Design and formulation of target specific non-invasive delivery system for pure isolates obtained from traditionally used medicinal plants to be useful for the treatment of diabetes and cancer

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CERTIFICATE FROM THE SUPERVISORS

This is to certify that this dissertation entitled **"Design and formulation of target specific non-invasive delivery system for pure isolates obtained from traditionally used medicinal plants to be useful for the treatment of diabetes and cancer"** submitted by **Mr. Md. Harun Al Rashid**. His registration for PhD has been confirmed at Jadavpur University on 05th August 2013 for the award of Ph.D. (Pharmacy) degree, is absolutely based upon his own work under the guidance of **Prof. (Dr.) Subhash C. Mandal, Dr. Vivekananda Mandal** and **Dr. A. T. Rajarajan**. Neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

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DECLARATION

I, hereby declare that the thesis **"Design and formulation of target specific non-invasive delivery system for pure isolates obtained from traditionally used medicinal plants to be useful for the treatment of diabetes and cancer"** is genuine record of research work carried out by me and no part of this thesis has been submitted to any University or Institution for award of any degree or diploma.

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Contents

Abstract

Diospyros melanoxylon Roxb. (*D. melanoxylon*) belongs to the family Ebenaceae and its leaves are very well known for making beedi throughout the World. *D. melanoxylon* is traditionally used medicinal plant. The studies in this dissertation were conducted to obtain experimental evidence on the preparation of nanoparticles and therapeutic efficacy of *D. melanoxylon* as an anticancer and antidiabetic agent. The leaves was extracted with n-hexane followed by ethyl acetate solvent and fractionated with N-hexane, chloroform, ethyl acetate and aqueous fraction and subjected to phytochemical screening. Qualitative phytochemicals analysis of the samples from *D. melanoxylon* was carried out for the detection of secondary metabolites. Total flavonoids, phenolics, triterpenoids and tannins content of were evaluated using colorimetric assay. Qualitative analyses of polyphenolic compounds were performed using HPLC method. The antioxidant activity was examined by assessing the various radical scavenging assays. Some human cancerous cell lines (HeLa, MCF-7, HCT-116, PC-3, RCC 45, and HEK293) and normal cell lines (NKE and WI-38) were used for anticancer activity for the assessment of cell death summary through methylene blue assay. This study also estimated the comparative extraction techniques and its *in-vitro* antidiabetic prospective of the leaves of *D. melanoxylon.* The *in-vitro* antidiabetic assay was performed using α-amylase and α-glucosidase assay. The results indicated that ethyl acetate fraction (EAF) followed by aqueous fraction (AQF) exhibited remarkable content of flavonoids, phenolics, triterpenoids and tannins. Both EAF and AQF have anticancer and antidiabetic activity with significant IC_{50} values but not showed significant antioxidant activity. However, the most powerful anticancer and antidiabetic activity was recorded by EAF followed by AQF at a dose dependent manner. It is also revealed that EAF and AQF exerted low or minimal toxicity against normal cell lines.

Above study confirmed the better anticancer and antidiabetic activity of ethyl acetate fractions obtained from *D. Melanoxylon*. To assessment the hypothesis if nanoparticle-encapsulated fraction could progress bioactivity, we prepared nanoparticle encapsulation based on poly (lactide-co-glycolide) (PLGA) and confirmed encapsulation by scanning electron microscopy (SEM). Sub ethyl acetate fraction (SEAF) and nanoparticles of subethyl acetate (NSEAF) were characterized for their anticancer and antidiabetic activity. Comparatively, NSEAF showed better activity on different cancerous cell lines HCT116 (IC_{50} 32.89 ± 1.71 µg/ml), MCF-7 (IC_{50} 36.13 ± 0.96 µg/ml) and PC-3 (IC_{50} 32.39 ± 1.91 µg/ml), and antidiabetic activity on the basis of α-amylase (IC_{50} 35.62 ± 2.56 µg/ml) and α-glucosidase (IC_{50} 73.52 ± 1.13 µg/ml) action at a dose dependent manner. It is also demonstrated that NSEAF exerted very low toxic effect against normal cell lines. For this reason, NSEAF studied in the present research may be considered as possible future drug candidates for the treatment and management of cancer and diabetes.

List of abbreviations

ROS	Reactive oxygen species
RNS	Reactive nitrogen species
DNA	Deoxy Ribo nuclic acid
RNA	Ribo nuclic acid
DM	Diabetes Mellitus
WHO	World Health Organization
PLGA	Polylactic Co-glycolic Acid
CS	Chitosan
MPS	Mononuclear phagocytic system
FDA	Food and drug administration
SLN	Solid lipid nanoparticles
PNPs	Polymeric nanoparticles
PLA	Poly (lactic acid)
PAMAM	Polyamidoamine
PG	Poly (L-glutamic acid)
PEI	Polyethyleneimine
PEG	Polyethylene glycol
ZnO	Zinc oxide
EDTA	Ethylene diamino tetra acetic acid
SWNTs	Single-walled nanotubes
MWNTs	Multi-walled nanotubes
MAE	Microwave-assisted extraction
PSA	Prostate specific antigen
MBT2	Murine bladder tumor
EGFR	Epidermal growth factor receptor
CDK	Cyclin dependent kinase
MAPK	Mitogen-activated protein kinase
NSAIDs	Nonsteroidal anti-inflammatory drugs
COX	Cyclooxygenase
PGs	Prostaglandins
PAF	Platelet aggravating factor
UA	Ursolic acid
PI3K	Phosphatidylinositol-3-kinase
FFA	Free fatty acid
TPA	12-O-tetracanoylphorbol-13-acetate
MIN	Minute

NAFLD	Nonalcoholic fatty liver disease
OA	Oleanolic acid
AOM	Azoxymethane
LDL	Lowdensity lipoprotein
EAF	Ethyl acetate fraction
CF	Chloroform fraction
AQF	Aqueous fraction
NHE	N-hexane extract
NHF	N-hexane fraction
DPPH	2, 2 diphenyl 1 picrylhydrazide
DMEM	Dulbecco's modified Eagle's medium
RPMI	Roswell Park Media Institute
SEM	Scanning electron micrographs
HPLC	High performance liquid chromatography
SDS	Sodium dodecyl sulphate
ATCC	American Type Culture Collection
SE	Soxhlet extraction
UAE	Ultrasound assistant extraction
ME	Microwave extraction
MAE	Microwave assisted extraction
MCF-7	Breast cancer cell
HCT 116	Human colon carcinoma cell
PC-3	Prostate cancer cells
HEK293	Human epithelial kidney
RCC 45	Rat colon cancer cells
NKE	Normal human kidney epithelium
NPs	Nanoparticles
V-SRC	sarcoma viral oncogene homolog
V-HARAS	Harvey rat sarcoma viral oncogene homolog
AI	adequate intake
UL	upper intake level
RT	transcriptase
FBS	Fetal bovine serum
PS	Penicillin-streptomycin
TLC	Thin layer chromatography
AA	Ascorbic acid
PVA	Polyvinyl alcohol
SEAF	Subethyl acetate fraction
NSEAF	Nanoparticle of subethyl acetate fraction
DTA	Data Transfer Assistance
PBS	Phosphate buffer serum
PDI	polydespersity index

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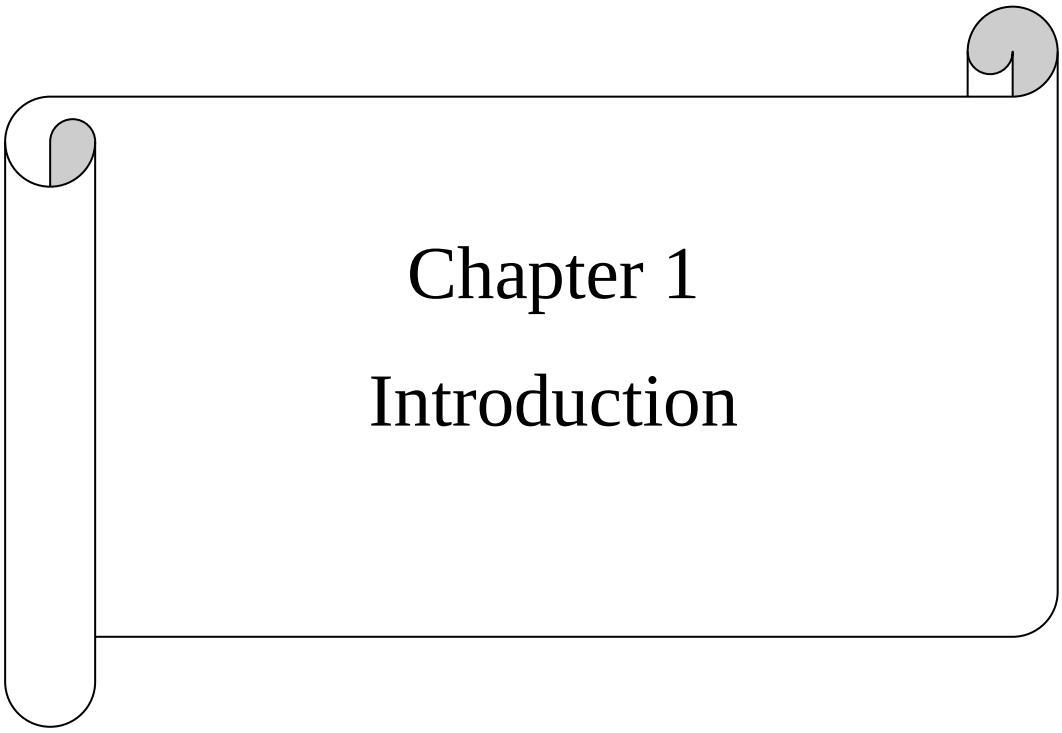


Chapter 1

Introduction

1. Introduction

1.1. Cancer

Cancer is one of the most stressful and life aggressive disease which implements cruel deaths globally. It is also the subsequent leading reason of death in urbanized countries after cardiovascular diseases and anticipated to declare nine million lives globally by 2015 (Rajesh et al., 2011). Although, chemotherapy is the widespread choice used for treatment of cancer, but it is frequently connected with number of negative aspects, viz., nonselective delivery of drugs, multidrug resistance, enhanced drug toxicity, adverse side effect to healthy tissue and inherent missing of advantageous respond of antioxidant and cytotoxic anticancer drug. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treatment of cancer, strongly points out that there is an imperative need of alternative strategies in cancer management (Das et al., 2009; Sahoo et al., 2007).

Oxidative stress or oxidative imbalance is the root cause for many life threatening diseases. The imbalance between reactive oxygen species (ROS) and reactive nitrogen species (RNS) primarily causes oxidative damage of DNA, RNA, proteins, sugars, and lipids which induce cell damage through endogenous mechanism system. The over production of ROS and RNS plays a perfect foundation for the onset of several life threatening ailments including cancer (Mihailovi et al., 2015; Valko et al., 2007; Wiseman and Halliwell, 1996).

Recently, medicinal herbs have been widely accepted with escalating awareness all over the world. Now-a-days, about 65 % of plant based medicine is used for cancer therapy (Harun et al., 2015; Nurhanan et al., 2008). In this regard, identification and characterization of the nontoxicity and efficacy of traditional herbs with antioxidative and antiproliferative action has been of utmost research interest and have open up a novel alternative to replace harsh and stressful chemotherapies for cancer treatment (Lo et al., 2002). Herbs act as a prosperous resource of antioxidant compounds viz., minerals, carotenoids, thiols, vitamins such as

ascorbic acid, tocopherols, phenolics, anthocyanins, stilbenes, flavonoids, lupeols (Mihailovi et al., 2015; Raza and John, 2008; Brewer, 2011) and also provides ample resource of antiproliferative compounds viz., podophyllotoxin, paclitaxel or vincristine, capable to dynamically reduce the growth of cancerous cells (Dewick, 2009). It is believed that eating of plant-based antioxidants could be linked with lesser occurrence of numerous human diseases associated to oxidative stress, including cancer (Halliwell and Gutteridge, 2007). In future, research on dietary antioxidants from plant sources can play a pivotal role in combating cancer and bringing in innovation in the treatment of cancer through a holistic approach.

1.2. Diabetes

Diabetes Mellitus (DM) is a rising health trouble. Currently about 250 million people are living with diabetes and this figure is anticipated to be more than 366 million by 2030 according to WHO reports (Hsieh et al., 2012). In calculation, nearly 3.2 million deaths per year are attributable to difficulties of diabetes; six deaths every minute (Chaudhary et al., 2012). DM is a recurrent metabolic disorder leads to the deficiency in the formation of insulin by the pancreas and has resulted significant morbidity and mortality because of microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular complications (heart attack, stroke and peripheral vascular disease) of patients (Umar et al., 2010). DM is principally linked with carbohydrate, fat and protein metabolism which reduced production of insulin to its action (Gregoire et al., 1998). Treatments of insulin dependent diabetes or type 2 (T2DM) basically improve insulin sensitivity or reducing the rate of carbohydrate absorption from the gastrointestinal tract. Numerous side effects are observed of the drugs which used to treat T2DM, especially for those patients with liver and renal functional disorders (Israili, 2011). Mammalian α-amylase is a prominent enzyme in the pancreatic juice, breaking down large and insoluble starch molecules into absorbable molecules, ultimately maltose (Gupta et al., 2003). On the other hand α-glucosidase is a membrane-bound enzyme at the epithelium of the

small intestine and plays a key role in carbohydrate digestion. Inhibition of α-amylase and α-glucosidase leads to the delay or reduction of increased postprandial blood glucose levels. Thus, these two enzymes have been proposed as a potential therapeutic target for drug discovery in the treatment of T2DM (Fred-Jaiyesimi et al., 2009).

Currently existing available drugs for the treatment of diabetes is not free from side effects. So, it is very important to identify and assess usually available natural drugs as alternatives to currently used antidiabetic drug. In this view, plants are cheaper and much effective, and are completely free from adverse effects. Traditionally a number of medicinal plants are used as antidiabetic and some of them have shown their efficacy through pharmacological evaluation. In addition, depending on traditional medical practices, the World Health Organization encouraged to study for the treatment and prevention of diabetes diseases (Harun et al., 2015; Ezeigbo et al., 2012).

A large number of bioactive triterpenoids have shown multiple biological activities with apparent effects on glucose absorption, glucose uptake, insulin secretion, diabetic vascular dysfunction, retinopathy and nephropathy. The versatility of the pentacyclic triterpenes provides a promising approach for diabetes management and antiadipogenic activity (Alqahtani et al., 2013).

Basically polyphenolic compounds viz. Phenols and flavonoids having their antidiabetic properties (Soumyanath, 2006). Polyphenolic compounds might prove to be important for alternative diabetes treatments or reduction of the risk of the disease. Attempts have been made to determine their potential in preventing β-cell apoptosis, promoting β-cell proliferation and insulin secretion and enhancing insulin activity (Pinent et al., 2008). It is reported that the flavonoid compound rutin of *Ruta graveolens* is pharmacologically active and had the capability to control insulin activity and insulin resistance in type 2 diabetic rats (Ahmed et al., 2010). It has been demonstrated that the flavonoids act as insulin

secretagogues or insulin mimetics, probably by influencing the pleiotropic mechanisms of insulin signaling in diabetes mellitus (Dam et al., 2013). The antidiabetic activity of triterpenoid saponin is may be due to reversing of atropy of the pancreatic islet of β-cells, as a result of which there may be increased insulin secretion and increase in the hepatic glycogen level and these may attenuate hyperinsulaenimia. The α-adrenergic blocking effect might contribute to their insulin secretion and sensitizing effects (Raju and Balaram, 2008).

1.3. Nanotechnology

Recently nanotechnology offered different efficient and safe drug delivery system where a constant dose of therapeutic agents is delivered directly to the cells, tissues and organs over an extended period resulting in an alternative therapeutic approach. By confining the activities of the drugs within the tissues and cells such delivery systems are foreseen as potential therapy candidates leading to focused destruction of the target area by minimizing arbitrary drug distribution (Sahoo and Labhasetwar, 2003). Modern nanoparticulate dosage forms including polymeric nanoparticles, nanocapsules, liposomes, solid lipid nanoparticles, and nanoemulsions, all of which can improve drug solubility. In general, nanoparticulate drug delivery enhances solubility and bioavailability, improving pharmacological activity and tissue macrophage distribution, while preventing physical and chemical degradation (Kumar, 2010; Raffa et al., 2010).

Applying nanotechnology to plant extracts has revealed an advantageous strategy for herbal drugs considering the numerous features that nanostructured systems have to offer, including solubility, bioavailability, and pharmacological activity enhancement, protection from toxicity, sustained delivery, and protection from physical and chemical degradation (Ajazuddin and Saraf, 2010; Bonifácio et al., 2014). Currently, nanotechnological processes involving medicinal plants have provided several innovative delivery systems, including polymeric nanoparticles. These materials, made from biodegradable and biocompatible

polymers such as Polylactic Co-glycolic Acid (PLGA) and chitosan (CS), represent an option for controlled drug delivery (Bonifácio et al., 2014). Hereby we select *D. melanoylon* which has a potent antiproliferative and antidiabetic potential apart from other medicinal significances such as, antiinflammatory, antiatherosclerotic, antimutagenic, anticarcinogenic, antibacterial, and antiviral activities (Rathore et al., 2014; Aiyegoro and Okoh, 2010).

1.4. Challenges of nanotechnology

In order to overpower this life-threatening disease several treatment modalities have been discovered to date. The predominant option for treating disease is cancer therapy. This approach may be successful to some extent, but they have their own drawbacks such as the limited accessibility of drug to the various tissues, the requirement of high doses, undesirable cytotoxicity, the development of multiple drug resistance and non-specific targeting. Thus, there is an urgent need to develop new and innovative technologies that could help to overcome the drawbacks associated with conventional cancer therapy treatment (Seigneuric et al., 2010).

During the rapid development of nanotechnology, the demand of nanoparticulate drug delivery systems is increasing (Kurmi et al., 2010; Kumar, 2010). Modern nanoparticulate dosage forms including polymeric nanocapsules, liposomes, nanoemulsions, nanoparticles, and solid lipid nanoparticles, all of which can enhance the drug solubility. Generally, nanoparticulate drug delivery enhances solubility and bioavailability, developing pharmacological activity and tissue macrophage distribution, improve lack of drug releasing profile and poor absorption. (Raffa et al., 2010; Anand et al., 2010).

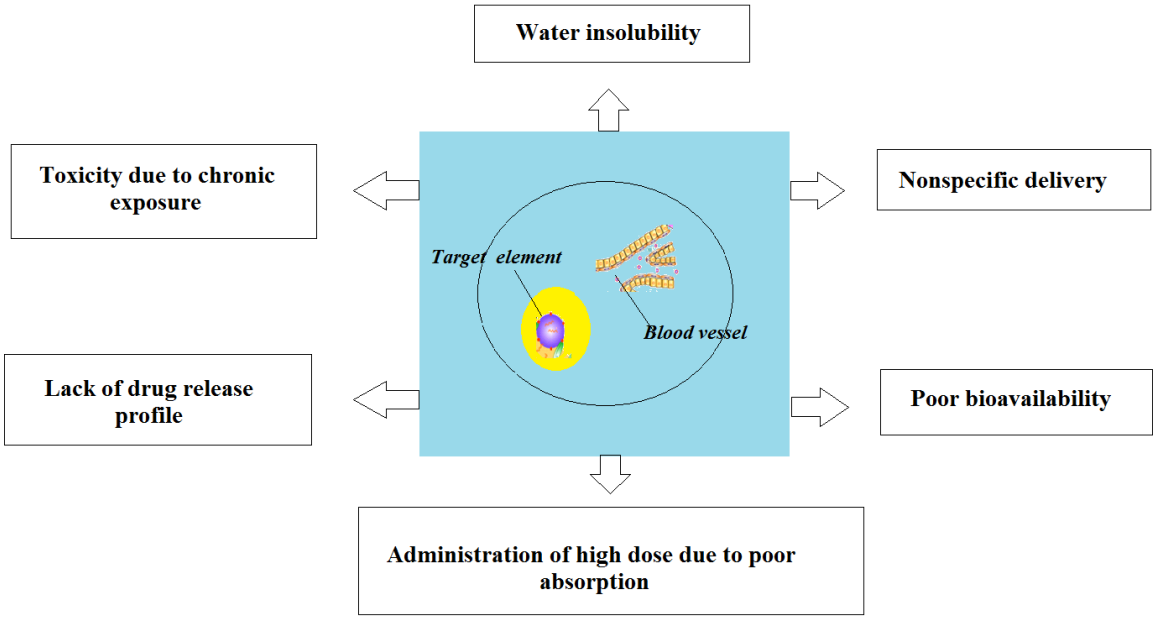


Figure 1 Challenges of nanotechnology of herbal drugs

In cancer therapy, localized delivery of the drug to various tissues is the key challenge. To wage an effective war against oxidation and microbial therapy, the new treatment approach should have the ability to selectively attack the target cells and tissues, while saving the normal cells from excessive burdens of drug toxicity. However, many drugs are designed to simply kill microorganism, often in a semi-specific fashion, and the distribution of antioxidant, antimicrobial and anticancer drugs in healthy organs or tissues is especially undesirable due to their potential for severe side effects. As a result, systemic application of drugs often causes severe side effects in other tissues which greatly limit the maximum allowable dosage of the drug. Further, fast elimination and extending over a wide area distribution into non-targeted organs and tissues requires the administration of a drug in large quantities, which is often not economical and sometimes complicated due to non-specific toxicity. Although conventional cancer therapy has been considered as one of the main modalities of treatment for oxidant and microbial attack, its triumph is largely hindered due to inadequate accessibility of anticancer agents to cells, tissue and organ, their intolerable

concentration-dependent toxicity, requiring high doses, rapid response, poor solubility and inconsistent bioavailability. Thus to mitigate the difficulty associated with conventional cancer therapy there is a call for developing an efficient drug delivery system that optimizes the pharmaceutical action of a drug while reducing its toxic side effects .

Nanotechnology is expected to play a critical role in creating a novel and efficient drug delivery system that can overcome the problems associated with conventional cancer treatment. The application of nanotechnology for drug delivery is widely expected to create novel therapeutics capable of changing the landscape for pharmaceutical application (Zhinan et al., 2003).

1.5. Approaches of nanotechnology

In recent years, the nanonization of herbal medicines have attracted a good deal of attention (Ratnam et al., 2006). Generally, nanoparticles may be defined as solid colloidal particles that include both nanospheres and nanocapsules. The particles are prepared by the methods of polymerization and synthesis with preformed polymers (Fattal and Vauthier, 2002; Vauthier and Bouchemal, 2008). One of their fundamental characteristics is the size, which is generally around 5-10 nm with an upper size limit of 1000 nm (Alle´mann et al., 1993). Generally the normal range of nanoparticles is 100-500 nm (Quintanar et al., 1998a). Nanoparticles systems with mean particle size well above the 100 nm standard have been reported in literature, including nanonized curcuminoids (Tiyaboonchai et al., 2007), paclitaxel (Arica et al., 2006) and praziquantel (Mainardes and Evangelista, 2005) which have a mean particle size of 450, 147.7, and >200 nm, respectively. In addition, nanoparticles could also be defined as being submicronic colloidal systems (Brigger et al., 2002). The nanospheres have a matrix type structure in which the active ingredient is dispersed throughout (the particle), whereas, the nanocapsules have a polymeric membrane and an active component core. Nanonization has several advantages viz., increasing ingredient

solubility, reducing medicinal doses, and improving the absorbency of herbal medicines compared to the respective crude drugs preparations (Katti et al., 2009).

1.6. Types of nanoparticles

1.6.1.Liposomes

Liposomes are concentric bilayered vesicles. The lipid bilayer is mainly composed of natural or synthetic phospholipids. An aqueous volume is entirely enclosed in the liposome by the lipid bilayer. Liposomes are mainly determined in terms of their size, surface charge and number of bilayers. Liposomes show number of advantages such as biocompatibility, amphiphilic character, and ease of surface modification making them a suitable drug delivery system for biotech drugs (Abhilash, 2010). Liposomes find their applications mainly in the field of biochemistry, biology, and medicine since its origin. All these parameters can change the pharmacokinetic profile of loaded drug mainly in case of proteins and peptides and can be easily modified by surface attachment of polyethylene glycol-units (PEG) making it as stealth liposomes and thus increase its circulation half-life (Rawat et al., 2006).

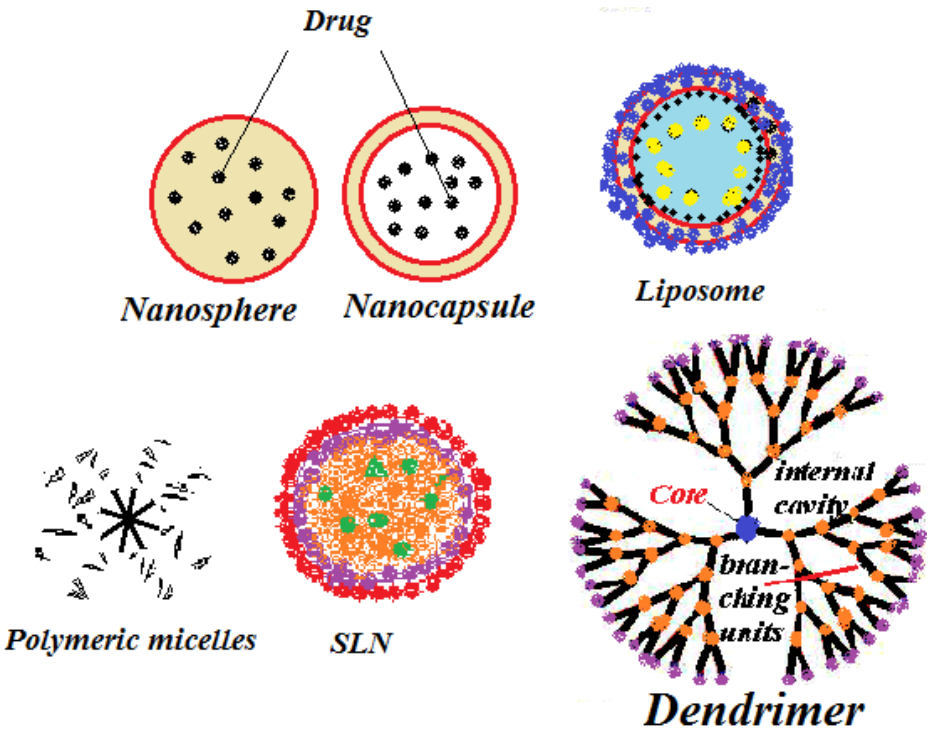


Figure 2 Different types of nanoparticles.

1.6.2. Nanocrystals and nanosuspension

Collection of hundreds or thousands of molecules that combine in a crystalline shape, comprising pure drug with only a thin covering of surfactant or combination of surfactants are called nanocrystals. Poorly soluble drugs that exhibit reduced bioavailability, unseemly absorption model and problems of preparing the parenteral dosage form may be overcome when formulated as nanocrystals. It has several benefits unlike carrier-based nanoparticles in which extent of loading may be low. For the preparation of nanocrystals small amount of surfactants are used for static and electrostatic surface stabilization. Due to slow dissolution rate, administration of high drug levels with depot release can be achieved with nanocrystals. As pure drug is used and no carrier is needed, potential toxicity issues associated with the carrier molecule is eliminated. Nanoparticles offer the potential for targeting the mucosa of the gastrointestinal tract after oral administration, and aiming the cells of the mononuclear phagocytic system (MPS) to treat infections such as fungal and mycobacterial infections and leishmaniasis, thus serving as a favourable delivery system for drugs like amphotericin B, tacrolimus, etc. (Abhilash, 2010). The size of nanocrystals allows for safe and effective drug delivery route through capillaries. Potential of nanocrystals can be inferred by the FDA approval of Rapamune®, containing sirolimus which is an immunosuppressant drug to prevent graft rejection in children after liver transplantation and Emend®, which contains aprepitant, MK 869, is used in the treatment of emesis associated with the cancer chemotherapy (Abhilash, 2010; Rabinow, 2004; Junghanns and Müller, 2008).

1.6.3. Polymeric nanoparticles

In comparison to solid lipid nanoparticles (SLN) or nanosuspensions, polymeric nanoparticles (PNPs) consist of a biodegradable polymer. Biocompatibility is an important characteristic for potential application in tissue engineering, drug and gene delivery and new vaccination process. Most biodegradable polymers mainly consists of synthetic polyesters

like polycyanoacrylate or poly (D, L-lactide) and related polymers like poly (lactic acid) (PLA) or poly (lactide-co-glycolide) (PLGA). Latest developments also include natural polymers like chitosan, gelatin, and sodium alginate to overcome some toxicological problems with the synthetic polymers. Polymeric nanoparticles represent a significant improvement over traditional oral and intravenous methods of administration in terms of efficiency and effectiveness (Abhilash, 2010). The advantages of using PNPs in drug delivery are many, being the most important that they generally increase the stability of any volatile pharmaceutical agents and that they are easily and cheaply fabricated in large quantities by a multitude of methods (Abhilash, 2010). Also, polymeric nanoparticles may have engineered specificity, allowing them to deliver a higher concentration of pharmaceutical agent to a desired location. Mostly under the term of nanoparticle, nanospheres are understood. From its definition nanospheres are considered as a matrix system in which the matrix in uniformly dispersed. It should be mentioned, that besides these spherical vesicular systems, nanocapsules are also known, where a polymeric membrane surrounds the drug in a matrix core (Abhilash, 2010). The choice of polymer and the ability to modify drug release from polymeric nanoparticles have made them ideal candidates for cancer therapy, delivery of vaccines, contraceptives and delivery of targeted antibiotics (Kayser et al., 2005; Abhilash, 2010). Moreover, polymeric nanoparticles can be easily incorporated into other activities related to drug delivery, such as tissue engineering, and into drug delivery for species other than humans. From the polymer chemistry viewpoint, there will be in the future a challenging field to create new polymers matching hydrophilic and lipophilic properties of upcoming drugs for smart formulation (Kayser et al., 2005; Abhilash, 2010).

1.6.4. Dendrimers

Dendrimers, a unique class of polymers, are highly branched macromolecules whose size and shape can be precisely controlled. Dendrimers are synthesized from monomers using either

convergent or divergent approach of polymerization (Abhilash, 2010). The well defined structure, monodispersity of size, surface functionalization capability, and stability are properties of dendrimers that make them attractive drug carrier candidates (Hughes, 2005). Drug molecules can be included into dendrimers via either complexation or encapsulation. Dendrimers are being investigated for both drug and gene delivery, as carriers for penicillin, and for use in anticancer therapy. Dendrimers used in drug delivery studies typically incorporate one or more of the following polymers: polyamidoamine (PAMAM), melamine, poly (L-glutamic acid) (PG), polyethyleneimine (PEI), poly (propyleneimine), polyethylene glycol (PEG) and Chitin (Hughes, 2005; Abhilash, 2010).

1.6.5. Gold nanospheres

Gold particles are of highest quality and can be used in the production of diagnostic tests as well as conjugation studies of proteins and antibodies. The particles have a narrow size distribution (CV between 5% and 15% depending on size) and are available from 2 to 250 nm. The number of particles/mL is given in the product/ ordering table. The solutions are stabilized with HAuCl4 gold and silver colloids or sols are available in a number of different sizes. There are 14 different gold colloid sizes and are offered in four packing sizes. The products are best stored at room temperature, although storage at 4°C is an option. However, temperatures too close to freezing will destabilize the sol, causing aggregation and product loss. (Bernkop-Schnurch et al., 2003).

1.6.6. Zine oxide nanoparticles

Zinc oxide (ZnO) is considered to be a technologically prodigious material having a wide spectrum of applications such as that of a semiconductor (Eg = 3.37 eV), magnetic material, electroluminescent material, piezoelectric sensor and actuator, naanostructure varistor, field emission displaying material, thermoelectric material, gas sensor, constituent of cosmetics etc. Often chemical synthesis methods like sol-gel process, micelle, chemical precipitation,

hydrothermal method, pyrolysis, chemical vapour deposition etc. lead to the presence of some toxic chemical species adsorbed on the surface that may have adverse effect in medical applications. Some reactions require high temperature and/ or high pressure for initiating the reaction, while some reactions require inert atmosphere protection, and/toxic matters such as H_2S, toxic templeate and stabilizer, and metallic precursors (Hudlikar et al., 2010). In nanoparticles synthesis chemicals used are toxic and lead to non-ecofriendly by products (Singhal et al., 2011). Use of biological organisms such as micro organisms, plant extract or plant biomass could be an alternative to chemical and physical methods for the production an eco-friendly manner on of nanoparticles (Bhattacharaya and Rajinder, 2005). Several biological systems including bacteria, fungi and yeast have been used in synthesis of nanoparticles (Alagumuthu et al., 2012). Synthesis of nanoparicles using microorganism involves elaborate process of maintaining cell cultures, intracellular synthesis and multiple purification steps. In this regard using "green" methods in the synthesis of Zinc oxide nanoparticles has increasingly become a topic of interests as conventional chemical compounds/organic solvents as reducing agents (Mason et al., 2012).

1.6.7. Silver nanoparticles

Silver nanoparticles are broadly applied in shampoos, soaps, detergents, cosmetics, toothpastes and medical and pharmaceutical products and are hence directly encountered by human systems (Bhattacharya and Murkherjee, 2008; Bhumkar et al., 2007). Earlier, the antifungal properties of silver and silver nitrate were well incorporated in the field of medical science. Also, the medicinal importance of innumerable plants and plant parts were known. But the plant-mediated silver nanoproduct is a relatively newer concept. Nanobiotechnology and their derived products are unique not only in their treatment methodology but also due to their uniqueness in particle size, physical, chemical, biochemical properties and broad range of application as well. This current emerging field of nanobiotechnology is at the primary

stage of development due to lack of implementation of innovative techniques in large industrial scale and yet has to be improved with the modern technologies. Hence, there is a need to design an economic, commercially feasible as well environmentally sustainable route of synthesis of Ag NPs in order to meet its growing demand in diverse sectors.

Various approaches available for the synthesis of silver NPs include chemical (Sun et al., 2002), electrochemical (Yin et al., 2003), radiation (Dimitrijevic et al., 2001), photochemical methods (Callegari et al., 2003) and Langmuir-Blodgett (Zhang et al., 2006; Swami et al., 2006) and biological techniques (Naik et al., 2002). In this race of Ag NP preparation, plant-mediated green biomimetic synthesis of silver nanoparticle is considered a widely acceptable technology for rapid production of silver nanoparticles for successfully meeting the excessive need and current market demand and resulting in a reduction in the employment or generation of hazardous substances to human health and the environment.

1.6.8. Silica nanospheres

These mono-disperse silica particles with a density of 2.0 gm/cm3 are simpler to dispense and to separate. These are useful for coupling of DNA, oligonuleotides, oligopeptides, proteins, lectins, and antibodies. The silica particles are also available with different functional groups as –NH2, and –COOH, albumin, protein A, epoxy, NHS, NTA, and EDTA (Li et al., 2004).

1.6.9. Carbon nanotubes

In the last 15 years, it has been an exciting time for the field of carbon nanomaterials. The discoveries of fullerenes and carbon nanotubes have attracted the attention of many researchers all over the world **(**Zhou et al., 2002**)**. Iijima **(**Iijima et al., 1991**)** first discovered these in 1991. They are now commercially available and can be manufactured on large scales **(**Maurin et al., 2001**)**. They are hexagonal networks of carbon tubes, 1 nm in diameter and 1-100 nm in length. There are two types of nanotubes: single-walled nanotubes (SWNTs) and

multi-walled nanotubes (MWNTs), which differ in the arrangement of their graphene cylinders. These are small macromolecules that are unique for their size, shape, and have remarkable physical properties. Nanotubes offer some distinct advantages over other drug delivery and diagnostic systems due to very interesting physicochemical properties such as ordered structure with high aspect ratio, ultra-light weight, high mechanical strength, high electrical conductivity, high thermal conductivity, metallic or semi-metallic behavior and high surface area **(**Sinha and Yeow, 2005).

1.7. Plant materials

Plants are potent biochemists and have been components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components (Das et al., 2010). The systematic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many laboratories. Scientific analysis of plant components follows a logical pathway. Plants are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found (Parekh et al., 2006). Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. Many authors had reported about plant extract preparation from the fresh plant tissues. The logic behind this came from the ethno medicinal use of fresh plant materials among the traditional and tribal people. But as many plants are used in the dry form (or as an aqueous extract) by traditional healers and due to differences in water content within different plant tissues, plants are usually air dried to a constant weight before extraction. Other researchers dry the plants in the oven at about 40°C for 72 hrs. In most of the reported works, underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively compared with other above ground parts in search for bioactive compounds possessing

antimicrobial properties (Ncube et al., 2008; Das et al., 2010).

1.8. Herbal medicine

Ever since the birth of mankind there has been a relationship between life, disease and plants. Primitive men started studying diseases and treatments (Lyons and Pertrucelli, 1987). There is no record that people in prehistoric times used synthetic medicines for their aliments but they tried to make use of the things they could easily procure. The most common thing they could find their environment i.e. the plants and animals (Singh and Abarar, 1990). They started using plants and found that majority of plants were suitable as food, where as other were either poisonous or medicinally useful (Fuller and Henrick, 1985). By their experience, this knowledge of herbal remedies was transferred to generation as folk medicine. So the history of herbal medicine is as old as human history. Herbal medicine is still the mainstay of about 75–80% of the world's population, mainly in developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. Aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine and vinblastine are a few important examples of what herbal medicine have given us in the past. Most of these plant-derived drugs were originally discovered through the study of traditional cures and folk knowledge of indigenous people and some of these could not be substituted despite the enormous advancement in synthetic chemistry. Consequently, plants can be described as a major source of medicines, not only as isolated active principles to be dispensed in standardized dosage form but also as crude drugs for the population. Today in many countries modern medicine has displaced plants with many synthetic products but almost 30% of pharmaceutical preparations are still obtained directly or indirectly from plants. The modern

era has seen some decline in use of medicinal plants and their extracts as therapeutic agent, particularly in developed countries, many of which either been discarded by the medical profession or now given in the form of isolated compound. The strategy of isolating the active principles calls herbal medicine which is responsible for pharmacological activities.

1.9. Extraction and phytochemical screening

Choice of suitable extraction technique is very important for extract preparation. Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al., 2008).

Extraction is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such preparations have been popularly called galenicals, named after Galen, the second century Greek physician (Remington, 2005).

Extraction methods used pharmaceutically involves the separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity (Ncube et al., 2008).

The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further

processed to be incorporated in any dosage form such as tablets and capsules. These products contain complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans (Handa et al., 2008). The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, Soxhlet extraction, sonication and microwave extraction etc.

Traditional extraction methods need lengthy time and also less effective. Some natural product degraded during the extraction methods when increase temperature as they are thermally unstable (Hemwimon et al., 2007). Traditionally, for the extraction of triterpenoids, heat reflux and Soxhlet extraction techniques had been the first line of choice (Banik et al., 2008; Zhang et al., 2007) but they are sometime not advantageous as require more time as well as organic solvent. Due to the consumption of huge energy resources it also adds up to the huge carbon load which is a severe problem affecting the entire mankind. In contrast, microwave-assisted extraction (MAE) is known for its better efficacy, good reproducibility, low consumption of organic solvents and time, and low carbon dioxide production. MAE depends on conventional heating where heat is transfer from the cell matrix into the extracting solvent (Mandal et al., 2008). Several applications of MAE for biologically active compounds have appeared in the literatures, such as extraction of coumarin and related compounds from *Melilotus officinalis* (Martino et al., 2006), extraction of tanshinones from *Salvia miltiorrhiza* (Pan et al., 2001), extraction of flavonoids from *Radix astragali* (Xiao et al., 2008), and extraction of oleanolic acid from *Gymnema sylvestre* (Mandal and Mandal, 2010).

1.10. Choice of solvents

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion

of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants (Eloff, 1998). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non- toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Ncube et al., 2008; Das et al., 2010).

1.11. Plant profile

Taxonomical classification

Kingdom: plantae
Order: Ericales
Family: Ebenaceae
Genus: Diospyros
Species: *D. melanoxylon*

Vernacular names

Sanskrit: dirghapatraka
Bengali: kendu, kend
Oriya: Kendu
English: coromandel ebony persimmon,ebony
Gujrati: amrug,tamru
Hindi: abnus,tendu,nallatumki,timburni, kendu,karundumbi
Tamil: karai,tumbi,tumki,thumbi,karundumbi
Telugu: Tumuki
Kannada: Abanasi
Malayalam: Kari

Diospyros melanoxylon Roxb. (*D. melanoxylon*) also known as “kendu” belonging to the family Ebenaceae contains a number of phyto elements such as saponins, alkaloids,

glycosides, flavonoids, terpenoids, carbohydrates and tannins which are renowned in displaying diverse biological activities together with antiinflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, and antiviral activities (Aiyegoro and Okoh, 2010). The dried flowers of *D. melanoxylon* are evidenced in Unani medicine to be used for urinary discharges, inflammation of the spleen and enhancement of the blood. The bark extract is used in Ayurveda as an astringent lotion for the eyes. This plant contains an array of bioactive constituents namely, Aurone, Lupeol, Betulin, Betulinic acid, Ursolic acid, α and β-amyrin, Uvavol, Bauerenol, Oleanolic acid, Hentriacotanol, β-sitosterol, 7-methyljuglone, Dimelquinone, Dihydroxytriterpenic acid, Methoxyderivative of -1, 4-Naphthquinone, Disinidigo A and B, Pentacylicquinone Biramentaceone, Dimethoxyderivative of 1-naphthol Hentriancontane, 8-hydroxy-octadec-10(Z)-enoic acid Malvalic acid, Bicylic sesquiterpene, Sterculic acid etc (Mallavadhani et al.,1998). The leaves are highly esteemed for wrapping “bidis” which is a cheaper rural version of “cigarettes”. Their flavor, flexibility and resistance to decay are the key factors in making their use valuable in the “bidi” industry. *D. melanoxylon* leaf trade is one of the important tribal activities of India and is a good revenue earner for Government, besides providing gainful employment to several tribal peoples (Chopra et al., 1949; Sastry, 1952).

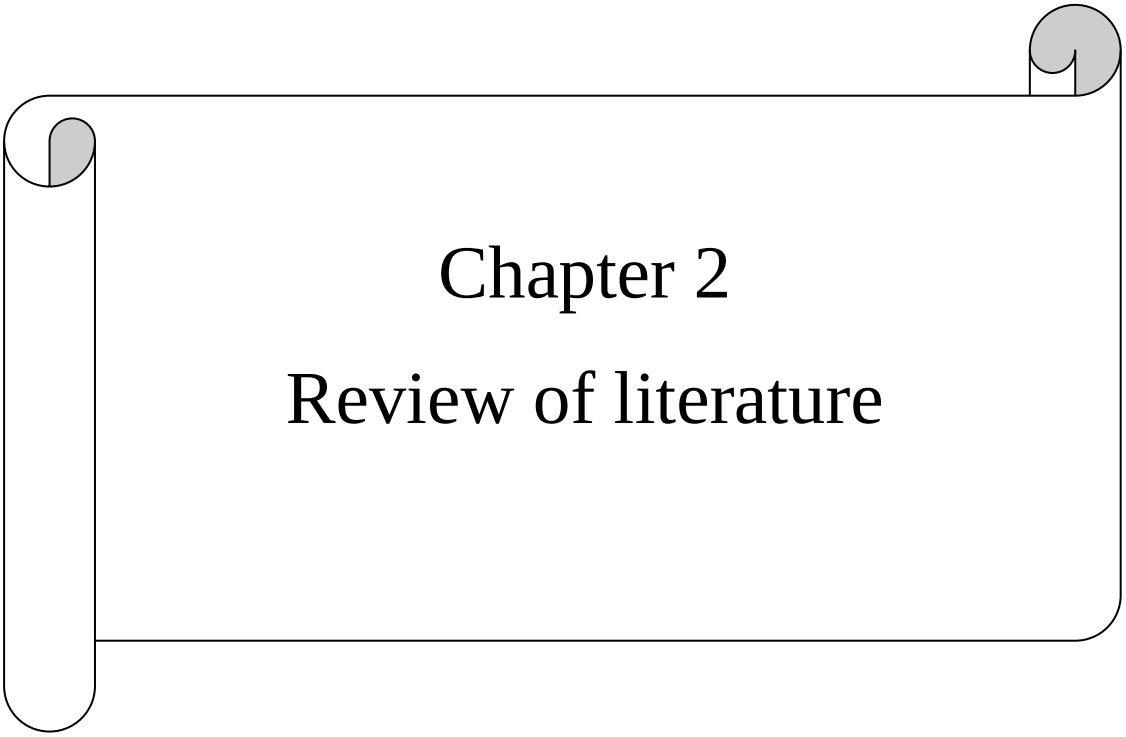
The traditional medicinal importance of the plant is ascertained as diuretic, carminative, laxative, styptic, good in epitaxis and night blindness, improves the eyesight, used in opthalmia, trichiasis, burns, tuberculosis glands, scabies, and old wounds (Gupta et al., 2009: The Wealth of India ,2006: Kirtikar and Basu, 2006).

The preliminary phytochemical screening of *D. melanoxylon* shows the presence of steroids, triterpenoids in petroleum ether extract and flavonoids, tannins, phenolic compounds, sterols, triterpenoids in ethyl acetate extract (Nadkarni, 2007). Flavonoids, tannins, phenolic compounds, steroids in alcoholic extract and carbohydrates, proteins, amino acids,

flavonoids, tannins, phenolic compounds, and tartaric acid as an organic acid in aqueous extract are also present (Gupta et al., 2013). The active phytoconstituents present in petroleum ether extract of *Diospyros meloxylon* (Roxb) furnished were ceryl alcohol, lupeol, betulin and β-sitosterol (Gupta and Rao, 1964).

A large number of bioactive triterpenoids have shown multiple biological activities with apparent effects on glucose absorption, glucose uptake, insulin secretion, diabetic vascular dysfunction, retinopathy and nephropathy. The versatility of the pentacyclic triterpenes provides a promising approach for diabetes management and antiadipogenic activity (Alqahtani et al., 2013). Steroids have the ability to release insulin by the stimulation of regeneration process and revitalization of the remaining β cells. Lupeol (triterpenoid) and β-sitosterol (steroid) cause decrease in glycated hemoglobin, serum glucose and nitric oxide with a concomitant increase in serum insulin level (Soumyanath, 2006; Pinent et al., 2008). Lupeol minimizes the lipid abnormalities and possesses cardioprotective effects which will be beneficial in hypercholesterolemic condition (Ahmed et al., 2010).

D. melanoxylon is used in the management of diverse diseases and treatment of diabetes, anaemia, inflammation of spleen (Dam et al., 2013) dyspepsia, diarrhoea, scabies, hypotensive and used as carminative, laxative and astringent. The beneficial effects of flavonoids have been studied in relation to diabetes mellitus which may act as insulin secretagogues or insulin mimetics, probably by influencing the pleiotropic mechanisms of insulin signaling (Raju and Balaram, 2008).



Chapter 2

Review of literature

2. Review of literature

2.1. Herbal management of cancer

2.1.1. East Asia

Different types of herbal medicines have been demonstrated to exhibit anticancer activity of different cancerous cell lines from China and other parts of East Asia. The plant *Paeoniae radix*, commonly known as red peony, has been used in the treatment of liver diseases for the centuries in China. Presently, it was demonstrated that the plants having the activity against the human hepatoma cell lines HepG2 and Hep3B after exposure to *P. radix* by the induction of apoptosis through a pathway independent of tumor suppressor gene, p53 (Lee et al., 2002). Aqueous solution of *Astragalus redix* is another example of an ancient Chinese herbal remedy, commonly known as milk vetch. The growth of the gastric cancer cell lines AGS and KATO-III, colon cancer cell HT29, breast cancer cell lines MEL7 and MEL14 were inhibited using this herbs extracts. The inhibitions of the cell proliferation were associated with the gastric cancer cell lines with 68% and 62% inhibition of AGS and KATO-III, respectively. At a specific concentration and time, an AGS cell lines showed growth reduction which was found not to be due to apoptosis (Lin et al., 2005). Another traditional Chinese medicine is *Panax ginseng* which is now found frequently in Korean, Japanese, and Chinese prescriptions for chronically ill patients (Lin et al., 2003).

Aegle marmelos is an important ancient herbal medicinal plant from Bangladesh that has revealed inhibitory activity on the proliferation of a variety of cancer cell lines (Chang et al., 2003). Several cell lines viz., leukemic cell line K562, T-lymphocyte Jurkat cells, MCF-7 breast cancer cells, and melanoma Colo38 cells were tested. Three extracts of *A. marmelos* have been reported to exhibit the greatest anti-proliferative effects on K562 leukemia cells. All cell lines were tested against these extracts in a dose-dependent manner and showed the activities. Mixtures of traditional natural herbs have also been used in the treatment of cancer.

PC-SPES is a patented mixture of eight herbs. PC stands for prostate cancer, and SPES is the Latin word for hope. Commercially available mixture of 8 herbs of PC-SPES is composed of: chrysanthemum (*Dendranthema morifolium*), licorice (*Glycyrrhiza glabra*), isatis (*Isatis indigotica*), scutellaria (*Scutellaria baicalensis*), saw palmetto (*Serenoa repens*), *Panax pseudoginseng*, *Rabdosia rubescens*, and *Ganoderma lucidum* (Wang et al., 2007). The ingredients in this mixture have been used in Chinese medicines with some scientific research to support its use (Lampronti et al., 2003). For example, in China, *P. pseudoginseng* (pseudoginseng or mountain paint) has been used for centuries as a herbal medicine and has presently found to have antioxidant activities (DiPaola et al., 1998). *G. lucidum* (lingzhi mushroom) has been used to treat disorders of the immune system in Chinese medicines and has been shown to inhibit proliferation of human colorectal cancer cells (Halicka et al., 1997). *R. rubescens* (blushred rabdosia), a herbal remedy is commonly used for medicinal purposes. It has been used against esophageal cancer for its anticancer activity (Manosroi et al., 2006). It was found that prostate cancer, PC-SPES decreases testosterone levels in patients during use and that the levels increase 3 weeks after its discontinuation (Lansky E 52). PSA (prostate specific antigen) levels had a concomitant decrease after the first treatment of PC-SPES in each 8 patients who participated in the study. After the treatment was halted the concentration of PSA increased within 3 weeks. Several studies have demonstrated that PC-SPES inhibits the growth of MCF-7 cells *in vivo* (Bisi-Johnson et al., 2011).

2.1.2. Middle East

Ficus species is the one of the most widely used herbs for medicinal purposes in the Middle East for millennia (Chiang et al., 2005). It is commonly known as fig. Various types of compounds have recently been extracted from the stems of *F. formosana* (Moraceae) and have been shown to have cytotoxic activity against HepG2, PLC/PRF/5 hepatocellular, and Raji lymphoblastoid cancer cells *in vitro* (Rubnov et al., 2001). Moreover triterpenes were

derived from *F. microcarpa* roots and eleven were tested against the proliferation of HONE-1 nasopharyngeal, KB oral epidermoid, and HT29 colorectal- carcinomas, with eight having IC_{50} values ranging from 4.0-9.4 μM (Aburjai et al., 2007). On the other hand, 6-*O*-acyl-*B*-D-glucosyl-*B*-sitosterols were derived from *F. carica* and tested against various cancer cell lines (Sfaxi et al., 2009). DJ-75 Burkitts lymphoma cells and Jurkat T-cell lymphoma cells were inhibited at levels of 87% and 81%, respectively at concentrations of 50 μg/mL. Jurkat and DU-145 prostate cancer cells were inhibited by 62% and 61%, respectively at concentrations of 25 μg/mL. Every study validates the use of *Ficus* species in traditional medicine by Middle Eastern and other populations.

In Jordan, garlic (*Allium sativum*) is the most widely used herbs as a safe medicinal plant (Li et al., 2002). A manganese superoxide dismutase derived from this herb has inhibitory effects on the growth of porcine endothelial cells and B16 mouse melanoma cells *in vitro* (Lamm et al., 2000). Another active compound ajoene is derived from *A. sativum* that has been tested against MCF-7, KB, Bel 7402 hepatocellular, BGC 823 gastric, HCT colon, HL60 promyeloleukemic, and HeLa cervical cancer cells (Rubnov et al., 2001). The lowest IC_{50} values ranged from 5.2 μM to 26.1 μM, with HL60 cells. Furthermore, an *in vivo* study determined the activity of aged garlic extract against the MBT2 murine bladder tumor model with significant inhibitions of tumor growth after exposure to doses of 50 and 500 μg/mL and improvement in survival seen in the latter (Ramasamy and Agarwal, 2008). Collectively, these studies further support *in vivo* research involving *A. sativum* in preventing or treating different forms of cancer in humans.

In the Middle East, and Europe, milk thistle (*Silybum marianum*) has been used to treat gall bladder and liver ailments (Kim et al., 2009). *S. marianum* contain a flavonolignan known as silymarin which has been found to interfere with cell cycle regulators and apoptotic proteins (Kienle and Kiene, 2010). It inhibits epidermal growth factor receptor (EGFR) signaling

along with suppressed expression of cyclin dependent kinase (CDK). There is a coinciding upregulation of the CDK inhibitors, CDK-interacting protein 1 (p21) and CDK inhibitor 1B (p27), and growth arrest at G1 and G2. The presence of silymarin leads to apoptosis through the mitogen-activated protein kinase (MAPK)/C-JUN N-terminal kinase (JNK) pathway. The most active compound of silymarin is silibinin, which has been shown to interfere with metastasis (Sabová et al., 2010).

2.1.3. Europe

Although not as widespread as in Asia and the Middle East, there is evidence indicating prevalence in the use of ancient herbal remedies to treat cancer among Europeans. Extracts of *Viscum album*, widely known as mistletoe, are among the most prescribed medicinal herbs in Europe (Hunziker-Basler et al., 2007). An aqueous extract of *V. album* was investigated for its cytotoxic effect against Jurkat cells. Enhanced cytotoxic effects have been reported when *V. album* was combined with doxorubicin (Khil et al., 2007). It has been shown that aqueous extracts of this herb exhibit a concentration-dependent Cytotoxicity on bladder cancer cells (Fukuda et al., 1999). Another study has reported that *V. album* coloratum agglutinin is effective at eliminating the human colorectal cancer cell line COLO in a time and dose dependent manner by apoptosis as shown through caspase-8 activation (Sheng et al., 1997).

Ellis (2005) and Arayne et al. (2007) demonstrated that *Coptis chinesis* (goldthread) and *Berberis vulgaris* (Pepperidge bush) played important roles in various types of health problems in Europe, North America, and China. Berberine is an isoquinoline alkaloid derived from both *Coptis* and *Berberis*. It inhibits the enzyme cyclooxygenase-2 (COX-2) thereby restraining the growth of DLD-1 colon cancer cells (Howe et al., 2001). The transcription of the COX-2 gene, which codes for the enzyme that plays an important role in the tumor genesis of colon cancer cells, is also suppressed. It had previously been reported that the cyclooxygenase-2 (COX-2) enzyme, enhanced by the expression of oncogenes such as

sarcoma viral oncogene homolog (V-SRC), Harvey rat sarcoma viral oncogene homolog (V-HARAS), and wingless-type MMTV integration site family (WNT), is activated in colon cancer cells, resulting in an increase in prostaglandins (Conese and Blasi, 1995). The mechanism of COX-2 inhibition through berberine, have also been found to help inhibit breast cancer cells (Santibanez et al., 2000).

2.2. Herbal management of diabetes mellitus

The systematic study of herbal medicines and the investigation of the biologically active principles of phytomedicines including their clinical applications, standardization, quality control, mode of action and potential drug interactions have emerged as one of the most exciting developments in modern therapeutics and medicine. Healthcare practitioners and medical scientists have come to accept that herbal medicines are different from the pharmacologically active molecules that they may contain (Rai and Carpinella, 2006). Several comparative clinical studies have been published to show that herbal medicines could have full therapeutic equivalence with chemotherapeutic agents while retaining the simultaneous advantage of being devoid of serious adverse effects. Developments in molecular biology and information technology have enhanced the understanding of the mechanism of action of many herbal drugs and the associated phytomedicines which differ in many respects from that of synthetic drugs or single chemical entities (Rai and Carpinella, 2006). Herbal medicinal products are now generally available in both developed and developing countries.

Phytochemicals from roots of ginseng have been used for over 2000 years in Far East because of their health promoting effects in diabetic cases. The ginseng species most commonly used include *Panax ginseng* (Asian ginseng) and *Panax quinquefolius* (American ginseng) (Xie et al., 2004). Their pharmacological activity is mostly attributed to ginsenosides, a family of steroids named steroidal saponins (Dey et al., 2002). The most

commonly reported side effects of ginseng are nervousness and excitation but these diminish with continued use or dosage reduction (Xie et al., 2004).

The hypoglycaemic activity of some medicinal plants have been identified and experimentally demonstrated in *in-vivo* and *in-vitro* diabetic models and documented in several studies. These plants include; *Azadirachta indica* (Prabbakar et al., 2013), *Cassia occidentalis* linn. (Laxmi et al., 2010), *Colocynthis citrullus* (Abdel-Hassan et al., 2000), *Ocimum gratissimum*, *Momordica charantia* (Joseph and Jini, 2013) and *Zingeber officinale* (Asha et al., 2011) among many others. The chemical composition and potency of herbal products depends on the plant extract derivative, the age of the plant part used season when harvested and the method of processing (Kunle et al., 2012).

Spices commonly used as diet adjuncts that contribute to the taste and flavour of foods have been demonstrated to have hypoglycemic activity (John et al., 2011). Among the spices, fenugreek seeds (*Trigonella foenumgraecum*), garlic (*Allium sativum*), onion (*Allium cepa*) and turmeric (*Curcuma longa*) have been experimentally documented to possess hypoglycemic potential (John et al., 2011).

Herbal medicines are usually perceived by the public as being natural, safe and free from side effects and the rationale for their continued use has largely rested on long-term clinical experience (Wheelwright, 1994). Plants contain hundreds of constituents and some of them may have toxic side effects which make it necessary to carry out toxicity studies. The continued use of herbal medicines will necessitate a thorough scientific investigation, and should go a long way in validating their folkloric usage.

2.3. Antidiabetic mineral elements from herbal medicine

Herbal medicines are good and balanced sources of essential micronutrients minerals that are valuable in the management of diabetes mellitus. These micronutrients function as essential coenzymes and cofactors for metabolic reactions and thus help support basic cellular

reactions such as glycolysis, the citric acid cycle, lipid and amino acid metabolism required to maintain energy production and life (Shils, 1999).

Micronutrients have been investigated as potential preventive and treatment agents for both type I and type II diabetes and for common complications of diabetes (Mooradian, 1994). For instance, magnesium is a cofactor in the glucose-transporting mechanism of the cell membrane and various enzymes in carbohydrate oxidation, and is thought to play a role in the release of insulin. A deficiency of magnesium is significantly more common in type II diabetes than the general population (Mooradian, 1994). Magnesium deficiency has been associated with complications of diabetes particularly retinopathy. Studies have shown patients with most severe retinopathy also presents with the lowest magnesium levels (Pandey et al., 2011). On the other hand, manganese is a cofactor of various enzymes critical in cellular biochemical reactions such as activation of manganese superoxide dismutase, an antioxidant enzyme involved in the protection of cell membranes and tissues from degeneration and disruption helping the body to catabolize carbohydrates, lipids, proteins and in energy production (George, 2004).

Zinc is involved in the regulation of insulin receptor-initiated signal transduction mechanisms and insulin receptor synthesis (Ezaki, 1989). Zinc plays a key role in the regulation of insulin production by pancreatic tissues and glucose utilization by muscles and fat cells (Song, 1998). The abilities to synthesize and secrete insulin and use glucose are impaired in the zinc deficient state (Ezaki, 1989). Intestinal zinc absorption rates and plasma zinc levels are reduced in diabetic patients (Rosner, 1968). The trace element trivalent chromium (Cr^{3+}) is required for the maintenance of normal glucose metabolism. Experimental chromium deficiency leads to impaired glucose tolerance which improves upon the addition of chromium to the diet. Chromium plays an important role in glucose and lipid metabolism, and dietary deficiency can cause impaired glucose tolerance which is of great importance to

diabetes. Oral supplementation with chromium corrects these problems in-patients with type II diabetes and in children with protein-energy malnutrition. Such supplements have no effect in people with normal chromium intakes.

Chromium is an essential micronutrient which functions as a cofactor in insulin-regulating activities. It facilitates insulin binding and subsequent uptake of glucose into the cell and therefore decreases fasting glucose levels, improves glucose tolerance and lowers insulin levels. It also lowers total cholesterol in normal, elderly and type II diabetic subjects (Mooradian, 1994; Baker, 1996). Without chromium, insulin's action is blocked and glucose levels are elevated (Mooradian, 1994). Chromium picolinate is a form of chromium that exhibits biological activity (Mertz, 1969).

Trivalent chromium has long been considered to be a safe nutritional supplement (Castro, 1998). Although the hexavalent form of chromium is a known human respiratory tract carcinogen when inhaled in high-exposure industrial settings, there is no evidence of any carcinogenic effects in humans from the trivalent form of chromium found in chromium supplements. A reasonable amount of supplemental chromium dose is 200g/day (Castro, 1998). The current adequate intake (AI) for chromium is 25 μg for women and 35 μg for men. No tolerable upper intake level (UL) which has been established. Previous recommendations placed a daily intake at ≤ 200 μg/day within a safe and adequate range (Anderson, 1998).

Another important mineral element is vanadium which is known to play critical role in regulation of intracellular signaling and as a cofactor of enzymes essential in energy metabolism. It reduces the rate of gluconeogenesis and increases glycogen deposition (Cohen, 1995). A reasonable amount of supplemental vanadium is 20 gm/day. Vanadyl sulfate at a dose of 100 mg/day is effective in improving insulin sensitivity (Cohen, 1995). Molybdate is an effective anti-hyperglycemic agent in diabetics with severe insulin

resistance. It is associated with substantial reduction of hyper-insulinaemia and an increase in pancreatic insulin stores. The glucose-lowering effect of molybdenum may be partly related to reduction of hepatic glucose production, and possibly to increased glucose usage (Reul, 1997).

Medicinal plants toxicities could be attributed to the high levels of mineral elements. For instance, trivalent chromium sources are not toxic. However, hexavalent chromium toxicity from industrial exposure through inhalation has been associated with increased incidence of lung cancer. In experimental animals, ingestion of chromate resulted in liver and kidney damage (Fishbein, 1988). Epigastric pain, diarrhea and vomiting have been observed from high zinc intake from food stored in galvanized containers. Supplements of as little as 25 mg of zinc have resulted in diminished absorption of copper, presumably because of competition (Kaplan, 1989). Lead toxicity produces neurological, gastrointestinal, renal, immunological, endrocrinological and hematopoietic changes in humans (Kaplan, 1989). Supplementation of human volunteers with vanadyl compounds at oral doses of 50-125 mg/day caused cramps, loosened stools, green tongue in all patients, fatigue and lethargy in some individuals (Dimond, 1963).

2.4. Literature on *Diospyros melanoxylon* Roxb.

2.4.1. Analgesic activity

Gupta et al. (2013) showed that the ethnobotanical uses of *Diospyros melanoxylon* (Roxb.) examine analgesic properties in the stem bark and root bark by ethanolic extracts in mice (Gupta et al., 2013). The analgesic effect of ethanolic extracts was evaluated by 'hot plate' and 'acetic acid-induced writhing test' in mice by using pentazocine (5 mg/kg) and asprin (50 mg/kg) simultaneously as reference drugs. *Diospyros melanoxylon* (Roxb.) stem bark's ethanolic extracts possess analgesic activity, but here we also used root bark to check the extent of difference in activity. The ethanolic extract of stem bark (200 mg/kg) possessed

significant analgesic activity, as in acetic acid-induced writhing test, the writhing count was reduced significantly compared to standard and root bark ethanolic extracts. In the hot plate method with ethanolic extracts of stem bark (200 mg/kg), the basal reaction time was increased significantly (p<0.01) and the percentage increase in threshold to pain was also significant as compared to standard.

Nonsteroidal anti-inflammatory drugs (NSAIDs) trim down pain with repressing the formation of prostaglandins, by hindering the action of the enzyme Cyclooxygenase (COX). So containment of fusion of prostaglandins (PGs) by NSAIDs truly lessens the fight of the mucosa to damage as well as snooping with restore developments. Selective COX-2 inhibitors were thought to be the result to this puzzle as it is obligatory that NSAIDs repress prostaglandin synthesis at sites of inflammation. It has been reported that extracts of stems bark and root bark screening for their analgesic activity by acetic acid-induced writhing method and hot plate method, albino mice were divided into four different groups (six animals each) (Bachhav et al., 2009; Ramaswamys Pillai et al., 1985). Leaf of *D. Melanoxylon* contains flavonoids having analgesic activity (Hossinzadeh, 2002).

2.4.2. Anti-inflammatory activity

Kendu leaves have the valuable flavones, and pentacyclic triterpines and flavonoids have anti inflammatory activity (Rosa, 1972). Flavonoids are incredibly imperative secondary metabolite mainly inhibit the enzyme prostaglandin synthatase, additional purposely the endoperoxidase and accounted to produce anti-inflammatory property (Agrawal et al., 2010). This investigation observed that the flavonoids and pentacyclic triterpenoids of *D. melanoxylon* illustrated important anti-inflammatory activity in dose dependant way. This recommends that the further experiment to be carried out on this plant and their lead compounds with the vision of characterizing the potential activities.

2.4.3. Antiulcer activity

Inequity among aggressive and protective factors causing gastric ulcer which is the most prevalent disease (AlKofahi and Atta, 1999). Medicine, healing of ulcers is embattled at either neutralizing aggressive factors (acid, pepsin, active oxidants, platelet aggravating factor "PAF", leukotrienes, endothelins, bile or exogenous factors including NSAIDs) or motivating the mucosal defences (mucus, bicarbonate, normal blood flow, prostaglandins (PG), nitric oxide) (Borelli and Izzo, 2000).

Pyloric ligation is one type of method mostly used for the evaluation of anti-ulcer drugs and increased the acid secretion, which in turn causes increase in gastric volume, low pH, increased free and total acidity ensuing into increase in ulcer index (Maruthappan Sakthi, 2010).

A recent investigation has been demonstrated that the anti-ulcer activity against pylorus ligation induced gastric ulcer model using a methanolic leaf extract of *D. melanoxylon*. The methanolic extract 200mg/ kg body weight reduces the ulcer index significantly ($p< 0.05$) comparison to control group (Gupta et al., 2009). It also lessens the total Acidity, Acidity free, and pH of the gastric content and viewing protection index 32% and 58% at the dose of 100 mg/ kg and 200 mg/kg. Correspondingly, the standard drug ranitidine (50mg/ kg) proved 85 % of ulcer protection.

It showed that the ethanol extract treated animals showed significant epithelialisation and wound contraction of the excision wound (Ande et al., 2012). Acute toxicity studies were conducted for the ethanol extract of *D. melanoxylon* leaves. The maximum tolerated dose was found to be 2000 mg/kg b.w when the extract was administered orally. Excision wound model was assessed for wound healing activity. Three groups of six animals in each group were anesthetized by open mask method with anaesthetic ether. The rats were depilated on

back. One excision wound was inflicted by cutting away 500 mm full thickness of skin on ethanol sterilized dorsal thoracic region of rats. The wound was left undressed to the open environment. Group I: served as control and received gum tragacanth suspension orally. Group II: served as standard and treated externally with Povidone iodine ointment. Group III: treated with ethanol extract of the *D. melanoxylon* Ointment. The ointment was topically applied daily till the complete epithelialization starting from the day of operation. The parameters studied were wound closure and time of epithelialization. The wounds were traced on mm 2 graph paper on the days of 4th, 8th, 12thand 16th and thereafter on alternate days until healing were complete. The percentage of wound closure was calculated.

2.4.4. Antihyperglycemic activity

It has been demonstrated that the extracts showing significant antihyperglycemic activity as compared to standard drug. Ethanolic extract (200mg/kg) show beneficial effects on blood glucose and hyperlipidemia associated with diabetes, which might be due to presence of steroids, tannins, alkaloids and triterpenoids in the extract. Ethanolic extract could serve as a good adjuvant to other oral hypoglycaemic agents and seems to be promising for the development of phytomedicines for diabetes mellitus (Topno, 1997).

An additional study reported that the antihyperglycaemic activity is *D. melanoxylon* Roxb. which was selected on the basis of its abundant availability in Maharashtra state and on the basis of ethnomedicinal information that the tribe of the Chotta Nagpur region (Orissa) use it extensively as antidiabetic (Ashutosh et al., 2013).

2.4.5. Antimicrobial activity

For the period of the previous few years, antimicrobial activities of plant extracts and natural products have been severely observed as require for safe drugs which has augmented due to

the abuse of antibiotics and an increase in immuno-deficiency disease (Grayer and Harborne, 1994).

The leaves of *D. melanoxylon* were explored for its antimicrobial activity and estimated by determining the zone of inhibition of *S. areas* (gram positive) and *E. coli* (gram negative) in different extracts like ethanolic and petroleum ether. Standard Ciprofloxacin was viewing zone of inhibition (mm) 24 and 30 for *S. aureus* and *E. coli* in that order. In ethanolic extract, it was found 25 and 24 while in petroleum ether extract zone of inhibition was found with a value of 18 and 14 respectively. This revision demonstrated that the possibility of different extracts above microbes for progress of novel antimicrobial mediator (He and Liu, 2006).

2.4.6. Anticancer activity

It has been revealed that *D. melanoxylon* leaves contain ursolic acid (0.56%). Ursolic acid is a pentacyclic triterpenoid acts as a chemopreventive and chemotherapeutic agent in various types of cancer by inhibiting the initiation, promotion and metastasis of cancer (Jadhav et al., 2009). Ursolic acid inhibits the cell proliferation of HepG2 human liver cancer cells (Novotny et al., 2001). It has been demonstrated the *in-vitro* antitumor activity in 2001 of ursolic acid (Es-saady et al., 1996a). Ursolic acid lessens the cell proliferation of many tumor cell lines and mechanisms of action have been tackled. It has been showed an early G1cytostatic effect for ursolic acid in B16 cells and MCF-7 breast carcinoma cells (Es-saady et al., 1996b; Wang et al., 2013).

2.5. Pharmacological activities of some important compounds

Many compounds have been isolated from the different parts of the plants known as *D. Melanoxylon* those having pharmacological activity. Here pharmacological uses of some important compounds are described below.

2.5.1. Ursolic acid

Ursolic acid (UA) not only inhibits cell growth but also induces apoptosis through modulation of the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathway in human prostate cancer cells. This finding suggests that UA may be a new chemotherapeutic candidate against prostate cancer (Meng et al., 2015). The antifibrotic effect of UA is partially due to its oxidative stress attenuating effect through manipulation of NADPH oxidase 4 activities and expression. This result suggests that UA may be a promising antifibrotic agent (He et al., 2015). A multi-inlet vortex mixer is a robust and pragmatic tool for tailoring the particle size of a UA nanosuspension. Particle size appears to be a critical determinant of the anticancer activity of the UA nanoparticles (Wang et al., 2015).

UA increases free fatty acid (FFA) burning by enhancing skeletal muscle FFA uptake via uncoupling protein 3/AMP-activated protein kinase dependent pathway, which provides a novel perspective on the biological function of UA against obesity and IR (Chu et al., 2015). UA from *Prunella vulgaris* enhances sleep duration through GABA receptor activation and could be a therapeutic candidate for insomnia treatment (Jeon et al., 2015).

The greater ability of the combination of UA and resveratrol to inhibit skin tumor progression was attributable to the greater inhibitory effects on growth factor and inflammatory signaling, skin inflammation, and epidermal hyperproliferation induced by 12-O-tetracanoylphorbol-13-acetate (TPA) treatment (Cho et al., 2015). The combination of UA and artesunate can reduce both triglyceride and cholesterol, and the effects were more potent than of either agent alone, which indicates a strong synergistic effect (Yuliang et al., 2015). The beneficial effects of UA on nonalcoholic fatty liver disease (NAFLD) may be due to its ability to increase lipid oxidation and to inhibit hepatic endoplasmic reticulum stress. Together, UA may be further considered as a natural compound for NAFLD treatment (Li et al., 2015).

In vitro, in silico, and *in vivo* results indicate that UA is a promising, inexpensive, widely available natural lead, which can be designed and developed into a macrofilaricidal drug. This is the first ever report on the anti-filarial potential of UA from *E. tereticornis (*Kalani et al., 2014).

The antihyperglycaemic role of UA is mediated through insulin secretion and insulinomimetic effect on glucose uptake, synthesis, and translocation of GLUT4 by a mechanism of cross-talk between calcium and protein kinases. UA is a potential antidiabetic agent with pharmacological properties for insulin resistance and diabetes therapy (Castro et al., 2015). Combination treatment with UA and rosiglitazone downregulated lipogenic genes and upregulated fatty acid oxidative genes in high-fat diet-fed mice. This study suggests that UA in combination with rosiglitazone reduced lipid accumulation in liver (Sundaresan et al., 2014). Low-dose UA had preventive potency for diabetic renal complications, which could be mediated by changes in hepatic glucose metabolism and the renal polyol pathway. High-dose UA was more effective anti-dyslipidemia therapy in non-obese type 2 diabetic mice (Lee et al., 2014).

2.5.2. Oleanolic acid

It has been demonstrated that oleanolic acid (OA) induced apoptosis of lung adenocarcinoma cells through down-regulating Bcl-2 expression, and up-regulating Bax and Bad expression (Feng et al., 2011).

OA having the activity on its cytotoxic, antitumor, antioxidant, anti-inflamatory, anti-HIV, acetyl cholinesterase, alpha-glucosidase, antimicrobial, hepatoprotective, anti-inflammatory, antipruritic, spasmolytic activity, anti-angiogenic, antiallergic, antiviral and immunomodulatory activities (Sultana and Ata, 2008). OA extracted from wild loquat leaves can significantly inhibit the viability of A549 cells (human lung adenocarcinoma epithelial cell line) (Yuan et al., 2015).

OA inhibits tumor promotion in mouse skin (Sharma et al., 2010) it also inhibits azoxymethane (AOM)-induced colonic aberrant crypt foci and multi-crypt aberrant crypt/foci in a dose dependent manner (Tokuda et al., 1986) and suppress preneoplastic lesions induced by 1, 2-dimethylhydrazine in rat colon (Janakiram et al., 2008).

OA causes a dose and a time dependent cell kill of the human colon carcinoma cell line HCT15, inhibits proliferation and arrested the cells in G0/G1 phase (Barh and Viswanathan, 2008). It induces apoptosis in human leukemia cells HL60 through caspase activation (Li et al., 2002). OA selectively inhibits growth of ras oncogene-transformed R6 cells (Zhang et al., 2007) and induces apoptosis in human liver cancer HepG2, Hep3B, Huh7 and HA22T cell lines (Wu et al., 2009). It also inhibits growth of ascitic tumors in mice (Patt et al., 1949).

2.5.3. Lupeol

Lupeol has been extensively studied for its inhibitory effects on inflammation under *in vitro* conditions and in animal models of inflammation. A comprehensive study conducted by Fernandez *et al*. showed that topical application of lupeol (0.5 and 1 mg/ear) alleviated 12-*O*-tetradecanoyl-phorbol acetate (TPA)-induced inflammation in an ear mouse model (Martelanc et al., 2007).

Lupeol-induced apoptosis of pancreatic cells is mediated through the activation of caspase-3, -8 and -9. The observation that expression levels of procaspase-3 did not exhibit any significant change upon lupeol treatment could be explained through a possible involvement of a feedback mechanism, which restores depleted procaspase-3 levels at intracellular level (Saleem et al., 2005).

Lupeol and its derivatives are cytotoxic against human leukemias, melanomas, neuroblastomas and normal fibroblast cells (Liu et al., 2004).

Lupeol has been investigated for its cardioprotective effects and was demonstrated to provide 34.4% protection against *in vitro* low density lipoprotein (LDL) oxidation. Lupeol and lupeol

acetate have also shown hypotensive activity, which may make them possible preventative agents in this cardiac disorder and other consequent cardiovascular diseases (Saleem et al., 2003).

2.5.4. β-Sitosterol

Water extract shows significant antioxidant activity and free radical scavenging activity followed by methanol and dichloromethane extracts, due to high content of flavonoids and beta-Sitosterol identified for the first time by LC/MS and GC/MS, respectively (Mohamed et al., 2010). Tuber of *M. jalapa* Linn. contain β-sitosterol identified for the first time by LC/MS and GC/MS, respectively having the antibacterial (Vankar et al., 2010) and antifungal activity (Mohamed et al., 2010). *Beta*-sitosterol has been reported to show anti-inflammatory, antineoplastic, antipyretic, and immunomodulating activity (Fraile et al., 2012).

2.5.5. Betulinic acid and betulin

Ahmad et al. (2013) isolated two medicinal pentacyclic triterpenoids, betulinic acid and betulin from methanolic bark extract. Betulinic acid has valuable biological potential, such as inhibitors of HIV-1 entry, HIV protease, or of reverse transcriptase (RT), whereas betulin had significant anticancer effect on adenocarcinoma, cervix carcinoma, hepatoma, and breast cancer (Ahmed et al., 2013).

Betulinic acid has been shown to inhibit HIV-1 replication. Based on its chemical structure, betulinic acid derivatives have been reported as inhibitors of HIV-1 entry, HIV-protease or of reverse transcriptase (RT) (Fujoka et al., 1994).

Dalme et al. (2013) has demonstrated the *in vivo* antitumor activity of betulinic acid on MCF-7 breast cancer tumors in nude mice. The antitumor effect of betulinic acid can further be enhanced by use of combination therapy and novel drug delivery systems, thus making it a promising candidate for management of breast cancer patients (Damle et al., 2013).

Betulinic acid and betulin obtained from aqueous extract of *Diospyros peregrine* fruits showed *in vivo* antidiabetic activity on rat model (Vikrant and Sharma, 2011).

2.5.6. β-amyrin

β amyrin obtained from aqueous ethanolic seed extract of the plant *Caesalpina bonducella* has hypoglycaemic activity reported in streptozotocin induced diabetic rats increases the release of insulin from pancreatic cells (Sharm et al., 1997).

Beta amyrin obtained from an *n*-hexane extract of *Bombax malabaricum* flowers showed antimicrobial and antifungal activity (El-Hagrassi et al., 2011).

Petroleum ether extract of *Moringa oleifera* and *Phyllanthus emblica* contain β amyrin analysis by GC-MS having antibacterial, antioxidant, potential antiplatelet components, hypoglycemic, hypolipidemic effects, Sedative action, and hepatoprotective activities (Subarnas et al., 1993).

Triterpene beta-amyrin also seems to exert a similar anti-inflammatory activity (Ekpo and Pretorius, 2007). The ethanol extract of *E. hirta* and its component beta-amyrin produced a remarkable anti-inflammatory effect, and are able to block most of the iNOS protein functions and NO induction, presenting a great potential as a new selective NO inhibitor for the treatment of arthritis inflammation (Shih et al., 2010).

2.5.7. Aurone

Aurone obtained from root extract of *Pfaffia paniculata* have been demonstrated the biological activity such as Antisickling, analgesic, anti-arthritic, antitumor, anti-inflammatory, sexual stimulant, increase blood circulation and increase oestrogen production (Mpiana et al., 2007; Gosmann et al., 2003; Mazzanti and Braghiroli, 1994).

Chapter 3

Aims and Objectives

Aims and Objectives

The primary objective of this research work was to develop nanoparticles of active fraction and evaluate the anticancer and antidiabetic activity obtained from the traditionally used medicinal plant *Diospyros melanoxylon* Roxb. also known as Kendu. This present work was aimed with the following objectives.

- Preparation of different fractions of *Diospyros melanoxylon* Roxb. Leaves with qualitative and quantitative biophytochemicals.
- Bioactivity profiling of fractions on different cancerous cell lines using methylene blue assay and antidiabetic activity on the basis of α-amylase and α-glucosidase enzyme assay.
- Preparations of polymeric nanoparticles of most active fraction for developing anticancer and antidiabetic activity.

Chapter 4

Experimental and results-part 1

Anticancer activity of *Diospyros melanoxylon* Roxb. and correlation with their polyphenolic profiles

Background and principal finding

In this experiment, qualitative phytochemical analysis reflects the presence of carbohydrates, alkaloids, steroids, flavonoids, tannins, terpenoids, triterpenoids and saponins of different fractions obtained from *Diospyros melanoxylon* Roxb. leave. It is also estimated the total phenolic, flavonoid, triterpenoid and tannin contents. A search for possible antioxidant was carried out in different fractions of *Diospyros melanoxylon* Roxb. Antioxidant activities of different fractions were tested using different free radical scavenging methods. Anticancer screening of different fractions was estimated using methylene blue assay on different cancerous cell lines and normal cell lines. Significant cytotoxicity and IC_{50} values were observed against cancerous cell lines. Among all, comparative ethyl acetate fraction displayed a good anticancer activity and showing the lowest IC_{50} values.

4. 1Material and methodology

4.1.1. Plant materials

The leaves of *D. melanoxylon* were collected from Dhenkanal District, Bhubaneswar, Odisha. The leaves were washed properly, shade dried for 20 days and cut into small pieces. The dried leaves were ground using mechanical grinder, passed through sieve number 60 to obtain a homogeneous leaf powder and stored in an airtight container for experimental trial.

4.1.2. Preparation of extract and fractions

The powdered plant material 1.5 kg (300 gm × 5 assembly) was extracted with 5 litres (1 litre × 5 assembly) of n-hexane using Soxhlet apparatus for 28 hours by hot percolation method for removing fatty and waxy substances. The residue was dried at room temperature and

again extracted in above same assembly using ethyl acetate for 36 hours using Soxhlet apparatus. Traditionally, Soxhlet and heat reflux extraction methods have been the first line choice of extraction when it comes to preparation of crude extracts for ethnopharmacological studies (Banik and Pandey, 2008). Subsequently with the ethyl acetate fraction (EAF), two more fractions such as chloroform fraction (CF) and aqueous fraction (AQF) was prepared using liquid-liquid partitioning system. Mixing was completed by inverting the funnel 6 times (pressure in the separating funnel was discharged after each invert), where after the mixture was allowed to form two layers, and the lower-polar, and the upper-less polar, layers were collected separately. The process was repeated six times. The different solvent fractions and n-hexane extract (NHE) were concentrated under vacuum using rotary vacuum evaporator at 40^0C and the dried fractions stored in a refrigerator for further use. The entire fractions and NHE were made solvent free by vacuum evaporation.

4.1.3. Chemicals and reagents

2, 2 diphenyl 1 picrylhydrazide (DPPH) was obtained from Sigma-aldrich (St. Louis, MO). naphthylethylenediamine dihydrochloride, hydrogen peroxide (H_2O_2), 2-deoxyribose, EDTA, $Fecl_3$, and ascorbic acid were obtained from LOBA Chemicals, Mumbai, India. Dulbecco's modified Eagle's medium (DMEM), and Fetal bovine serum (FBS) were obtained from Gibco, Grand Island, NY 14072, USA 1-716-774-6700. Roswell Park Media Institute (RPMI) 1640, non-essential amino acids, penicillin-streptomycin (PS), L-glutamine, and gentamicin were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Aluminium chloride ($AlCl_3$), Potassium acetate, Folin-Ciocalteu reagent, Na_2CO_3, trichloroacetic acid, perchloric acid, and glacial acetic acid were obtained from Merck specialties private Limited, Mumbai, India. Quercetin and Gallic acid were obtained from Sisco Research Laboratories Pvt. Ltd, Maharashtra, India and SD fine-chem limited, Mumbai, India respectively. All the chemicals and reagents were of analytical grade obtained commercially.

4.1.4. Scanning electron micrographs

In order to elucidate extraction procedure and to understand the extraction mechanism, scanning electron micrographs (SEM) of the marc obtained from Soxhlet extraction method was taken. After removing the solvent, the remaining *D. melanoxylon* leaf sample was plunged in liquid nitrogen and then cut with a cold knife. The sectioned particles were fixed on a specimen holder with aluminium tape and then sputtered with palladium. The specimen was examined with a QUENTA FEG 250 (Netherland) scanning electron microscope under high vacuum condition and at an accelerating voltage of 20 kV (3500× and 30000× magnification).

4.1.5. Qualitative phytochemicals analysis

Previously described method by Asaduzzaman et al. (2014) was used with slight modification. The tests were performed to find out the presence of active chemical constituents such as carbohydrates, alkaloids, steroids, flavonoids, saponins, glycosides, terpenoids, and tannins (Asaduzzaman et al., 2014).

4.1.6. High performance liquid chromatography (HPLC) analysis

The various test samples (1mg) of each fraction (ethyl acetate, aqueous and chloroform) and n-hexane extract were dissolved in 2 mL methanol and was vortexed for three hours for proper mixing. The final solution was filtered using 0.45 µm membrane filter (Biotech, Germany) prior to high performance liquid chromatography (HPLC) analysis.

The identification of possible bioactive compounds (phenolics and flavonoids) was performed on HPLC (FRC-10A, UFLC, SHIMADZU, JAPAN) with PDA detector using C18, 4.2 mm × 250 mm, 5 µm column. The chromatographic separation was carried out using solvent HPLC grade methanol (100 %) and TFA 0.1%. UV-Vis detected at 280 nm. Injection volume was 10 µL.

The identification of each compound was established by comparing the retention time of the peaks with those previously obtained by injection of pure standard compounds (gallic acid, ellagic acid, ferulic acid, rutin, and quercetin) and identified the compounds.

4.1.7. Antioxidant assay

4.1.7.1. Determination of total flavonoid content

Previously described method by Ordonez et al. (2006) was used with slight modification (Ordonez et al. 2006; Fatiha et al., 2015). Total flavonoid content (TFC) was determined based on the formation of a complex flavonoid-aluminium in different samples of *D. melanoxylon* in triplicate assay. Total flavonoid content was expressed in milligrams of Quercetin Equivalent per gram of dried sample (mg QE/gm sample) using the equation obtained from the calibration curve $Y = 0.0006x + 0.001$, $R^2 = 0.9953$, where Y is the absorbance and X is the concentration of Quercetin Equivalent.

4.1.7.2. Determination of total phenolic content

Previously described method by Koncić et al. (2010) was used with slight modification (Koncić et al., 2010). The method was performed in triplicate using Folin-Ciocalteu reagent to determine total phenolic content (TPC) in different samples of *D. melanoxylon*. The Folin-Ciocalteau assay method depends on the basis of transmission of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically at 765 nm. Total phenol content was expressed in milligrams of Gallic acid equivalents per gram of dried sample (mg GAE/gm sample) using the expression from the calibration curve $Y = 0.0556x - 0.1909$, $R^2 = 0.9929$, Where Y is the absorbance and X is the concentration of Gallic acid equivalent in mg/gm.

4.1.7.3. Determination of total triterpenoid content

Previously described method by Fan and He (2006) was used with slight modification (Fan and He, 2006). 100 µL of different fractions and NHE (0.25-1.25mg/mL) were mixed individually with 150 µL (7.5 % w/v) vanillin-glacial acetic acid solutions and 500 µL of perchloric acid solution. The sample solutions were heated for 30 min at 60^0C and then cooled in an ice-water bath to ambient temperature. After the addition of 2.25 mL glacial acetic acid, each sample solution's absorbance was measured at 548 nm using a UV-visible spectrophotometer. Total triterpenoid content (TTC) was expressed in milligrams of Lupeol equivalents per gram of dried samples (mg LPE /gm dry sample) using the equation obtained from the calibration curve $Y = 0.0143x + 0.1546$, $R^2 = 0.9947$.

4.1.7.4. Determination of total tannin content

Total tannin content of various samples was evaluated according to Russo et al. (2015) with slight modifications (Russo et al., 2015). 500 µL of bovine serum albumin (BSA) solution in 0.2 mol/L acetic buffer, pH 5.7 with 0.17 mol/L NaCl (1.5 mg/mL) was added to various concentrations of samples (0.25-1.25 mg/mL) and mixed carefully. After 20 min, the samples were centrifuged at 5000 rpm for 20 min. The supernatant was removed, and the pellet dissolved in 1 mL of 1% aqueous solution of sodium dodecyl sulphate (SDS) and 4% triethanolamine followed by addition of 250 µL of 0.01 mol/L $Fecl_3$ in 0.01 mol/L HCl. After 45 min the absorbance was recorded at 510 nm. Total tannin content (TTC) was expressed as mg of tannic acid equivalent/gm of sample (mg TAE/gm of sample) using the equation obtained from the calibration curve $Y = 0.046x + 0.0796$, $R^2 = 0.9974$.

4.1.7.5. DPPH scavenging assay through TLC

The antioxidant activity of different samples obtained from *D. melanoxylon* was evaluated by a previously reported method of Singh and Kumari (2015) with slight modification (Singh and Kumari, 2015). Various samples (3 mg, diluted in methanol, 1:10) were spotted on TLC

plate. The TLC plate was then developed in methanol and ethyl acetate in the ratio of 60:40 (volume by volume). The prepared DPPH solution (0.2% in methanol) was sprayed on the developed plate and allowed to stand for 45 min at room temperature. Purple color of DPPH was lightened and formation of yellow spot showed the presence of antioxidant activity.

4.1.7.6. Antioxidant assay through DPPH

Previously described method by Sharma and Bhat (2009) was used with slight modification (Sharma and Bhat, 2009; Ashrafa et al., 2015). Total antioxidant activities of different fractions and NHE were measured on the basis of electron donating ability by bleaching a purple solution of 2, 2-diphenyl 1 picryl-hydrazyl (DPPH) at different concentrations (100-500µg/mL). Samples were added to 0.5 mL of 0.2 mmol/L DPPH solution. The samples were kept at dark for 45 min and absorbance was taken against a blank at 517 nm. Ascorbic acid (AA) was used as a positive control and results were expressed as half maximum inhibitory concentration (µg/mL) in triplicate assay. The percentage of scavenging ability of different fractions and NHE were calculated using the following equation.

DPPH scavenging activity (%) = [(Abs control – Abs sample)]/ Abs control)] × 100

Where Abs control is the Abs DPPH+ Methanol and Abs sample is the Abs of DPPH radical + sample or standard.

4.1.7.7. Nitric oxide scavenging assay

Nitric oxide scavenging activity of entire fractions and NHE were performed by the method previously described by Sreejayan and Rao (1997) with slight modification (Sreejayan and Rao, 1997). Sodium nitroprusside (10mM), in phosphate-buffered saline, was mixed with samples of *D. melanoxylon* at different concentrations (100-500µg/mL). The mixture was dissolved in water and incubated at room temperature for 150 min. Griess reagent (0.5 mL), which contain 1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine

dihydrochloride, was added to the mixture after incubation period. The absorbance of the chromophore formed was read at 546 nm. Ascorbic acid and the same reaction mixture without samples were employed as positive and negative control, respectively.

4.1.7.8. Reducing power assay

The reducing power of different samples of *D. melanoxylon* was estimated by the technique of Luqman et al. (2009) with little modification (Luqman et al., 2009). The different concentrations of samples (100-500 µg/mL) were mixed with 0.75mL (0.2M) phosphate buffer (pH 6.6) and 0.75 mL (1%) K_4FeCN_6 and incubated for 30 min at 45ºC in a water bath followed by precipitation with 0.75 mL (10%) trichloroacetic acid. The supernatant (1.5 mL) was diluted with the same volume of double distilled water and ferric reducing abilities of the samples were confirmed by addition of 0.1 mL (0.10%) $FeCl_3$. The absorbance of reaction mixture was noted at 700 nm against control. More absorbance of the reaction mixture pointed to better reducing power.

4.1.7.9. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was measured through a previously reported method by Irsad et al. (2012) by means of Fenton reaction (Irshad et al., 2012). 1.5 mL of various concentration of samples (100-500 µg/mL) were mixed with 60 µL (1.0mM) $Fecl_3$, 90 µL (1mM) 1, 10-phenanthroline, 2.4 mL (0.2M) phosphate buffer (pH 7.8), and 150 µL (0.17M) H_2O_2. The absorbance of reaction mixture was recorded at 560 nm after incubation period of 10 min at 40ºC temperature in triplicate method. Ascorbic acid was used as standard sample. The hydroxyl radicals scavenging activity was calculated according to the following equation.

% inhibition= [(Ae-Ao)/Ao] × 100

Where, Ae is the absorbance of control and Ao is the absorbance of tested samples.

4.1.7.10. Hydrogen peroxide scavenging assay

Previously described method by Rahmat et al. (2012) was used with slight modification (Rahmat et al., 2012). 2 mM solution of hydrogen peroxide was prepared in 50 mM phosphate buffer (pH 7.4). The reading of hydrogen peroxide concentration was noted calorimetrically at 230 nm by means of the molar extinction coefficient for H_2O_2 of 0.1 mL (81 $mol^{-1}cm^{-1}$). Samples in different concentration (100-500μg/mL) and standard ascorbic acid were transferred into the ependorff with the help of micropipette and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). Then transfer the solution in tubes and vortexed after adding 0.6 mL hydrogen peroxide solution. Absorbance of hydrogen peroxide was recorded at 230 nm after 15 min, against negative control. 50 mM phosphate buffer without hydrogen peroxide was used as negative control. Experiments were performed in triplicates. Hydrogen peroxide scavenging capacity was intended by the formula:

% inhibition= $(1 - Ae/Ao) \times 100$

Where, Ao is the absorbance without sample, and Ae is absorbance with sample.

4.1.8. Cell lines and culture conditions

The cell lines were ordered from the American Type Culture Collection (ATCC) and those used in this study were breast cancer cell (MCF-7), human colon carcinoma cell (HCT 116), cervical cancer cells (HeLa), prostate cancer cells (PC-3), Human epithelial kidney (HEK293), and rat colon cancer cells (RCC 45); normal human kidney epithelium cells (NKE) and normal lung tissue cells (WI-38). MCF-7, HCT-116, HEK293, and WI-38 cells were grown in DMEM medium with 10% FBS and 1% penicillin-streptomycin. HeLa, PC-3, NKE, and RCC45 cells were grown in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin. All cells were supplemented in a humidified atmosphere at 37^0C in 5% CO_2. Afterward, the exhausted medium was removed, replaced with fresh medium, and incubated

again for 24 hours. The cell cultures were then washed with PBS and were suspended using trypsin. Fresh medium was added to the cells.

4.1.9. Cytotoxicity assay

The methylene blue cytotoxicity assay was carried out according to the method previously described by Rathore et al. (2014) with slight modification (Rathore et al., 2014). Cells were seeded at 1.5×10^4 cells in each well of 96-well plate using 100 µL of fresh culture medium and were allowed to attach for overnight. Cell counts were made to determine cell viability by using the Tryptan Blue exclusion method (Kedar and Chakrabarti, 1982). For screening, the cells (60 - 70% confluence) were treated with the fractions at different concentrations. Afterward, in order to obtain a curve, the most active fractions were tested for cytotoxicity at 20, 40, 60, 80, 100 µg/mL concentrations. After 48 hours of treatment, the medium was removed from each well, methylene blue was added and incubated for 90 min on a shaker followed by decanting and washing with water to remove extracellular dye. A solution (0.1 N HCl) was then added for detaching the dyed cells. The colorimetric assay at 580 nm was performed on 96 wells micro plate. The cytotoxicity effects of different samples were calculated as a percentage of cell growth inhibition with respect to control and IC_{50} value calculated. The assay was performed in triplicates.

Statistical analysis

All tests were performed as three independent experiments; each carried out in triplicate. Results were expressed as mean values with standard deviations (mean ± SD) and were subjected to two-way analysis of variance (ANOVA). All the data were calculated using GraphPad Prism version 7.02 software (Graph Pad Software, Inc., La Jolla, CA, USA).

4.2. Results

In this study four samples were prepared from a traditionally used medicinal plant *D. melanoxylon* for the management of oxidative stress-related ailments and were examined for *in vitro* antioxidant and anticancer activity. Two out of these exhibited momentous anticancer potential against various cancerous cell lines.

4.2.1. Extraction mechanism

In order to study cell damage during extraction, the *D. melanoxylon* leaf samples was examined by scanning electron microscopy. Fig 3A and B represent the micrographs of the Soxhlet extraction sample at different magnification, both at the surface and cellular levels. The changes observed for Soxhlet extraction showed ruptures took place at the surface of leaf samples accompanied by widening of the cellular channels. However, the surface of the sample was destroyed after extraction followed by massive widening or opening up of the cellular pores (Mandal et al., 2009).

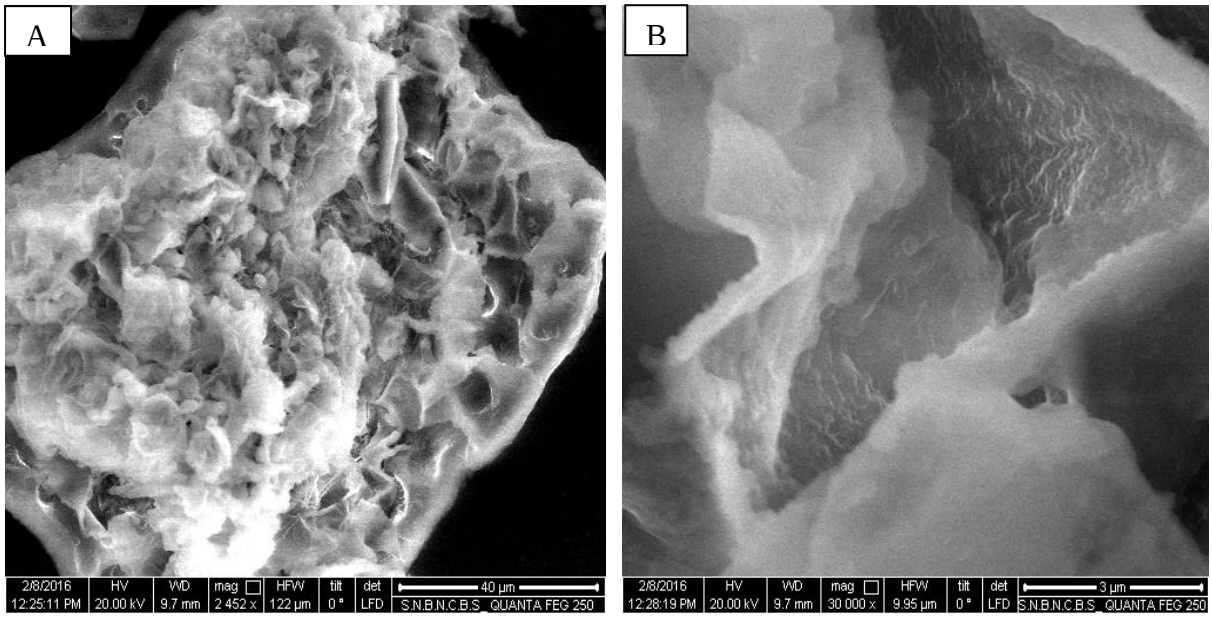


Figure 3 Scanning electron micrographs of *D. melanoxylon* leaf sample after Soxhlet extraction. (A) Disruption of surface morphology and (B) Widening of cellular channels and internal pores to facilitate leaching of the phytoconstituents

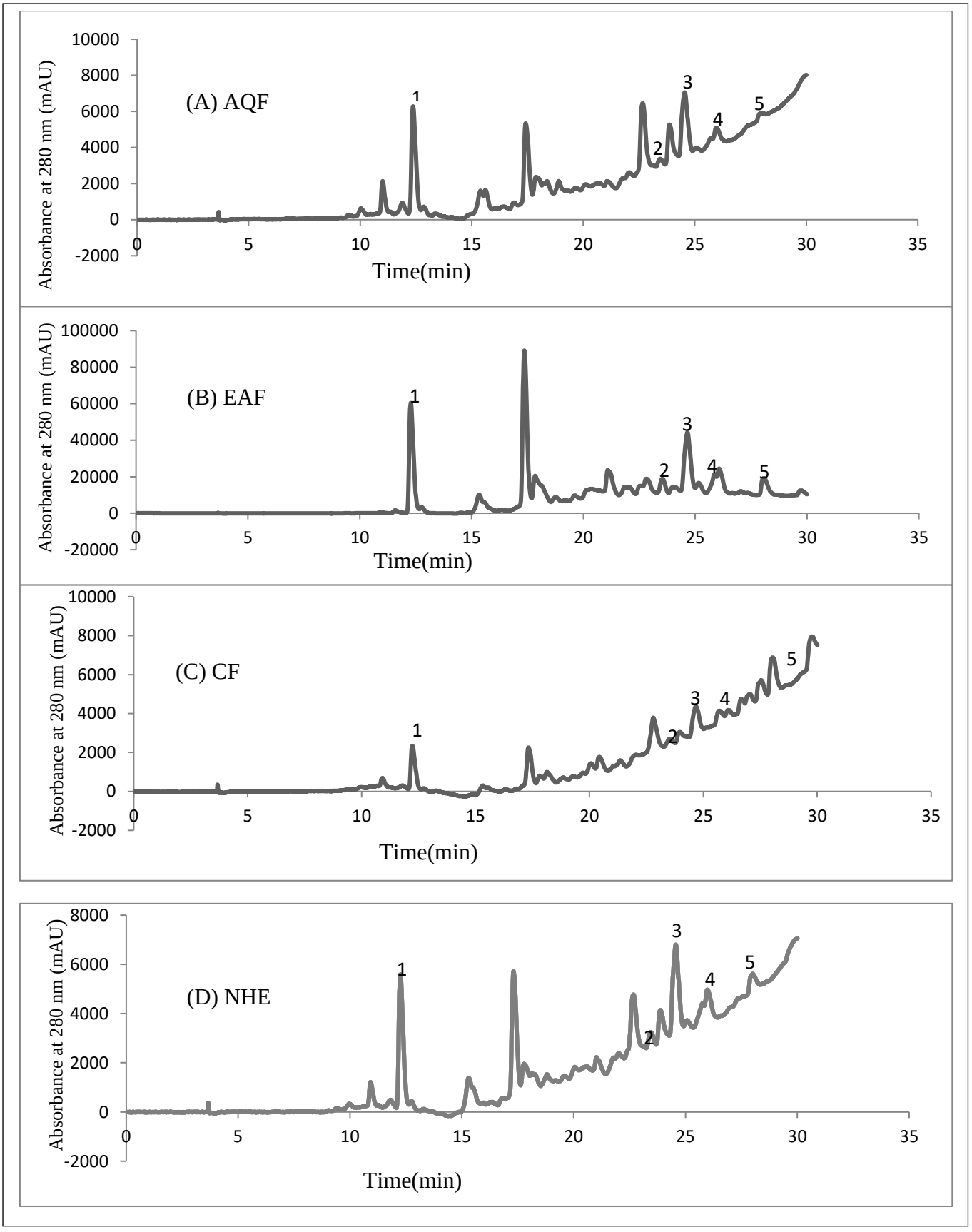
4.2.2. Qualitative Phytochemicals analysis

The different samples of *D. melanoxylon* viz., EAF, AQF, CF and NHE were scrutinized qualitatively for the presence of various phytochemicals. The various samples confirmed the presence of carbohydrates, flavonoids, saponins, glycosides, tannins and terpenoids. Alkaloids and steroid were found to be absent in the various samples. Quantitative analysis revealed that the EAF contained the highest amount of phenolics, flavonoids, and triterpenoids followed by AQF, CF and NHE.

4.2.3. High performance liquid chromatography (HPLC) analysis

Various samples (EAF, AQF, CF and NHE) obtained from *D. melanoxylon* leaves were analyzed for identification of polyphenolic (phenolic acids and flavonoids) compounds by HPLC-DAD analysis. Typical chromatograms of different samples and standards are presented in figure 4. Compounds were identified by comparison of retention times with standard reference compounds viz., four phenolic compounds (gallic acid, ellagic acid, ferulic acid, and rutin) and one flavonoid (quercetin).

Phenolic and flavonoid derivatives were detected in the HPLC-DAD profiles of the four samples. In the group of phenolic acids, gallic acid was the major compound detected in all the samples with retention time 12.277 min. Ferulic acid was the minor compound detected in all the samples with retention time 23.017 min. Subequent to this, rutin was the second major compound in all the samples with retention time 24.708 min. Ellagic acid was present in the entire sample with retention time 25.454. As revealed from the area under the curve ellagic acid content was found to be more in EAF comparatively when compared to quercetin whose retention time was 28.08 min. Among the different fractions of *D. melanoxylon* leaf, EAF was exposed as an effective solvent for extraction of most of phenolic acids and flavonoid.


(A) AQF
Absorbance at 280 nm (mAU)
Time(min)
(B) EAF
Absorbance at 280 nm (mAU)
Time(min)
(C) CF
Absorbance at 280 nm (mAU)
Time(min)
(D) NHE
Absorbance at 280 nm (mAU)
Time(min)

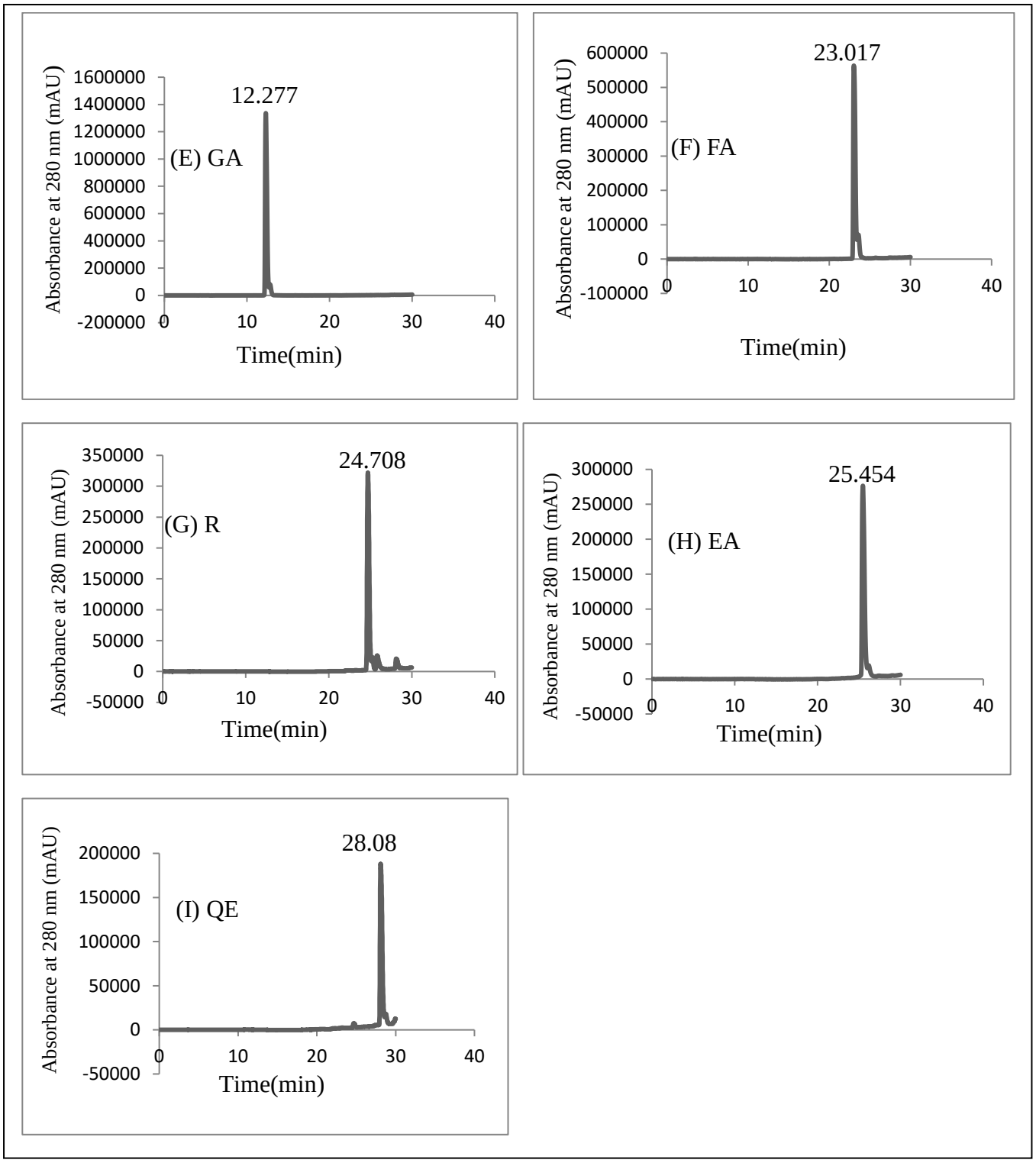


Figure 4 Identification of the major polyphenolic compounds in chromatograms of (A) AQF=Aqueous fraction (B) EAF=Ethyl acetate fraction, (C) CF=Chloroform fraction, (D) NHE=N-hexane extract, (E) GA=Gallic acid, (F) Ferulic acid, (G) R=Rutin, (H) EA=Ellagic acid, and (I) QE=Quercetin. Peak 1=Gallic acid; 2 =Ferulic acid; 3 =Rutin; 4 =Ellagic acid; 5 =Quercetin.

4.2.4. Total flavonoid and phenolic content

Total flavonoid and phenolic content was analyzed systematically and results are shown in Table 1. The contents of flavonoid compounds in different fractions varied as such EAF> AQF> NHE > CF with values 53.04 ± 1.93; 48.96 ± 0.285; 13.75 ± 0.54 and 11.97 ± 0.31 mg QE/gm dry sample, respectively. The contents of phenolic compounds so determined for different plant fractions varied in the following sequence EAF> AQF> NHE> CF, with values 131.4 ± 0.44; 124.8 ± 0.24; 15.87 ± 0.04 and 7.73 ± 0.18 mg GAE/gm dry sample, respectively. The results are also in agreement with the findings of HPLC analysis.

4.2.5. Total triterpenoid and tannin content

Total triterpenoid and tannin content was analyzed systematically and results are shown in Table 1. The contents of triterpenoid compounds varied in different fractions accordingly, EAF> AQF> CF> NHE with values 53.98 ± 1.95; 33.95 ± 2.4; 20.19 ± 1.66 and 10.31 ± 1.75 mg LPE /gm dry sample, respectively. The contents of tannin compounds varied in different fractions accordingly, EAF> AQF> NHE> CF with values 79.60 ± 0.60; 72.40 ± 0.18; 12.85 ± 0.10 and 12.12 ± 0.11 mg LPE /gm dry sample, respectively.

Table 1 Total flavonoid, phenolic, triterpenoid, and tannin content in different samples of *D. melanoxylon*

Fractions	Total flavonoids compounds as Quercetin equivalent (mg QE/gm sample)	Total phenolic compounds as mg Gallic acid equivalent (mg GAE/gm sample)	Total Triterpenoid Compound as Lupeol equivalent (mg LPE/gm sample)	Total Tannin Compound as tannic acid equivalent (mg LPE/gm sample)
EAF	53.04 ± 1.93	131.4 ± 0.44	53.98 ± 1.95	79.60 ± 0.60
AQF	48.96 ± 0.285	124.8 ± 0.24	33.95 ± 2.41	72.40 ± 0.18
CF	11.97 ± 0.31	7.73 ± 0.18	20.19 ± 1.66	12.12 ± 0.11
NHE	13.75 ± 0.54	15.87 ± 0.04	10.31 ± 1.75	12.85 ± 0.10

Each value in the table is represented as Mean ± SD (n = 3).

4.2.6. DPPH scavenging assay

The discoloration of the purple color of the DPPH on TLC plates confirmed the positive antioxidant activity of the EAF, AQF and CF. It was also seen that NHE did not show any color change. Different fractions of *D. melanoxylon* confirmed significant scavenging activities for DPPH free radicals as shown in Figure 5. EAF showed the highest scavenging activity (lowest IC_{50}; 346.11 ± 1.33µg/mL) followed by AQF (430.3 ± 0.87 µg/mL), CF (463.27 ± 0.95 µg/mL) and NHE (672.43 ± 4.44 µg/mL) in Table 2.

4.2.7. Nitric oxide scavenging assay

This is supported by the principle that sodium nitroprusside in samples at various concentrations which produces nitric oxide and interacts with oxygen to produce nitrite ions that can be calculated using Griess reagent. Samples mainly scavenge the nitric oxide and fight with oxygen which decreased creation of nitrite ions. Nitric oxide scavenging activity is

shown in Figure 5. EAF was found to be more potent (IC_{50} 349.38 ± 7.33 33µg/mL) when compared to other fractions (Table 2).

4.2.8. Hydroxyl scavenging assay

An enormously reactive entity in biological systems is the OH radical. It is one of the most destructive genuses in free radical pathology, capable of damaging bimolecular integrity of the living cells. These radical unites among nucleotides in DNA and cause strand breakage leading to carcinogenesis, mutagenesis, and cytotoxicity. Hydroxyl radical (OH) scavenging aptitude of different samples is openly linked to its antioxidant activity. EAF was mainly efficient (IC_{50} 325.17 ± 5.76 µg/mL) followed by AQF (IC_{50} 358.1 ± 4.04 µg/mL), CF (IC_{50} 397.67 ± 4.66 µg/mL), and NHE (IC_{50} 476.89 ± 0.06 µg/mL), shown in Table 2. The hydroxyl scavenging activity of ascorbic acid was found to be 80.43 ± 2.23 µg/mL, shown in Figure 5.

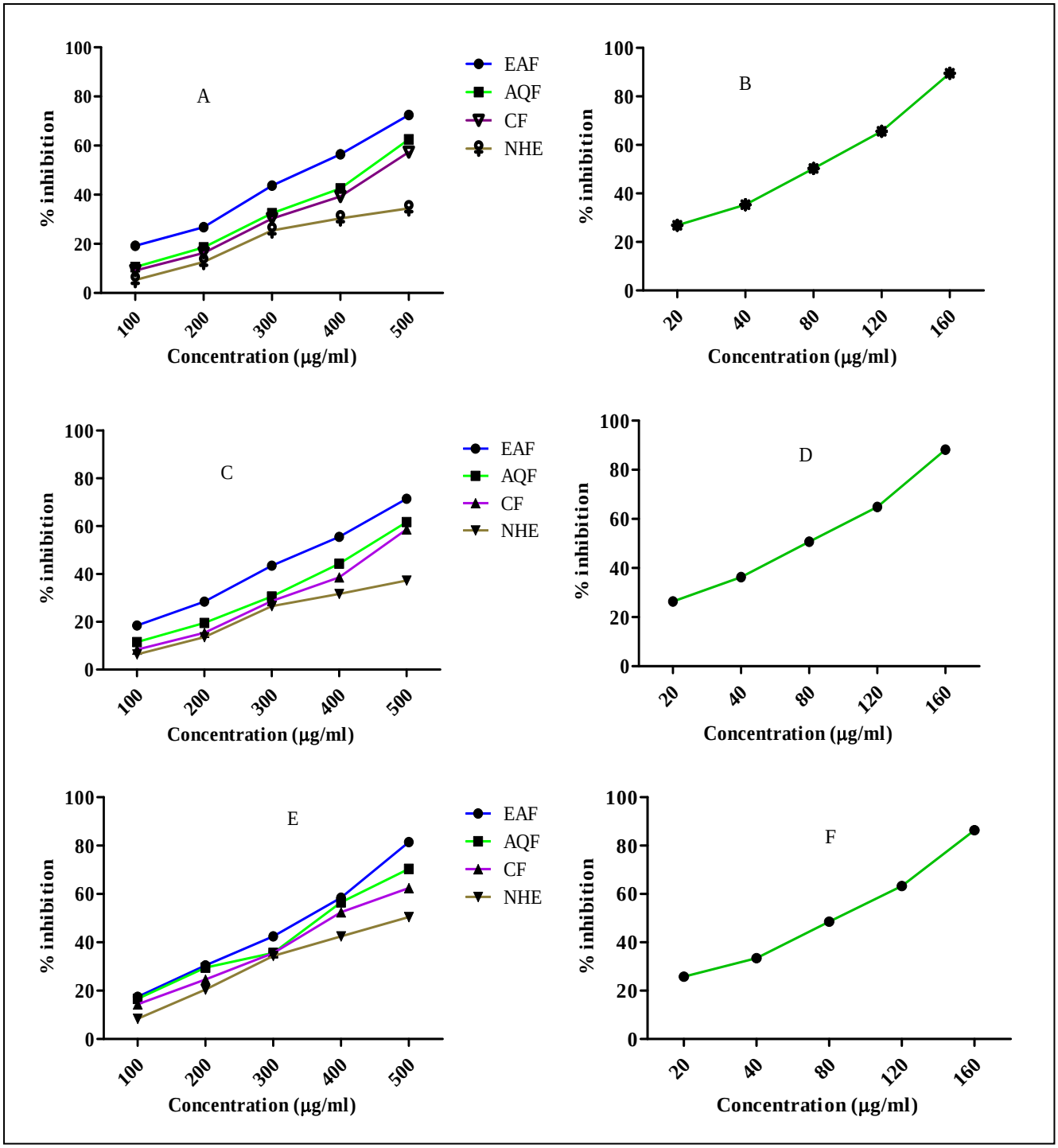
4.2.9. Hydrogen peroxide scavenging assays

Hydrogen peroxide is transformed into free radical termed OH in living cells and counter with biomolecules subsequently causing tissue injury and cell death. So it is anticipated that these are noxious to living cells sometimes, but it is basically non reactive. Table 2 shows the IC_{50} of hydrogen peroxide scavenging effect by various samples along with ascorbic acid and Figure 5 shows the percentage of inhibition. EAF (lowest IC_{50} 332.69 ± 2.36 µg/mL) noticeably scavenged the free radicals and was mainly effective than other samples. Inhibitory concentration of standard ascorbic acid was found to be 80.07 ± 0.83µg/ mL.

4.2.10. Reducing power assay

Reducing power assay is based on the principle of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Yildirim et al., 2001). In this assay, the presence of antioxidants in the samples would result in the reducing of ferric (Fe^{3+}) to ferrous form (Fe^{2+}) by donating an electron. Amount of ferrous (Fe^{2+}) complex can then be

monitored by measuring the absorbance of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in the reductive ability. Figure 5 shows dose dependent response for reducing power assay and IC_{50} values are depicted in Table 2.


A
% inhibition
Concentration (µg/ml)
EAF
AQF
CF
NHE
B
% inhibition
Concentration (µg/ml)
C
% inhibition
Concentration (µg/ml)
EAF
AQF
CF
NHE
D
% inhibition
Concentration (µg/ml)
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NHE
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% inhibition
Concentration (µg/ml)

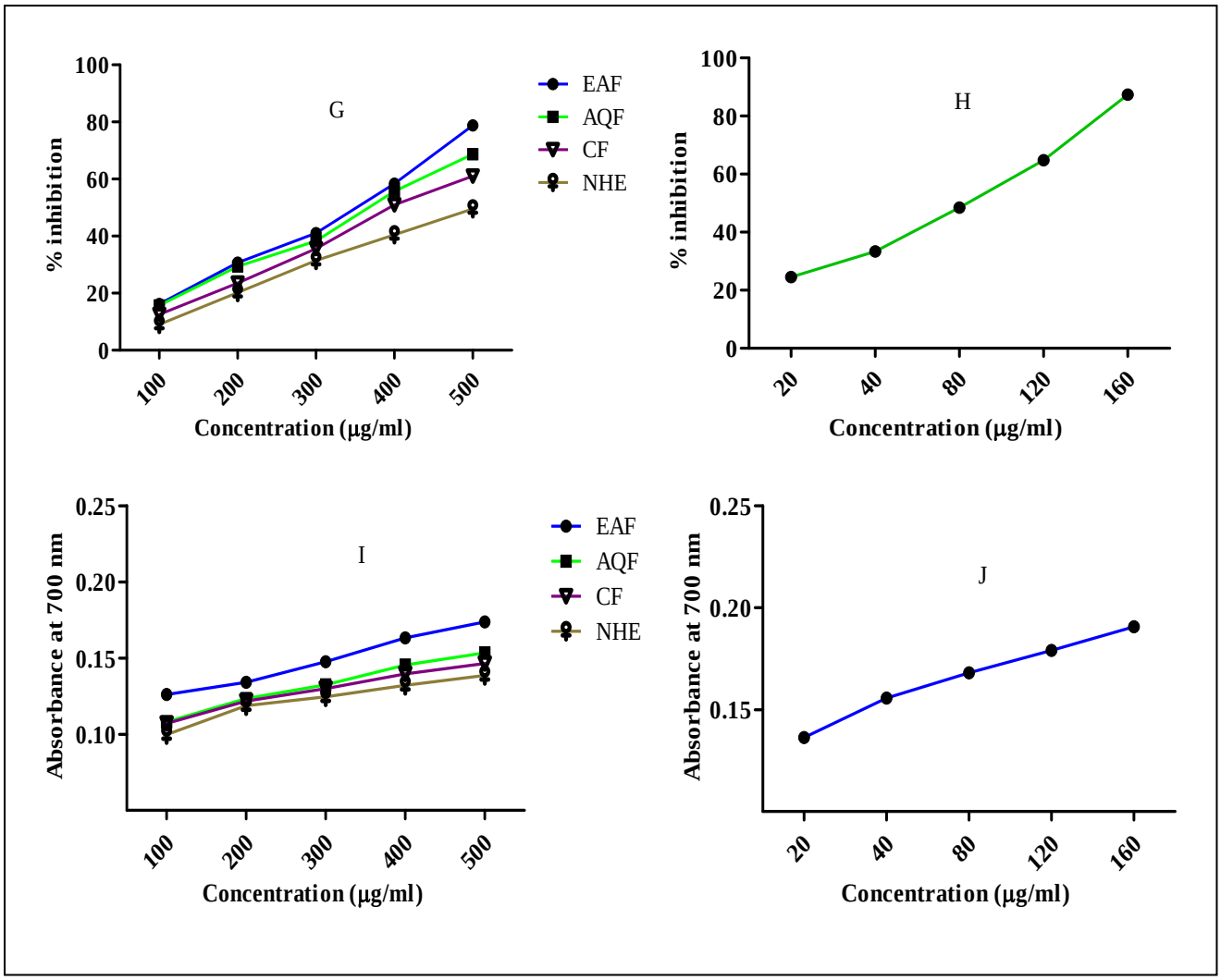


Figure 5 *In-vitro* antioxidant screening of (A) fractions on DPPH, (B) ascorbic acid on DPPH, (C) nitric oxide assay of fractions, (D) nitric oxide assay of ascorbic acid, (E) hydroxyl scavenging assay of fractions, (F) hydroxyl scavenging assay of ascorbic acid, (G) hydrogen peroxide assay of fractions, (H) hydrogen peroxide assay of ascorbic acid, (I) reducing power assay of fractions, and (J) reducing power assay of ascorbic acid

Table 2 IC_{50} (μg/mL) of different fractions of *D. melanoxylon* for various antioxidant systems

Treatment	DPPH radical scavenging assay	Hydroxyl scavenging assay	Hydrogen peroxide scavenging assay	Reducing power assay	Nitric oxide scavenging assay
EAF	346.11 ± 1.33	325.17 ± 5.76	332.69 ± 2.36	237.94 ± 13.93	349.38 ± 7.33
AQF	430.3 ± 0.87	358.1 ± 4.04	361.46 ± 2.14	375.39 ± 9.08	431.61 ± 7.35
CF	463.27 ± 0.95	397.67 ± 4.66	398.06 ± 6.35	424.50 ± 29.07	462.45 ± 7.58
NHE	672.43 ± 4.44	476.89 ± 0.06	496.98 ± 19.38	493.24 ± 15.22	637.59 ± 12.37
AA	76.02 ± 0.39	80.43 ± 2.23	80.07 ± 0.83	22.33 ± 1.30	76.23 ± 0.99

Each value in the table is represented as Mean ± SD (n = 3).

4.2.11. Anticancer activity

The methylene blue assay was used to screen the possible cytotoxic activity of different samples against five human cancer cells lines (HeLa, MCF-7, HCT116, PC-3, and HEK-293), one rat colon cancer cell line (RCC 45) and two normal cell lines (NKE and WI-38). Different samples caused loss of cell viability in a dose dependent manner.

EAF and AQF of *D. melanoxylon* leaves exhibited the highest cytotoxicity on all the tested cell lines as shown in Figure 6. NHE demonstrated moderate anticancer activity against observed cancer cells in this study. To the best of knowledge, these two fractions had not been investigated for their anticancer activity. Interestingly, all the samples showed poor

cytotoxicity against the normal cell lines as depicted in figure 6. Further, the most active samples were selected to study the dose response cytotoxicity effect. The median inhibitory concentration (IC_{50}) values for the most active fractions were calculated for all the tested cell lines and the values are shown in Table 3.

Interestingly, all the samples showed either mild or negligible cytotoxicity against both the normal cell lines (NKE and WI-38) which were used as the model normal cell lines.

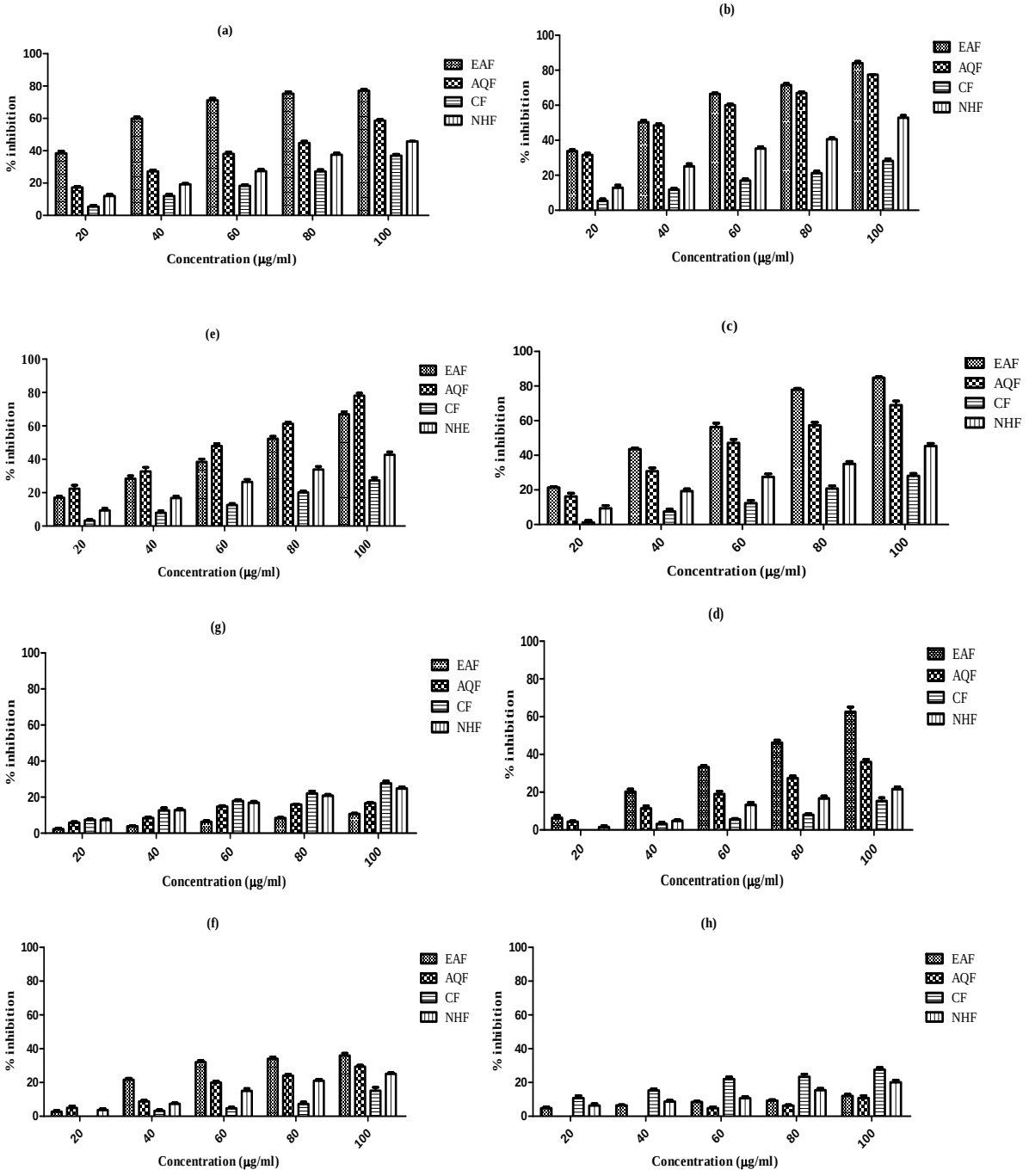


Figure 6 Cell toxicity studies of different samples on (a) HeLa, (b) MCF-7, (c) HCT116, (d) PC-3, (e) HEK293, (f) RCC45, (g) WI-38, and (h) NKE

Table 3 IC_{50} (μg/mL) of different fractions of *D. melanoxylon* for various anticancer systems

Treatment	Carcinoma cell lines						Normal cell lines	
	HeLa	**MCF-7**	**HCT116**	**PC-3**	**HEK293**	**RCC45**	**NKE**	**WI-38**
EAF	28.96 ± 2.17	41.52 ± 1.4	51.59 ± 0.85	83.68 ± 4.08	75.18 ± 1.83	122.77 ± 3.7	562.00 ± 114.54	468.44 ± 15.29
AQF	85.63 ± 2.69	47.41 ± 1.10	69.2 ± 2.79	136.86 ± 6.93	62.04 ± 2.10	161.59 ± 2.15	390.97 ± 48.02	318.42 ± 5.95
CF	136.86 ± 5.63	182.72 ± 19.71	168.32 ± 14.38	207.7 ± 12.85	180.34 ± 19.54	329.79 ± 81.25	207.31 ± 21.23	190.68 ±14.65
NHF	110.32 ± 2.67	95.02 ± 3.62	111.77 ± 3.25	311.47 ± 45.40	116.30 ± 1.89	186.73 ± 13.60	282.51 ± 24.25	216.18 ± 1.66

Each value in the table is represented as Mean ± SD (n = 3).

4.3. Discussion

SEM images could give a clear evidence of widening of cellular channels allowing easy entry of the solvent into the plant matrix. Solid-liquid extraction is thus the best method for the transfer of solute from one phase to another. This transfer mechanism is driven by capillary flow and depends upon the viscosity of the solvent. In soxhlet extraction, mass transfer happens from the inside to the outside while heat transfer happens from the outside to the inside. This observation suggests that soxhlet treatment affects the structure of the cell due to the sudden temperature rise (Mandal and Mandal, 2010).

The qualitative phytochemical screening for various fractions of *D. melanoxylon* was carried out. The results of phytochemical screening reveals that the leaves used for the extraction

procedure were imperative and adequate full-fledged to proliferate and contain metabolites needed to confirm various protective mechanisms. The preliminary phytochemical studies indicated the presence of carbohydrates, alkaloids, flavonoids, saponins, triterpenoids, glycosides, and tannins in the various sample. A number of scientific reports indicate triterpenoids; steroids and phenolic compounds such as tannins, coumarins and flavonoids have chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis (Blois, 2002; Bala et al., 2010). Furthermore, flavonoids have been also shown to posses antimutagenic and antimalignant effect (Fotsis et al., 1997).

Qualitative HPLC analysis revealed that gallic acid and rutin are abundantly present in the different fractions of *D. melanoxylon* leaf extract. So *D. melanoxylon* leaf extract can be thought to be developed as a possible drug candidate.

After an extensive scan of available literatures, it is noteworthy that widespread phenolic compounds such (−)-epigallocatechin-3-gallate, caffeic acid and chlorogenic acid can inhibit DNA methyltransferases and restore the expression of certain genes, which are silenced in cancer cells. Lee and Zhu (2006) demonstrated that caffeic acid or chlorogenic acid partially inhibited the methylation of promoter region of the *RARβ* gene in breast cancer cells MCF7 and MDA-MB-231 (Lee and Zhu, 2006; Lee et al., 2005). Yoshioka et al. (2000) also proved that flavonoids are responsible for inhibitory activity against cancerous cell lines (Yoshioka et al., 2000). Aires et al. (2013) have demonstrated that phenolic compounds such as gallic acid and trans-resveratrol inhibited colon cancer cell growth through apoptosis induction (Aires et al., 2013). These are some of the evidences which definitely give a strong indication of their involvement in the prevention and treatment of oxidative stress related diseases or disorders including cancer.

As a consequence, it is inferred from the above results that the flavonoid, phenolic, triterpenoid and tannin composition of different samples of the *D. melanoxylon* leaf extract are mainly responsible for their strong potency against different cancerous cell lines. For example, in this study it was seen that two fractions namely EAF and AQE are more active against cancerous cell lines in a concentration dependent manner. EAF have shown IC_{50} values of 41.52 ± 1.4, 51.59 ± 0.85, 83.68 ± 4.08, 75.18 ± 1.83, 122.77 ± 3.7; and AQF showed IC_{50} values of 47.41 ± 1.10, 69.2 ± 2.79, 136.86 ± 6.93, 62.04 ± 2.10, and 161.59 ± 2.15 against cancerous cell lines of MCF-7, HCT116, PC-3, HEK293, RCC45, respectively.

Above results indicate possible involvement of phenolics and flavonoids for the antiproliferative activity of EAF and AQF. This fact has already been generalized in the first half of the discussion portion. Moreover, this fact has also been duly supported in a review article which exemplifies a strong positive relation between phenolics/flavonoids and anticancer activity (Roleira et al., 2015). CF and NHE did not show significant antiproliferative activity against all cancerous cell lines. According to Taylor et al. (2001), this can be accounted to the presence of insufficient quantities of the active principles in the CF and NHE and hence no activity was seen with the dose levels used (Taylor et al., 2001).

A wide variety of methods have been used to determine the antioxidant activity of samples and no single assay provides an accurate method to determine the capacity to scavenge free radicals. Therefore, it is essential to use diverse methods to assess different aspects of the oxidation process. Furthermore, different compounds may act as antioxidants through different mechanisms. Therefore, it has been recommended that at least two different assays should be used in evaluating antioxidant activity of herbs (Moon and Shibamoto, 2009). Therefore, various samples were tested for their antioxidant potential using DPPH radical scavenging, hydroxyl radical scavenging, hydrogen peroxide scavenging, reducing power and

the nitric oxide scavenging methods. All the four fractions so prepared were screened for *in vitro* antioxidant potential.

Phenolic compounds have been shown to be responsible for the antioxidant activity of plant materials (Rice-Evans et al., 1996). However, in this case results are interesting when it comes to formulate a relationship between total phenolic content and observed antioxidant activity for the EAF and AQF. These two fractions gave the highest yield of phenolics, flavonoids, triterpenoids and tannins contents and also showed good anticancer action as evident from their low IC_{50} values. But when it comes to antioxidant potential, all fractions demonstrated significant high IC_{50} values of almost 5 times higher than the standard which was ascorbic acid. Higher phenolic content necessarily always does not indicate higher antioxidant potential. Many studies have focused on the biological activities of phenolics which are also potent antioxidants and free radical scavengers. The antioxidant activity of phenolics including flavonoids being mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Rice-Evans et al., 1996). The antioxidant activity of fractions could therefore not only be explained on the basis of their phenolic content, but required also their proper characterization.

Furthermore, the samples are very complex mixtures of many different compounds with distinct activities. The lack of relationship observed in this study is in agreement with other report in the literature (Ghasemi et al., 2009). Sengul et al. (2009) also reported a negative correlation between total phenolic content and antioxidant capacities of a number of medicinal plants extracts (Sengul et al., 2009).

The high IC_{50} values obtained in the active fraction of EAF and AQF probably indicates that oxidative mechanisms may not have been so predominant in proliferation of cancer cells. The mechanism for the observed cytotoxic effects may probably be due to cell cycle arrest followed by apoptosis. Depicted results definitely question the commonly assumed

involvement of the antioxidants in cancer treatment. Further committed studies are underway to elucidate the mechanisms involved. However, no doubts can be raised on the antiproliferative action of the plant *D. melanoxylon.* The fractions (EAF/AQE) which tested positively have a bright prospect to be developed as further drug candidates with high selectivity and efficacy.

The general consequences obtained from this study indicates that several fractions such as EAF and AQF of the plant *D. melanoxylon* have a bright prospective to be used as an anticancer agent.

Chapter 5

Experimental and results-part 2

Antidiabetic activity of different fractions obtained from *Diospyros melanoxylon* Roxb. leaf

Background and principal finding

The present study intends to screen novel α-amylase and α-glucosidase inhibitors from natural sources like plant to control hyperglycemia. The present study aimed to investigate the different phenolic profiles of leaf of different fractions of *D. melanoxylon* and their antidiabetic activities that could lead to the finding of more effective agents for the treatment and management of diabetes. In this experiment we have prepared four fractions from each four different extraction methods. Antioxidant activity was not performed as in our previous work did not show significant values. Antidiabetic activity was investigated by α-amylase and α-glucosidase inhibition tests. To understand the contribution of metabolites, phenolic, flavonoid, triterpenoid and tannin content were performed. Among all, comparative ethyl acetate fraction displayed a good α-amylase and α-glucosidase inhibition, showing the lowest IC_{50} values. The results of this study revealed that the different fractions from different methods of extracts could be determinant for the medicinal properties of this plant especially for antidiabetic activities.

5.1. Materials and methodology

5.1.1. Apparatus and condition

The extraction system comprised of microwave extractor (CATA R) manufactured by Catalyst Systems (Pune, India) equipped with a magnetron of 2450 MHz with a maximum power of 700W (100%), a reflux unit, 10 power levels (140 W (20%) to 700W (100%)), time controller, temperature sensor, exhaust system, beam reflector and a stirring device. Ultrasound assistant extraction (UAE) was conducted with an ultrasound probe (Labsonic WM, Sartorious, Goettingen, Germany).

5.1.2. Extraction and fractionation

5.1.2.1 Soxhlet extraction

Exhaustive Soxhlet extraction was performed using a classical Soxhlet apparatus with accurately weighed 20 gm sample of the drug powder screened through sieve number 60 for 25 hours by hot percolation method using a hydro-methanolic mixture (95:05). After extraction, the extract was passed through Whatman no. 1 mm membrane filter, evaporated under reduced pressure and preserved for experimental trials.

5.1.2.2. Maceration extraction

Maceration was carried out in a closed conical flask of 250 mL for 5 days. 20 gm powdered leaf sample was taken and extracted with 100 mL methanol: water (95:05). Occasionally shaking of the conical flask was carried out. Heat was not applied in either of the cases. The marc was separated by centrifugation (for15 min at 4^0C and 4000 rpm, R-8C, REMI, Mumbai, India) and the supernatant was then evaporated under reduced pressure and preserved for experimental analysis.

5.1.2.3. Ultrasound assisted extraction(UAE)

20 g of powdered leaf sample was extracted with (5gm × 4 assembly) 100 mL (25 mL × 4 assembly) of the previously mentioned solvent. Extract was concentrated in the same manner as explained earlier.

5.1.2.4. Microwave assisted extraction(MAE)

20 gm leaf powdered sample was extracted with 150 mL extraction solvent as mentioned earlier. The microwave operating conditions were as follows, Power: 500 Watt, extraction time: 8 min, solvent: sample ratio loading- 30:4 mL/gm and preleaching time: 10 min. Preleaching time is the contact time between sample matrix and extracting solvent before microwave irradiation. 10 min preleaching time is favourable for enhancing the extraction yield and allows sufficient swelling of the plant matrix. This increased hydrated status helps

is bursting of the cell due to internal thermal stress and also extension of the cellular pores thus facilitating leaching of the target analyte (Mandal, Mandal et al., 2010). After extraction, sample was concentrated as explained earlier. Methanol was used as the extracting solvent due to its better solubilising capacity for triterpenoids. A literature search revealed that methanol has been used frequently for the extraction of triterpenoids. Moreover, due to its better dissipation factor (tan d = 0.6400), methanol would heat up better in the MAE process.

5.1.3. Preparation of different fractions

All the extract viz. soxhlet extract, macerated extract, ultrasound extract, and microwave treated extract were dissolved one by one in double distilled water separately and passed through 1 mm membrane filter paper. Subsequently with the aqueous fraction (AQF) three more fractions such as n-hexane (NHF), chloroform fraction (CF) and ethyl acetate fraction (EAF) was prepared using liquid-liquid partitioning system with the help of a separating funnel. Mixing was completed by inverting the funnel 6 times (pressure in the separating funnel was discharged after each invert), where after the mixture was allowed to form two layers and both layers were collected separately. The process was repeated six times. The entire fractions were made solvent free by vacuum evaporation and refrigerated till further use.

5.1.4. Scanning electron micrographs

In order to understand the extraction mechanism, marc obtained from various extraction methods were subjected to scanning electron micrographs. After removing the solvent, the remaining *D. melanoxylon* leaf samples were plunged in liquid nitrogen and then cut with a cold knife. The sectioned particles were fixed on a specimen holder with aluminium tape and then sputtered with platinum. All the specimens were examined with a JEOL JSM-6700F (Akishima, Tokyo, Japan) scanning electron microscopy under high vacuum condition at different magnification.

5.1.5. Phytochemical analysis

Phytochemical analysis was carried out to identify secondary metabolites of *D. melanoxylon* fractions obtained from different extraction methods. The fractions were tested for alkaloids, glycosides, carbohydrates, terpenoids, flavonoids, saponins and tannins. Phytochemical analysis was performed based on previously reported methods with slight modification (Ayoola et al., 2008; Yadav and Agarwala, 2011).

5.1.6. High performance liquid chromatography (HPLC) analysis

The various test fractions (1 mg) of each extraction method (Soxhlet extract, Macerated extract, ultrasound extract and microwave assistant extract) was dissolved in 2 mL methanol and was vortexed for two hours for proper mixing. The final solution was filtered using 0.45 µm membrane filter (Biotech, Germany) prior to high performance liquid chromatography (HPLC) analysis.

The identification of compounds on HPLC (FRC-10A, UFLC, SHIMADZU, JAPAN) with PDA detector was performed using C18, 4.2 mm × 250 mm, 5 µm column. The chromatographic separation was carried out using solvent methanol (100 %) and TFA 0.1%. UV-Vis detected at 280 nm. Injection volume was 10 µL.

The identification of each compound was established by comparing the retention time and UV-Vis spectra of the peaks with those previously obtained by injection of pure standard compounds (gallic acid, ellagic acid, ferulic acid, rutin and quercetin).

5.1.7. Determination of total polyphenol content

Total polyphenolic content was performed by using the method Koncić et al. (2010) with slight modification (Koncić et al., 2010). The method was performed in triplicate using Folin-Ciocalteu reagent to determine total phenolic content in different fraction of different method obtained from *D. melanoxylon*. The Folin-Ciocalteau assess depend on the basis of transmission of electrons in alkaline medium from phenolic compounds to

phosphomolybdic/phosphotungstic acid complexes. Momentarily, 75 µL of diluted fraction and 425 µL of double distilled water were added to 500 µL Folin-Ciocalteu reagent and 500 µL of Na_2CO_3 (10% *w/v*). The combination was mixed and incubated for 45 min in the dark at 37^0C temperature. After incubation, the absorbance was measured at 765 nm using a UV-Vis spectrophotometer. Total phenol content was expressed in milligrams of Gallic acid equivalents per gm of dried sample (mg GAE/gm sample) using the expression from the calibration curve Y = 0.0556x - 0.1909, R^2= 0.9929.

5.1.8. Determination of total flavonoid content

Total flavonoid content was performed by method Russo et al. (2015) with slight modification (Russo et al., 2015). It was based on the formation of a complex flavonoid-aluminium in different fraction of *D. melanoxylon* in triplicate assay. Briefly, 150 µL of different fractions was added to 45 µL of 3% $NaNO_3$ into microcentrifuge tube. After 10 min, 90 µL of 2% $AlCl_3$ was added and at the 6th min, 300 µL of 1 M NaOH solution was added and the total volume was made up to 1.5 mL with double distilled water. The mixture was mixed well and the absorbance was measured against reagent blank at 465 nm after 30 min of incubation at 45^0C temperature. Quercetin was used as standard drug to plot the calibration curve. Total flavonoid content was expressed in milligrams of Quercetin Equivalent per gm of dried sample (mg QE/gm sample) using the equation obtained from the calibration curve Y = 0.0006x + 0.001, R^2= 0.9953.

5.1.9. Determination of Total Triterpenoid content

Total triterpenoid content was determined by the method of Fan and He (2006) with slight modification (Fan and He, 2006). 100 µL of different fractions (0.25-1.25mg/mL) were mixed individually with 150 µL (7.5 % w/v) vanillin-glacial acetic acid solutions and 500 µL of perchloric acid solution. The sample solutions were heated for 30 min at 60^0C and then cooled in an ice-water bath to ambient temperature. After the addition of 2.25 mL glacial

acetic acid, each sample solution's absorbance was measured at 548 nm using a UV-visible spectrophotometer. Total triterpenoid content was expressed in milligrams of Lupeol equivalents per gm of dried samples (mg LPE /gm dry sample) using the equation obtained from the calibration curve $Y = 0.0143x + 0.1546$, $R^2=0.9947$.

5.1.10. Determination of Total Tannin Content

Total tannin content of various samples was evaluated according to the method of Russo et al. (2015) with slight modification (Russo et al., 2015). 500 µL of bovine serum albumin (BSA) solution in 0.2 mol/L acetic buffer, pH 5.7 with 0.17 mol/L NaCl (1.5 mg/mL) was added to various concentrations of sample (0.25-1.25 mg/mL) and mixed carefully. After 20 min, the samples were centrifuged at 5000 gm for 20 min. The supernatant was removed, and the pellet dissolved in 1 mL of 1% aqueous solution of sodium dodecyl sulphate (SDS) and 4% triethanolamine. After that, 250 µL of 0.01 mol/L $FeCl_3$ in 0.01 mol/L HCl was added. After 45 min the absorbance reading was taken at 510 nm. Total tannin content was expressed as mg of tannic acid equivalent/gm of sample (mg TAE/gm of sample) using the equation obtained from the calibration curve $Y = 0.046x + 0.0796$, $R^2=0.9974$.

5.1.11. α-amylase Inhibitory activity

α-amylase inhibition assay was carried out using microplate reader according to the method of Sudha et al. (2011) based on the starch-iodine test with little modification with slight modification (Sudha et al., 2011). 75 µL of different concentrations of fractions (100-500 mg/mL) were added to 150 µL 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) and 75 µL α-amylase solution, and incubated at 37°C for 15 min. Take 100 µL from each sample reaction solution, 750 µL soluble starch (1%, w/v) and 500 µL phosphate buffer solution was added and incubated at 37°C for 45 min. Take 25 µL of above mixture and added 2.5 mL of iodine reagent (5 mM I_2 and 5 mM KI) and mixed properly for uniform mixture. The color change was observed and the absorbance was taken at 565 nm on

a microplate reader. Absence of sample is the control reaction representing 100% enzymatic activity. To eliminate the absorbance produced by plant fractions, appropriate fraction controls without the enzyme were also included. The standard drug acarbose (α-amylase inhibitor) was used as a positive control. It is observed the dark-blue color which indicates the presence of starch, a brownish color indicates partially degraded starch and a yellow color indicates the absence of starch in the reaction mixture. In the presence of inhibitors from the fraction the starch added to the enzyme assay mixture is not degraded and gives a dark blue color complex whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolysed by α-amylase.

5.1.12. Inhibition of α-glucosidase activity

The α-glucosidase enzyme inhibition activity was carried out according to the method of kuppusamy et al. (2011)(kuppusamy et al., 2011). It was determined by incubating100 μL of α-glucosidase enzyme (1 U/mL) solution with100 μL of phosphate buffer (pH 7.0) which contains 100 μL of enzyme inhibitor such as acarbose (3.6-50 μg/mL) at 37^0C for 60 min in maltose solution. To stop the α-glucosidase action on maltose, the above reaction mixture was kept in boiling water for 3 min and cooled. To this, 2 mL of glucose reagent was added and its absorbance was measured at 540 nm to measure the amount of liberated glucose by the action of α-glucosidase enzyme. The percentage inhibition and 50% inhibitory concentration (IC_{50}) value was calculated.

Statistical Analysis, Determination of Percentage Inhibition and IC_{50} Value

In this study, data were expressed as mean ± standard deviation (SD). Graph pad prism version 7.02 was used (two-way analysis of variance) for ANOVA to access the mean differences. The percentage inhibition of α-amylase and α-glucosidase was calculated using the following formula:

% inhibition= (Absorbance of control-Absorbance of test)/ Absorbance of control× 100.

5.2. Results and discussion

5.2.1. Effect of microwave power and irradiation time

Microwave power of 500W with irradiation time 8 min was used for the extraction of *D. melanoxylon* leaf sample. The above operating conditions were chosen based on the earlier reports of our research group. MAE is directly correlated with effects of microwave energy on phytomolecules by ionic conduction and dipole rotation which result in power dissipated in a volumetric fashion inside the solvent and plant material and then generate molecular movement and heating.

5.2.2. Effect of preleaching time and solvent to material ratio

Preleaching time can be defined as the contact time between sample matrix and extracting solvent before microwave irradiation. Preleaching time of 10 min is favourable enough to allow sufficient swelling of the plant matrix for enhancing the extraction yield. This increased hydrated status helps is bursting of the cell due to internal thermal stress and also enlargement of the cellular pores thus facilitating leaching of the target analyte (Pan et al., 2001).

The solvent volume always must be sufficient to ensure that the entire sample is immersed during the extraction process. Generally in conventional extraction techniques a higher volume of solvent will increase the extraction performance, but in MAE a higher solvent volume may give lower yield (Hao et al., 2002; Mandal et al., 2001). According to Mandal and Mandal 2010, before the ratio of solvent to material reached, at which the yield reached its highest value. And then it fell down slightly due to an inadequate stirring of the solvent when the microwaves are applied at larger volumes. Additionally, big volume of solvent cause more absorption of microwave energy and satisfactory microwave energy not available for helping the cell fracture for efficient leaching out of the intent analyte (Pare et al., 1991; Pan et al., 2003).

5.2.3. Extraction mechanism

In order to study the extraction mechanism the marc of *D. melanoxylon* obtained after each extraction technique were examined by a scanning electron microscopy. Figure 7 A, B, C, D, E, F, G, H represent the micrographs of the maceration extraction sample, ultrasound treated sample, soxhlet extraction sample, and microwave treated sample, respectively both at the surface and cellular levels. The changes observed for macerated sample were not intense and only few slight ruptures took place on the surface of the sample accompanied by slight widening of the cellular channels. Soxhlet extraction process revealed more ruptures has taken place on the surface as well as internally at the cellular level. On the other hand the surface of the sample was greatly destroyed after microwave treatment followed by massive widening or opening up of the cellular pores. This observation suggests that microwave treatment affects the structure of the cell due to the sudden temperature rise and the internal pressure increase. The higher temperature attained by the cell wall, during MAE, causes dehydration of cellulose and reduces its mechanical strength, which allows the solvent to gain an easy entry inside the cellular channels (Mandal et al., 2009).

But, another interesting theory of synergism between mass transfer and heat transfer can also be put forward in support of the accelerated extraction due to microwave effect. Solid-liquid extraction may be thought as a phase transfer of solute from one phase to another. The transfer mechanism is governed by capillary flow and depends upon solvent viscosity. In conventional solvent extraction, mass transfer occurs from the inside to the outside while heat transfer occurs from the outside to the inside. For microwave solvent extraction, due to improved hydration status of the plant cell, internal heating inside the plant cell takes place thus making the two transport phenomena work in the same direction from the inside of the extracted material to the bulk solvent. The acceleration of extraction rates under microwaves could be due to a synergy combination of the two transfer phenomena mass and heat acting in

the same direction. In microwave solvent extraction, heat is dissipated volumetrically inside the irradiated medium, while in conventional solvent extraction; heat is transferred from the heating medium to the interior of the sample.

Table 4 Comparison of MAE with other conventional techniques (extraction time, solvent volume, percentage of yield, and solvent ratio)

Extraction method	**Extraction time**	**Solvent Volume (mL)**	**Percentage of yield**	**Solvent ratio**
Soxhlet	16 hours	200	17.25	Methanol: Water (95:05 v/v)
Maceration	4 days	200	7.22	
USE	20 min	150	12.25	
MAE	8 min	100	22.72	

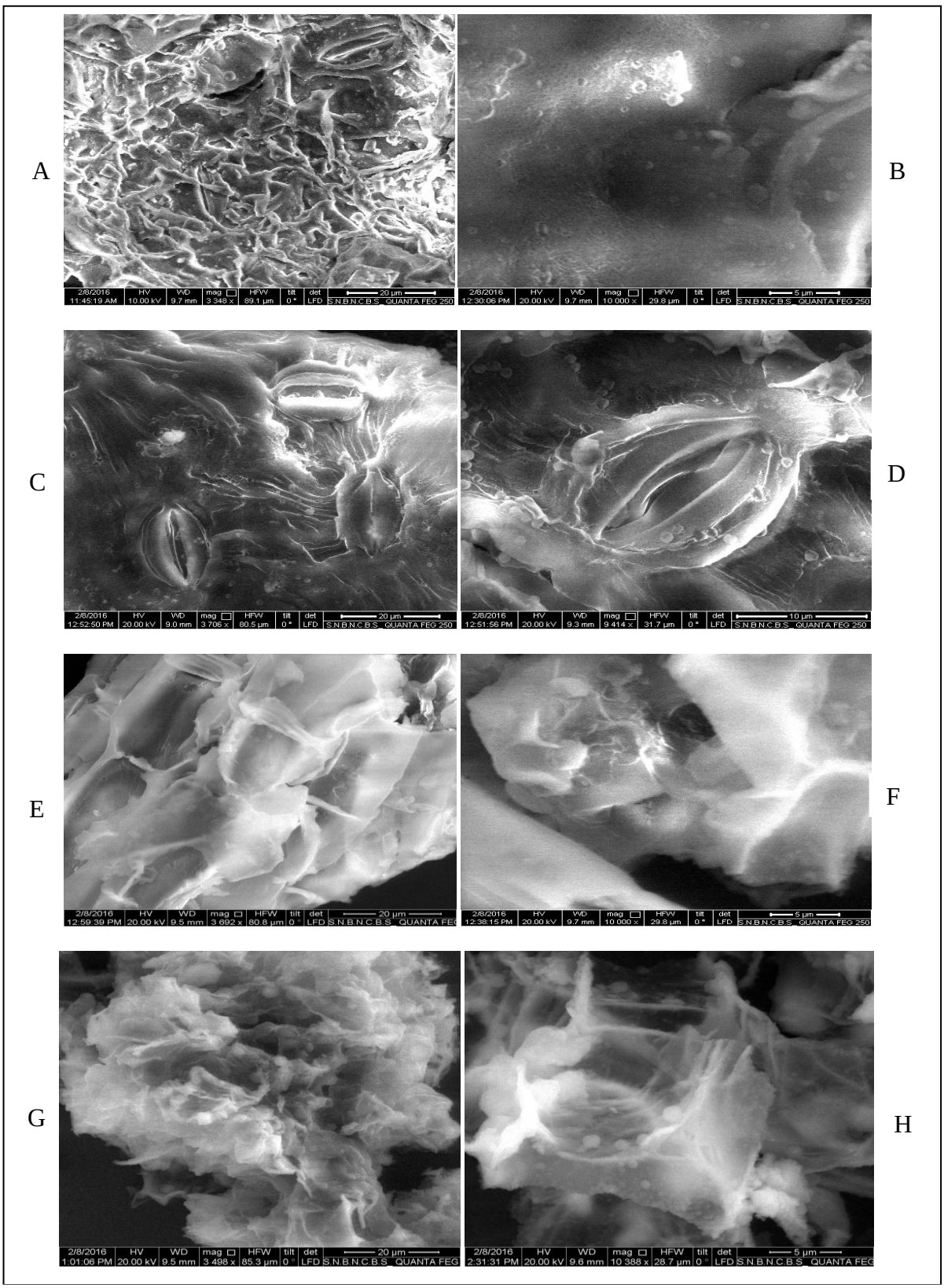


Figure 7 Scanning electron micrographs of leaf sample. (A) Surface view, (B) cellular channels and internal pores of maceration sample. (C) Surface view, (D) cellular channels and internal pores of ultra sound leaf sample. (E) Surface view, (F) cellular channels and internal pores of soxhlet leaf sample. (G) Surface view, (H) cellular channels and internal pores of microwave assistant leaf sample.

5.2.4. Comparison of MAE with other conventional techniques

The selection of an extraction method would mainly depend on the advantages and disadvantages of the processes, such as extraction yield, complexity, production cost, environmental friendliness, pharmacological activity and safety. MAE is a relatively new method, which has received increasing attention as an alternative method. The principle of heating during MAE is based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation. Ionic conduction is the electrophoretic migration of ions when an electromagnetic field is applied. The resistance of the solution to this flow of ions will result in friction and therefore heat the solution.

Dipole rotation means realignment with the applied field. At 2.45 GHz, which is the frequency used in commercial systems, the dipoles align, randomize and jostle 4.9×109 times per second and this results in heating (Camel, 2000). In the current study, MAE was compared with the other conventional extraction techniques for the extraction of *D. melanoxylon*. The conditions of different techniques and their results are summarized in Table 4 and showed that in terms of yield of target analyte. The best results were obtained by MAE, which gave significantly more. On extraction time, MAE was also the fastest extraction method with only 8 min of extraction time and preleaching time of 10 min. Maceration and soxhlet extractions are time consuming processes based on heat or mixing to increase the mass transfer rate. Ultrasound extraction is not so time consuming but not so effective method. MAE was found to be 85.47%, 32.11% and 214.68% more effective when compared to ultrasound extraction, soxhlet extraction and maceration extraction, respectively. These features along with an ease of operation and implementation would position MAE as a valuable and cost effective technology suitable for today's highly competitive industries with growing demand for increased productivity, improved efficiency and reduced cycle time.

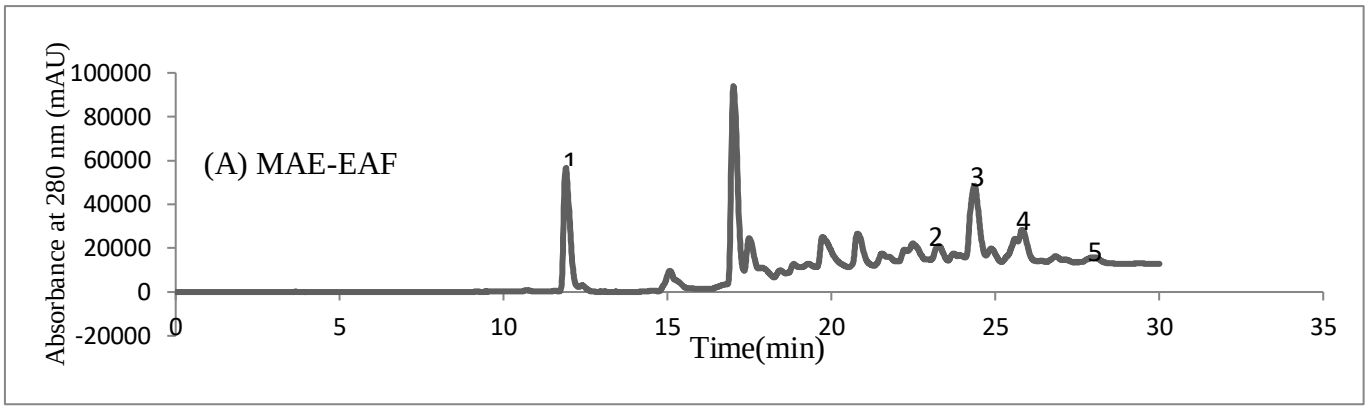
5.2.5. Phytochemical compositions

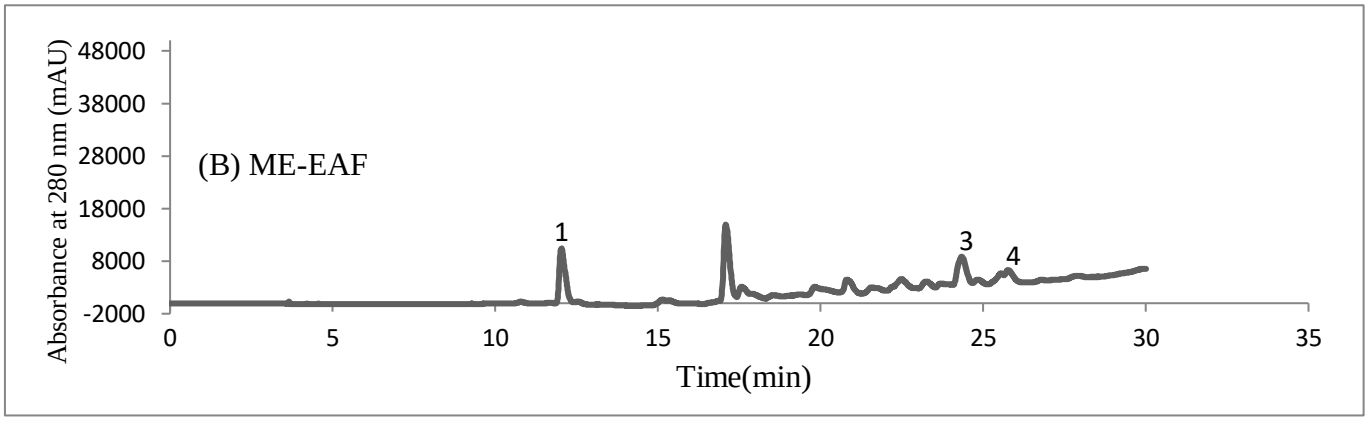
The fractions of *D. melanoxylon* leaf obtained from this study contained carbohydrates, terpenoids, flavonoids, saponins and tannins but not alkaloids and steroid.

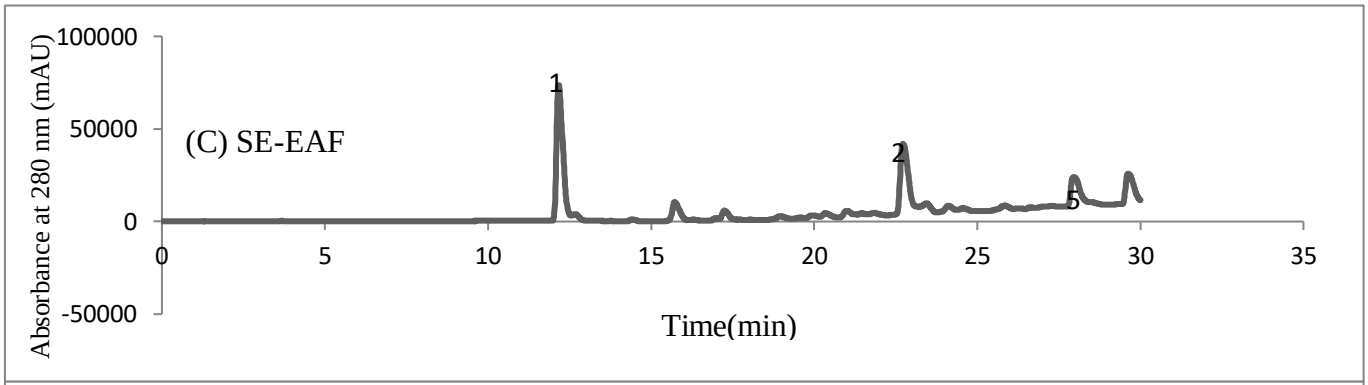
5.2.6. High performance liquid chromatography (HPLC) analysis

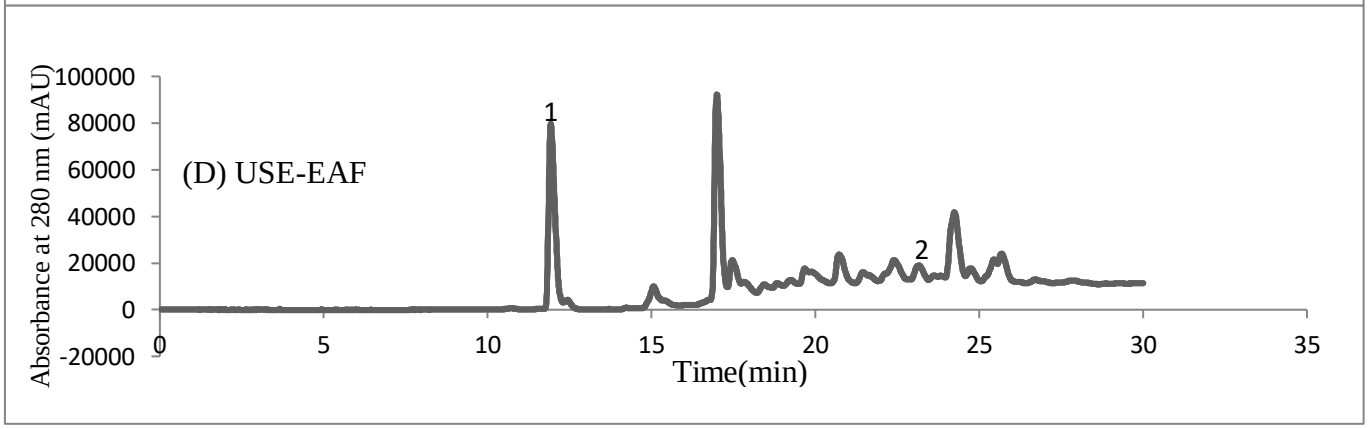
EAF from various extraction methods obtained from *D. melanoxylon* leaves was analyzed for identification of polyphenolic (phenolic acids and flavonoids) compounds by HPLC-DAD analysis. We have determined the HPLC analysis of only EAF as our previous work proved that this fraction is more active. Typical chromatograms of EAF and standards are presented in figure 8. Compounds were identified by comparison of retention times with standard reference compounds viz., four phenolic compounds (gallic acid, ellagic acid, ferulic acid, and rutin) and one flavonoid (quercetin).

Phenolic and flavonoid derivatives were detected in the HPLC-DAD profiles of the four samples. In the group of phenolic acids, gallic acid was the major compound detected in all the samples with retention time 12.277 min. Basically in MAE-EAF content higher concentration of gallic acid. Ferulic acid was the minor compound detected in all the samples with retention time 23.017 min. Then rutin was the second major compound in all the samples with retention time 24.708 min. Ellagic acid was present in the entire sample with retention time 25.454 min. Ellagic acid content is more in MAE-EAF comparatively. Minor concentration of quercetin compound was present to the entire fractions with retention time 28.08 min. Among the different extraction method of *D. melanoxylon* leaf, MAE-EAF was exposed as an effective solvent for extraction of most of phenolic acids and flavonoid.


(A) MAE-EAF
Absorbance at 280 nm (mAU)
Time(min)
1
2
3
4
5


(B) ME-EAF
Absorbance at 280 nm (mAU)
Time(min)
1
3
4


(C) SE-EAF
Absorbance at 280 nm (mAU)
Time(min)
1
2
5


(D) USE-EAF
Absorbance at 280 nm (mAU)
Time(min)
1
2

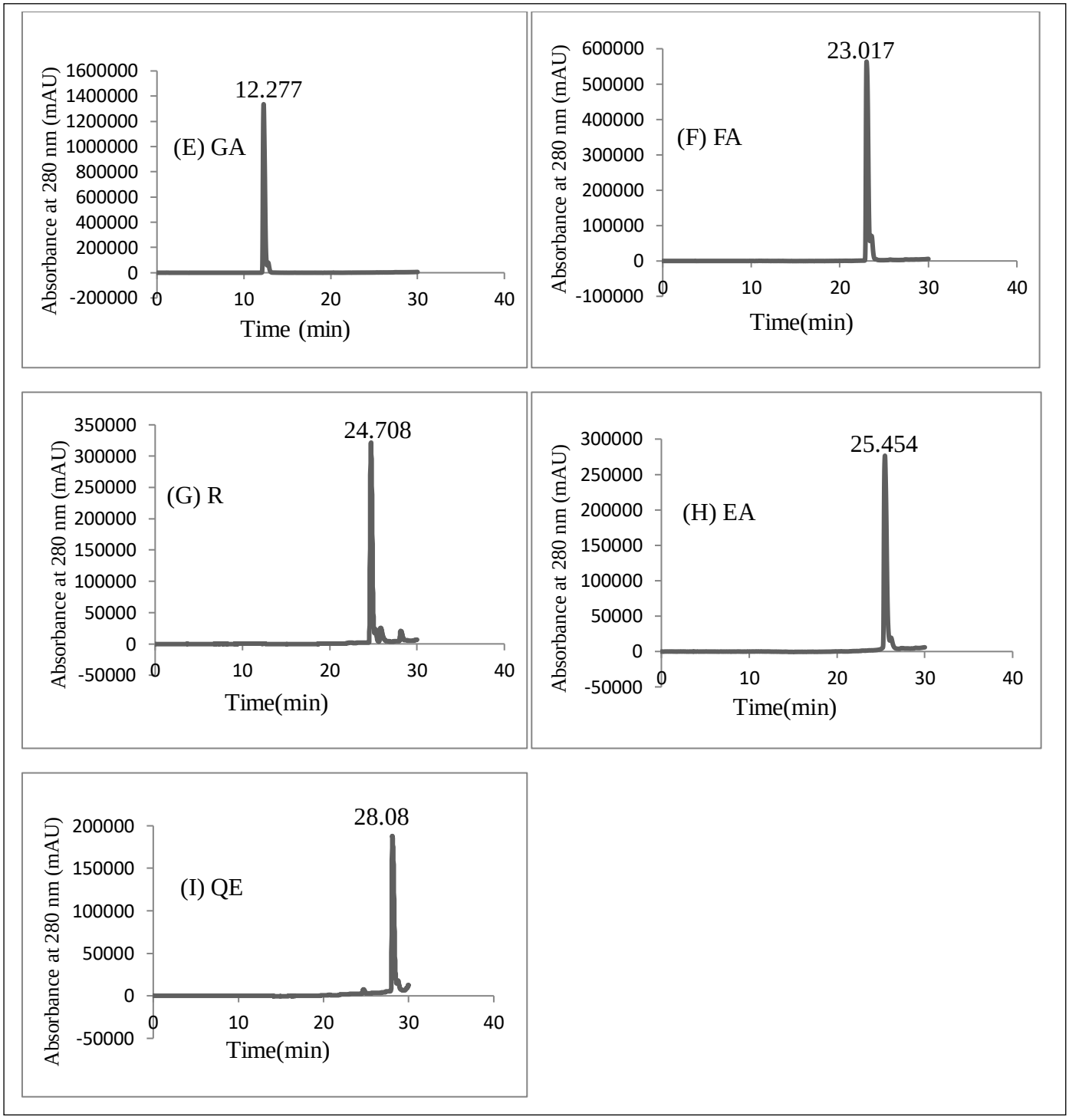


Figure 8 Identification of the major polyphenolic compounds in chromatograms of Ethyl acetate fraction(EAF) of (A) MAE-EAF=Microwave assistant extract, (B) ME-EAF=Maceration extract, (C) SE-EAF=Soxhlet extract, (D) USE-EAF=Ultrasound extract. Standard compounds (E) GA=Gallic acid, (F) Ferulic acid, (G) R=Rutin, (H) EA=Ellagic acid, and (I) QE=Quercetin. Peak 1=Gallic acid; 2 =Ferulic acid; 3 =Rutin; 4 =Ellagic acid; 5 =Quercetin.

5.2.7. Total polyphenolic and flavonoid content

Total polyphenolic and flavonoid content was analyzed systematically. Results are shown in Table 5. The total polyphenolic content is expressed as gallic acid equivalent (mg GAE/gm dry sample) and results were calculated from the standard gallic acid calibration curve (R^2= 0.992). The total flavonoid content is expressed in Quercetin equivalents (mg QE/gm dry sample) and results were calculated from the standard Quercetin calibration curve (R^2= 0.995). The results are also in agreement with the findings of HPLC analysis.

5.2.8. Total triterpenoid and tannin content

The total triterpenoid content is expressed in milligrams of Lupeol equivalents per gram of dried samples (mg LPE/gm dry sample) and results were calculated from the standard Lupeol calibration curve (R^2= 0.994). Tannin content was expressed as mg tannic acid equivalent (TAE)/gm of dried sample by using a standard curve (R^2= 0.9974) and results are reported in Table 5.

Table 5 Results of total polyphenol content, total flavonoid content, total triterpenoid content, and total tannin content of different fractions of different extraction method of leaf of *Diospyros melanoxylon*

Treatment		Total polyphenol compounds as mg Gallic acid equivalent (mg GAE/gm sample)	Total flavonoid compounds as Quercetin equivalent (mg QE/gm sample)	Total Triterpenoid Compounds as Lupeol equivalent (mg LPE/gm sample)	Total Tannin Compounds as tannic acid equivalent (mg TAE/gm sample)
Soxhlet extract	**NHF**	11.12 ± 0.34	353.12 ± 6.56	14.36 ± 0.15	12.49 ± 0.17
	CF	9.56 ± 0.33	291.65 ± 7.20	12.54 ± 0.21	10.3 ± 0.18
	EAF	19.52 ± 0.29	847.88 ± 8.30	22.4 ± 0.21	20.56 ± 0.18
	AQF	15.40 ± 0.37	641.56 ± 6.21	16.30 ± 0.16	14.2 ± 0.16
Maceration extract	**NHF**	8.31 ± 0.14	256.17 ± 4.88	12.26 ± 0.22	10.32 ± 0.33
	CF	7.73 ± 0.18	202.68 ± 7.75	10.72 ± 0.26	8.3 ± 0.21
	EAF	15.49 ± 0.29	786.81 ± 3.74	19.61 ± 0.20	18.38 ± 0.26
	AQF	12.17 ± 0.08	591.63 ± 2.84	16.32 ± 0.37	12.58 ± 0.24
Ultrasound extract	**NHF**	9.63 ± 1.02	303.82 ± 5.15	13.65 ± 0.24	11.52 ± 0.18
	CF	8.79 ± 0.47	235.86 ± 5.57	11.68 ± 0.32	9.6 ± 0.25
	EAF	17.84 ± 1.13	829.26 ± 6.30	21.34 ± 0.41	19.74 ± 0.31
	AQF	14.54 ± 0.32	625.45 ± 4.07	17.30 ± 0.28	13.9 ± 0.19
Microwave assistant extract	**NHF**	10.64 ± 0.23	363.64 ± 10.55	13.28 ± 0.12	11.51 ± 0.12
	CF	9.40 ± 0.18	297.96 ± 7.92	13.04 ± 0.12	10.94 ± 0.09
	EAF	21.48 ± 0.19	905.88 ± 14.90	24.59 ± 0.12	23.81 ± 0.06
	AQF	16.37 ± 0.29	660.18 ± 9.47	15.41 ± 0.18	15.59 ± 0.12

Each value in the table is represented as Mean ± SD (n = 3).

5.2.9. *In vitro* antidiabetic activity

Drugs that inhibit hyperglycaemia by suppressing hydrolysis of starch for α-amylase and carbohydrate for α-glucosidase have been extensively used in the management of diabetes mellitus (Mandal et al., 2008; Martino et al., 2006). Many herbal extracts have been reported for their antidiabetic activities and are currently being used in Ayurveda for the treatment of diabetes. However, such medicinal plants have not gained much importance as medicines due to the lack of sustained scientific evidence.

In the present study, different fractions from various extraction methods of *D. melanoxylon* leaves were screened for their α-amylase and α-glucosidase inhibitory activity. Several studies performed on this plant state them to be hypoglycaemic, but no reports exist on study for pancreatic α-amylase inhibitors in order to justify their hypoglycaemic property. The rationale for performing extractions from non-polar to polar solvents is to confirm and validate the inhibitory activity in the aqueous extractions performed in the traditional manner as well as to search for newer, more potent inhibitory compounds in the organic solvents. Primary screening for α-amylase inhibition was performed based on starch-iodine colour complex formation. On the other hand α-glucosidase is a membrane-bound enzyme at the epithelium of the small intestine and plays a key role in carbohydrate digestion.

Catherine et al. (2011) have reported the antidiabetic effect (α-amylase and α-glucosidase inhibition activities) of tannins and results have revealed cheering effects (Catherine et al., 2011). Flavonoids have also been demonstrated to suppress glucose level considerably as a strong inhibitor of diabetes (Kim et al., 2000). Koneri et al. (2014) reported that the triterpenoid saponin of *M. cymbalaria* possesses potential antidiabetic activity which may be endorsed to modulation of calcium channel and β-cell rejuvenation. So a number of scientific reports indicate phenolics, flavonoids, triterpenoids and tannins have antidiabetic role through their effects on different possible mechanism (Koneri et al., 2014).

Amongst the entire fraction, ethyl aetate fraction from soxhlet extract (IC_{50} 67.30 ± 0.75 µg/mL), maceration extract (IC_{50} 94.52 ± 0.85 µg/mL), ultrasound extract (IC_{50} 74.27 ± 0.89 µg/mL) and microwave extract (IC_{50} 52.39 ± 1.21 µg/mL) have shown better α-amylase enzyme inhibitory activity.

Results were expressed as IC_{50}. Acarbose was used as standard drug with an IC_{50} value is 72.22 ± 0.94 of α-amylase and 87.36 ± 0.86 of α-glucosidase for both inhibition assays and the standard curves are shown in figure 11. Data obtained showed in table 6 that ethyl acetate fraction obtained from microwave extraction was stronger inhibitors of α-amylase than α-glucosidase, as presented in figure 9 and 10. Among all the fractions, the most active was found to be EAF for both enzyme inhibitory activities.

The results authenticate that a high phenolic, flavonoid, triterpenoid and tannin content are necessarily associated with a strong potency of the tested *D. melanoxylon* leaf fractions against diabetes.

The ethyl acetate fraction from each extraction method exhibited strong i.e., ≥ 50% inhibition against α-amylase and α-glucosidase activity. A dose dependent effect was observed on increasing the concentrations of the fraction solution, suggesting a competitive type of inhibition. Plots of percent inhibition vs log concentration of fractions showed typical sigmoidal dose response curves (Figure 9 and 10). It was noted that EAF exhibited an IC_{50} value less than acarbose suggesting that it could be a promising lead fractions. It could thus be speculated that this fraction possess significant antidiabetic activity and microwave assisted extraction is the best method for the preparation of said fraction.

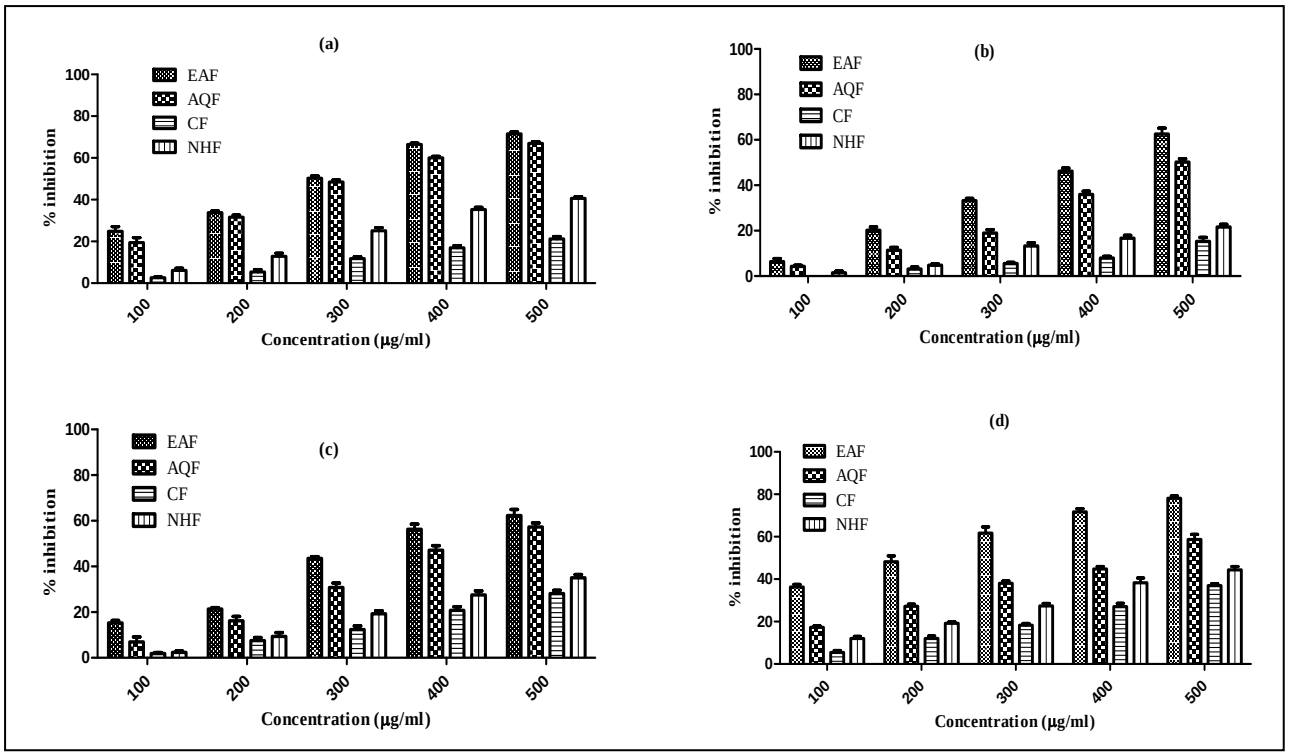


Figure 9 Alfa amylase activities of different samples on (a) Soxhlet extract, (b) Maceration extract, (c) Ultrasound extract and (d) Microwave assistant extract.

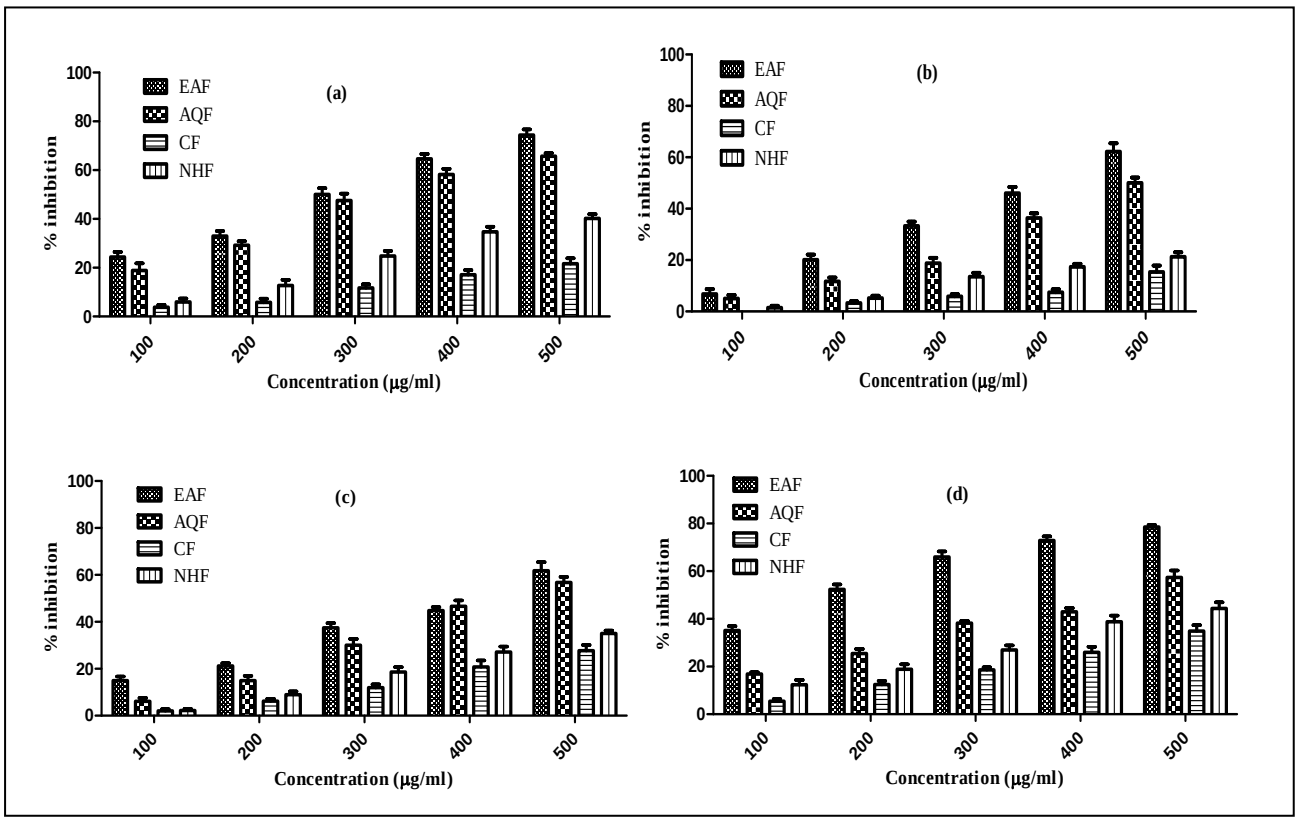


Figure 10 Alfa glucosidase activity of different samples on (a) Soxhlet extract, (b) Maceration extract, (c) Ultrasound extract and (d) Microwave assistant extract.

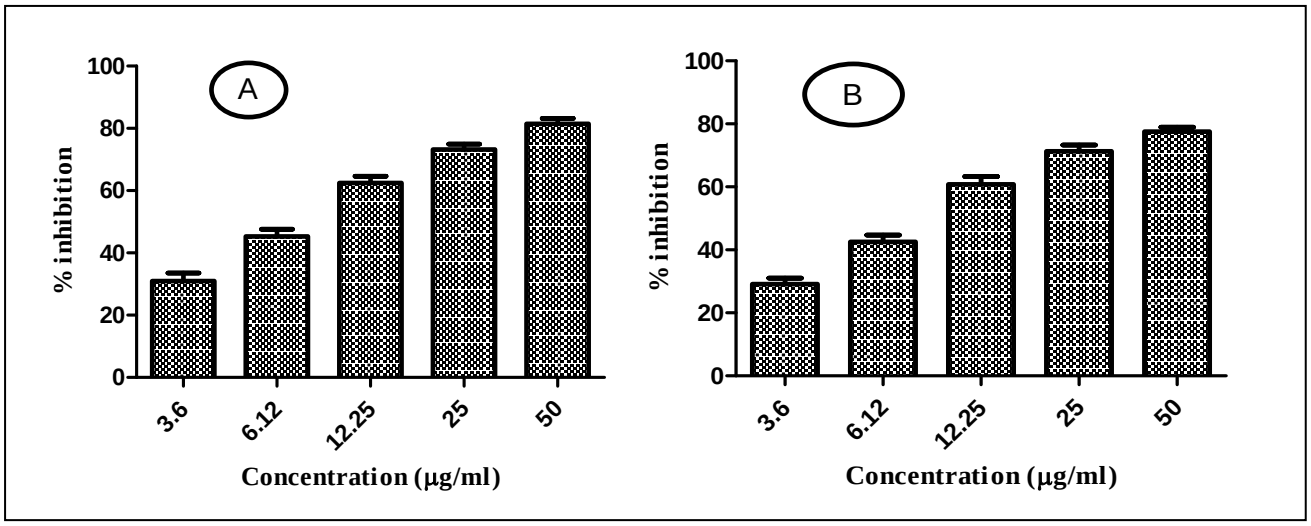


Figure 11 Standard curve of acarbose for (A) α-amylase and (B) α-glucosidase.

Table 6 Results (IC_{50}) of α-amylase and α-glucosidase activity of different fractions obtained from different extraction method of *Diospyros melanoxylon* leaf.

Treatment		**α-amylase inhibitory activity (µg/mL)**	**α-glucosidase activity (µg/mL)**
Soxhlet extract	**NHF**	164.30 ± 2.57	172.09 ± 1.70
	CF	172.89 ± 1.63	174.93 ± 1.71
	EAF	67.30 ± 0.75	86.24 ± 1.03
	AQF	106.29 ± 0.92	103.28 ± 1.20
Maceration extract	**NHF**	201.06 ± 2.71	176.48 ± 0.67
	CF	225.93 ± 2.28	181.62 ± 1.35
	EAF	94.52 ± 0.85	116.17 ± 1.12
	AQF	145.71 ± 0.54	126.49 ± 0.78
Ultrasound extract	**NHF**	189.67 ± 2.00	161.38 ± 0.95
	CF	204.08 ± 1.74	159.32 ± 1.34
	EAF	74.27 ± 0.89	93.24 ± 1.10
	AQF	120.56 ± 0.95	111.29 ± 1.22
Microwave assistant extract	**NHF**	141.67 ± 1.36	156.66 ± 1.43
	CF	170.12 ± 1.28	185.08 ± 1.56
	EAF	52.39 ± 1.21	73.52 ± 1.13
	AQF	96.34 ± 0.82	89.21 ± 1.08
Acarbose		72.22 ± 0.94	87.36 ± 0.86

Each value in the table is represented as Mean ± SD (n = 3).

Chapter 6

Experimental and results- part 3

Anticancer and antidiabetic activity of polymeric nanoparticles of subethyl acetate fraction

Background and principal finding

It is seen from the previous work that ethyl acetate fraction is more active against cancerous cell lines and diabetes. In this regard ethyl acetate fraction has been more fractionated with the help of column chromatography using suitable solvent system. Sub EAF (SEAF) and nanoparticles of sub ethyl acetate (NSEAF) were characterized for their anticancer and antidiabetic activity. Anticancer activity was performed using the methylene blue assay. Antidiabetic activity was performed on the basis of α-amylase and α-glucosidase assay. Comparatively, NSEAF showed better activity on different cancerous cell lines and antidiabetic activity on the basis of α-amylase and α- glucosidase action at a dose-dependent manner. It is also demonstrated that NSEAF exerted very low toxic effect against normal cell lines. So, NSEAF studied in the present research may be considered as possible future drug candidates for the treatment and management of cancer and diabetes.

6.1. Materials and methodology

6.1.1. Extraction and fractionation methods

After done the Soxhlet extraction process, ethyl acetate fraction (EAF) was collected and loaded in silica gel column chromatography (mesh size 230-400, bed volume ~130 mL, L = 25 cm, D = 4 cm) as our previous works has been proved that comparatively EAF having more anticancer as well antidiabetic activity. Mobile phase was selected by checking movement of the compounds on a thin layer chromatography (TLC) plate. For 40 gm of EAF, 350 mL each of the following solvents mixture was applied: N-hexane: ethyl acetate (NH:EA) 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70, 25:75 and 20:80. N-hexane: ethyl acetate: dichloromethane (NH: EA: DCM) 15:80:05, 10:80:10, 05:85:10. Ethyl acetate: dichloromethane: methanol (EA: DCM: MEOH) 90:05:05. Ethyl acetate: methanol (EA: MEOH) 90:10. Those fractions showed the same spots in TLC plate were collected in conical flask, dried in a vacuum evaporator and tested for cell toxicity study against MCF-7. SEAF 2, 4, 6 and 7 showed maximum cell toxicity. Mixed these active SEAF properly and prepared nanoparticles for anticancer and antidiabetic activity.

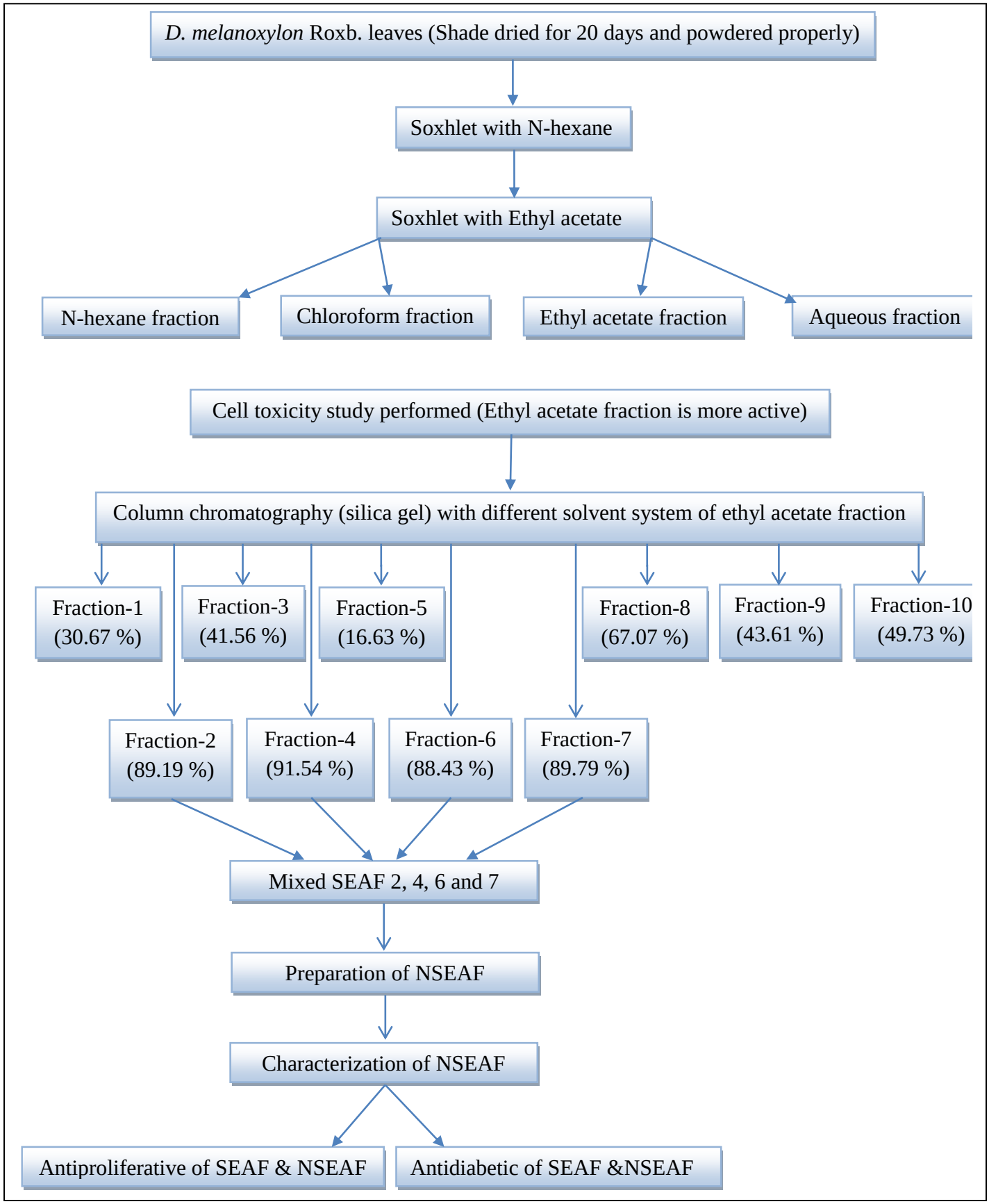

D. melanoxylon Roxb. leaves (Shade dried for 20 days and powdered properly)
Soxhlet with N-hexane
Soxhlet with Ethyl acetate
N-hexane fraction
Chloroform fraction
Ethyl acetate fraction
Aqueous fraction
Cell toxicity study performed (Ethyl acetate fraction is more active)
Column chromatography (silica gel) with different solvent system of ethyl acetate fraction
Fraction-1 (30.67 %)
Fraction-3 (41.56 %)
Fraction-5 (16.63 %)
Fraction-8 (67.07 %)
Fraction-9 (43.61 %)
Fraction-10 (49.73 %)
Fraction-2 (89.19 %)
Fraction-4 (91.54 %)
Fraction-6 (88.43 %)
Fraction-7 (89.79 %)
Mixed SEAF 2, 4, 6 and 7
Preparation of NSEAF
Characterization of NSEAF
Antiproliferative of SEAF & NSEAF
Antidiabetic of SEAF &NSEAF

Figure 12 Schematic diagram for fractionations and activity performed of *D. melanoxylon*

6.1.2. Chemicals and reagents

PLGA obtained as gift sample from Evonik India Pvt. Ltd., Mumbai-400072. (MW 50,000–75,000; poly D, L-lactide-co-glycolide ratio 75:25), Polyvinyl alcohol (PVA, MW 125,000; S.D. Fine Chem. Pvt. Ltd., Mumbai, India), hydroxypropyl-β-cyclodextrin (HPβCD); HiMedia Laboratories, Mumbai. Dulbecco's modified Eagle's medium (DMEM), and Fetal bovine serum (FBS) were obtained from Gibco, Grand Island, NY 14072, USA 1-716-774-6700. Roswell Park Media Institute (RPMI) 1640, non-essential amino acids, penicillin-streptomycin (PS), L-glutamine, and gentamicin were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used were of reagent grade and used as purchased without further purification.

6.1.3. Preparation of nanoparticles

NSEAF were prepared by emulsion solvent evaporation method according to Maji et al. (2014) with some modifications (Maji et al., 2014). SEAF and subsequently PLGA (75:25) (~250 mg) were dissolved in 1.5 mL of methanol and 1.5 mL of dichloromethane mixture (phase 1). PVA was dissolved in water (2.0 % w/v) (phase 2), and 0.5 mL of phase 2 was added drop-wise into phase 1 with homogenization at speed (8,000 rpm) using a high-speed homogenizer (IKA Laboratory Equipment, Model T10B Ultras-Turrax, Staufen, Germany). The prepared primary emulsion was then added slowly into 75 mL of 1.5% (w/v) PVA solution (phase 3) with a continuous homogenization (8,000 rpm), which produced a secondary emulsion and then sonicate for 2 min. The secondary emulsion was then placed on a magnetic stirrer and stirred overnight for evaporation to remove organic solvent and solidification of the particles. The NPs were then first separated by centrifugation at 5,000 rpm for 4 min to separate larger particles and

then the supernatant was collected and recentrifuged at 18,000 rpm for 1 hour. The solid particles, thus separated, were resuspended in Milli-Q water, and centrifuged to wash the particles to remove the excess PVA attached on the surface of the NPs and to remove the free drug. The washing was repeated two times. The separated NPs were frozen at -20°C and lyophilized (Laboratory Freeze Dryer, Instrumentation India Ltd., Kolkata, India) for 8-9 hours to obtain a solid product.

6.1.4. Determination of drug loading

For the determination of drug loading, an accurately weighed amount of NSEAF (2 mg) was placed into a centrifuge tube, and 2 mL of 5% SLS-NaOH solution was added. It was continuously shaken for 3-4 hours at 37°C in an incubator shaker. The dispersed phase was separated from the continuous phase by centrifugation. Then the supernatant was collected and the released drug was assayed spectrophotometrically at 278 nm. The percentage of drug loading and entrapment efficacy were calculated using Equation 1 and equation 2:

$$\text{Amount of drug loading} = \frac{\text{Actual drug loading in nanoparticles}}{\text{Weight of nanoparticles sample}} \times 100\ \% \qquad 1$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of drug loading}}{\text{Theoretical drug loading}} \times 100\ \% \qquad 2$$

6.1.5. Determination of particle size and zeta (ζ) potential

Average particle size, polydespersity index (PDI) and zeta potential of different formulations was determined based on quasi-elastic light scattering method using Data Transfer Assistance (DTA) software by Zetasizer Nano ZS 90 (Malvern Instruments, Malvern, UK) at 25°C. Briefly,

1mg of sample was dissolved in 2 mL Milli Q water and sonicated for 30 seconds in an ice bath. All measurements were performed in triplicate.

6.1.6. Surface morphology and particle size measurement using scanning electron microscopy (SEM)

Particle size and external morphology of the nanoparticles were studied by scanning electron microscopy (SEM) (Model-JSM-6360; JEOL, Tokyo, Japan).

6.1.7. Cell lines and culture conditions

The cell lines were ordered from the American Type Culture Collection (ATCC) and those used in this study were Human colon cancer cells (HCT116), breast cancer cell (MCF-7) and prostate cancer cells (PC-3), normal human kidney epithelium cells (NKE) and normal lung tissue cells (WI-38). HCT116, MCF-7 and WI-38 cells were grown in DMEM medium with 10% FBS and 1% penicillin-streptomycin. PC-3 and NKE cells were grown in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin. All cells were supplemented in a humidified atmosphere at 37^0C in 5% CO_2. Afterward, the exhausted medium was removed, replaced with fresh medium, and incubated again for 24 hours. The cell cultures were then washed with PBS and were suspended using trypsin. Fresh medium was added to the cells.

6.1.8. Antiproliferative assay

The methylene blue cytotoxicity assay was carried out according to the method previously described by Rathore et al. (2014) with slight modification (Rathore et al., 2014). Cells were seeded at 1.5×10^4 cells in each well of 96-well plate using 100 μL of fresh culture medium and were allowed to attach for overnight. Cell counts were made to determine cell viability by using the Tryptan Blue exclusion method (Kedar and Chakrabarti, 1982). For screening, the cells (60 - 70% confluence) were treated with the fractions at different concentrations. Afterward, in order

to obtain a curve, the most active fractions were tested for cytotoxicity at different concentrations (6.25-100 µg/mL). After 48 hours of treatment, the medium was removed from each well; methylene blue was added and incubated for 90 min on a shaker followed by decanting and washing with water to remove extracellular dye. A solution (0.1 N HCl) was then added for detaching the dyed cells. The colorimetric assay at 580 nm was performed on 96 wells micro plate. The cytotoxicity effects of different samples were calculated as a percentage of cell growth inhibition with respect to control and IC_{50} value calculated. The assay was performed in triplicates.

6.1.9. Inhibition of α-amylase activity

α-amylase inhibition assay was carried out using a microtitre plate according to Sudha et al. (2011) based on the starch-iodine test with slight modification (Sudha et al., 2011). 75 µL of different concentrations of NPs (6.25-100 mg/mL) were added to 150 µL 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) and 75 µL α-amylase solution, and incubated at 37°C for 15 min. Take 100 µL from each sample reaction solution, 750 µL soluble starch (1%, w/v) and 500 µL phosphate buffer solution was added and incubated at 37°C for 45 min. Take 25 µL of above mixture and added 2.5 mL of iodine reagent (5 mM I_2 and 5 mM KI) and mixed properly for uniform mixture. The color change was observed and the absorbance was taken at 565 nm on a microplate reader. Absence of sample is the control reaction representing 100% enzymatic activity. To eliminate the absorbance produced by NSEAF, appropriate NSEAF controls without the enzyme were also included. The standard drug acarbose (α-amylase inhibitor) was used as a positive control. It is observed the dark-blue color which indicates the presence of starch, a brownish color indicates partially degraded starch and a yellow color indicates the absence of starch in the reaction mixture. In the presence of inhibitors from the

fraction the starch added to the enzyme assay mixture is not degraded and gives a dark blue color complex whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolysed by α-amylase. The assay was performed in triplicates.

6.1.10. Inhibition of α-glucosidase activity

The α-glucosidase enzyme inhibition activity was carried out according to kuppusamy et al. (2011) with little modification (Kuppusamy et al., 2011). It was determined by incubating100 μL of α-glucosidase enzyme (1 U/mL) solution with100 μL of phosphate buffer (pH 7.0) which contains 100 μL of enzyme inhibitor such as acarbose (6.25-100 μg/mL) at 37^0C for 60 min in maltose solution. To stop the α-glucosidase action on maltose, the above reaction mixture was kept in boiling water for 3 min and cooled. To this, 2 mL of glucose reagent was added and its absorbance was measured at 540 nm to measure the amount of liberated glucose by the action of α-glucosidase enzyme. The percentage inhibition and 50% inhibitory concentration (IC_{50}) value was calculated. The assay was performed in triplicates.

Statistical analysis

All tests were performed as three independent experiments; each carried out in triplicate. Results were expressed as mean values with standard deviations (mean ± SD) and were subjected to two-way analysis of variance (ANOVA). All the data were calculated using Graph Pad Prism version 7.02 software.

6.2. Results

6.3. 6.2.1. Particle size and surface morphology using SEM

SEM photographs of NSEAF are shown in figure 13. It is manifest from the photographs that most of the particles were in the submicron size range. NSEAF are spherical and smooth surface.

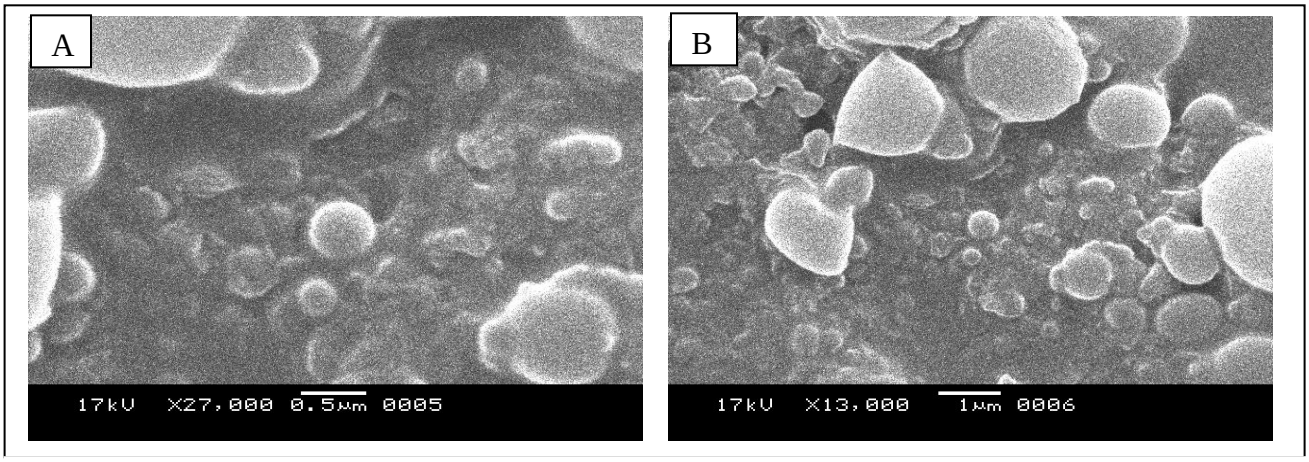


Figure 13 Scanning electron microscopic images.

Notes: Experimental formulations: (**A**) Formulation NP at ×27000; (**B**) Formulation NP at ×13000

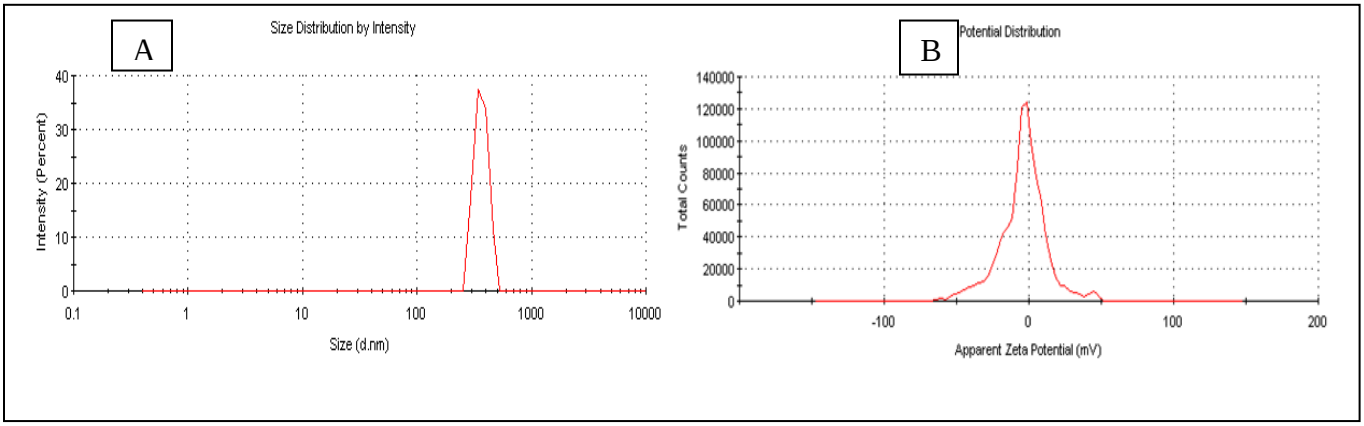


Figure 14 (A) Average particle size: 365.7nm (**B**) Zeta potential: -4.07 mV of NSEAF.

6.3.2. Determination of drug loading and entrapment efficiency

Drug loading amounts of NSEAF is 26% and theoretical drug loading is 42.85%. Entrapment efficiency of NSEAF is 60.67%. Drug loading was found to be directly proportional to the amount of the drug added during the preparation of nanoparticulate formulation (Maji et al., 2014).

6.3.3. Determination of particle size and zeta (ζ) potential

Particle sizes and zeta potentials of NSEAF are shown in Figure 14. An average particle size of NSEAF was 365.7 nm with the PDI of 0.689. Zeta potential was found to be -4.07 mV.

6.3.4. Antiproliferative activity

Different concentrations of SEAF and NSEAF of *D. melanoxylon* leaves were tested for their antiproliferative activity. The methylene blue assay was used to screen the possible cytotoxic activity of different samples against three human cancer cells lines (HCT116, MCF-7 and PC-3) and two normal cell lines (NKE and WI-38). Different samples caused loss of cell viability in a dose dependent manner. NSEAF exhibited the highest cytotoxicity on all the tested cell lines when compared with SEAF as shown in figure 15. When increasing the concentration of samples, percentage of cell death also increasing as shown in figure 15. SEAF demonstrated moderate antiproliferative activity against observed cancer cells in this study.

Interestingly, all the samples showed poor cytotoxicity against the normal cell lines. Further, the most active samples were selected to study the dose response cytotoxicity effect. The median inhibitory concentration (IC_{50}) values for the most active samples were calculated for the tested cell lines and the values are shown in Table 7.

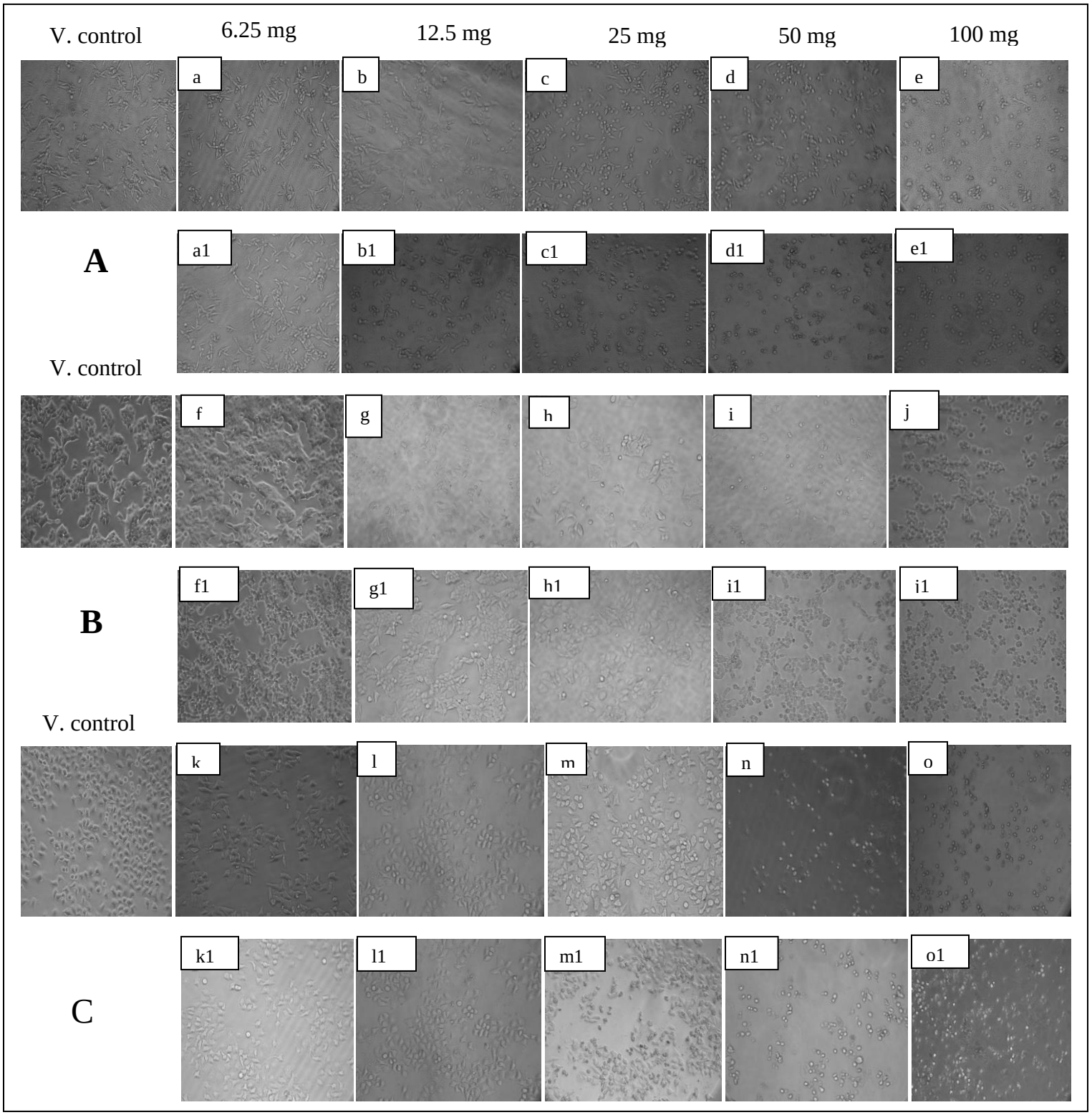

V. control
6.25 mg
12.5 mg
25 mg
50 mg
100 mg
a
b
c
d
e
A
a1
b1
c1
d1
e1
V. control
f
g
h
i
j
B
f1
g1
h1
i1
i1
V. control
k
l
m
n
o
C
k1
l1
m1
n1
o1

Figure 15 Microscopic observation of samples at different concentrations on various cancerous cell lines for 72 h. V. control= Vehicle control

A. Toxicity on HCT. Cell toxicity study of SEAF (a-e) and NSEAF (a1-e1) at different concentrations.
B. Toxicity study on MCF-7. Cell toxicity study of SEAF (f-j) and NSEAF (j1-j1) at different concentrations.
C. Toxicity study on PC-3. Cell toxicity study of SEAF (k-o) and NPs (k1-o1) at different concentrations.

6.4. Antidiabetic activity

In the present study, NSEAF were screened for their α-amylase and α-glucosidase inhibitory activity. Primary screening for α-amylase inhibition was performed based on starch-iodine colour complex formation. On the other hand α-glucosidase is a membrane-bound enzyme at the epithelium of the small intestine and plays a key role in carbohydrate digestion. Amongst the entire sample, NSEAF (IC_{50} 35.62 ± 2.56 µg/mL) have shown better α-amylase enzyme inhibitory activity compare to α-glucosidase (IC_{50} 73.52 ± 1.13 µg/mL) activity shown in table 7. Results were expressed as IC_{50}. Acarbose was used as standard drug with an IC_{50} value is 121.5 ± 1.20 of α-amylase and 130.36 ± 1.39 of α-glucosidase for both inhibition assays. Data obtained showed that NSEAF were stronger inhibitors of α-amylase than α-glucosidase, as presented in table 7. A dose dependent effect was observed on increasing the concentrations of samples solution, suggesting a competitive type of inhibition. Plots of percent inhibition vs log concentration of samples solution showed typical sigmoidal dose response curves shown in figure 16. It was noted that NSEAF exhibited an IC_{50} value less than acarbose suggesting that it could be a promising lead sample. It could thus be speculated that NSEAF possess significant antidiabetic activity.

Table 7 IC_{50} (μg/mL) of SEAF and NSEAF of *D. melanoxylon* for various anticancer and antidiabetic systems

Treatment	Cancerous cell lines			Normal cell lines		Antidiabetic activity	
	HCT116	**MCF-7**	**PC-3**	**NKE**	**WI-38**	**α-amylase**	**α-glucosidase**
SEAF	47.71 ± 3.09	49.57 ± 1.25	47.75 ± 3.25	319.95 ± 3.36	464.14 ± 1.62	47.60 ± 2.76	89.21 ± 1.08
NSEAF	32.89 ± 1.71	36.13 ± 0.96	32.39 ± 1.91	453.88 ± 5.37	564.99 ± 1.67	35.62 ± 2.56	73.52 ± 1.13

Each value in the table is represented as Mean ± SD (n = 3).

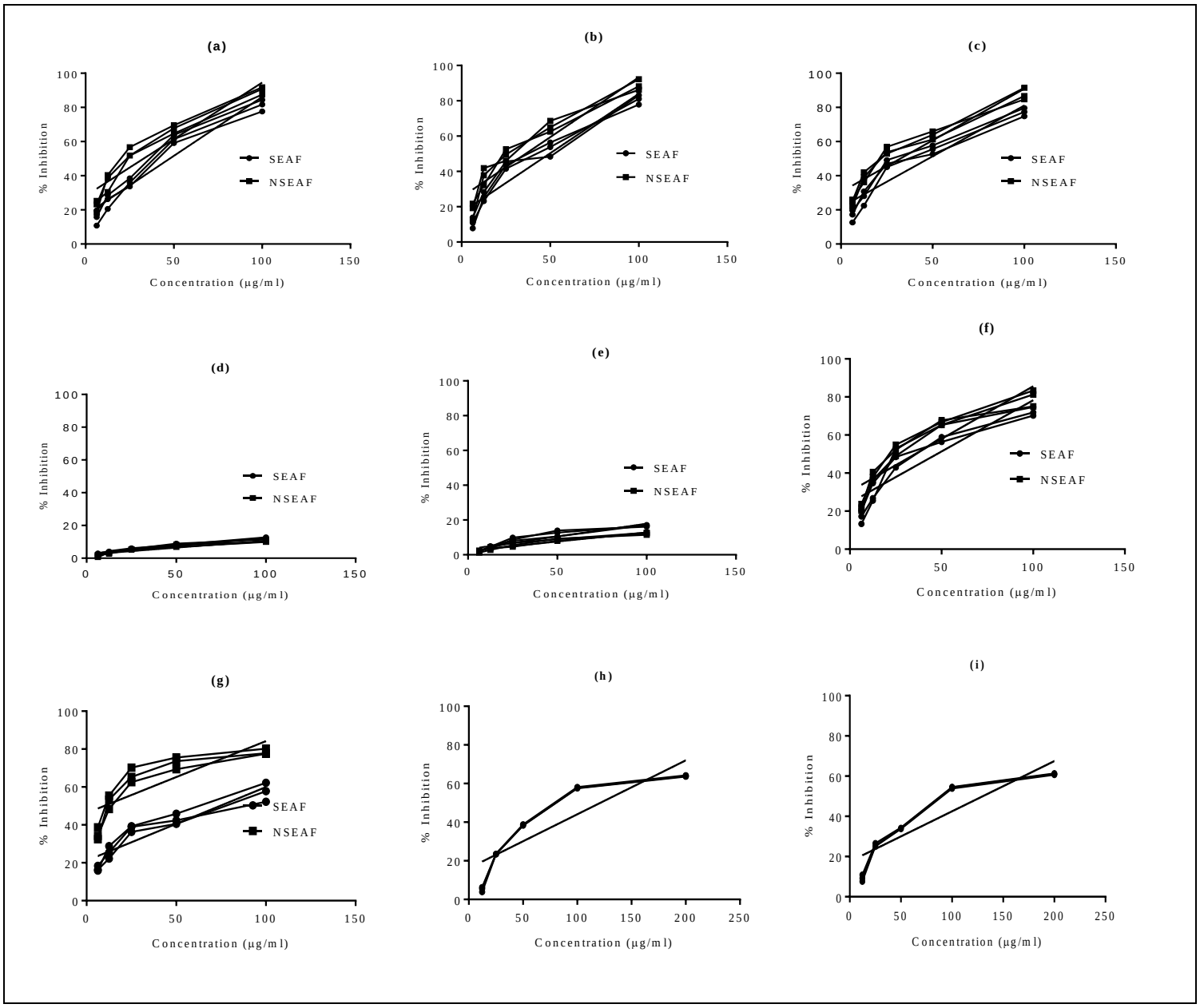


Figure 16 Cell toxicity studies of different samples in (a) HCT116, (b) MCF-7, (c) PC-3, (d) WI-38, and (e) NKE. Antidiabetic activity of different samples in (f) α-amylase and (g) α-glucosidase. Standard curve of Acarbose in (h) α-amylase and (i) α-glucosidase

7. Discussion

In this study, it is checked the activity of SEAF and NSEAF and examined in cancerous cell lines (HCT116, MCF-7 and PC-3) and antidiabetic activity (α-amylase and α-glucosidase). It was found that NSEAF inhibited cancer cell growth and having antidiabetic activity significantly better than SEAF shown in figure 16.

Chemotherapy agents used for cancer therapy have limited efficacy mainly due to their low specificity toward cancer cells and poor pharmacobioavailability *in vivo*, resulting in incidence of normal tissue toxicity and major side effects (Yallapu et al., 2010; Markman, 2008; Herzog and Pothuri, 2006). To address the problem, there is a high demand to explore therapeutic modalities with no or minimal side effects to normal cells. In this case, the study of using targeting molecules for cancer-specific therapy, linking to nanosized drug carriers, has been a promising strategy for targeted drug delivery and controlled drug release in the battle against cancer (Farokhzad et al., 2004: Langer, 2001).

On the other hand, drugs that inhibit hyperglycaemia by suppressing hydrolysis of starch for α-amylase and carbohydrate for α-glucosidase have been found in the control of diabetes mellitus (Mandal et al., 2008; Martino et al., 2006). Many herbal extracts have been reported for their anti-diabetic activities and are currently being used in Ayurveda for the treatment of diabetes.
More than 800 plants with antidiabetic properties according to the ancient Indian literature intelligences and that more than 1200 plants according to ethnopharmacological assessments used for hypoglycaemic action (Mishra et al., 2010). Two carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase are mainly responsible for postprandial hyperglycaemia. α-amylase begins the process of carbohydrate digestion by hydrolysis of 1, 4 glycosidic linkages of

polysaccharides viz. starch and glycogen to disaccharides and α-glucosidase catalyzes the disaccharides to monosaccharides, which leads to postprandial hyperglycaemia (Hara and Honda, 1990; Matsui et al., 2007). Therefore, inhibitors of α-amylase and α-glucosidase are helpful in the management of hyperglycaemia as they hindrance carbohydrate digestion, which subsequently decrease the postprandial plasma glucose level.

In this study, it was wanted to check if the efficacy of the drug could be improved by nanoparticle encapsulation. This would be of further benefit because the encapsulated drug could be directly carried into the cells, making it more bioactive. Here, in this circumstance it was tested to the hypothesis on different cancerous cell lines, α-amylase and α-glucosidase of SEAF as well as NSEAF. In this present study showed that NSEAF having high drug loading efficacy and entrapment efficacy. SEM images indicate that the NSEAF were spherical in shape and had a smooth surface. Average particle size of NSEAF was 365.7 nm with PDI 0.689 mV. So, the majority of the particles were found to be in submicron size with a relatively narrow variety of distribution as maintained by the PDI significance. The Zeta Potential of NSEAF was found to be around -4.07 mV. The electrostatic potential at the surface of a particle was determined by the zeta potential. Zeta potential value of -30 mV to +30 mV recommends that the particles would stay in a suspended state for a longer time and are not disposed to rapid agglomeration in the liquid circumstances (Meißner et al., 2009; Basu et al., 2012). Therefore, zeta potential value of our NSEAF proposes that should be stored in a lyophilized state and should be reconstituted only before experimental use.

Previous study confirmed that EAF having better anticancer and antidiabetic potentials obtained from *D. melanoxylon* in *in vitro* model. Further, the role of some nanoparticles was also predicted in making slight changes in molecular orientation of the drug molecules and thereby

biological activities. Therefore, the results of the present study assume further significance. In the present study, it was confirmed the anticancer and antidiabetic potentials of PLGA loaded NPs in five dose dependent manner. The outcome recommended that the higher dose was more competent as compared with the lower dose. In this study, the cytotoxicity and antidiabetic activity of NSEAF was more than that of SEAF. The possible mechanism underlying the enhanced efficacy of NSEAF against different cancerous cell lines may include the enhanced intracellular drug accumulation by nanoparticle uptake (Li et al., 2008; Zhang et al., 2007; Zhang et al., 2004).

By the way, to encapsulate orthodox drugs, PLGA has former been used including co enzyme Q (Ankola et al., 2007) taxol (Kim et al., 2004) and camptothecin (Vasey et al., 1999). Though, in the field of herbal medicine has also been reported to have enhanced cellular uptake and increased bioavailability of nanoparticle encapsulated the yellow curcumin present in turmeric known as *Curcumin longma* (Bisht et al., 2007; Anand et al., 2010). So, in this consequence it is seen that NSEAF was establish to be still more powerful and dynamic as compared with SEAF may be due to enhanced cellular uptake and increased bioavailability of nanoparticle encapsulated.

To the best information, this is the first effort to encapsulate SEAF of *D. malanoxylon* that is notorious to have anticancer and antidiabetic potentials of NSEAF. The results of exposure to cancerous cell lines viz. HCT116, MCF-7 and PC-3 *in vitro* are encouraging for their possible therapeutic use as an anticancer agent. Interestingly, all the samples showed either mild or negligible cytotoxicity against both the normal cell lines (NKE and WI-38) which were used as the model normal cell lines.

Advance research will also be essential to investigate if the value of the principal pharmacologically active compound(s) can also be improved by PLGA nanoparticles encapsulation by carry out experiment in both *in vivo* and *in vitro* methods. In this field, this work is rapidly gaining and should prove satisfying in the areas of toxicology and therapeutics and also in drug development for frightful diseases like cancer and diabetes.

Chapter 7

Summary and conclusion

Summary and conclusion

The present study has been focused about the development of polymeric nanoparticles obtained from traditionally used medicinal plant *Diospyros melanoxylon* Roxb. and evaluation of their anticancer and antidiabetes activity. The experimental work performed in this dissertation consists of three parts.

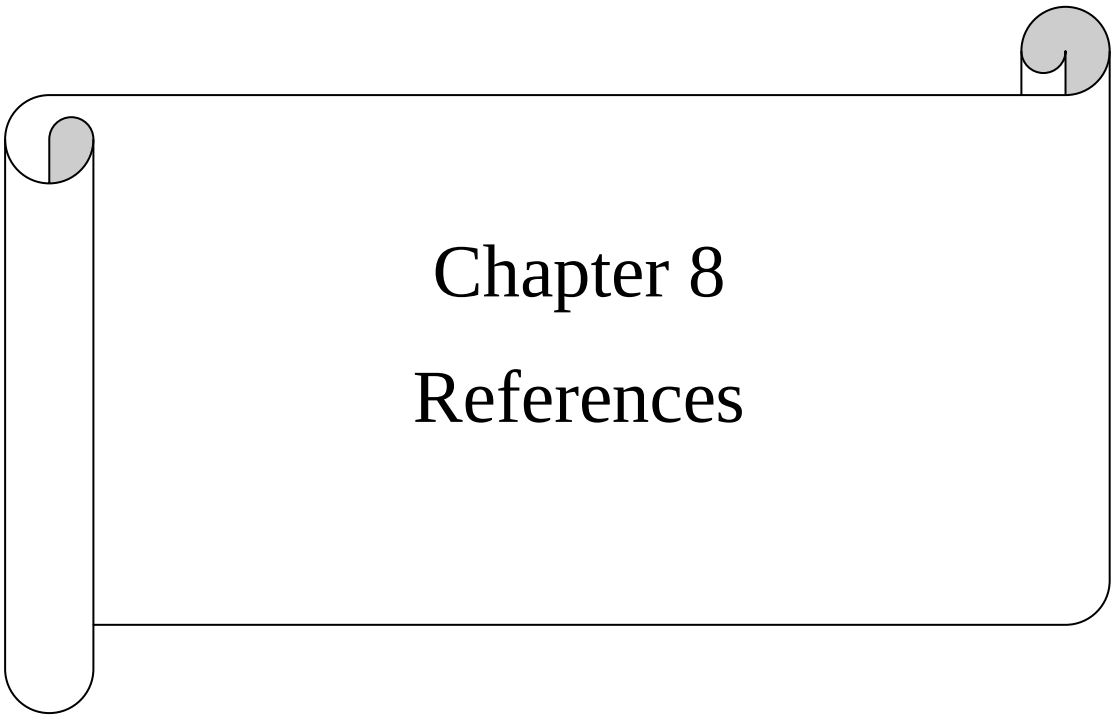
The first part of the study was carried out in anticancer activity of different fractions of the plant extract of *D. melanoxylon* leaf. Also this effort has gathered experimental confirmation that *D. melanoxylon* leaf fractions contained significant amount of flavonoids, polyphenols, triterpenoids and tannins. Qualitative HPLC analysis exposed that gallic acid and rutin are richly present in the different fractions of *D. melanoxylon* leaf extract. So *D. melanoxylon* leaf extract can be thought to be developed as a possible drug candidate. Moreover, it has been demonstrated that the *D. melanoxylon* leaf fractions are potential anticancer agents with minimal toxic effect on normal cell lines. The anticancer activity might be due to the presence of bioactive compounds in plant. Additionally, this experiment indicated that the plant contained potential activities and seems to serve as a prospective material for further development of novel plant-based anticancer mediators, which if properly and extensively studied, could provide many chemically interesting and biologically active drug candidates. So, it is inferred from the above results that the flavonoid, phenolic, triterpenoid and tannin composition of different samples of the *D. melanoxylon* leaf extract are mainly responsible for their strong potency against different cancerous cell lines. Particularly, in this study it was seen that two fractions namely EAF and AQE are more active against cancerous cell lines in a concentration dependent manner.

The second part of the study was carried out in antidiabetes activity of different fractions from various extraction methods of *D. melanoxylon* on the basis of α-amylase and α-glucosidase inhibitory activity. Primary screening for α-amylase inhibition was performed

based on starch-iodine color complex formation. On the other hand α-glucosidase is a membrane-bound enzyme at the epithelium of the small intestine and plays a key role in carbohydrate digestion. As a result, it is confirmed that the phenolic, flavonoid, triterpenoid and tannin composition of different fractions from different extraction methods are mainly responsible for their antidiabetic effect. Furthermore, the results authenticate that a high phenolic, flavonoid, triterpenoid and tannin content are necessarily associated with a strong potency of the tested *D. melanoxylon* leaf fractions against diabetes. Thus of the above mentioned ethyl acetate fraction from each extraction method exhibited strong i.e., ≥ 50% inhibition against α-amylase and α-glucosidase activity. It was noted that EAF exhibited an IC_{50} value less than acarbose suggesting that it could be a promising lead fractions. It could thus be speculated that this fraction possess significant antidiabetic activity.

The third part of the study was carried out the anticancer and antidiabetes activity of SEAF and NSEAF of *D. melanoxylon* leaf extract. EAF has chosen as in the first and second part has already been proved that comparatively EAF having more anticancer as well as antidiabetic activity. Preparation of PLGA loaded nanoparticle was effectively proficient by *D. melanoxylon* fraction. The biodegradable polymer PLGA based nanoparticles were successfully developed. This particular pharmaceutical product development was formulated with the biodegradable polymer PLGA (75:25) to achieve desired specific characters such as particle size, zeta potential, PDI, drug loading and successful entrapment of SEAF, and for the betterment in cancer and diabetes therapy. This one step, eco-friendly efficient process involves plant based bio-resource serving as both reducing as well as stabilizing agents. It is reported here the admirable inhibitory potential of SEAF as well as NSEAF against different cancerous cell lines, α-amylase and α-glucosidase activity. These activities are considered to be significant pharmacological targets for their anticancer properties. Similarly, it exhibited superior against α-amylase and α-glucosidase towards the exploration of its antidiabetic

potential. This is the first ever report on different cancerous cell lines and antidiabetic activity on the basis of α-amylase and α-glucosidase inhibitory action of PLGA loaded nanoparticles of SEAF obtained from *D. melanoxylon*. The high potency of these biogenic NSEAF against cancerous cell lines and glycosidase inhibitory activities *in vitro* provided strong scientific evidence for anticancer and antidiabetic potential which intensely rationalize its use in the therapy and management of cancer and diabetes.



Chapter 8

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