	0.5	0.54	0.49	0.52	0.48	9.86		
6:4	SM64	0.6	0.4	0.65	0.39	0.62	0.38	10.93		
7:3	SM73	0.7	0.3	0.76	0.30	0.72	0.28	12		
8:2	SM82	0.8	0.2	0.86	0.20	0.81	0.19	12.97	0.1	0.4
9:1	SM91	0.9	0.1	0.97	0.10	0.91	0.09	14.04		
9.5:0.5	SM955	0.95	0.05	1.03	0.05	0.95	0.05	14.46		
1	T80	1	0	1.08	0	1	0	15		

6B.2.1.2 Determination of co-surfactant (CoS) and oil

After the suitable surfactant ratio has been identified, it was used for further experiments.

The Smix, oil phase and CoS were mixed according to the **Table 6B.2**. Around 400 µl of milliQ water was added gradually to each mixture under constant stirring and the visual characteristics were noted. The sample with the desired transparency and viscosity was chosen for further experiments.

Table 6B. 2 Composition of pre-titration mix for determination of suitable CoS and oil

CoS		**Smix (ml)**	**Oil (ml)**
Types	**Volume (ml)**		
1-Butanol Iso-butanol Ethanol Iso-propanol PEG 400 1-Octanol	4	1	0.1

6B.2.1.3 Determination of oil-to-Smix ratio and Smix-to-CoS ratio

With the selected Smix ratio and the selected CoS, water titration method was done at different Smix-to-CoS (v/v) ratio (4:1, 1:1, 1:4), oil-Smix ratio (1:12, 1:11, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1) and water percentage (10% to 95%) were checked. The different mixtures were formulated according to **Tables 6B.3** to **6B.8**.

Table 6B. 3 Composition of pre-titration mix at Smix : CoS of 4:1

Oil : Smix (v/v)	Oil (ml)	Smix (ml)	CoS (ml)	Total volume of mixture (ml)
1:12	0.083	1	0.25	1.333
1:11	0.091			1.341
1:10	0.1			1.35
1:9	0.111			1.361
1:8	0.125			1.375
1:7	0.143			1.393
1:5	0.2			1.45
1:4	0.25			1.5
1:3	0.333			1.583
1:2	0.5			1.75
1:1	1			2.25

Table 6B. 4 Water titration volumes for pre-titration mix at Smix : CoS of 4:1

Oil : Smix (v/v)	Total volume of mixture (ml)	Volume of water to be added (ml) at									
		10%	20%	30%	40%	50%	60%	70%	80%	90%	95%
1:12	1.333	0.148	0.333	0.571	0.889	1.333	2.000	3.110	5.332	11.997	25.327
1:11	1.341	0.149	0.335	0.575	0.894	1.341	2.012	3.129	5.364	12.069	25.479
1:10	1.35	0.150	0.338	0.579	0.900	1.350	2.025	3.150	5.400	12.150	25.650
1:9	1.361	0.151	0.340	0.583	0.907	1.361	2.042	3.176	5.444	12.249	25.859
1:8	1.375	0.153	0.344	0.589	0.917	1.375	2.063	3.208	5.500	12.375	26.125
1:7	1.393	0.155	0.348	0.597	0.929	1.393	2.090	3.250	5.572	12.537	26.467
1:6	1.417	0.157	0.354	0.607	0.945	1.417	2.126	3.306	5.668	12.753	26.923
1:5	1.45	0.161	0.363	0.621	0.967	1.450	2.175	3.383	5.800	13.050	27.550
1:4	1.5	0.167	0.375	0.643	1.000	1.500	2.250	3.500	6.000	13.500	28.500
1:3	1.583	0.176	0.396	0.678	1.055	1.583	2.375	3.694	6.332	14.247	30.077
1:2	1.75	0.194	0.438	0.750	1.167	1.750	2.625	4.083	7.000	15.750	33.250
1:1	2.25	0.250	0.563	0.964	1.500	2.250	3.375	5.250	9.000	20.250	42.750

Table 6B. 5 Composition of pre-titration mix at Smix : CoS of 1:1

Oil : Smix (v/v)	Oil (ml)	Smix (ml)	Cos (ml)	Total volume of mixture (ml)
1:12	0.083			2.083
1:11	0.091			2.091
1:10	0.1			2.1
1:9	0.111			2.111
1:8	0.125			2.125
1:7	0.143	1	1	2.143
1:6	0.167			2.167
1:5	0.2			2.2
1:4	0.25			2.25
1:3	0.333			2.333
1:2	0.5			2.5
1:1	1			3

Table 6B. 6 Water titration volumes for pre-titration mix at Smix : CoS of 1:1

Oil : Smix (v/v)	Total volume of mixture (ml)	Volume of water (ml) to be added at									
		10%	20%	30%	40%	50%	60%	70%	80%	90%	95%
1:12	2.083	0.231	0.521	0.893	1.389	2.083	3.125	4.860	8.332	18.747	39.577
1:11	2.091	0.232	0.523	0.896	1.394	2.091	3.137	4.879	8.364	18.819	39.729
1:10	2.1	0.233	0.525	0.900	1.400	2.100	3.150	4.900	8.400	18.900	39.900
1:9	2.111	0.235	0.528	0.905	1.407	2.111	3.167	4.926	8.444	18.999	40.109
1:8	2.125	0.236	0.531	0.911	1.417	2.125	3.188	4.958	8.500	19.125	40.375
1:7	2.143	0.238	0.536	0.918	1.429	2.143	3.215	5.000	8.572	19.287	40.717
1:6	2.167	0.241	0.542	0.929	1.445	2.167	3.251	5.056	8.668	19.503	41.173
1:5	2.2	0.244	0.550	0.943	1.467	2.200	3.300	5.133	8.800	19.800	41.800
1:4	2.25	0.250	0.563	0.964	1.500	2.250	3.375	5.250	9.000	20.250	42.750
1:3	2.333	0.259	0.583	1.000	1.555	2.333	3.500	5.444	9.332	20.997	44.327
1:2	2.5	0.278	0.625	1.071	1.667	2.500	3.750	5.833	10.000	22.500	47.500
1:1	3	0.333	0.750	1.286	2.000	3.000	4.500	7.000	12.000	27.000	57.000

Table 6B. 7 Composition of pre-titration mix at Smix : CoS of 1:4

Oil : Smix (v/v)	Oil (ml)	Smix (ml)	Cos (ml)	Total volume of mixture (ml)
1:12	0.083			5.083
1:11	0.091			5.091
1:10	0.1			5.1
1:9	0.111			5.111
1:8	0.125			5.125
1:7	0.143	1	4	5.143
1:6	0.167			5.167
1:5	0.2			5.2
1:4	0.25			5.25
1:3	0.333			5.333
1:2	0.5			5.5
1:1	1			6

Table 6B. 8 Water titration volumes for pre-titration mix at Smix : CoS of 1:4

Oil : Smix (v/v)	Total volume of mixture (ml)	Volume of water to be added (ml) at									
		10%	20%	30%	40%	50%	60%	70%	80%	90%	95%
1:12	5.083	0.565	1.271	2.178	3.389	5.083	7.625	11.860	20.332	45.747	96.577
1:11	5.091	0.566	1.273	2.182	3.394	5.091	7.637	11.879	20.364	45.819	96.729
1:10	5.1	0.567	1.275	2.186	3.400	5.100	7.650	11.900	20.400	45.900	96.900
1:9	5.111	0.568	1.278	2.190	3.407	5.111	7.667	11.926	20.444	45.999	97.109
1:8	5.125	0.569	1.281	2.196	3.417	5.125	7.688	11.958	20.500	46.125	97.375
1:7	5.143	0.571	1.286	2.204	3.429	5.143	7.715	12.000	20.572	46.287	97.717
1:6	5.167	0.574	1.292	2.214	3.445	5.167	7.751	12.056	20.668	46.503	98.173
1:5	5.2	0.578	1.300	2.229	3.467	5.200	7.800	12.133	20.800	46.800	98.800
1:4	5.25	0.583	1.313	2.250	3.500	5.250	7.875	12.250	21.000	47.250	99.750
1:3	5.333	0.593	1.333	2.286	3.555	5.333	8.000	12.444	21.332	47.997	101.327
1:2	5.5	0.611	1.375	2.357	3.667	5.500	8.250	12.833	22.000	49.500	104.500
1:1	6	0.667	1.500	2.571	4.000	6.000	9.000	14.000	24.000	54.000	114.000

6B.2.1.4 Physico-chemical characterization of the ME formulation

6B.2.1.4.1 Visual properties

Different visual properties *viz*. color, transparency and viscosity of the various formulations were observed through naked eyes and were noted carefully. In the "Handbook of Non Invasive Drug Delivery Systems", ME were defined as clear, free flowing, isotropic liquids, comprising of a mixture of oil, water and surfactant (Narsimha Murthy and Shivakumar 2010). Thus, the formulations which were transparent or translucent and had free flowing/watery consistency were selected for further studies.

6B.2.1.4.2 Physical stability

The physical stability of the selected formulations was checked by centrifugation at 2000, 4000, 6000, 8000, 10000 and 12000 rpm for 30 mins. Those formulations in which no phase separation was observed were selected for the next test.

6B.2.1.4.3 Dye dissolution test using methylene blue stain

Aqueous methylene blue stain was used to test the nature of the ME selected from the previous tests. Around 100 µl of methylene blue was added very carefully along the walls of the microcentrifuge tubes (Eppendorf), which were allowed to stand, un-distrurbed. The nature of diffusion of the stain in the formulation helps to determine the type of ME formed.

6B.2.1.4.4 Thermal stability

Two tests were conducted on the selected ME to determine their thermal stability:

6B.2.1.4.4.1 Stability at 25°C and 37°C

The samples were kept for 24 hrs at each temperature and the changes in their physical appearances were carefully noted.

6B.2.1.4.4.2 Stability under freeze thaw cycle at -4°C

The samples were incubated at -4°C for 24 hrs, after which they were allowed to thaw at 25°C and the physical appearance of the samples were noted.

Those formulations which were stable and retained their original optical isotropic characteristics i.e. those which did not undergo any change in their visual properties were chosen for further experiments.

6B.2.1.5 Preparation of labdane - loaded ME

After the stable ME formulations have been prepared and identified, one of the formulations was chosen to be used for further bioassays. Around 1 mg of labdane diterpene was dissolved in 1 ml of the suitable oil phase by sonication at 40°C. Oil-in-water (o/w) ME with 50% water was prepared using this solution as the oil phase. The physicochemical characteristics of the labdane ME were determined as given above **(section 6B.2.1.4)**.

6B.2.1.6 Field emission transmission electron microscopy (FETEM)

Morphological analysis i.e. determination of the size and dimensions of the labdane loaded ME was done using FETEM. The sample for FETEM was prepared as per the protocol given previously (Fan et al. 2014). Briefly, 10 µl of labdane – loaded ME was placed on a clean copper grid (carbon coated) or TEM grid and allowed to air dry at room temperature (25ºC). Then 10 µl of 2% phosphotungstic acid (HiMedia, India) was added to the same spot on the grid and allowed to dry at room temperature. Phosphotungstic acid is an electron dense substance that is opaque for electrons and hence, used as a negative stain for electron microscopy (Mosgoeller et al. 2012). The copper grid was then taken for analysis using multipurpose FETEM (JEOL JEM-2100F). The analysis for the FETEM image of the ME was done using Image J software.

6B.2.2 AST of labdane - loaded ME

The antibacterial potential of the synthesized ME (loaded with labdane) was tested upon the Gram negative bacteria, *E. coli* (MTCC 723) and Gram positive bacteria, *S. aureus* (ATCC 6538). The assay was performed as per previous protocol (CLSI 2015) with minor modifications.

6B.2.2.1 MIC determination using broth micro-dilution method

6B.2.2.1.1 Preparation of microbial cultures

6B.2.2.1.1.1 Revival from glycerol stock

The bacterial strains were revived from their glycerol stocks in sterilized Luria Bertini broth (LB broth) and incubated at 37°C for 24 hrs. The overnight cultures were sub-cultured on nutrient agar plates and incubated overnight at 37°C. The nutrient agar plate cultures were used as stock for further experiments.

6B.2.2.1.1.2 Turbidity standard for inoculum preparation

The inoculum density must be standardized for a susceptibility assay. The standardization was done by measuring O.D. at 530 nm and adjusting it to 0.09-0.1 (equivalent to 0.5 McFarland Standard or ≈ 2×10^8 CFU/ml). Different methods were used for the turbidity standardization of the 2 bacteria:

S. aureus - Direct colony suspension method was followed for *S. aureus.* Around 3-5 isolated colonies from the nutrient agar plate cultures were selected and suspended in sterile phosphate buffer saline (pH 7.4). The O.D. was adjusted to 0.09-0.1 at 530 nm.

E. coli - Growth method was used in case of *E. coli.* A broth culture was made in nutrient Muller Hinton broth (MHB) from the nutrient agar plate cultures and incubated at 37°C till an O.D. of 0.09-0.1 or more at 600 nm was achieved. Then, the turbidity was adjusted to 0.09-0.1 with sterile phosphate buffer saline (pH 7.4).

6B.2.2.1.2 Antibiotic stock preparation

The antibiotic, Ampicillin (HiMedia, India) was used as a positive control for antimicrobial assays. On an already calibrated weighing balance, 100 mg of ampicillin sodium salt was accurately weighed. It was dissolved in 75 ml of phosphate buffer (pH 8) (as per the calculation using the formula given below). Further dilution, as per requirement, was done in the MHB used for the antibacterial susceptibility testing.

$$Weight\ (mg) = \frac{Volume\ (ml) \times Concentration\ (\mu g/ml)}{Potency\ (\mu g/ml)}$$

Or,

$$Volume\ (ml) = \frac{Weight\ (mg) \times Potency\ (\mu g/ml)}{Concentration\ (\mu g/ml)}$$

6B.2.2.1.3 Broth dilution assay

The assay was performed using a sterile, round bottom, 96-well micro-dilution plate (Eppendorf). The wells were loaded as per **Table 6B.9.** The plate was incubated within 15 mins of loading the inoculum. The incubation was done for 24 hrs at 37°C with shaking (180 rpm).

6B.2.2.1.3.1 Determining the MIC value of labdane ME from broth micro-dilution end point

The MIC was read as the lowest concentration of the antimicrobial agent at which the growth of the microorganism was completely inhibited and no visible growth (≤ 2mm in diameter) was observed.

Table 6B. 9 Composition for broth microdilution AST of labdane ME

Volume of labdane ME (µl)	Volume of MHB (µl)	Inoculum (µl) (OD_{600} = 0.09 – 0.1)	Volume of PBS (µl)	Total volume (µl)	Concentration of labdane (µg/ml)
10	100	10	80	200	10
20			70		20
30			60		30
40			50		40
50			40		50
60			30		60
70			20		70
80			10		80
90			0		90
(Ampicillin; Stock – 20 µg/ml) 50			40		0
Negative control 0			90		0
(Carrier control; Empty ME) 90			0		0

6B.2.2.2 FC analysis

The physiological state of the bacterial populations after treatment with the labdane loaded ME was analyzed and determined using flow cytometry.

6B.2.2.2.1 Inoculum preparation

The bacterial cultures for flow cytometric studies were prepared as given above **(section 6B.2.2.1.1)**. A loopfull of colony from the overnight grown culture was inoculated in 2 ml of MHB and incubated at 37°C overnight. The bacterial cells were pelleted down by centrifugation at 5000 rpm for 5 mins. The cells were re-suspended in 3 ml of sterile PBS buffer (pH 7.4). The OD at 600 nm was adjusted to 0.09-0.1. A 100 µl aliquot of the cell suspension was inoculated in fresh 2 ml MHB and incubated at 37°C till cells entered log phase (≈12-16 hrs). The log phase bacterial cells were pelleted down by centrifugation at

5000 rpm for 5 mins. Broth macrodilution was done as per the **Table 6B.10**. The MHB cultures were incubated at 37ºC for 24 hrs.

6B.2.2.2.2 Staining

6B.2.2.2.2.1 Preparation of dyes

On an already calibrated weighing balance, 5 mg of PI was weighed and dissolved in 1 ml of sterile and filtered PBS buffer to obtain a 5 mg/ml stock. It was further diluted with PBS buffer and working dye solution (100 µg/ml) was obtained.

Further, a 1 mg/ml stock was first prepared by dissolving 1 mg of 6-carboxyfluorescein diacetate (CFDA) powder in 1 ml of acetone. Further, it was diluted with PBS buffer to obtain working dye solution (25 µg/ml).

6B.2.2.2.2.2 Dual staining procedure

The bacterial cells prepared as per **Table 6B.10** were pelleted down by centrifugation at 5000 rpm for 5 mins. The cells were re-suspended in PBS buffer and OD at 600 nm was adjusted to 0.09-0.1. About 1 ml aliquot of the cell suspension was kept aside for resuscitation method of viability assessment as described below (**section 6B.2.2.3)**. To the above cell suspension, 20 µl of CFDA working solution was added and mixed thoroughly. The samples were incubated at 37°C for 5 mins in dark condition. Cells were again pelleted down by centrifugation at 5000 rpm for 5 mins. The supernatant was thrown away and the pellet was re-suspended in fresh PBS buffer. The PI working solution aliquot of 20 µl was added to the above suspension and mixed thoroughly and incubated for 10 mins in dark condition.

Table 6B. 10 Composition for broth macrodilution

E. coli					
Samples	**Volume of MHB (ml)**	**Volume of PBS (µl)**	**Antimicrobial agent (µl)**	**Total volume (ml)**	**Inoculum (µl)**
Positive control	1	-	**(Ampicilin, Stock 40 µg/ml)** 1000	2	100
Negative control		1000	-		
ME control		600	400		
Labdane loaded ME		600	400		
S. aureus					
Samples	**Volume of MHB (ml)**	**Volume of PBS (µl)**	**Antimicrobial agent (µl)**	**Total volume (ml)**	**Inoculum (µl)**
Positive control	1	-	**(Ampicilin, Stock 40 µg/ml)** 1000	2	100
Negative control		1000	-		
ME control		700	300		
Labdane loaded ME		700	300		

6B.2.2.2.3 FC setup

The flow cytometric analysis was carried out in CytoFlex (Beckman Coulter, USA) instrument. The quality control (QC) analysis of the instrument was carried out using the CytoFlex Daily QC beads. The threshold was set on side scatter (SSC) and the photomultiplier (PMT) voltages were adjusted using unstained live bacterial cells so that the bacterial population is entirely on scale on a forward scatter (FSC) *vs* SSC plot. The FSC and the SSC histograms were also set so that the bell-shaped curves were not cut off from display. The FL1-FITC and FL2-PE PMT voltage were adjusted to obtain the unstained population in the lower quadrant of FL1-FITC-A *vs* FL2-PE-A contour plot.

6B.2.2.2.4 Data acquisition and analysis

Data acquisition and analysis of the samples were performed using an SSC threshold. A total number of events were kept as 20,000 for each sample. Data analysis was done using the FloJo software (v10.0.7) (Tree Star, Stanford, USA).

6B.2.2.3 Viability assessment by resuscitation method

Resuscitation method is a classical technique to determine the revival of bacterial cells after the removal of a stress (particularly an antimicrobial agent) to which it had been previously subjected to (Bissonnette et al. 1975).

As discussed previously **(section 6B.2.2.2.2.2)**, bacterial cells prepared for broth macrodilution (**Table 6B.10)** were kept aside for viability assessment. For this assay, 50 µl of the bacterial cells were spread on plate count agar media (HiMedia, India) using a sterile, glass spreader. The plates were properly sealed, incubated under static condition at 37°C and observed for growth after 24 hrs.

6B.2.2.4 Interaction study of labane - loaded ME with antibiotic

Qualitative measurement of the interaction of antimicrobial agents can be performed by using agar well or disk diffusion method. The interaction between labdane - loaded ME and ampicillin was carried out by agar well diffusion as previously described (Verma 2007).

For this assay, 100 µl of the bacterial cells was aliquoted and spread on MHA (HiMedia, India) using a sterile, glass spreader. Different concentrations of labdane – loaded ME and antibiotic (below their individual MICs) were loaded in the wells on the

agar plate. The plates were properly sealed, incubated under static condition at 37°C and observed for zone of inhibition after 24 hrs.

6B.3 Results and discussion

6B.3.1 Preparation of ME

6B.3.1.1 Determination of Smix ratio

The formulations prepared were visually observed for 2 properties – viscosity and transparency. Those samples, which were watery or less viscous, transparent or minimal translucent and did not stick on the walls of the glass vials were MEs and chosen for further experiments.

From the experiment, the above criteria were observed in Smix ratio of 9.5:0.5 (Tween 80: Span 80) (Sample code – SM955) as shown in **Fig 6B.1**. For all the subsequent experiments, this ratio was maintained throughout.

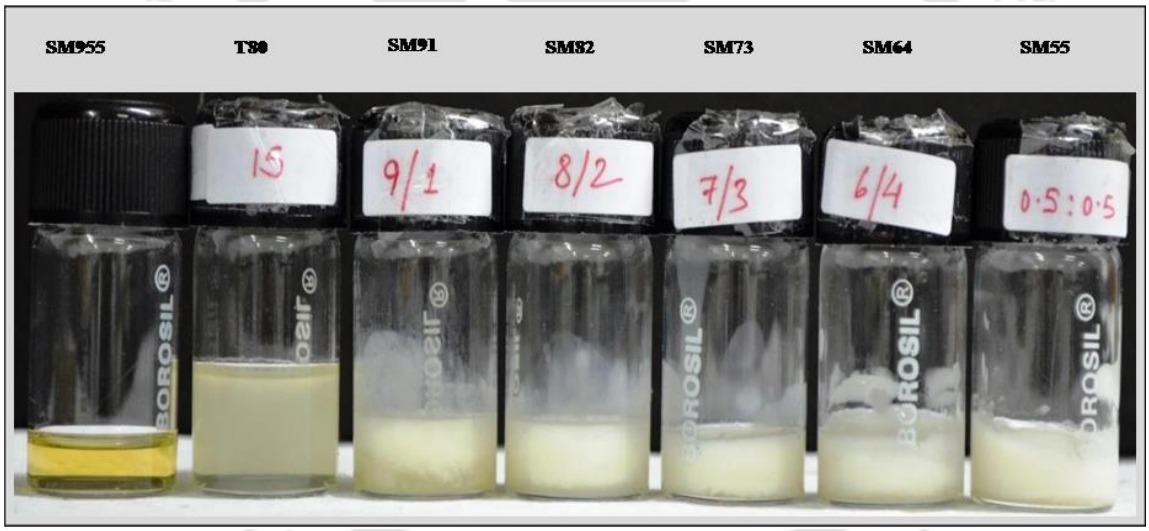


Fig 6B. 1 Formulations at different Tween 80: Span 80 ratio (v/v)

6B.3.1.2 Determination of CoS and oil

After determination of the Smix ratio, 2 more variables *viz*. oil and CoS, with suitable concentrations, need to be determined for the synthesis of ME with the SM955 mix.

Using **Table 6B.2**, it was found that ethanol was the most suitable solvent and ethyl oleate was the most suitable oil among those listed which produced maximum ME along with SM955. Thus, ethanol was chosen as the CoS for further optimization procedures.

6B.3.1.3 Determination of oil-to-Smix ratio and Smix-to-CoS ratio

Smix-to-CoS ratio of 1:4 with ethyl oleate and oil-to-Smix (v/v) ratio from 1:10 to 1:12 was found to be the most suitable for producing ME. The combination produced water-in-oil (w/o) ME up to 30% water content. Oil-in-water (o/w) ME was produced above 40% water content with oil-to-Smix (v/v) ratio from 1:10 to 1:12. Smix-to-CoS ratios of 1:1 and 4:1 mostly produced white, opaque emulsions irrespective of percentage of water or oil-to-Smix ratios.

6B.3.2 Physico-chemical characterization of ME

O/W ME at ethyl oleate-to-SM955 (v/v) ratios of 1:12, 1:11 and 1:10 were selected for the characterization and further bioassays.

6B.3.2.1 Visual characteristics

The ME formed by the suitable combinations of Smix, oil and CoS were optically isotropic, either transparent or minimally translucent **(Fig 6B.2)** and were chosen for further physicochemical characterization.

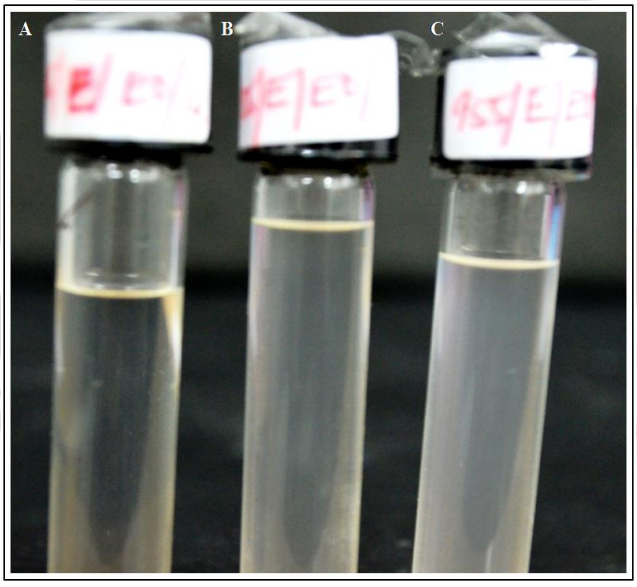


Fig 6B. 2 ME formed with 9.5: 5 Smix ratio and 1: 4 Smix : CoS with oil : Smix ratio of **(A)** 1:12 **(B)** 1:11 and **(C)** 1:10

6B.3.2.2 Physical stability

The MEs were found to be physically stable at all the centrifugation rpms and there were no phase separation in any of them.

6B.3.2.3 Dye dissolution test

Water soluble dyes such as methylene blue diffuse uniformly throughout in o/w ME but clumps in one area in case of w/o ME (Al-Adham et al. 2000; Guimarães et al. 2014). In the ME chosen above, methylene blue was distributed uniformly in the formulation, thus, showing that they were indeed o/w ME **(Fig 6B.3 A)**. A random w/o ME has also been shown in **Fig 6B.3 (B)** for reference.

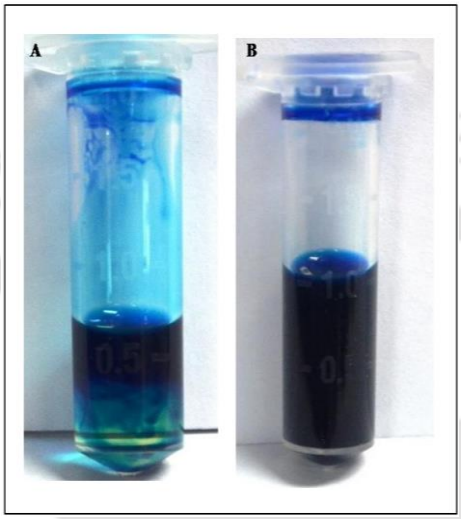


Fig 6B. 3 Methylene blue dye dissolution test with **(A)** w/o ME and **(B)** o/w ME

6B.3.2.4 Thermal stability

The ME did not develop cloudiness after 24 hrs of incubation at 37°C. They also retained their optical transparency after the freeze thaw cycle between -4°C and 25°C. Thus, stable ME were obtained, out of which, the formulation consisting of 1:4 (v/v) ratio of SM955 to ethanol, 1:12 (v/v) ratio of ethyl oleate to SM955 and ≥ 40% water by volume was chosen for the preparation of labdane-ME formulation and further bioassays **(Fig 6B.4)**.

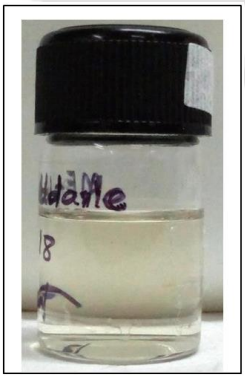


Fig 6B. 4 Labdane loaded ME

6B.3.2.5 FETEM

The FETEM based morphological characterization of labdane - loaded ME revealed that this o/w ME droplets had a double - layered hexagonal perimeter **(Fig 6B.5)**. The inner core would be hydrophobic ethyl oleate core which was surrounded by the Smix - CoS outer membrane. Their dimensions are listed below in **Table 6B.11**. However, this analysis was not able to reveal the overall 3 – dimensional (3D) structure of the ME droplets as the drying step in the sample preparation disrupted its 3 D geometry. A cryo – TEM analysis would be required to understand the 3 D geometry of the ME.

Table 6B. 11 Dimensions of the labdane – loaded ME

Parameters	Mean ± standard deviation (nm)	Maxima (nm)	Minima (nm)
Length of hexagonal inner layer	121.757 ± 13.2	106.567	104.552
Length of hexagonal outer layer	165.645 ± 33.431	200.038	109.281
Inter layer distance	40.566 ± 8.539	53.458	30.911
Angles	120.555º ± 4.737º	125.422º	112.311º

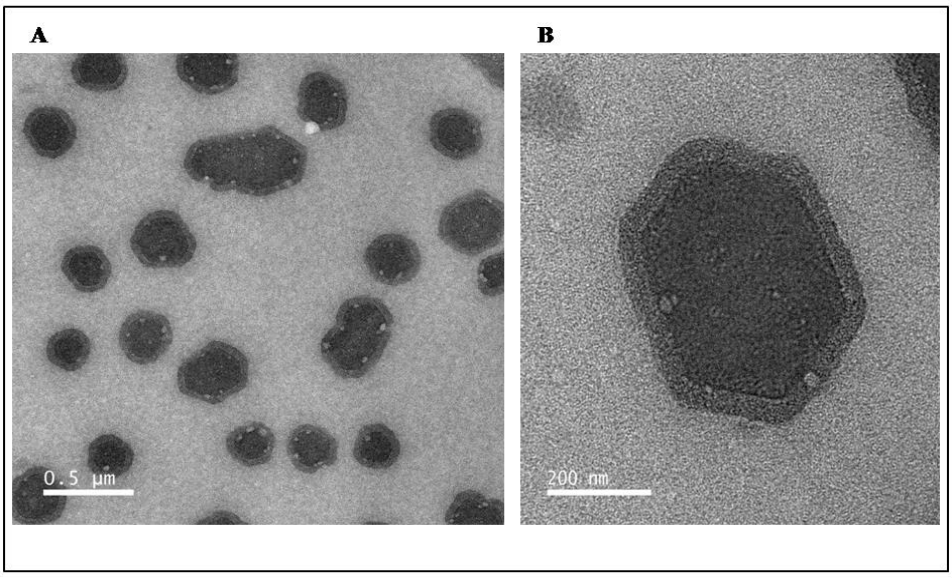


Fig 6B. 5 (A) Labdane - loaded ME; **(B)** Single ME

6B.3.3 AST of labdane – loaded ME

AST was performed using broth micro – dilution method to determine the MIC of the labdane – loaded ME against Gram negative bacteria, *E. coli* and Gram positive bacteria, *S. aureus*. As evident from **Fig 6B.6**, the MIC value of the labdane – loaded ME was 40 µg/ml and 30 µg/ml for *E. coli* and *S. aureus* respectively.

However, it was observed that the empty or unloaded ME also had a minimal inhibitory affect on the bacterial growth. Therefore, further studies would be required to determine the minimum susceptible volume of the unloaded ME.

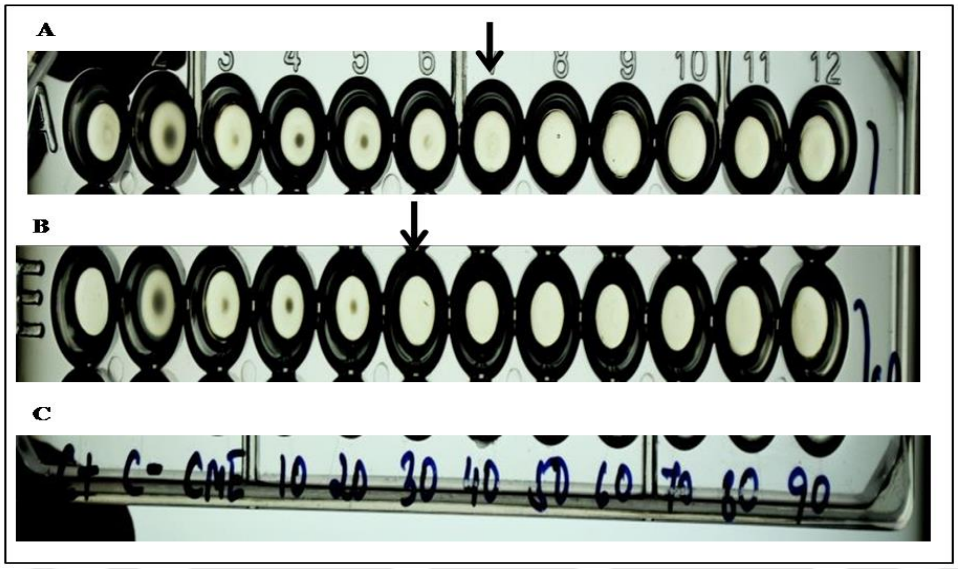


Fig 6B. 6 Broth microdilution of labdane – loaded ME AST against **(A)** *E. coli*; arrow indicates the MIC value of 40 µg/ml and **(B)** *S. aureus*; arrow indicates the MIC value of 30 µg/ml. **(C)** Different concentrations of labdane – ME used; C+ indicates positive control, C- indicates negative control and C-ME indicates empty ME without labdane

6B.3.4 FC analysis

Traditional methods for antibacterial assay are based on the visualization of the bacterial growth in a qualitative manner. Such methods however, do not provide any insight into the physiological state of the bacteria under treatment. In the present study, FC based investigation has been carried using dual staining technique to understand the physiological state and accurately quantify the heterogenous population of bacterial cells. PI is a fluorescent and intercalating agent that is widely used as a DNA stain. On the other hand, cFDA is used for esterase activity detection. Combining PI with cFDA in

flow cytometric analysis can help in the detection and clear discrimination of 3 subpopulations (dead, viable and injured) of cells (Balouiri et al. 2016).

Contour plots of FC investigation after treatment with labdane – loaded ME, along with appropriate controls are shown in **Fig 6B.7 – 6B.9**. It was observed that 2 sub – populations of *S. aureus* existed in the presence of positive control **(Fig 6B.8 and 6B.9 F)** However, this population was not present when treated with the labdane – loaded ME. In case of *E. coli* **(Fig 6B.7 – 6B.9 A-D)**, no such subpopulations were observed and there was complete inhibition. The labdane – ME was more effective against *E. coli* and possibly against other Gram negative bacterial pathogens. This is in coherence with the antibacterial activity of labdane against Gram negative bacteria as observed in the previous chapter. Moreover, it is interesting to note that labdane, which was ineffective against Gram positive bacteria as observed in the previous chapter **(section 5B 1.3.1 – 5B 1.3.4)**, was effectively inhibiting its growth when packed in a ME. Thus, labdane – loaded ME looks promising as an antibacterial gent against *S. aureus* and *E. coli.*

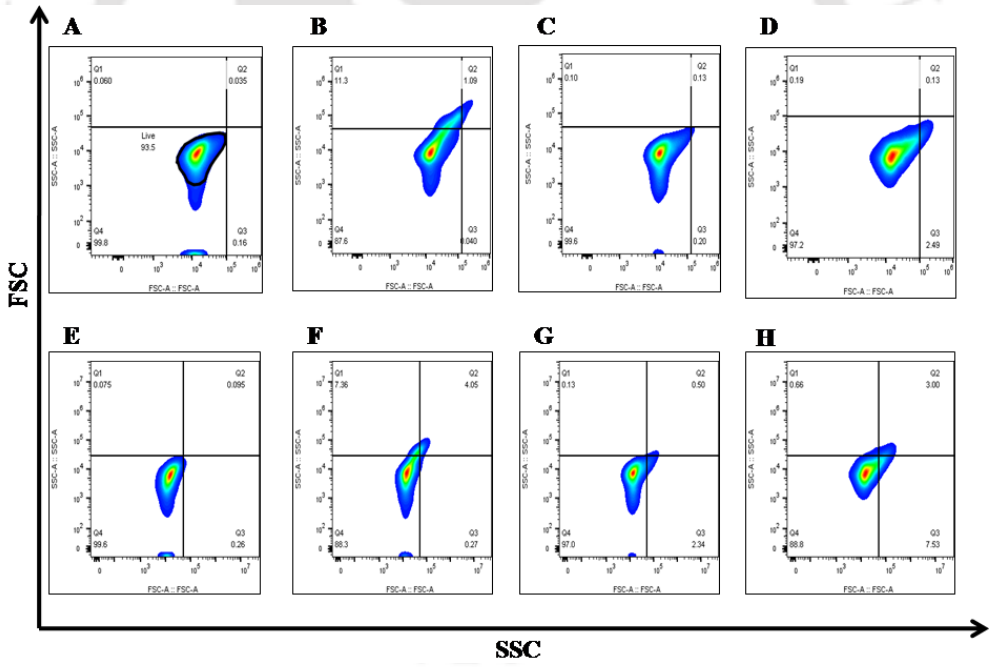


Fig 6B. 7 FC contour plots (FSC vs SSC) of dual stained *E. coli* (**A – D)** and *S.aureus* **(E – H)** after treatment. **A** and **E** represents bacteria after treatment with negative control; **B** and **F** represents bacteria after treatment with positive control; **C** and **G** represents bacteria after treatment with empty ME and **D** and **H** represents bacteria after treatment with labdane loaded ME

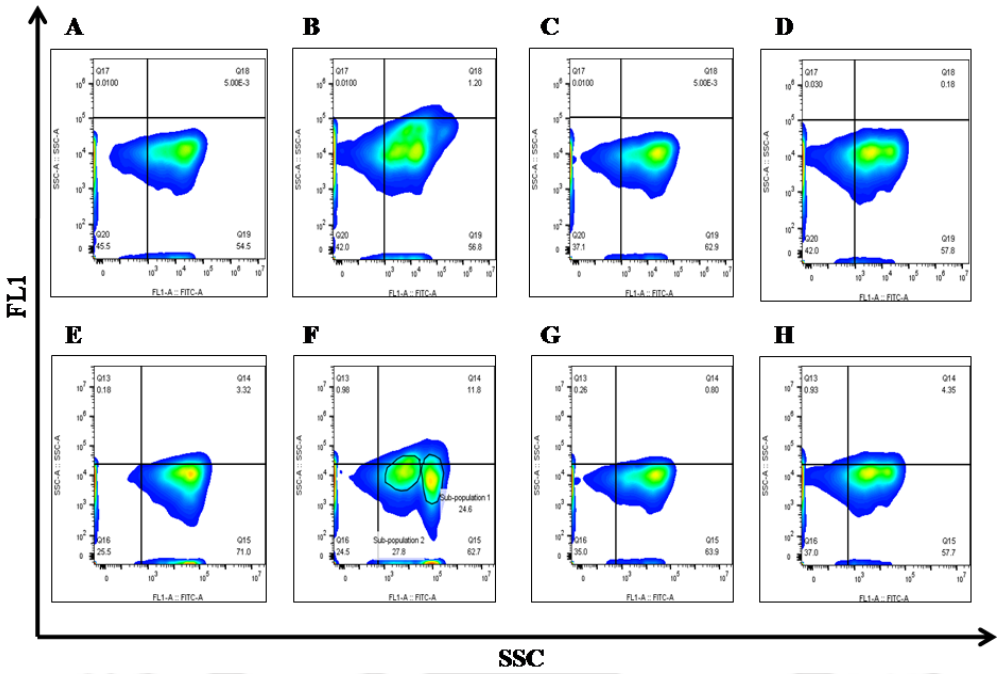


Fig 6B. 8 FC contour plots (FL1 vs SSC) of dual stained *E. coli* **(A – D)** and *S.aureus* **(E – H)** after treatment. **A** and **E** represents bacteria after treatment with negative control; **B** and **F** represents bacteria after treatment with positive control; **C** and **G** represents bacteria after treatment with empty ME and **D** and **H** represents bacteria after treatment with labdane loaded ME

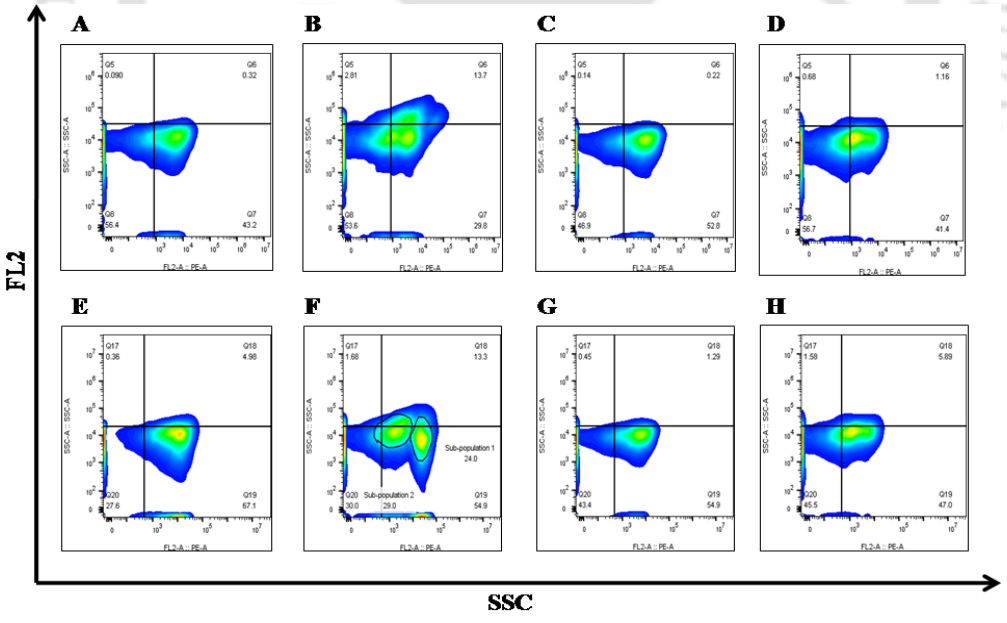


Fig 6B. 9 FC contour plots (FL2 vs SSC) of dual stained *E. coli* **(A – D)** and *S.aureus* **(E – H)** after treatment. **A** and **E** represents bacteria after treatment with negative control; **B** and **F** represents bacteria after treatment with positive control; **C** and **G** represents bacteria after treatment with empty ME and **D** and **H** represents bacteria after treatment with labdane loaded ME

FC analysis has become a promising tool in a wide range of applications including antibacterial activity studies by detecting the change in bacterial membrane potential, permeability and even for the development of rapid antibacterial drug discovery in the recent years (Gnanadhas et al. 2013; Scanlon et al. 2014). Previously, FC investigation on *L. innocua*, *E. coli* and *S. aureus* revealed the antibacterial effect of various plant essential oils (Nguefack et al. 2004; Ghosh et al. 2013a). Though there are no reports on the FC investigation on the antibacterial activity of ME encapsulated plant extracts, this tool has been used in the investigation of ME – based drug delivery in cell lines (Ma et al. 2013; Qu et al. 2015).

6B.3.5 Resuscitation assay

The term 'resuscitation' literally means 'revival.' Mostly used in medical science, resuscitation is the process of correcting physiological disorders in an acutely unwell patient; a well known example is mouth – to – mouth resuscitation. Resuscitation in microbiology is not unheard of. Back in 1983, J.S. Abbiss had highlighted that the food – borne microbes are able to show resuscitation or revival even after injury caused to microbes by the various treatments provided for food processing and storage (Abbiss 1983). Absence of proper tests for pathogens, under some antimicrobial agent, may often give false sense of security until attention is paid to the sub-lethally damaged, yet viable cells.

For the present study, plate count agar was used for the growth of the microorganisms. The 2 sub – populations of *S. aureus* were reported from the flow cytometric analysis under ampicillin treatment **(section 6B.3.3)**. From the resuscitation assay, these 2 sub – populations were identified to be an ampicillin resistant population and a susceptible population; as such, the resistant sub-population had a luxuriant growth in the resuscitation assay. It was also observed that this population was well inhibited by the labdane - ME and had lesser growth in the plate count agar **(Fig 6B.10 A)**. In case of *E. coli*, no subpopulations were observed under antibiotic treatment. However, a low intensity growth of the antibiotic - treated bacteria (as compared to *S. aureus*) was observed in the plate count agar from the reseuscitation assay **(Fig 6B.10.B)**. Interestingly, even this low growing *E. coli* population was very effectively inhibited by the labdane – loaded ME. Thus, the labdane – loaded ME can be considered as a promising drug delivery and antibacterial agent against most pathogenic bacteria (both Gram negative and positive bacteria) including methicilin resistant *S. aureus* (MRSA).

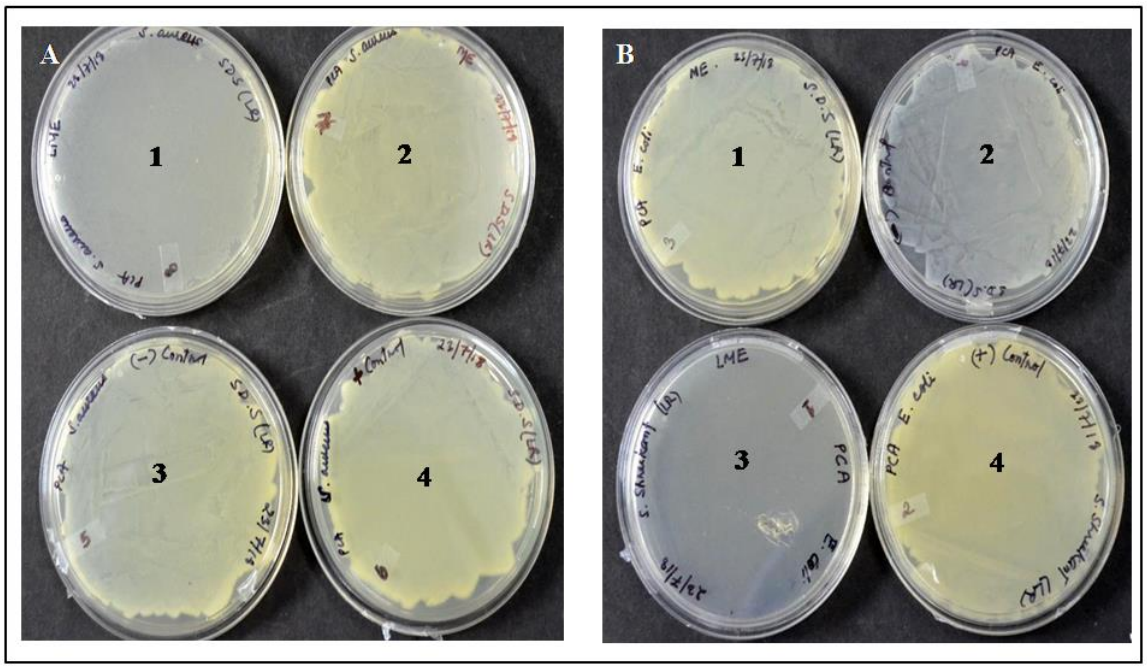


Fig 6B. 10 Resuscitation assay of **(A)** *S. aureus* after treatment with **(1)** labdane loaded ME **(2)** Empty ME **(3)** Negative control **(4)** Positive control and **(B)** *E. coli* after treatment with **(1)** Empty ME **(2)** Negative control **(3)** labdane loaded ME **(4)** Positive control

6B.3.6 Interaction study of labane loaded ME with antibiotic

Combination therapy with multiple antimicrobial agents is ideally used to treat polymicrobial infections when treatment with a single agent may be inadequate. Reduction in antimicrobial dosage-related toxicity might also be theoretically achieved by antimicrobial combinations, permitting the use of drugs at lower doses than if the drugs were administered independently (Verma 2007).

In the present study, the concentration of ampicillin and labdane – loaded ME used was less than their MIC. It was observed that for both *E. coli* and *S. aureus*, the combination of antibiotic and labdane – loaded ME showed additive or indifferent affect **(Fig 6B.11 A and B)**. This suggested that, even though the labdane – loaded ME was able to inhibit the growth antibiotic resistant population of bacteria, there was no increase in its individual effect when used in combination with an antibiotic. A similar indifferent behavior is also exhibited by epigallocatechin gallate, obtained from *Camellia sinesnis* (tea leaves), in combination with β-lactam antibiotics (Hu et al. 2002). It may be hypothesized that the increased permeability of the cell wall/membrane to antibiotics caused by the labdane – loaded ME-induced damage of the cell wall might be the main factor for the additive effects (Hemaiswarya et al. 2008).

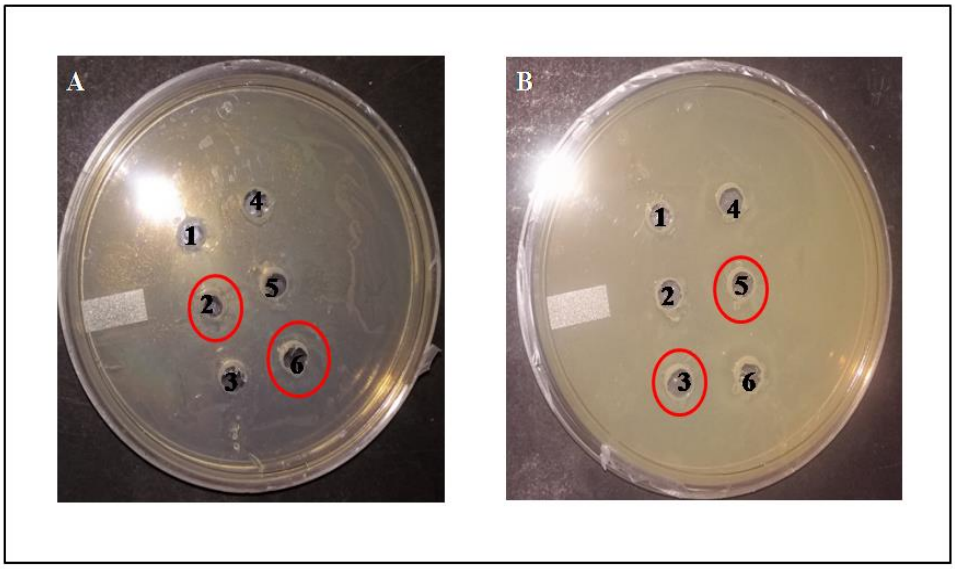


Fig 6B. 11 Additive effect of ampicillin with labdane – loaded ME against **(A)** *S. aureus* and **(B)** *E. coli*; concentrations used: 3, 2 and 1 µg/ml for ampicillin **(1-3)** and labdane loaded ME **(4-6)** respectively

6B.4 Conclusion

In the current part of the present chapter, the solubility hindrance of labdane diterpene has been successfully addressed. A physically and thermally stable oil – in - water microemulsion was prepared using a surfactant mix ratio of 9.5:0.5 (Tween 80:Span 80) and ethanol as co - surfactant for the delivery of labdane (dissolved in ethyl oleate oil phase) in a biological system. A successful anti-bacterial susceptibility assessment against two common bacteria, *E. coli* and *S. aureus*, representative species of the Gram negative and Gram positive bacteria was also performed; the labdane loaded ME can be very promising futuristic antibacterial agent. It was observed from the previous chapter that the labdane diterpene alone was not able to inhibit the growth of any Gram positive bacteria in the slightest even at high concentrations (possibly due to its hydrophobic nature). But, it is interesting to note that the labdane loaded ME was successfully able to inhibit the growth of ampicillin resistant bacteria population of both Gram positive and Gram negative bacteria. Both the antimicrobial agents i.e. the antibiotic and labdane loaded ME however, cannot be used in combination therapy as they exhibited indifference or additive affect against both Gram positive and Gram negative bacteria.

However, it would be very interesting to understand as to how the labdane diterpene produces its potential anti- bacterial activity and whether it would be effective against other fastidious and non-fastidious bacterial pathogens.

Chapter 7: Conclusion and Future prospects

This chapter summarizes the entire work undertaken in this doctoral thesis and provides good leads for future work in this field

Chapter 7: Conclusion and future prospects

7.1 Conclusion

Investigations of plant based secondary metabolites, based on ethnopharmacological knowledge, is of utmost importance in order to understand their complex chemical structures and their pharmacological potential in the quest of new and effective drugs. The traditional communities living in NE India have a lot of 'traditional knowledge' handed down through generations, about the use of the plants as medicines. Use of different plants (many of which are endangered or threatened) for ethnomedical purposes has been documented, many of which find their usage by the ethnic groups of this region. Zingiberaceae is considered to be "blessing of nature" as it a resource for many useful products like spices, medicines, perfumes, dyes, aesthetics etc. Members of this rhizomatous family are recognized by their characteristic odour which is due to the presence of volatile, essential oils and oleoresins. India is one of the richest abodes of Zingiberaceae, with the NE region of India housing the greatest abundance. Members of the largest genus of this family, *Alpinia*, have been subjected to fractionation process to yield volatile oils, extracts and bioactive components. A lesser explored member of the genus *Alpinia* is *Alpinia nigra*, a medium sized rhizomatous, herbaceous plant, that is rich in secondary metabolites like flavanoids, terpenoids, alkaloids etc. *Alpinia nigra*, a medium sized biennial, herbaceous plant that propagates by vegetative means through rhizomes, was the plant under investigation in the current work. This plant bears racemose type of inflorescence with a character of flexistyly. It is well known as a home remedy for curing various diseases among the ethnic and tribal groups of NE India. The rhizomes are aromatic and contain essential oils.

The research work carried in the present thesis work is focused broadly on the major compounds from the seeds of the plant, *A. nigra*. Overall the thesis is divided into 7 chapters with appropriate section and headings. The research work undertaken to fulfill the specified objectives has been explained in Chapters 3-6.

In **Chapter 3**, the work undertaken for the achievement of the first objective of the thesis has been explained. Seeds obtained from different growing stages of *A. nigra* were subjected to organic solvent extraction using different solvents and maximum extract yield was obtained from the middle stage or seeds from red fruits using ethyl acetate using mechanical room temperature extraction; the seed-to-solvent ratio for

maximum extract yield was optimized. The extracts were blackish in color, sticky and highly viscous. The extracts lacked crystalline nature, thermally stable and were hydrophobic.

Chapter 4 addresses the second objective of the present investigation, which was in continuation with work undertaken in the previous objective. A pure compound was isolated from the seeds of *A. nigra* and identified as labdane diterpene, (E)-labda-8(17), 12-diene-15, 16-dial; the maximum yield was sourced from hexane extract and the seed-to-solvent ratio was optimized. The compound was golden colored, highly hydrophobic, amorphous, sticky oil that was found to be thermally stable. Due to the low intensity vibrations of Raman spectroscopy, the compound was subjected to SERS study using copper nanoparticles, which showed an enhancement of 1000 fold in the Raman bands. *In-silico* studies revealed that labdane diterpene was unsuitable for oral administration but was suitable for IV administration theoritically.

Chapter 5 describes the biological potential of the isolated labdane diterpene. This chapter has been divided broadly into **2 sections**. Erythrocytes are considered to be model cells for hemato-compatibility studies. Since labdane diterpene was found to be suitable for IV administration theoritically, the highest compatible concentration of labdane with RBCs was determined in the **first section** of this chapter. The **second section** of this chapter, which was further divided into 2 sub-sections, described antimicrobial studies of labdane diterpene against bacterial and yeast. All the antimicrobial studies were carried out using the hemato-compatible concentrations of the compound. Labdane diterpene caused lysis of Gram negative bacteria at high concentrations but was not effective against Gram positive bacteria. However, the compound had very low efficacy at lower concentrations. Labdane diterpene exhibited antagonistic activity against *Candida albicans*, the notorious pathogen. The minimum effective concentration of the compound was determined; this concentration range was used for the further anti-*Candida* studies. Various growth kinetics and analytical studies lead to an interesting observation – labdane diterpene inhibited the growth of *C. albicans* in a dose dependent manner by binding to cell surface transporters of the fungus and preventing substrate uptake.

Chapter 6 described an important, yet usually ignored aspect of drug development – its solubility. Labdane diterpene had a very low solubility in aqueous

medium; hence possible solutions to overcome this hindrance are described in this chapter in **2 sections**. Nanoparticles are well-known tools for drug delivery. Hence, in the **first section**, copper and silver nanoparticles were used for IV administration of labdane diterpene and their hemato-compatible range was determined. This targeted delivery of labdane using nanoparticles increased the antibacterial efficacy of the ineffective, yet water soluble, concentrations of the compound. However, this combination was not effective against Gram positive bacteria. In the **second section** of this chapter, microemulsions were designed with suitable combinations of surfactant, co-surfactant and oil phase, for the delivery of labdane diterpene in aqueous medium. These double-membrane bound labdane-loaded microemulsions were found to kill bacteria – both Gram negative and Gram positive. Interestingly, these labdane-loaded microemulsions were able to kill the antibiotic resistant population of bacteria.

7.2 Future prospects

The current research work has enabled the isolation and characterization of a bioactive compound, labdane diterpene, from the seeds of *A. nigra*. It has also successfully addressed the long-standing problem of solubility associated with hydrophobic compounds. This investigation has opened news avenues, some of which are listed below, that can be addressed infuture:

- Characterization of *A. nigra* - derived extracts may establish a case study for the medicinal property profiling of *A. nigra* varieties of NE India
- Different parts of *A. nigra* can be further exploited as a source for other important bioactive compounds
- Labdane diterpene can be exploited greatly in the field of synthetic chemistry for preparation of suitable, biologically effective analogues. This can lead to the development of more effective drug candidate against pathogenic microbes
- Comparative analysis on the SERS profiling of labdane diterpene can be carried out, when the compound is adsorbed on different metallic nanoparticles
- Labdane diterpene has a strong characteristic (spicy) odour. Hence, this compound can be developed has a potential and effective mosquito repellent
- Study can be carried out on how labdane diterpene produces its potential anti - bacterial activity and whether it would be effective against other fastidious and non-fastidious pathogens
- Labdane-loaded microemulsion is lipophilic and has helped to effectively overcome its solubility hindrance. Thus, this labdane-ME can be used on insect cell lines to test its insecticidal activity

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RESEARCH OUTPUT

Publications

RESEARCH PAPER

Manuscripts Published

1. **Chakrabartty I**, Baruah PK, Panda AN, Khare A, Rangan L* (2018) Hybrid formulation of Cu nanoparticles and labdane diterpene from *Alpinia nigra*: a vibrational spectroscopic study**; Journal of Applied Spectroscopy**, 85 (5), 983-990

2. Basak S, **Chakrabartty I**, Hedaoo V, Shelke RG, Rangan L* (2018) Assessment of genetic variation among wild *Alpinia nigra* (Zingiberaceae) population: genome mining and molecular marker approach; **Molecular Biology Reports**, pp 1-13 doi/org/10.1007/s11033-018-4458-3

Manuscripts under review

1. **Chakrabartty I**, Vijayasekhar A, Rangan L* (2018) Therapeutic potential of labdane diterpene from *Alpinia nigra*; **Medicinal Chemistry Research**

2. **Chakrabartty I**, Kumar V, Rangan L* (2018) Physicochemical characterization and antimicrobial screening of rhizome essential oil from *Alpinia nigra*; **Phytotherapy Research**

Manuscripts under preparation

1. **Chakrabartty I**, Kalita NK, Barooah P, Katiyar V, Rangan L* Physico – rheological characterization of organically derived seed samples from *Alpinia nigra*, an ethnic culinary item of North – East India

2. Sadokpam S#, **Chakrabartty I#**, Rangan L* Antibacterial susceptible assessment of microemulsion formulation of labdane diterpene from *Alpinia nigra* (#Equal contribution)

3. Baruah PK#, **Chakrabartty I#**, Rangan L, Baruah AK, Khare A* Efficacy of cellulose paper treated with Cu and Ag nanoparticles synthesized via pulsed laser ablation in distilled water in the annihilation of bacteria (#Equal contribution)

PROCEEDINGS PAPER

Published

- Baruah PK, Raman MA, **Chakrabartty I**, Rangan L, Sharma AK, Khare A* (2018) Antibacterial effect of silk treated with Ag and Cu nanoparticles synthesized by pulsed laser ablation in distilled water; **AIP Conference Proceedings 1953**; doi: 10.1063/1.5032399

In communication

- Rangan L*, Tushar, Basak S, **Chakrabartty I**, Ghosh S, Das A, Kumar M, Sadokpam S, Vijayasekar A, Hedaoo VS, Kumar V, Ramachandran S (2018) Maintaining Biodiversity in North-east India: Ethno-medicinal usage of Zingiberaceae; **BVA Proceedings**

CONFERENCES

- **Chakrabartty I*** and Rangan L (2018) *Alpinia nigra*: The unexplored ore of Zingiberaceae for future therapeutics; *Indo-Japan Bilateral Symposium for Future Perspectives of Bioresource Utilization in North East India (IJBS'17)* , 1st-4th February 2018, IIT Guwahati, pp 68 (Oral presentation)
- **Chakrabartty I,** Panda AN, Khare A and Rangan L* (2017) FT-IR, FT-Raman, NMR and SERS studies of labdane diterpene from *Alpinia nigra*; *National Workshop on Fluorescence and Raman Spectroscopy (FCS)*, 17th – 21st December 2017, IIT Guwahati, pp 123
- **Chakrabartty I** and Rangan L* (2017) Understanding the unique inhibitory potential of (E)- labda – 8 (17), 12 – diene – 15, 16 – dial, a bioactive compound from *Alpinia nigra*, on the growth kinetics of *Candida albicans*; *Bioprocessing India*, 9th – 11th December 2017, IIT Guwahati , pp 84
- **Chakrabartty I,** Vijayasekhar A. and Rangan L* (2017) Viability assessment of bacteria under the treatment of (E)-labda-8(17), 12-diene-15, 16-dial, a bioactive compound from the seeds of *Alpinia nigra**;*** *Translational Research on Natural Products for Therapeutic Uses (TRNPTU)*, 21st November 2017, IASST Guwahati, pp 13
- Sadokpam S**,** **Chakrabartty I** and Rangan L* (2017) Formulation strategies and anti-candidal assessment of a labdane-type diterpene from *Alpinia nigra; Translational Research on Natural Products for Therapeutic Uses (TRNPTU)*, 21st November 2017, IASST Guwahati , pp 24

- **Chakrabartty I**, Khare A, Panda AN and Rangan L* (2017) Vibrational spectroscopic studies of bioactive labdane diterpene from seeds of Alpinia nigra in conjugation with Cu nanoparticles; *International Conference on "Sophisticalted Instruments in Modern Research"* (*ICSIMR)*, 30th June-1st July 2017, IIT Guwahati, pp 98
- **Chakrabartty I** and Rangan L* (2017) (E)-labda-8(17), 12-diene-15,16 dial from *Alpinia nigra*: Is it a potential agent against *Candida albicans*??; *3rd Research Conclave* , 16th- 19th March 2017, IIT Guwahati , pp 106
- Vijayshekhar A, **Chakrabartty I,** Khare A and Rangan L* (2017) Effect of labdane diterpene dialdehyde –a bioactive compound from the seeds of *Alpinia nigra*, on erythrocytes; *3rd Research Conclave* , 16th- 19th March 2017, IIT Guwahati , pp 96
- **Chakrabartty I**, Khare A and Rangan L* (2016) Investigating the inhibitory effect and possible mode of action of (E)-Labda-8(17), 12-diene-15,16-dial from *Alpinia nigra* on *Candida albicans; International Conference on Current Trends in Biotechnology (ICCB) organized by BRSI*, 8th – 10th December 2016, VIT Vellore, pp 344
- **Chakrabartty I** and Rangan L* (2016) Isolation, identification and flow cytometric investigation of DNA leakage from *Candida albicans* upon treatment with (E)-labda-8 (17), 12-diene- 15, 16- dial from *Alpinia nigra*; *9th Annual TCS Event and Flow cytometry workshop on flow applications in basic, applied and clinical biology (FABACTCS)*, 3rd – 5th November 2016, IIT Guwahati, pp 60
- **Chakrabartty I**, Khare A, Panda AN and Rangan L* (2016) Raman spectroscopic studies of labdane diterpene, a bioactive compound from the seeds of *Alpinia nigra*; *22nd Indian Society for Chemists and Biologists* (*ISCB 2016) International Conference* , 6th-8th February 2016, Uka Tarsadia University, Surat, pp 150

WORKSHOPS ATTENDED

- Workshop on "*Advanced Techniques in Cell and Molecular Biology*" organized by Department of Biotechnology, IIT Guwahati on June 2014 at IIT Guwahati
- Workshop on "*Flow Cytometry Data Analysis*" organized by Department of Biotechnology, IIT Guwahati on February 2015 at IIT Guwahati
- "*Indo – Japan Workshop on Translational Agriculture – Avenues for International Cooperation*" organized by DBT and Department of Biosciences and Bioengineering,

IIT Guwahati on January 2017 at IIT Guwahati

- 9th TCS annual event and flow cytometry workshop on "*Flow applications in basic, applied and clinical biology*" organized by TCS and Department of Biosciences and Bioengineering, IIT Guwahati on November 2016 at IIT Guwahati
- Workshop on "*ZE5 & Droplet Digital PCR – QX200*" organized by Department of Biosciences and Bioengineering, IIT Guwahati on November 2017 at IIT Guwahati
- "*Sensitization Workshop on Technological Empowerment of Women*" organized by Department of Biosciences and Bioengineering, IIT Guwahati on November 2017 at IIT Guwahati
- Workshop on **"***Research Methodology*" organized during Reflux on January 2018 at IIT Guwahati
- Workshop on "*Capacity Building in Effective Management of Intellectual Property Rights (IPRs) in Biotechnology by Universities and Research Institutes in Assam*" organized by Biotech Consortium India Limited (BCIL), New Delhi on November 2014 at College of Veterinary Science, Assam Agricultural University, Guwahati
- Workshop on "*Protection of Traditional Product Names in the North East Region using Geographical indications. Its importance in enhancing business and promoting Regional Art and Craft*" organized by Ministry of Commerce and Industry, Department of Industrial Policy Promotion, GI Registry, Chennai, Govt. of India on February 2015 at NEDFi Auditorium, Guwahati
- Workshop on "*Intellectual Property Rights*" organized by IPR Cell, R&D Section, IIT Guwahati on November 2016 at IIT Guwahati
- Workshop on "*Intellectual Property Rights- hands on training on patent search*" organized by Research Conclave 2017, IIT Guwahati on March 2017 at IIT Guwahati.