Metabolite profiling of *Ocimum sanctum* L. leaves and *Hemidesmus indicus* (L.) R. Br. roots and their pharmacological activities

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By

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DECLARATION

I hereby declare that the work presented in this thesis entitled "**Metabolite profiling of *Ocimum sanctum* L. leaves and *Hemidesmus indicus* (L.) R. Br. roots and their pharmacological activities"** has been carried out by me under the supervision of **Dr. Sarada D. Tetali in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad** and this has not been submitted earlier for any degree or diploma of any other University or Institutes.

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CERTIFICATE

This is to certify that Mr. Sudhansu Sekhar Choudhury has carried out research work presented in the present thesis entitled **"Metabolite profiling of *Ocimum sanctum* L. leaves and *Hemidesmus indicus* (L.) R. Br. roots and their pharmacological activities",** for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

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Sudhansu Sekhar Choudhury

Abbreviations

AA: Arachidonic acid

AE : Alcoholic extract

A-FR: Alcoholic fraction

ANOVA : Analysis of variance

AAE : Ascorbic acid equivalents

βC: β-caryophyllene

BD : Becton, Dickinson and Company

CVD : Cardiovascular disease

DAPI : 4',6-diamidino-2-phenylindole

DCF : Dichlorofluorescein

dL: deciliter

DMSO : Dimethyl sulfoxide

DPPH : 2,2-diphenyl-1-picrylhydrazyl

dw: dry weight

E-FR: Ethyl acetate faction

ELISA : Enzyme-linked immuno assay

ESI : Electrospray ionization

EUG: Eugenol

FBS : Fetal bovine serum

GA : Gallic acid

GAE : Gallic acid equivalents

GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

h: (hour)s

HDL-C: High density lipoprotein-cholesterol

H_2DCFDA : 2',7'-dichlorodihydrofluorescein diacetate

Hep : Heparin

HFD: High fat diet

HIAE: *Hemidesmus indicus* alcoholic extracts

HIWE: *Hemidesmus indicus* water extract

HLPO: Heart lipid peroxidation

HPLC : High pressure liquid chromatography

kg: kilogram

HFD: High fat diet

IC_{50} : Inhibitory concentration fifty

IL-6: Interleukin-6

IL-8: Interleukin-8

IMT: Imatinib

LC-MS : Liquid chromatography-mass spectrometry

LDL : Low density lipoprotein

LDL-C: low density lipoporotein -cholesterol

LLPO: Liver lipid peroxidation

LM: Limolene

LPS: Lipopolysaccharide

MCP-1 : Monocyte chemoattractant protein-1

MDA: Malondialdehyde

MIP-1α: Macrophage inflammatory protein-alpha

µM: Micro molar

µg: Micro gram

mg : Milligram

min : Minute(s)

ml : Millilitre

mM : Milli molar

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezoliumbromide

NF-κB : Nuclear transcription factor kappa B

OSAE: *Ocimum sanctum* alcoholic extract

OSWE: *Ocimum sanctum* water extracts

PCR : Polymerase chain

PMA: Phorbol-12-myristate-13-acetate

QE : Quercetin equivalents

QTOF-MS : Quadrupole time-of-flight mass spectrometer

RA: Rosmarinic acid

ROS: Reactive oxygen species

RPA: Reducing power activity

rpm : Revolutions per minute

RT : Retention time

RT-PCR: Real time polymerase chain reaction

SD: Standard deviation

TAC: Total antioxidant capacity

TBA: Thiobarbituric acid

TC: Total cholesterol

TFC: Total flavonoid content

TG: triglycerides

THP-1: Human monocytic leukemia cell line

TLR-2: Toll like receptor-two

TLR-4: Toll like receptor-four

TNF-α : Tumor necrosis factor-alfa

TPC: Total phenol content

VCAM-1: Vascular cell adhesion molecule

v/v: Volume/volume

WE: Water extract

W-FR: Water fraction

WHO: World health organization

w/v:Weight/volume

Chapter 1

INTRODUCTION

Chapter 1

Introduction

Medicinal Plants

Medicinal plants are well recognized for their significant benefits in health care since ancient times and their various parts are used to treat several health alignments across the world (Jain *et al.,* 2006). Presence of various types of phytoconstitutents in plants helps to play a significant role in curing several types of disorders. It is well convinced that plant derived drugs have been in discovery with evolution of human healthcare for thousands of years (Jagatheeswari *et al*., 2013). Systematic observations on the use and efficacy of medicinal plants significantly contributed to the recognition of therapeutic properties. Therefore, they are frequently prescribed, even if their chemical constituents are not completely known. All over the globe, the use of medicinal plants has been significantly increasing for the benefit of primary health care (Maciel *et al.,* 2002). Plants continue to serve as possible sources of new drugs and various chemicals (Srivastav *et al.,* 2011). Medicinal plants are recognized as source of medicinal compounds and an impressive number of modern drugs have been isolated and tested. Many of these isolations are based on the use of the plants in traditional medicine. The ancient texts like Rig Veda (4500-1600 BC) and Atharva Veda mentioned the use of several plants as source of medicines. Several books on Ayurvedic medicine such as Charaka Samhita and Susruta Samhita explained the medicinal uses of more than 700 herbs (Jain, 1991). India has rich flora that is widely distributed throughout the country. Plant based drugs and their derivatives are commonly used and well familiar worldwide. As per the scientific literature survey, nearly 88 % of the global population turn to plant derived medicines as their first line of defense for maintaining health and combating diseases. Many people of Asia and India are utilizing plants as a part of their routine health management (Samy *et al.,* 2008). More than thousands of several plant species are recozonised to have

medicinal value. Indeed, metabolite profiling of medicinal plants disclosed the link between traditional and modern medicines. The use of traditional medicines and medicinal plants in most of the developing and developed countries as therapeutic agents for the maintenance of good health has been widely observed. The ongoing growing recognition of medicinal plants is due to several reasons, including escalating faith in herbal medicines (Kala *et al.*, 2006). The medicinal properties of plants is believed to be because of their antioxidant, antiinflammatory, antipyretic effects of the several types of phytochemicals present in them (Cowman, 1999; Adesokan *et al.*, 2008).

According to World Health Organization, medicinal plant can be designed as any type of plant, which in one or more of its organs contain substances that are either used for therapeutic purposes or as precursors for the synthesis of useful drugs. Therefore, such plants should be investigated for better understanding of their properties, safety and efficacy of use (Nascimento *et al.*, 2000). According to WHO reports, around eighty percent of the global population still relies on botanical drugs. Furthermore, WHO (2001) also defines herbal preparation as product produced by subjecting plant materials to extraction, purification and concentration or any other physical or chemical processes which may be used for immediate consumption or as a crude basis for herbal final products.

Different medicinal plants produce bioactive compounds which are more applicable for several medicinal purposes. These compounds or phytoconstitutents in plant material either act on different physiological systems of animals including human being by interfering with the metabolism in human body. The identification, isolation, purification and characterization of active ingredients of medicinal plant extracts using various analytical methods is highly mandatory to develop certain theurapitic drugs. In India, the ayurvedic system has described a large number of such phytomedicines. Determination of pharmacological activities can provide better information on their active principles and

mechanism of action (Jain *et al.,* 2006). India is well familiar for its traditional systems of medicine i.e Ayurvedic, Siddha and Unani system of medicine. Many rural house holders in India with limited access to organized health service, practice home remedies whose formulae have been handed down from generation to generation. In Indian system of medicine, generally the medicines of plant origin are preferred over the medicines of animal origin due to the presence of abudent natural flora. Indian medicinal plants are rich sources of substances that have several therapeutic properties like cardioprotective, chemoprotective and many other effects.

According to the World Health Organization, the burden of chronic diseases, including coronary heart disease (CHD), cancers, diabetes and obesity contributed to 59 % of the 56.5 million deaths reported worldwide in 2001, with CHD ranking number one as the main contributor to morbidity and mortality worldwide, there is a significant interest in identifying plants that have cardioprotectant, cardiotonic activities and also the phytochemicals responsible for those activities. Natural plant products posses antioxidant property, which alleviate oxidative stress, and thus can help in preventing many of the human diseases including cardiovascular diseases (CVD), Alzheimer's disease, Parkinson's disease, diabetes, cancer etc. Several herbal medicines have been advanced to clinical use in modern times (Huo *et al.,* 2012).

Medicinal plants and herbal formulations

The term "herbal drug" refers to the part/parts of a plant (leaves, flowers, seeds, roots, barks, stems, etc.used for preparing medicines. Furthermore, World Health Organization (2001) defines medicinal plant as herbal preparations produced by subjecting plant materials to extraction, fractionation, purification, concentration or other physical or biological processes which may be produced for immediate consumption or as a basis for herbal products. WHO encourages, recommends and promotes traditional/herbal remedies and several poly herbal

drugs in natural health care programmes because these drugs are easily available at low cost, safe and people have faith in them (Rasheed *et al.,* 2012). Standardization and preparation of herbal formulations or polyherbal drugs is highly essential in order to assess toxicity as well as their interaction with others drugs. Based on the concentration of their active principles, physical, chemical, phytochemical, properties, *in vitro* and *in vivo* studies have to be performed. The quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine. The subject of herbal drug standardization is of deep interest. As mentioned earlier, traditional system of medicine and their use in every aspect of human health has been playing a major role in meeting global health needs in several countries. For most herbal drugs, the specific ingredient that causes a therapeutic effect is not clearly known as they contain many ingredients. It is assumed that several of the ingredient synergistically produce the desired medicinal effect and shows their good efficacy. Several factors influence the components present in the herbal drugs including the environment in which a plant grew how and when the plants or its parts processed (Dwivedi and Daspaul, 2013). In addition to that, often times plant formulations are made of extracts of more than one plant used rather than single plant. Polyherbal drugs including Caps HT2, PHF-AROGH, Jwarhar mahakashay are the combination of different parts of the several medicinal plants. Herbal formulation approaches have regained their popularity, with some of their efficacy and safety aspects being supported by controlled clinical studies (Mali and Dhake, 2011). In India, Ayurveda herbal drugs need extensive scientific investigation and validation in terms of efficacy and safety (Patwardhan *et al.,* 2005). Herbal drugs are subjected to a series of scientific investigations and are critically being assessed of their pharmacological activities like antioxidant and antiinflammatory properties since oxidative stress and inflammation play major role in pathophysiology of several diseases.

Oxidative stress and Inflammation

Inflammation is the body's immediate response to cells and tissue damage, pathogens, chemicals, or physical injury (Haworth and Levy, 2007). Excessive inflammation may lead to tissue injury and can cause pathophysiology, organ dysfunction and death. The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response (Fig. 1.1).

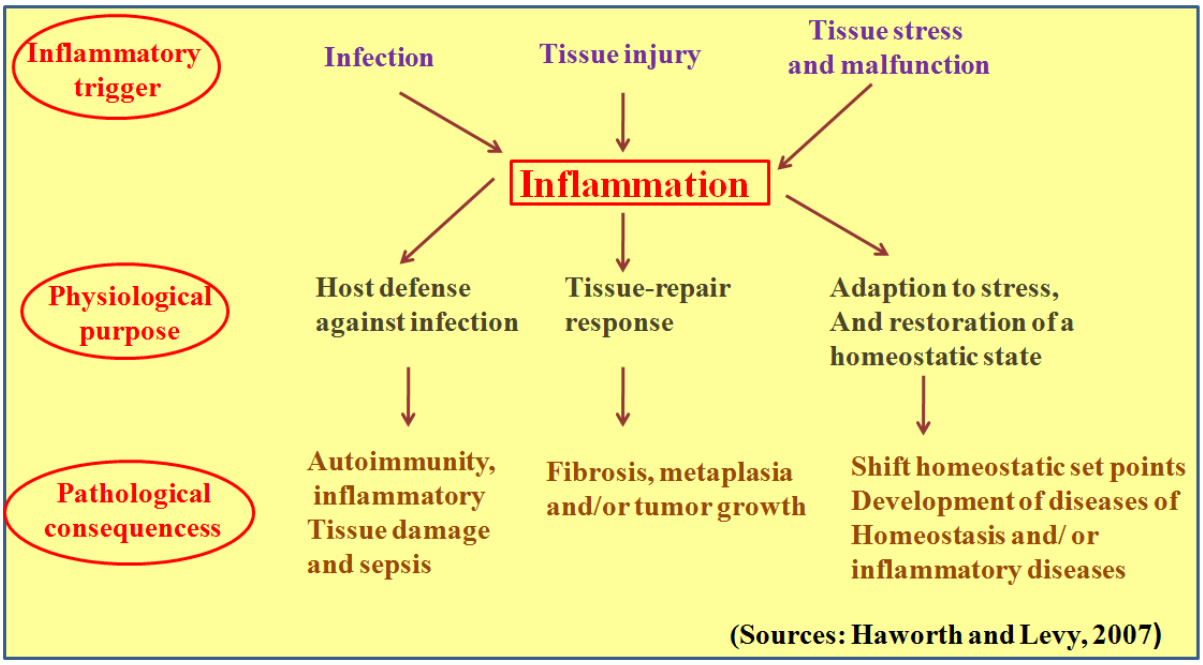


Fig. 1.1. Physiological and pathological outcomes of inflammation

Most important hallmarks of inflammation are oedema and leukocyte infiltration. Inflammation is characterized by redness and warmth at the site of injury. The white blood cells or leukcocytes take an important role in inflammation. The main feature of the inflammatory responses are vasodilation, i.e. widening of the blood vessels to increase the blood flow to the infected area; increased vascular permeability, which allows diffusible components to enter the site; other inflammatory responses include cellular infiltration by chemotaxis, or the directed movement of inflammatory cells through the walls of blood vessels to the site of injury. The degree to which these changes occur is normally proportional to the severity of the injury and the extent of infection. Based on timing and

pathological features, inflammation can be divided into two major categories-acute and chronic. Acute inflammation is a short-term response that usually results in healing: leukocytes infiltrate the damaged region, removing the stimulus and repairing the tissue. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Chronic inflammation, by contrast, is a prolonged, dysregulated and maladaptive response that involves active inflammation, tissue destruction and attempts of tissue repair. Such persistent inflammation is associated with many chronic human conditions and diseases, including allergy, atherosclerosis, cancer, arthritis and autoimmune diseases.

Cardiovascular diseases (CVD) and medicinal plants

As per the literature survey, cardiovascular diseases (CVD) are being considered as leading cause of mortality and disability. Developing countries like India are also struggling to manage the impact of CVD along with growing burden of obesity (Devasagayam *et al.,* 2004). One fifth of the deaths in India are beacuse coronary heart disease (CHD). According to health initiative forum, by the end of year 2020, it is assumed that one third of deaths will be due to CHD and India will have the largest CVD burden in the world. The most important clinical signature of CVD is an acute occlusion in the blood vessel due to blood clot formation by rupture of the lesion, it results in myocardial infarction (Lusis 2000). LDL oxidation and additional LDL atherogenic modifications determine the progression of atherogenesis (Stocker *et al.,* 1999). As per the scientific studies in symptomatic cardiovascular diseases, antioxidant levels are significantly lowered (Shanmugasundaram *et al.,* 1996). Hence, it is highly recommended to take antioxidants, especially lipid soluble and chain breaking antioxidants that accumulate in lipoproteins which are expected to have beneficial effects (Noguchi *et al.,* 2000). A large number of studies in experimental animals have shown that hypercholesterolemia and diabetes are the common risk factors for

development of atherosclerosis. They are also responsible for increased production of free radicals not only by endothelial cells but also by vascular smooth muscle cells. Medicinal plants are rich source of antioxidants and natural antioxidants are shown to attenuate the cascade of events as shown in Fig 1.2.

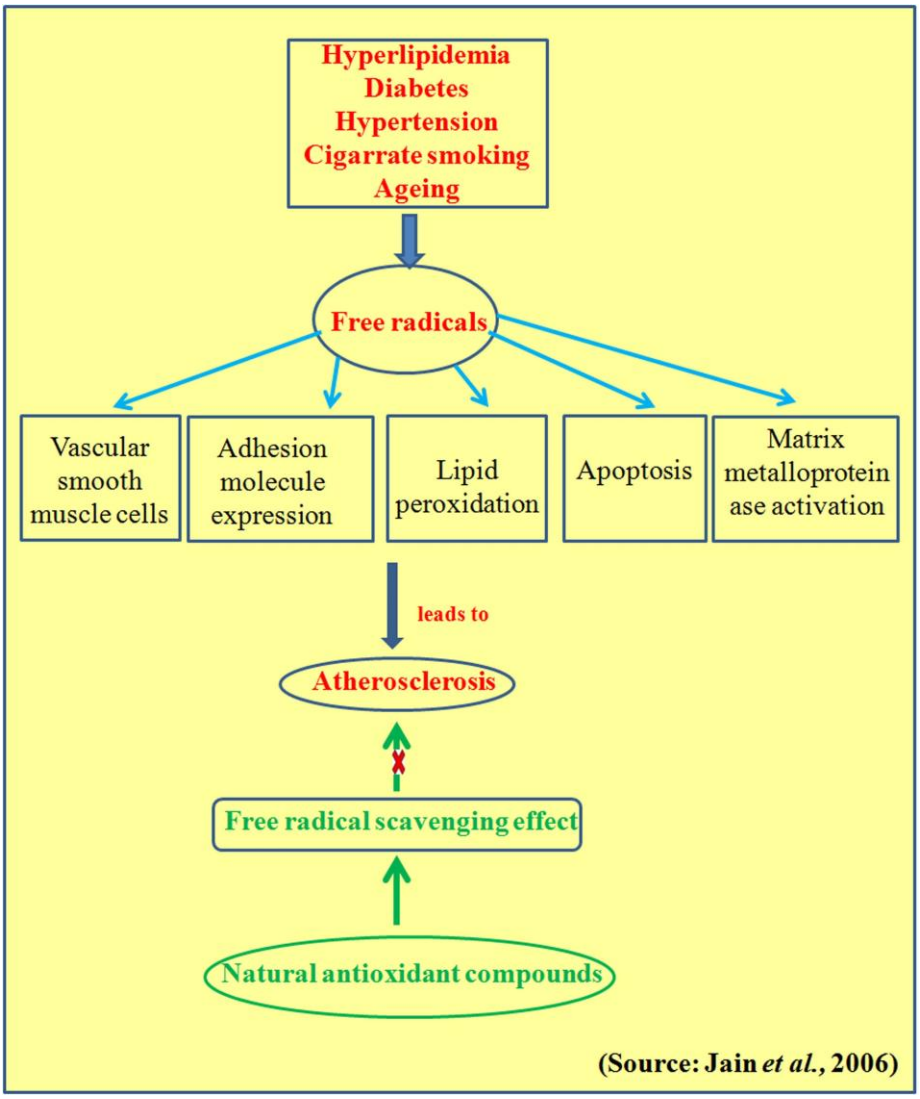


Fig. 1.2. Role of free radicals in cardiovascular diseases and its prevention by natural antioxidants.

A large number of scientific investigations proved that diets rich in antioxidants are associated with lower incidence of CVDs (Chopra *et al.*, 1996). In recent times, there is a lot of growing interest in phytoconstitutents from several medicnal and aromatic plants with several potential benefits.

Atherosclerosis

Atherosclerosis is considered as one of the most important type of cardiovascular diseases and it is also referred as chronic inflammatory blood vessel wall disease. It is characterized by the accumulation of lipids and fibrous elements in the vessel wall of large arteries. It is considered as a primary cause of heart disease and it develops due to oxidative stress, inflammation and thrombosis (Lusis, 2000). Apart from oxidative stress, several other conditions including hyperlipidemia, hyperglycemia, hyperuricemia and microbial infections are responsible for upregulation of several proinflammatory stimuli resulting in blood vessel wall permeability leading to the development of atherosclerotic lesions in the vessel wall (Libby, 2002).

Risk factors involved in development of atherosclerosis

Several risk factors like metabolic diseases, bacterial and viral infections play a pivotal role in the development of atherosclerosis. Epidemiologic studies supports that cigarette smoking is major health hazard as it contains free radicals that are associated with increasing oxidative stress and they set the stage for the cardiovascular dysfunction (Ambrose *et al.,* 2004). High fat diet fed in animal models showed increase in the level of LDL lipid profile marker serum which is considered as most important factor for development of atherosclerosis (Tomkin and Owens, 2012). Similarly it also clearly mentioned that, feeding of HFD causes upregulation of several proinflammatory markers like monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), E-selectin, vascular cell adhesion molecule 1 (VCAM-1) in blood and endothelial cells, which leads to the development of atherosclerostic lesions inside blood vessel wall (Shi *et al.,* 2005). Chronic hyperglycemia and insulin resistance in diabetes mellitus patients alters functions of smooth muscle cells, platelets render the arteries susceptible to atherosclerosis (Beckman *et al.,* 2002). It has been consistently reported that increasing degree of obesity is accompanied

by greater rates of cardiovascular diseases. Obesity is an independent risk factor for major coronary events although hypercholesterolemia and the metabolic syndrome are often associated with it. Obese subjects typically carry a proinflammatory state that may predispose them to acute coronary syndromes. This state is characterized by elevations of serum C reactive protein (CRP) that reflect high cytokine levels (Hubert *et al.,* 1983). Elevated circulating homocysteine (Hcy) level is also considered as important risk factor for occlusive disease in the coronary, cerebral, and peripheral vessels and predictive symtoms of survival in patients with stable coronary artery disease and finally it causes atherosclerosis (Ridker *et al.,* 2002). A part from that, infections due to bacteria or viruse can lead to the development of atherosclerosis. Pathogens, either as whole organism or through their structural components have potential to induce proatherogenic and prothrombotic response in the blood vessel leading to atherogenesis (Shah, 2001). Bacteria like *Chlamydia pneumonia, Helicobacter pylori* and viruses like human immunodeficiency virus, cytomegalovirus, Epistein-Barr virus, herpes simplex virus and measles are shown to play a role in the pathogenesis of atherosclerosis (Morre *et al.,* 2000).

Molecular aspects involved in atherosclerosis

As mentioned earlier, several factors like exposure to cigarette smoke, dust and pollution, feeding of high fat diet (HFD), hyperglycemia, hyperlipidemia are independent risk factors for atherosclerosis. These conditions activate the monocytes and platelets inside the blood. The activated blood cells release several proinflammatory cytokines and chemokines inside the blood plasma. Increased cytokine content in the blood plasma either directly or indirectly activates endothelial lining layer of the blood vessel wall. Further, activated endothelium expresses several cytokines, chemokines and cell adhesion molecules that directly leads to the adhesion of monocytes and platelets to the endothelium (Davis *et al.,* 2003). VCAM-1 expressed on endothelial cell surface plays a major role in monocyte recruitment, adherence

to endothelial cell monolayer and diapedesis between intact endothelial cells and penetration into subendothelial cell space. This type of leukocyte migration requires chemoattractant gradient, particularly MCP-1. Monocytes residing in the intima acquire characteristics of macrophages and express receptor that binds to lipoprotein particles, leading to foam cell formation which secrete proinflammatory cytokines (e.g., TNF-α and IL-6) and amplify local inflammatory responses and finally leading to fatty streak lesion/plaque formation in the blood vessel wall (Fig. 1. 3).

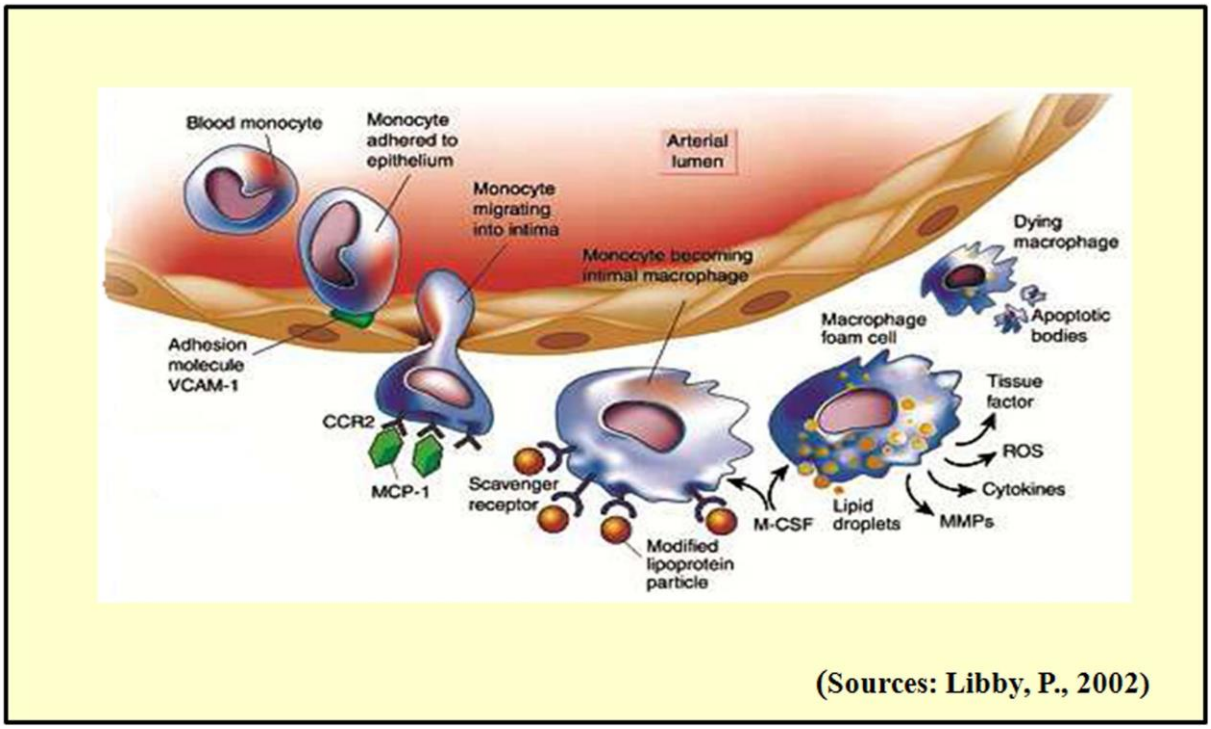


Fig. 1.3. Schematic representation of steps involved in inflammation and atherosclerosis development. Here blood monocytes get activated during the time of stress and releases several proinflammatory cytokines. Under normal circumstances, arterial endothelium resists prolonged contact with leukocytes and monocytes. Endothelial cells undergo inflammatory activation, they releases various leukocyte adhesion molecules. Further various chemokines and cytokines participate in the process of diapedeses leading to foam cell formation. Notably, these foam cells amplify local inflammatory lesions and reactive oxygen species by secreting proinflammatory cytokines finally playing a key role in thrombotic complications of atherosclerosis, hence producing atherosclerotic lesion.

Thus for maintaining normal cardiac health, antioxidants, statins and antiplatelet drug agents, TNF-α lowering drugs are recommended. Till date there are therapeutics developed which can manage many of the afore mentioned events, however, some of the drugs do have side effects. Therefore, screening and identification of medicinal plants having compounds which are antiinflammtory, antioxidant and antihyperlipidemic activity is highly important for the development of CVD therapeutics.

List of plant origin allopathic drugs used for treating CVD

‣Digitoxin and digoxin from *Digitalis purpurea:* It is one of the most effective and well known drug used for congestive heart failure, its over dosage is highly toxic.

‣Aspirin from *Salix alba*: remarkably fast and can help to survive during heart attack, over dosage can cause ulcers and stomach bleeding.

‣Statins from fungus, *Monascus purpurus*: It is a lipid lowering drug, saving millions of lives. However, side effects can be fatal including permanent damage to liver, muscles and nervous system.

The above mentioned allopathic drugs and several Ayurvedic formulations to heart diseases suggest that medicinal plants can be considered as best resource for developing new therapeutics to treating several inflammatory and cardiovascular diseases like atherosclerosis complexed diseases like CVD. On the basis of their traditional use in Ayurveda and different scientific studies, here we have chosen two plants namely OS/HI to check antioxidant and antiinflammatory properties.

Medicinal plants selected for the present study

The selection of the two medicinal plants for the present study is primarily based on their traditional use in Ayurveda for either treating cardiovascular or blood disorders and further on scientific reports for their safety in use. Leaves of *Ocimum sanctum* and roots of *Hemidesmus indicus* were selected for the present study and validated for their

antitherogenic effects as well as for their metabolite profile. *O. sanctum* is traditionally used for blood purifying purposes and treating various diseases of the blood and heart (Pattanayak *et al.,* 2010; Paranjpe, 2001). As per the scientific literature study in animal models, it clearly demonstrated that OS dry leaves possesses cardiotonic and cardioprotective effects (Sharma *et al.,* 2001). Simillarly, the dried roots of *H. indicus* are well known in Indian pharmacopoeia as the drug 'Anantmul', a common tonic prescribed for blood purification and other physiological disorders (Nagarajan *et al.,* 2001). *H. indicus* roots worked as a natural detoxifier and its cardiprotective effect in animal models was well studied (Zarei *et al.,* 2013).

Ocimum sanctum

Ocimum sanctum L. (OS) commonly called as Holy Basil/Tulsi is being used as wonderful herb for thousands of years in Ayurveda, traditional folklore and Sidha system of medicine (Pattanayak *et al.,* 2010). It is a popular home remedy for many ailments. OS also known as "the elixir of life" since it promotes longevity. The other name of OS called as Tulsi is derived from *'Sanskrit'*, which means "matchless one". The plant of genus *ocimum* belonging to family Lamiace are very important for their therapeutic potentials (Vishwabhan *et al.,* 2011). It is an important symbol of the Hindu religious tradition and considered as a Queen of herbs, the legendary 'Incomparable one' of India. It is one of the holiest and most cherished of the many healing and healthy giving herbs of the orient. In some aspects, it is also considered as a herbaceous sacred plant of India next to Kamal-the Lotus. It is renowned for its religious and spiritual sanctity especially in traditional system of medicine and made a lot of important contribution to the field of science from ancient times as also to modern research due to its large number of medicinal properties. The leaves of the plant are considered to be very holy and often form a consistent part of the Hindu spiritual rituals. Scientific reviews about this plants clearly mentioned that several medicinal properties are

attributed to the plant not only in Ayurveda and Siddha but also in Greek, Roman and Unani system of medicines and pharmacological systems of medicine (Mondal *et al.*, 2009). In most of the aspects, OS is used to cultivate for religious medicinal purposes and for its essential oil. The leaf extracts of this plant have been traditionally used for treating blood disorders and for cardioprotective effect (Joseph *et al.,* 2013; Kumar *et al.,* 2012).

Origin and distribution of *O. sanctum*

The plant grows widely in all dried parts and subtropical regions of India. It is found in Andhra Pradesh, Odisha, Madhya Pradesh and North Western part, as well as Andaman and Nicobar Islands to the Himalayas, up to 1800 meters above the sea level. A part from that it also abundantly found in other countries like Malaysia, Australia, West Africa and some of the Arab countries. This plant occupies a wide range of habitats.

Morphology

It is an erect, much branched, fragrant and erected plant attaining a height of about 30-60 cm when mature. Its aromatic leaves are simple, opposite, elliptic, oblong, obtuse or acute with entire or sub errate or dentate margins, growing up to 5 cm long.

Traditional uses

Tulsi is a popular home remedy for many ailments. In traditional systems of medicine, different parts like leaves, stem, flower, root, seeds and even whole plant of OS have been recommended for the treatment of bronchitis, bronchial asthma, malaria, diarrhea, dysentery, skin diseases, arthritis, chronic fever and insect bite etc. According to Ayurveda, especially juice of the leaves of OS plant is used as demulcent, stimulant and expectorant. An infusion of leaf is used as anti-spasmodic in gastric disorders of children. The leaves are good for nerves and to sharpen memory (Pattanaik *et al.,* 2010). Chewing of OS leaves also cures ulcers and infections of mouth (Kumar *et al.,* 2012). OS is good for heart, stimulates digestion, reduces breathing difficulties and cough (Nahak *et al.,* 2011). Os leaf powder is

also used for alleviating blood pressure (Ravi *et al.,* 2012). Thus, every part of the plant has very useful application in human health. Even today people use different parts of this plant for treatment of various ailments based on traditional knowledge.

Phytochemical constitutents

The chemical composition of OS leaves are highly complex because they contain many phytoconstitutents which have several biological activities. A part from that, as per the review of literature, the nutritional and pharmacological properties of the OS leaves in its natural form, as it has been traditionally used, result from synergistic interactions of many different active phytochemicals. The stem and leaves of holy basil contain a variety of constituents that may have biological activity, including saponins, flavonoids, triterpenoids, and tannins (Jaggi *et al.,* 2003). The leaves of OS contain eugenol, methyl eugenol, carvacrol , caryophyllene, linalool (Pattanayaka *et al.,* 2010) cirsilineol, circimaritin, isothymusin, apigenin and rosameric acid etc (Yanpallewar *et al.,* 2004). Flavonoids such as orientin and vicenin from aqueous leaf extract of OS have been isolated (Gupta *et al.,* 2002). Ursolic acid, apigenin, luteolin, apigenin-7-O-glucuronide, luteolin-7-O glucuronide, orientin and molludistin have also been isolated from the leaf extract. OS also contains a number of terpenes viz., bornyl acetate, β-elemene, neral, α and β-pinenes, camphene, campesterol, stigmasterol and β-sitosterol (Nair *et al.,* 1982).

Pharmacological activities of OS

Across the world, all over the world scientific research has expanded to evaluate and study the pharmacological activities, side effects and medicinal uses of OS leaves against different diseases especially inflammatory disorders. Leaf extracts of this plant have been used traditionally for its cardioprotective purposes (Joseph *et al.,* 2013; Kumar *et al.,* 2012; Pandey *et al.,* 2010; Pattanayak *et al.,* 2010; Rashid *et al.,* 2013). Recent scientific investigations have confirmed that OS possesses wide range of pharmacological benefits

such as hypolipidemic (Hussain *et al.*, 2001), hepatoprotective (Akilavalli *et al.,* 2011) and neuroprotective (Yanpallewar *et al.,* 2004) properties. Its anticoagulant (Khan *et al.,* 2011), antioxidant, antiinflammatory (Kath *et al.,* 2006; Kalabharathi *et al.,* 2011) and immunomodulatory (Mediratta *et al.,* 2002) effects have been reported using animal models. All these pharmacological activities of OS are attributed to its presence of active metabolites. A part from that, certain polyherbal drugs or herbal products of *O. sanctum* namely (e.g.Tulsi by Kirpal Export Overseases, India; Dry herbal extracts by Nikita Extracts, India; *Ocimum sanctum*-Beadlet by Borion Canada Inc., Canada) are available commercially in the market and are known majorly for their cardiac health benefits.

Hemidesmus indicus

Hemidesmus indicus (L) R.Br. (HI) belongs to family Asclepiadaceae commonly named as Indian Sarsaparilla. It has been used as folk medicine and considered as an ingredient in Ayurvedic and Unani systems of medicine against the diseases of inflammation and other blood related diseases (Lakshman *et al.* 2006). As per literature study, it is also clearly mentioned that HI is widely used in Indian system of medicine and considered as official drug in Indian Pharmacoepia (Austin, 2008). The dried roots of these plants are well known as the drug ‘Anantmul’, a common tonic prescribed for blood purification and other physiological disorders (Nagarajan *et al*., 2001). It is perennial climbing plant, used as the main ingredient in the preparation of the cool and refreshing drink called as sherbet. It is a treasure of tribal & forest wealth. HI has been ethnopharmacologically used as a therapeutic agent for a variety of diseases, as reviewed in several articles. Moreover, numerous research works have proven its uses beyond the ethnomedicinal uses in experimental animals.

Distribution of *Hemidesmus indicus*

It is growing in mesophytic to semi dry condition. Generally it founds in foothills of most mountainous terrains and upper gangetic plains and eastwards of Bengal central to south

India regions and some costal districts of Orissa (shanti *et al.*, 2010). It also found in south tropical Asian countries such as Pakistan and Sri Lanka, Bhutan and other countries (Shete and Bodhankar, 2010).

Morphology

It is a perennial climbing, slender, laticiferous, twinging, sometimes prostate or semi erect shrub (Austin 2008). Root part of this plant is woody, cylindrical in shape, irregularly bent, curved or slightly twisted. The diameter of the roots is 0.5–1.5 cm, brownish or purple in colour, aromatic odour, sweet in taste.

Traditional use of HI roots

The roots *H. indicus* have been used traditionally for curing various ailments like stomach pains, fever and veneral disease. One of the most important applications of this root part is used for blood purification (Shanthi *et al.*, 2010). The roots of this plant are generally used for preparation of soft drinks. The root extracts have been found to exhibit various pharmacological properties (Aneja *et al.*, 2008; Austin, 2008; George *et al.*, 2008).

Phytoconstitutents

Several phytochemical analyses have been carried out on HI plant. Mukhaerjee and Ray (1980) reported that roots of HI contains steroid, terpenoid, flavonoid and saponin. The presence of α-amyrin, triterpene and benzaldehyde in the root of HI was reported by Gupta (1981). From the roots of HI, hemidesmol, resin, glucoside, tannin, lupeol, α and β-amrin, β-sitosterol, lupeol acetate, coumarinolignoid like hesmidesmine, hemidesmin-1 and hemidesmin-2 were isolated (Mandal *et al.*, 1991, Das *et al.*, 1992). Alam *et al.*, (1994) reported the presence of benzoic acid and 2-hydroxy-4-methoxy benzenoid in the roots.

Pharmacological activities of HI roots

As mentioned earlier, **t**he root part of this plant have several protective effect in Indian traditional medicine. So far several scientific studies using animal model have been reported

on pharmacological benefits of *H. indicus* root, such as cardioprotective (Zarei *et al*. 2013), hypoglycemic (Murshed *et al.,* 2005) antiinflammatory (Shaikh, 2011), antioxidant (Saravanan *et al.,* 2007) and anti-viper venom (Alam and Gomes, 1998). ADP induced antiplatelet aggregation activity of HI roots also shown in human blood platelets (Mary *et al.*, 2003).

Chapter 2

OBJECTIVES AND STUDY DESIGN

Chapter 2

Objectives and study design

Objectives of the present study

1. Phytochemical profiles and antioxidant activities (*in vitro)* of alcoholic and water extracts of *O. sanctum* (OS) dry leaves and *H. indicus* (HI) roots.

2. Evaluation of antiinflammatory and/or antioxidant properties of OS *and* HI extracts in human monocytic (THP-1) cells.

3. Identification of active metabolite (s) from OS extracts responsible for it antiinflammatory properties in THP-1 cells.

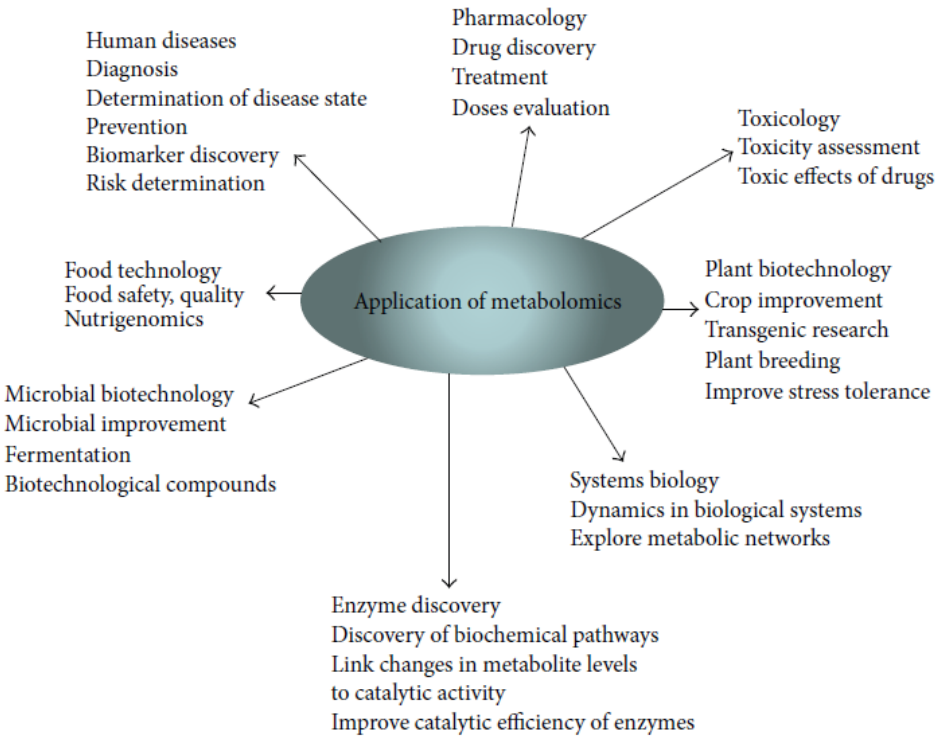
4. Evaluation of antilipidperoxidation and antihyperlipidemic effect of HI roots and pure compound, eugenol of OS in male Wistar rats (*in vivo* study).

Study Design

Objective 1: Phytochemical profiles and antioxidant activities (*in vitro)* of alcoholic and water extracts of *O. sanctum* (OS) dry leaves and *H. indicus* (HI) roots.

LC-MS based metabolite profiling of medicinal plant extracts

The term metabolomics reflects and provides accurate platform in identifying and holistic qualitative analysis of intracellular and extracellular metabolites present in cells and tissues. Plant metabolites are structurally diverse in nature and forming a highly complex spectrum of compounds of different size, solubility, volatility, polarity, quantity and stability (Gachon *et al.,* 2005; Yazaki *et al.,* 2009). Application of metabolomics technology to medicinal plants is advantageous as it permits the collection of information on various metabolites that have importance in pharmacological culinary, cosmetic areas (Okada *et al.,* 2010; Casati *et al.,* 2013) and other aspects also as shown in Fig. 2.1



(Source: Casati *et al.*, 2013)

Fig. 2.1. Application of metabolomics in different fields

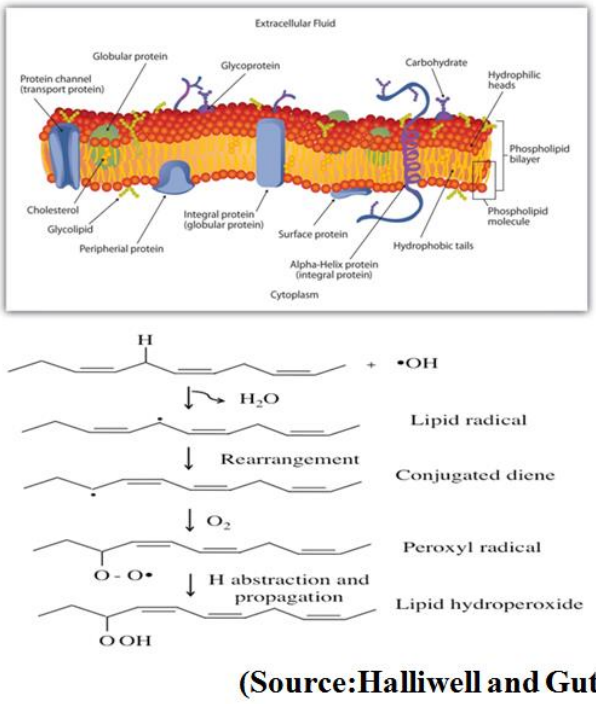
In Plant Biology field, several advanced and analytical technologies were discovered to analyze and identify the types of compounds present in plant extracts. Among them, liquid chromatography (LC) combined with mass spectrometry (MS) is one of the most widely used and well developed analytical techniques in plant metabolomics. Mass spectrometry (MS) is undergoing rapid development and extended use of its application for biological specimens, particularly of low molecular weight compounds. LC-MS methods required sample preparation applications were developed for plant flavonoid, phenolics and drug metabolism studies. The electrospray ionization (ESI) is a well developed, highly recommended and soft ionization technique extensively used for production of gas phase ions (without fragmentation) of thermally labile large

supramolecules. ESI assists the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis by utilizing electrical energy. Further the ionic species in solution can thus be analysed by ESI-MS on the bais of their increased sensitivity. Several databases such as Mass Bank, Metlin has been used for further confirmation of types and nature of metabolites present in the plant extracts. Identification and determination of accurate mass and their fragementation pattern obtained by MS/MS combined effects of electrospray ionization time-of-flight mass spectrometry playing a major role in identification of phenolic compounds, flavonoids and other secondary metabolites from the extracts. Mobile phase is the most important parameter in LC-MS method. Type of mobile phase used may have a big effect on the separation, retention and detection of different types of biomolecules. It can promote or suppress ionization of the analyte molecules. Proper selection of the mobile phase is the second most important step in the development of the separation method and identification of compounds. Solvents like water, acetonitrile, methanol, ethanol, chloroform, ammonium formate, formic acid and acetonotrile are used in reverse and normal phase LC are compatible with ESI.

Free radicals and lipid peroxidation

Generation of free radicals like H_2O_2, hydroxyl and superoxide radicals during the time of oxidative stress leads to the onset of several inflammatory disorder including cardiovascular diseases. Lipid peroxidation is considered as the major molecular mechanism involved in the oxidative damage to cell structures and functions. Thus it causes the pathogenesis of many diseases. Lipid peroxidation is a very complex process occurs in both plants and animal tissues. Generally, it involves the formation and propagation of lipid radicals upon reaction of unsaturated lipids with oxygen radical resulting in production of a variety of breakdown products, including alcohols, ketones,

alkanes, aldehydes and ethers (Dianzani & Barrera, 2008). Sphingolipids, sterol lipids and saccharolipids (Fahy *et al.,* 2005) are vulnerable to oxidation, yielding lipid hydroperoxides (Dixon *et al.,* 1998) as shown in Fig. 2.2.



(Source:Halliwell and Gutteridge, 1984)

Fig. 2.2. The membrane enclosing a typical animal cell is a phospholipid bilayer with embedded cholesterol and protein molecules.The lipid peroxidation process may be divided into the initiation, propagation and termination steps; only the double bond containing segment of a fatty acid is shown.

Human monocytic (THP-1) cells

THP-1 cells have become one of most widely used cell lines to understand the physiology of monocytes, then differentiated macrophages and their function in the cardiovascular system. The interaction between THP-1 cells with various vascular cells such as endothelial cells enables Scientists to investigate complex mechanism of communications between the cell types during vascular inflammation, particularly atherogenesis. THP-1 cells produces several proinflammatory cytokines, chemokines similar to peripheral blood monocytes, in response to LPS stimulation. Therefore THP-1 cells are (fig 2.3) considered

as good model system to study antiinflammatory properties of medicinal plant extracts and their pure compounds *(in vitro).*

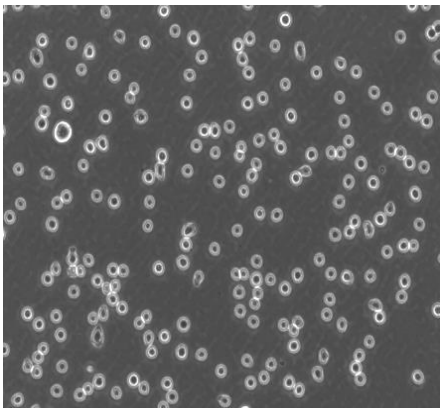


Fig. 2.3. Human monocytic THP-1cells, Photographs were taken under 10X magnification

In the present work, we have used THP-1 cells as experimental model to check the antioxidant and antiinflammatory properties of selected medicinal plant extracts and their pure compounds.

Arachidonic acid induced oxidative stress

Reactive oxygen species such as superoxide radical anion (·O2), singlet oxygen (^{1}O2), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) are produced as by-products of oxidative metabolism (Kourie, 1998). ROS production is associated with inflammation and leads to the cellular dysfunction. Human monocytic (THP-1) cells and macrophages are one of the main sources of ROS production in the human body (Stachowska *et al.*, 2005). 2,7-dichlorodihydrofluorescin diacetate (H_2DCFDA), a cell permeable ROS sensitive dye is widely used to determine intracellular ROS in various cell lines.

Arachidonic acid induced intracellular ROS generation has been considered as a well estabilished experimental system to test efficacy of antioxidants. Increased production of free arachidonic acid inside the cells occurs during the time of inflammation that leads to the activation of phospholipases (Katsuki and Okuda, 1995). Arachidonic acid acts as a

substrate for certain inflammatory enzymes like cycloxygenases and lipoxygenases. Metabolism of arachidonic acid through these two enzyme systems has been considered as a most important source for generation of free radicals and peroxides that leads to the oxidative damage.

Objective 2: Evaluation of antiinflammatory and/or antioxidant properties of OS *and* HI extracts in human monocytic (THP-1) cells.

Objective 3: Identification of active metabolite (s) from OS extracts responsible for its antiinflammatory properties in THP-1 cells.

Inflammatory markers and monocytes

Monocytes and macrophages play a central role in signal transduction and pathophysiology of several diseases of inflammation. These cell lines are involved in inflammatory processes, with a profound capacity to synthesize and secrete different proinflammatory cytokines and chemokines like IL-1β, IL-6, IL-8, TNF-α, MIP1α and MCP-1 (Cipollone *et al.,* 2003). Lipopolysacharide (LPS) a bacterial endotoxin plays a pivotal role in the initiation of variety of host responses and such action leads to systemic inflammatory response.

Tumor necrosis factor- alpha (TNF-α)

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine produced by various cell types. It is one of the earliest major proinflammatory mediators secreted by monocytes and macrophages when stimulated with LPS *in vivo* and *in vitro* (Nussler *et al.,* 1993). TNF-α has been implicated in the pathogenesis of several inflammatory diseases and its production has been suggested as a possible target for therapy to treat those diseases (Takashiba *et al.,* 1999). TNF-α has become a major target of numerous pharmaceutical investigations. It is a potent agonist in the activation of endothelial cells also. When cells are stimulated with appropriate agonists like LPS, mRNA level of TNF-α get induced and

a membrane-bound precursor protein with a relative molecular mass of 26 K is produced. In the present work, selected medicinal plants and some of their pure compounds have been tested if they can attenuate TNF-α production by LPS induced THP-1 cells, which could be considered as therapeutic as they can there by regulate downstream inflammatory responses.

Interleukin-6 (IL-6)

IL-6 is one of the most important and prominent proinflammatory marker secreted by monocytic and macrophage cells and has been identified as a local and circulating marker of coronary plaque inflammation (Ikeda *et al.*, 2001). IL-6 is considered as a pleiotropic cytokine and its role in the modulation of inflammation-related processes, particularly cytokine responses and tissue inflammatory cell infiltration are well studied.

Chemokines and atherosclerosis

The chemokine superfamily is generally composed of small molecular weight (8-11 kD) protein molecules. Chemokines secreted by monocytes can activate endothelial cells and thus play a major role in triggering the development of atherosclerosis. Chemokines are mainly responsible for cellular trafficking, vessel wall remodelling and atherothrombotic complications (Deshmane *et al.,* 2009). Latest scientific studies in animal models clearly suggest that blocking chemokine/chemokine receptor interactions may serve as a suitable approach to treat atherosclerosis (Braunersreuther *et al.,* 2007). Similarly, chemokine antagonists which inhibit leukocyte recruitment also could be interestingly studied to treat inflammation in response to myocardial infarction, which is considered as the major consequence of atherosclerosis. In the context of atherosclerosis, chemokines and chemokine receptors may coordinate communication between inflammatory cellular components of the peripheral blood and cellular components of the arterial wall, thereby

regulating leukocyte influx, capture, efflux, activation, and gene regulation as well as proliferation and/or apoptosis of resident cells in the plaque.

Macrophage inflammatory protein 1 alpha (MIP-1α)

Macrophage inflammatory protein 1 alpha (MIP-1 alpha), a low molecular weight protein is a well known interesting chemokine among the other chemokines, it exhibits a variety of proinflammatory activities including leukocyte chemotaxis. MIP-1α expression can be induced in a variety of cell types, including monocytes, macrophage cell lines, mast cell lines and T lymphocytes etc. MIP-1α expression is induced by LPS in macrophages and monocytes (Lukacs *et al.*, 1994). MIP-1α also induces ICAM-1 expression, mast cell degranulation, and production of other inflammatory markers like TNF-α, IL-1 and IL-6 (Cook, 1996). This wide variety of activities suggests that MIP-1α has an important role in inflammation.

Monocyte Chemoattractant Protein-1 (MCP-1)

The monocyte chemoattractant protein-1 (MCP-1) belongs to C-C chemokine family and it is considered as a potent chemotactic factor for monocytes. MCP-1 is secreted by a variety of cell types, either constitutively or after induction by oxidative stress (Deshmane *et al.*, 2009). It is produced by many cell types, including monocytic, endothelial, fibroblasts, epithelial, smooth muscle, mesangial, astrocytic and microglial cells (Cushing *et al.*, 1990; Standiford *et al.*, 1991). Literatures clearly indicates that monocyte/macrophages are found to be the major sources of MCP-1 production (Yoshimura *et al.*,1989). MCP-1 regulates the migration and infiltration of monocytes and natural killer (NK) cells. Among the other chemokines, MCP-1 is well studied and has been proved that its highly responsible for atherosclerosis (Kusano *et al.*, 2004) in animal models. Several studies have confirmed that MCP-1 is either directly or indirectly linked to cardiovascular disease.

In the present study, selected plant extracts and their pure compounds are tested for their effect in THP-1 cells on such cytokine and chemokine production as shown in Fig 2.4.

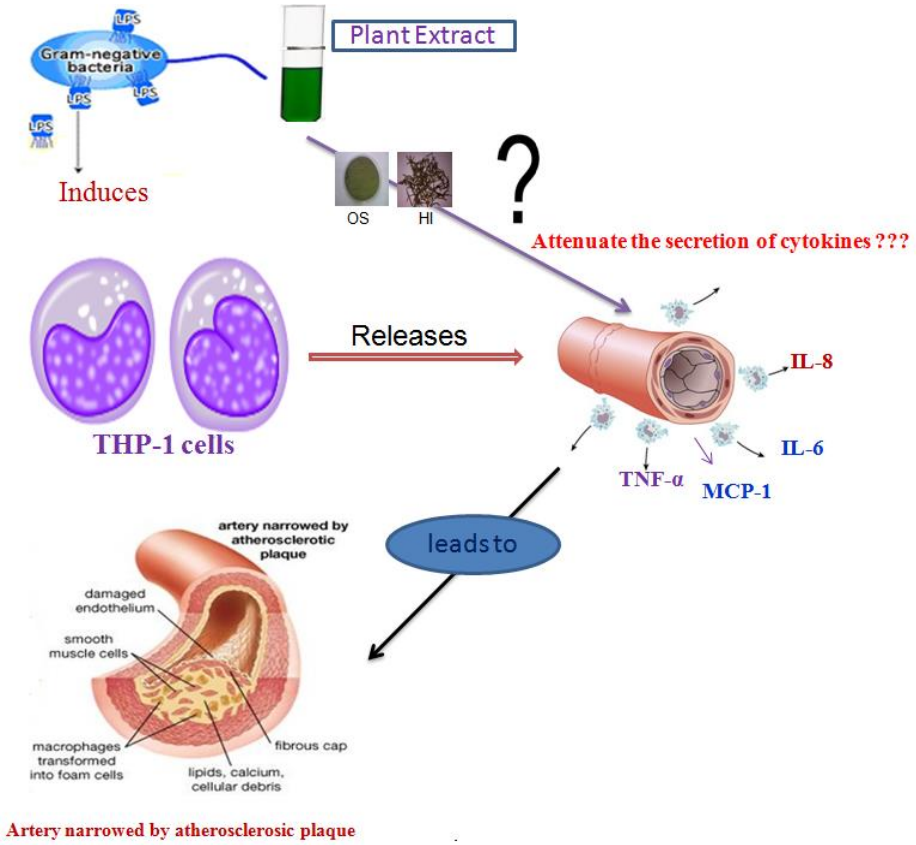


Fig. 2.4. Schematic diagram showing human monocytic THP-1 cells releases cytokines and certain chemokines that causes the inflammation in blood vessel and leads to the atherosclerosis. Pretreatment with selected plant extracts and their pure compounds attenuate the secretion of proinflammatory markers from THP-1 cells in the presence of LPS.

PMA induced monocytes to macrophage differentiation

Phorbol 12-myristate 13-acetate (PMA) is a phorbol ester that is commonly used to activate certain type of protein kinase C (PKC) by binding to C1 domain of protein kinase C and to induce membrane translocation and enzyme activation. It also activates MAP kinase pathways and promotes hematopoitic differentiation (Seger and Krebs, 1995; Clemens *et al.*, 1992). Prolonged treatment of cells with PMA at high concentrations

results in the downregulation of total PKC activity and is tumorigenic, whereas lower concentrations may be protective (Matthies *et al.,* 1987). PMA is the most commonly used phorbol ester. Monocytes as well as tissue macrophages provide immediate defence against foreign agents and assist during the setting off and development of the adaptive immune response. However, chronic inflammation due to activation of monocytes and macrophages also leads to several diseases including CVD. Inflammation due to tissue damage or infection results in resident macrophage activation, which increases the production of cytokines, chemokines, and other inflammatory mediators, as well as monocyte recruitment. Literature also clearly demonstrated that both monocytes and macrophages mediate immune and inflammation processes via the production and release of a variety of proinflammatory cytokines and chemokines (Schwende *et al.,* 1996). These biologically active agents are also known to modulate cell differentiation and proliferation. The human monocytic THP-l cells can be induced to differentiate along the monocytic pathway by treatment with phorbol esters by activating protein kinase C (Nishizuka, 1992). Differentiated macrophages are long-lived cells and perform specialized functions and are also highly atherogenic. To address this, THP-1 cells differentiation protocols have been developed using stimuli such as phorbol-12-myristate-13-acetate (PMA). Studies clearly demonstrates that, PMA induced differentiation mimics physiological differentiation including the expression or activation of several markers like CD14, TLR2 and TLR4 on the surface of macrophage cell line (Takashiba *et al.,* 1999).

Cluster of differentiation 14 (CD 14)

CD14 is considered as one of the most important LPS receptor (Wright *et al.,* 1990) and is anchored to the monocyte/macrophage cell membrane via glycosylphosphatidyl inositol (Ebong *et al.,* 2001). Several studies have clearly demonstrated that, cells bearing the CD14 receptor bind LPS in the presence of LPS-binding protein and are activated to

produce proinflammatory cytokines and chemokines and a variety of other molecules active in the innate immune response (Schumann *et al.,* 1990). It also regulates monocyte-lymphocyte interactions. Recently, it has confirmed that signal transduction via membrane CD14 requires a signaling complex which appears to include, the expression of toll like receptors like TLR 2 and TLR 4 (Chow *et al.,* 1999, Hoshino *et al.*, 1999). A part from that, it is shown that monocytic cells treated with PMA increases the expression of CD14 in their cell surface (Park *et al.,* 2007).

Toll like receptors 2 and 4 (TLR2 and TLR 4)

During the last few decades, there have been significant advances being observed in understanding of the biology of TLRs. Toll-like receptors (TLRs) of mammalian innate immune defense systems play a crucial role in the initiation of adaptive immune responses. TLRs are evolutionarily conserved receptor complexes in the pathogen-recognition process (Imler and Hoffmann, 2001). A critical and most important feature of macrophages for specific recognition and response includes the expression of optimum levels of different TLRs (West *et al.,* 2011, Zeromski *et al.,* 2008). Up on the interaction with specific pathogen-associated molecular patterns (PAMPs), TLRs trigger the signal transduction cascades that lead to cellular responses including induction of inflammatory cytokines and generation of ROS. During infection, binding of LPS to extracellular domain of TLR4 receptors on macrophages elicits activation of the TLR4/IRAK-2/NF-κB complex and release of several proinflammatory cytokines (Wan *et al.,* 2009). Deregulation of TLR expression and function causes defective immune responses (Leendertse *et al.,* 2008, Alexopoulou *et al.,* 2002) and the occurrence of chronic diseases (Sharif *et al.,* 2009).

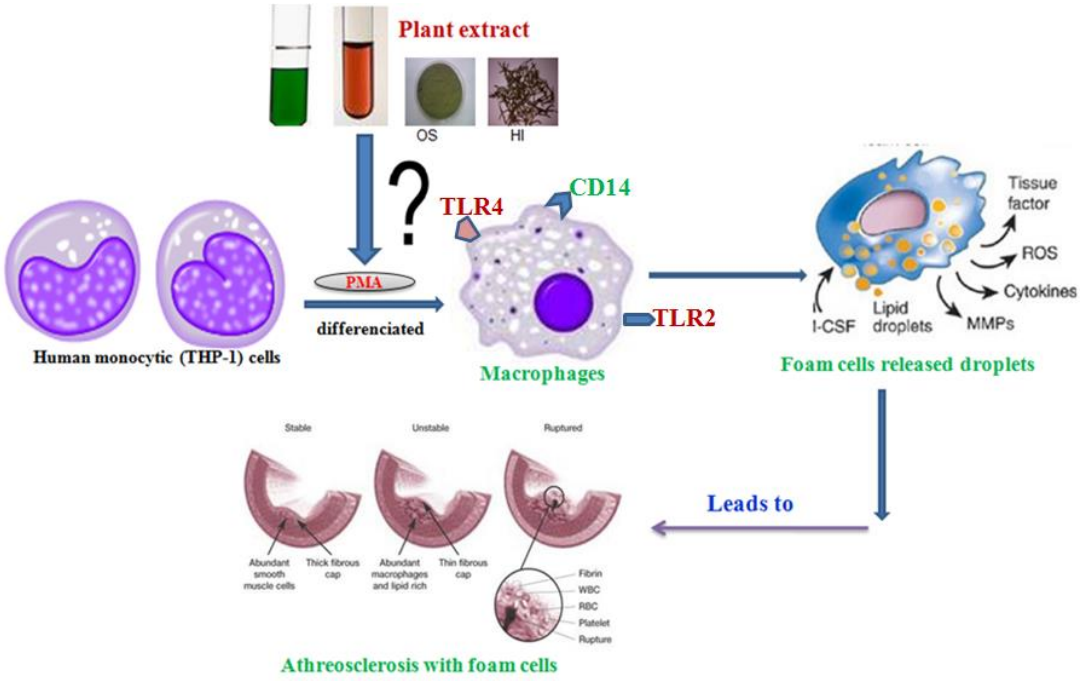


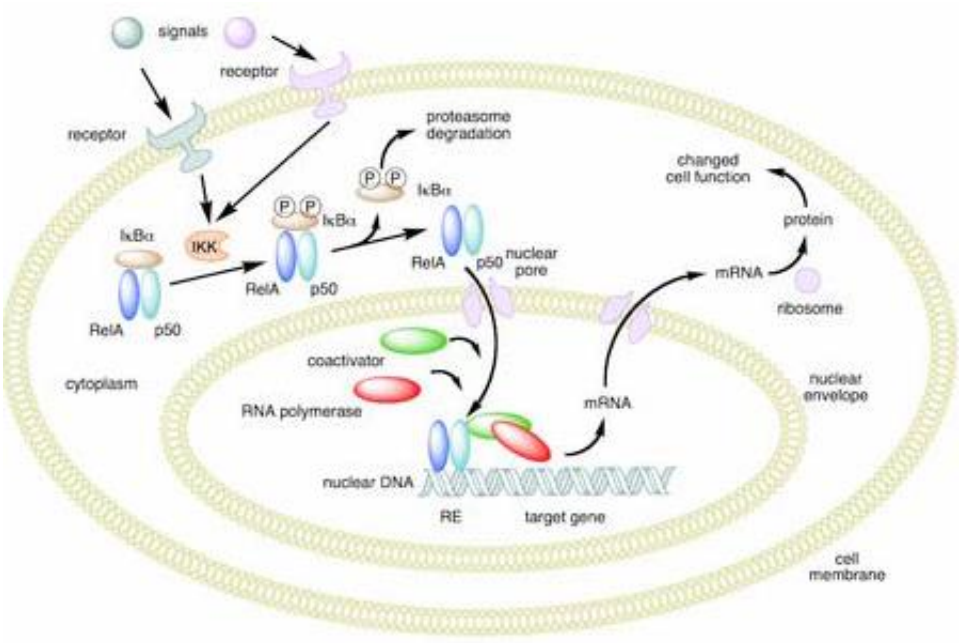
Fig. 2.5. Schematic diagram showing differentiation of human monocytic THP-1 cells into macrophages which express macrophage differentiated markers like CD14, TLR2/TLR4 in the presence of PMA and further differentiated to foam cell. Effect of the plant extracts and their pure compound are tested for their inhibitory effect on PMA mediated differentiation of monocytes to macrophages.

If we assume, inflammation is the hallmark of the disease, then studying the role of TLRs (Toll-like receptors) in atherosclerosis is reasonable and very important. According to reports, human monocytic (THP-1) cells are considered as well established model to study macrophage differentiation. The expression of TLR2 and TLR4 induced by PMA is well estabilished. In the present work we have evaluated the effect of selected medicinal plant extracts and their pure compounds on expression patterns of CD14, TLR2 and TLR4 in PMA induced THP-1 cells as shown in Fig. 2.5.

Phosphorylation and translocation of NF-κB

In any organism, cellular responses to infection with bacteria or viruses or stress requires rapid and accurate transmission of signals from cell-surface receptors to the nucleus. These signalling pathways totally depend on activation of specific transcription factors that stimulate or induce the expression of appropriate target genes inside the nucleus. Among

them Nuclear factor-kB (NF-kB) transcription factor is most common. NF-κB family proteins regulate genes of inflammation, immunity and cell proliferation. In normal physiological condition, NF-kB exists in the cytoplasm and requires a signalling pathway for activation. Such NF-κB activating pathways are triggered by a variety of extracellular stimuli such bacterial lipopolysaccharide, oxidized LDL or PMA that lead to the phosphorylation and subsequent proteasome-mediated degradation of inhibitory molecules (Karin *et al.*, 2000).



(Source: Prajapati *et al.*, 2010)

Fig. 2.6. A model for mechanism of NF-κB activation**.** Various stimuli and signals induce phosphorylation and subsequent polyubiquitination of IκBs, which are targeted for degradation and associated NF-κB dimmers are thereby released to translocate into the nucleus, where they modulate their expression of genes.

Activated NF-κB migrates into the nucleus and regulates the expression of multiple target genes as shown in Fig. 2.6. The NF-κB–IκB complex is mainly cytoplasmic in resting cells. Optimal induction of NF-κB target genes generally requires phosphorylation of NF-κB proteins, such as p65, within their transactivation domain by a variety of kinases (Viatour *et al.*, 2005). In mammals, the NF-κB family is composed of five related transcription factors: p50, p52, RelA (p65), c-Rel and RelB.

Objective 4: Evaluation of antilipidperoxidation and antihyperlipidemic effect of HI roots and pure compound eugenol of OS in male Wistar rats. (*in vivo* study)

Hyperlipidemia and atherosclerosis

The term hyperlipidemia clearly indicates that, it is a condition when abnormally high levels of lipoproteins are observed in the blood. This condition is also named as hyperlipidemia (Amit *et al.,* 2011). Hyperlipidemia has been ranked as one of the greatest risk factors contributing to prevalence and severity of coronary heart diseases, which is considered as primary cause of death. The elevation of serum total cholesterol and low density lipoprotein (LDL) cholesterol has been reported as a primary risk factor for cardiovascular disease. It has been proved that elevated plasma levels of cholesterol and of LDL are associated with atherosclerosis (Grundy and Vega, 1988).

Total cholesterol (TC)

Cholesterol circulates in the blood stream and is involved in structural function of the cells. Total cholesterol concentrations below 200 mg/dL have been considered as desirable, whereas, concentrations greater than 240 mg/dL are referred to as hyperlipidemic in humans (Salam *et al.,* 2013).

Triglycerides (TG)

Triglycerdies (TG) are esters consisting of a glycerol molecule coupled to three fatty acid residues of varying carbon chain (Kanakavalli *et al.,* 2014). TGs containing both saturated and unsaturated fatty acids are important components of cell membranes (Aminoff, 2004). Triglycerides are atherogenic because they are rich in apo C-III, which delays the lipolysis of VLDL and inhibits its uptake and clearance from plasma. In human body, concentration of triglyceride less than 150 mg/dL is reffered as normal, whereas, concentrations of > 200 mg/dL are considered as atherogenic and carry high risk for the development and progression of various CVDs (Ginsberg *et al.,* 2001). Some other scientific studies also

comment that, TGs, as far as is currently known, are not directly atherogenic in nature but represent an important biomarker of CVD.

LDL cholesterol

LDL-cholesterol is one of the most important lipid markers in serum and commonly known as the bad cholesterol. It produced by liver and it carries cholesterol and other lipids from the liver to different areas of the body. The LDL-receptor pathway is the major mechanism for mammalian cells to take in cholesterol. The high levels of LDL in blood indicate much more cholesterol in the blood stream than necessary and hence it is indicated as risk factor of heart disease. According to literature study, LDL cholesterol above >120 mg/dL may cause different types of chronic inflammatory diseases (Salam *et al.*, 2013).

HDL cholesterol

HDL is has been considered as good cholesterol, which is produced by the liver to carry cholesterol and other lipids from tissues back to the liver for degradation (Ridker *et al.*, 2010). High levels of HDL cholesterol (> 40 mg/dL) have been considered as an indicator of good health. In human body, when the level of LDL is high, they accumulate in the artery wall where they are oxidized and are taken up by foam cells in a process that leads to the development and progression of atherosclerosis, but HDL oppose atherosclerosis directly by removing cholesterol from foam cells, by inhibiting the oxidation of LDLs, and by limiting the inflammatory processes that causes the atherosclerosis (Barter, 2005). Lower levels of HDL-cholesterol were significantly and independently associated with an increased risk of coronary death. The concentrations of 40 mg/dL or higher have been considered as normal. Whereas, HDL concentrations below 40 mg/dL is considered as major risk factor for CVDs.

Role of herbal medicine in hyperlipidemia

Different medicinal plants and their herbal formulation play a major role in hypolipidemic activity. The advantages of using herbal medicines reported so far are effectiveness, safety, affordability and acceptability. Currently commercially available different hypolipidemic drugs are also associated with certain side effects. As per the scientific literature study, several medicinal plants have been used to confirm their antihyperlipidemic properties in animal model.

Causes of hyperlipidemia

The main cause of hyperlipidemia in developing and developed countries includes basically changes in lifestyle including taking high fat diet and other lifestyle factor like over body weight, smoking, lack of exercise or leading sedimentary life style. Heredity has also been a modifying factor for the progression of hyperlipidemia as it has been noted that the genes partly determine the amount of cholesterol body makes (Grauvogel *et al.*, 2010).

In this present work root extracts of *H. indicus* roots and a pure compound eugenol (EUG), a phenyl propanoid were tested for their antihyperlipidemic effect in high fat died (HFD) fed hyperlipidemic male Wistar rats as shown in Fig. 2.7.

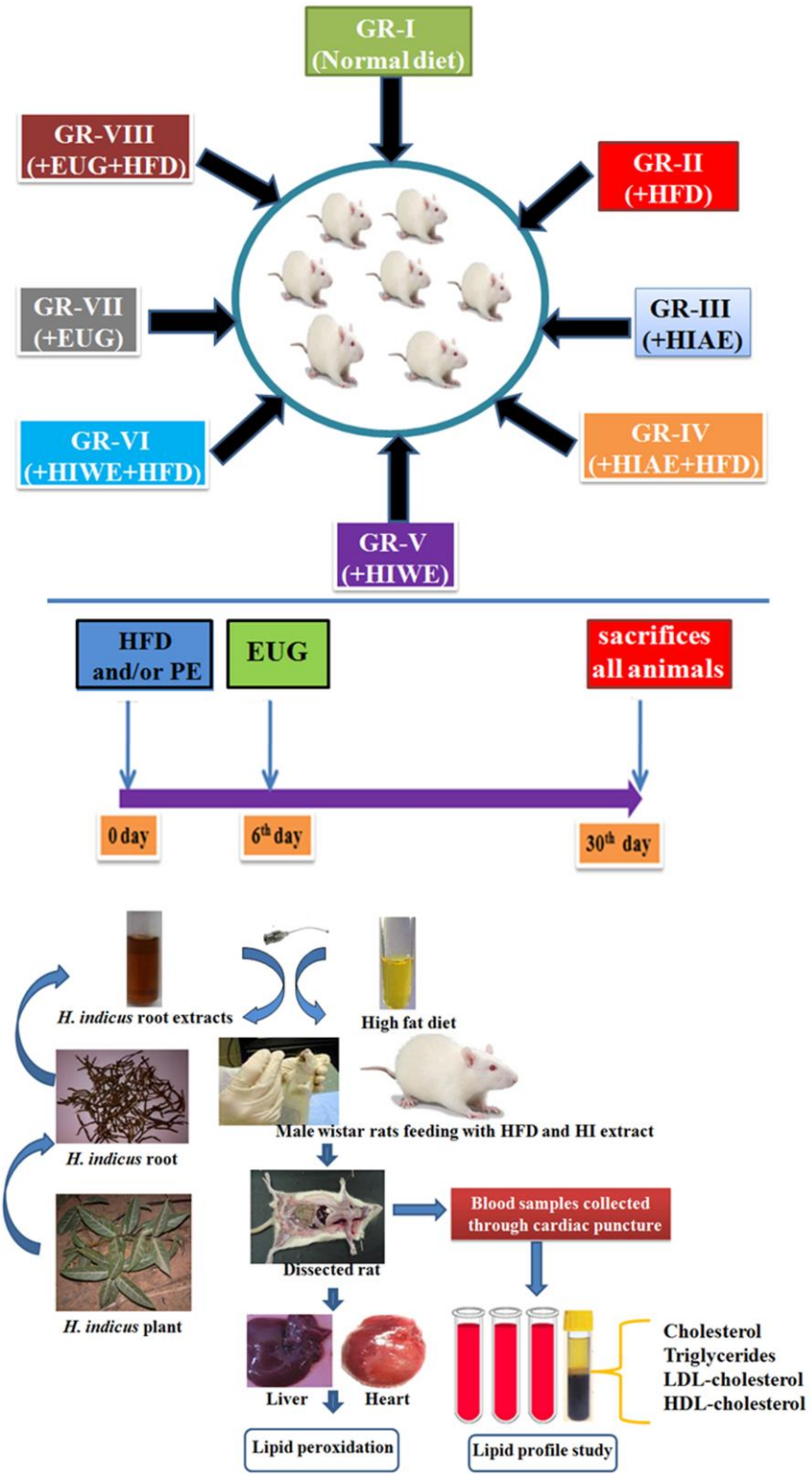


Fig. 2.7. Schematic diagram showed the antihyperlipidemic effect of HI roots and pure compound EUG in male Wistar rats. All are animals were divided into 8 groups with each groups having 7 animals. HFD, HI extracts were fed for 30 days and pure compound EUG fed for 26 days to the animals. After 30 days of treatment, all animals were sacrificed, serum of the blood samples was collected and performed lipid profile assay. Liver and heart tissues also were collected and preserved for further *in vitro* assays.

Chapter 3

MATERIALS AND METHODS

Chapter 3

Materials and methods

Materials

3.1. Plant material collection and authentication

The seeds of the *O. sanctum* plants (variety CIM-KANCHAN) voucher # 46803 were obtained from CIMAP, Hyderabad and authenticated by Prof. B. R. Rao, Scientist in charge, CIMAP. The plants were grown (Fig. 3.1A) in plant house located at UoH campus. The leaves were collected from one year old plants and shade dried for at least 7 to 10 days and stored in room temperature in the dark until further use and made into dried fine powder (Fig. 3.1 B) to prepare the alcoholic and water extracts. Similarly, the roots of the *H. indicus* (Fig. 3.2 A) voucher # 46808 were collected from UoH campus and authenticated by Dr. K. Venkata Ratnam.

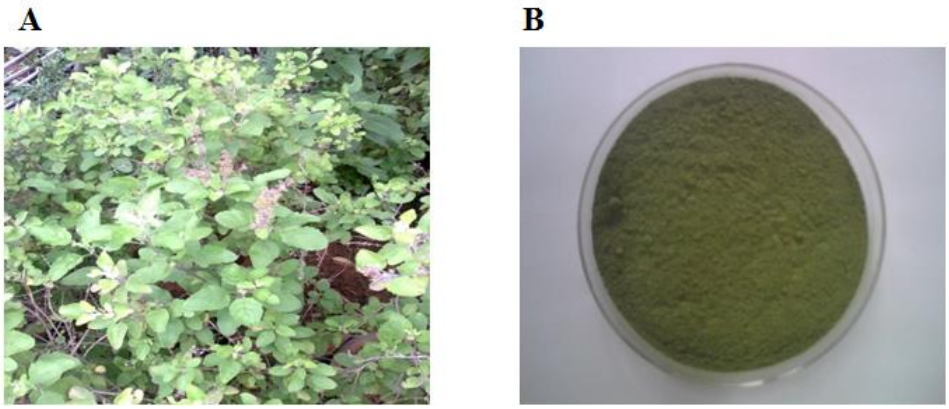


Fig. 3.1. *Ocimum sanctum* mature plant (A); dry leaf powder of *O. sanctum* (B).The seeds of the *O. sanctum* plants (voucher # 46803) were obtained from CIMAP, Hyderabad. The plants were grown in plant house located in UoH campus.

The roots were cut into small pieces (Fig. 3.2 B) sun dried and ground into fine powder, stored in an air tight container and appropriate quantities of plant material was weighed for preparation of different extracts and subjected to various *in vitro* (cell model) and *in vivo* (animal) assays in animal and cell model. Both the plant materials were deposited in

the Department of Botany, Sri Krishanadevaraya University Herbarium (SKU), Anantapur. India.

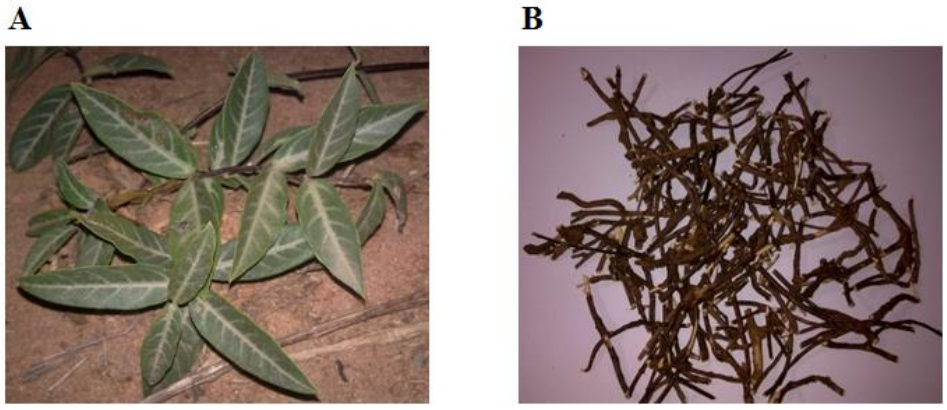


Fig. 3.2. *Hemidesmis indicus* mature plant (A); *Hemidesmus indicus* roots (B).The roots of the *H. indicus* (voucher # 46808) were obtained from UoH campus, Hyderabad.

3.2. Chemicals

3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrezolium bromide (MTT), sodium acetate trihydrate, were purchased from Hi-media, India. Arachidonic acid, ascorbic acid, β-caryophyllene, 2,7-dichlorofluorescin diacetate (H_2DCFDA), DEPC water, eugenol, gallic acid (GA), limolene, lipopolysaccharide-*Escherchia coli* (LPS), methanol, phorbol-12-myristate-13-acetate (PMA), rosmarinic acid, 2-thiobarbituric acid (TBA) and trypan blue were purchased from Sigma Aldrich (Germany). Alexa Fluor 594 goat anti-rabbit IgG (H+L), fetal bovine serum (FBS), penicillin-streptomycin, prolonged gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI), RPMI 1640 medium, trizol reagent, L-glutamine were purchased from Invitrogen (Germany). Human TNF-α and MCP-1 ELISA kits from BD Bioscience (USA). Power SYBR Green PCR Master Mix was obtained from Applied Biosystems (Carlsbad, CA, USA), iScript c-DNA synthesis kit was from Bio-Rad (Hercules, CA). Ferric chloride, hydrogen peroxide (H_2O_2) were obtained from Merck. Lipid profile kits were purchased from ERBA

diagnostic Mannheim GmbH, Mannheim, Germany. Imatinib (IMT) was obtained from Natco Pharma Limited. All other reagents used were of analytical grade.

Methods

3.3. Preparation of selected plant extracts

Both alcoholic and water extracts from *O. sanctum* dried leaves and *H. indicus* dried roots were prepared based on the method as described in Kokkiripati *et al.,* (2011). Alcoholic extract of both plant materials was performed by soaking the fine powder obtained from 200 mg of dried leaves (in case of *O. sanctum*) and 1g of root powder (in case of *H. indicus*) in 20 mL of 80% (v/v) ethanol and this process was continued by stirring at 40 ◦C for 5–6 h until the volume reached approximately to $1/5^{th}$ of the initial volume. However, water extract was prepared by soaking the same amount of the powder in 4 mL of distilled water for 12 to 16 h at room temperature. Later on, the extracts were centrifuged at 10,000 rpm for 10 min and supernatants were collected and stored until further use at 4ºC and used within 3 days. The respective extracts were dried completely under the vacuum to determine the yield. Concentration of the extracts in the above assays were expressed based on their respective dry weight basis. The yields obtained for OS dry leaves were 4 % (w/w) and 4.5 % (w/w) for alcoholic and water extracts, respectively. Yields obtained were 3.7 % (w/w) and 2.9 % (w/w) for *H. indicus* alcoholic and water extracts respectively.

3.4. Preparation of different fractions of alcoholic extracts of OS dry leaves

50 grams of OS dry leaf fine powder was weighed and soaked in 1 litre of 80 % v/v, ethanol and allowed for continuous stirring by maintaining the temperature 40-50 °C on a magnetic stirrer for 5–6 h until the volume reached approximately to $1/5^{th}$ of the initial volume. Further, concentrated hydro alcoholic samples were allowed for centrifugation at 10,000 rpm for 10 mins, residue was discarded and collected supernatant (filtrated)

was allowed to concentrate to get dry fine powder by using rotary evaporator (yield 6.5 %). To that dried fine powder (6.5 g), again 200 mL of ethyl acetate was added and kept if for continuous stirring on magnetic stirrer without any disturbance at room temperature for 48 h, then the entire ethyl acetate extracts was centrifuged at 10,000 rpm for 10 mins, supernatant was collected and concentrated to fine powder (yield 54 %) and labeled as ethyl acetate fraction (E-FR). To the left over residues of ethyl acetate extract, 200 mL of absolute alcohol was added and allowed for continuous stirring for 48 h, centrifuged and supernatant was collected and allowed to concentrate to get fine powder and labeled as alcoholic fraction (A-FR) A-FR yielded 63 %. Left over residues of A-FR were again soaked with 200 mL of autoclaved milli Q water for 48 h, again subjected to the same procedure and collected the supernatant of the water extracts. This extract was concentrated and labeled water fraction (W-FR, yield of W-FR, 92 %) and the left over residue were discarded. All the collected three different fractions were stored in separate glass vials with proper labeling for future use and preserved at 4 °C. The collected different fractions were allowed for LC-MS/MS metabolite analysis and further subjected to various in vitro assays using human monocytic (THP-1) cells to test antiinflamatory properties of different fractions on the basis of types of solvents used.

3.5. Phytochemical analysis of OS dry leaves and HI root extracts

3.5.1. Determination of total phenolic content

Preliminary phytochemical investigation was done by spectrophotometric method to quantify total phenolic and flavonoid contents in both the selected medicinal plant extracts. The concentration of the total phenolic content in water and alcoholic extracts of *O. sanctum* dry leaf and *H. indicus* root was determined by Folin-Ciocalteu (FC) method as described in Singleton (1999). Polyphenols in plant extracts reacts with specific redox reagents (Folin-Ciocalteu reagent) to form a blue complex that can be

quantified by testing absorbance at λ765nm (Scholfield *et al.*, 2001). Here the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. However, this reagent rapidly decomposes in alkaline solutions, which makes it necessary to use an enormous excess of the reagent to obtain a complete reaction. This excess can result in precipitation and high turbidity, making spectrophotometric analysis impossible. To solve this problem, Folin and Ciocalteu included lithium salts in the reagent, which prevented the turbidity (Folin and Ciocalteu, 1927). Tested plant extracts containing polyphenolic compounds were reduced by the Folin-Ciocalteu reagent by relying on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid reagent, there by producing blue colored complex which is released at λ765 nm.

3.5.2. Assay of total phenols using Folin-Ciocalteau reagent

3.5.2 a. Gallic acid standard solution:

Gallic acid was used as standard for preparation of calibration curve to estimate the total phenolic content in selected plant extracts. 50 mg pure gallic acid was dissolved in 1 mL of 80 % ethanol and diluted to 10 mL volume with double distilled water, labeled as stock solution 5 mg/mL and stored in refrigerator and used within 2 weeks.

3.5.2. b. Sodium Carbonate Solution.

Anhydrous sodium carbonate 20 % (w/v) was prepared with double distilled water in a beaker. Heated slightly for solubilisation and allowed for room temperature, after cooling, few crystals of sodium carbonate were added and left it at room temperature for 24 h, then filtered using whatman paper and stored at room temperature for further use.

Gallic acid working solution with varied concentrations (0, 50, 100, 150, 250 and 500 µg/mL) were prepared from the above mentioned stock solutions of 5

mg/mL. 20 µL of each of the working solution or different volumes of plant extracts were aliquoted into separate tubes, 0.79 mL of water was added to each tube, followed by adding 50 µL of FC reagent. After incubation of 30 sec to 8 min, 150 µL of sodium carbonate solution was added and vortexed to mix. All the solutions were left at 20 °C for 2 h and the absorbance of the reaction mixture was read at λ 765 nm against blank. Experiment was performed in triplicate. The amount of total phenol content was expressed as mg gallic acid equivalents (GAE)/g dwt of plant material. The total phenol content was subsequently calculated using gallic acid as standard.

3.6. Determination of total flavonoid content

A colorimetric assay was performed using aluminium chloride ($AlCl_3$) to determine the total flavonoid content in selected plant extracts according to the method as described in Saeed *et al.,* (2012) based on the formation of a flavonoid-aluminium complex. Quercetin was used as a reference standard for preparation of standard curve. Ten milligrams of quercetin was weighed and dissolved in 1 mL of absolute alcohol and varied concentrations from 0.1 mg to 1 mg/mL were prepared for standard graph. 1 mL of each concentration of quercetin and varied concentration of plant extract waere pipetted into glass tubes; 5 mL of distilled water and 0.3 mL of 5 % $NaNO_2$, (w/v) were added and incubated for 5 min at room temperature. After incubation, 0.6 mL of 10 % $AlCl_3$ (w/v) was added and continued for additional 5 min incubation, followed by addition of 2 mL of NaOH (1M) and made the volume to 10 mL with distilled water. The solution was mixed vigorously and absorbance was measured at λ 510 nm using a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The amount of total flavonoids was expressed in quercetin equivalents per g dwt of plant extracts. All samples were analyzed in triplicates.

3.7. ESI–LC–MS/MS metabolite analysis

ESI-LC-MS/MS was used as an analytical tool for qualitative or quantitative identification of several bioactive biological samples. ESI-MS coupled with HPLC has become a powerful technique in analyzing both small and large molecular weight molecules of assorted polarities in a complex biological sample (Ho *et al.,* 2003).

ESI-LC-MS/MS analysis of alcoholic and water extracts of OS leaf extracts, HI roots and different fractions of OS alcoholic extracts were performed on 6520 Accurate Q-TOF (Agilent Santa Clara, CA) mass spectrometer coupled to HPLC equipped with UV–VIS detector. Metabolites of the selected extracts were separated using HPLC according to the method as described in Dutta *et al.,* (2007). The column used for the separation of metabolites from the plant extracts was Zorbax Eclipse XDB- C 18, 4.6 × 50 mm, 1.8 µ. The solvent elution conditions used for OS extracts and different fractions of OS alcoholic extracts were as follows: solvent (A): water with acetic acid (0.1 % v/v) and solvent (B): methanol with acetic acid (0.1 %, v/v) respectively. The parameters used for LC-MS analysis of alcoholic and water extracts of HI roots in mobile phase were: solvent (A) ammonium formate with formic acid (0.1 % v/v) and (B) acetonitrile with formic acid (0.1% v/v) respectively according to the method as described in Surveswaran *et al.,* (2010). Controlled flow rate of 0.2 mL/min was maintained for following linear gradient elution interms of solvent B: (i) 20-55% from 0 to 15 min, (ii) 55-90% from 15-20 min (iii) 90% isocratic from 20 to 23 min (iv) 90-20% from 23 to 26 min (v) isocratic 20% from 26 to 35 min. Sample volume injected was 2 µL. ESI parameters: both on negative and positive ion mode; mass range 100-1700; spray voltage 4 KV; gas temperature 325ºC; gas flow 10 L/min; Nebulizer 40 psi.

3.8. Total antioxidant capacity

Total antioxidant capacities of the alcoholic and water extracts of selected plant species have been performed by phosphomolybdenum method as described in Raghavan *et al.,* (2003). The assay is based on the reduction of Mo (VI) to Mo (V) by the sample and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. Ascorbic acid was used to prepare the standard curve. Various concentration of standard solutions of ascorbic acid (2.5, 5, 10, 15, 20 and 25 μg/mL, w/v) or plant extracts were added to 1 mL of reagent (0.6 M sulphuric acid 28 mM sodium phosphate and 4 mM ammonium molybdate. The tubes containing reaction mixture were incubated in a thermal block at 95 °C for 90 min. The reaction mixture was cooled to room temperature and absorbance was measured at λ 695 nm against reagent blank. The antioxidant capacity was expressed as ascorbic acid equivalents.

3.9. Reducing power activity

Reducing power capacity assessment was performed according to the method as described in Oyaizu (1986). Varied concentrations of plant extracts were taken in an eppendorf tube. 0.25 mL of PBS (0.2 M, pH 6.6) was added to the plant extracts followed by adding 0.25 mL of 1 % (w/v) of potassium ferricyanide ($K_3Fe(CN)_6$) solution into the test tube. The test was incubated for 10 minutes at 50 ºC to complete the reaction. 0.25 mL of 10 % (w/v) trichloro acetic acid solution was added into the test tube. Finally 0.5 mL of 0.1 % (w/v) ferric chloride solution was added and made the volume to 1 mL with double distilled water. Then the absorbance of the solution was measured at 700 nm using a spectrophotometer against blank. Pure compound ascorbic acid of various concentrations was used as a standard. The reducing power activity was calculated per gram extract dry weight.

3.10. Free radical scavenging assays

3.10.1. DPPH radical scavenging test

The free radical scavenging activity of selected plant extract was measured by decrease in the absorbance of methanolic solution of 1,1-diphenyl-1-picrylhydrazyl (DPPH) according to the method as described in Nabasree and Bratati (2006). A stock solution of freshly prepared DPPH 0.004% (w/v) in methanol was used and 1 mL of this solution was added to plant extracts of different concentrations. After 30 min dark incubation, absorbance was measured at λ 517 nm against blank. Scavenging activity was expressed as percentage inhibition that is calculated using the following formula: [A λ517 nm of control– A λ517 nm of sample / A λ517 nm of control] x 100.

3.10.2. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of the selected plant extracts was measured by the method of Pedraza *et al.*, (2006). A solution of 40 mM hydrogen peroxide was prepared in PBS (0.1 M, pH 7.4) and xylenol orange reagent containing 250 mM ammonium iron (II) sulfate, 25 mM H_2SO_4, 100 mM sorbitol and 125 mM xylenol orange were prepared in amber bottle and stored at 4 °C. Various concentrations of plant extracts were pre-incubated with 10 µL of (40 mM) hydrogen peroxide for 10 min at room temperature and 200 µL of xylenol orange reagent was added to the above mixture and made up the volume to 1 mL with double distilled water and kept for 30 min incubation. Absorbance of hydrogen peroxide was determined at λ 584 nm. Ascorbic acid was used as a reference compound. The percentage of scavenging activity was calculated by using the formula A0-A1 / A0 × 100). A0 – absorbance of control (reagent with hydrogen peroxide but without plant extract), A1– absorbance in presence of plant extract.

3.11. Ferric chloride induced lipid peroxidation in rat liver and heart tissue homogenates of male Wistar rats

Lipid peroxidation is based on the decomposition of lipid membranes of cells which leads to the formation of malondialdehyde (MDA), aldehyde, enols as end-products which served as the index of lipid peroxidation. MDA formed at the end of reaction reacts with thiobarbituric acid (TBA) to form coloured complex which can be spectrophotometrically measured at λ 532 nm. Polyunsaturated fatty acids in cell membrane with three or more double bonds are particularly more susceptible to peroxidation. Once the reaction is initiated, the process proceeds as a free radical chain reaction. Male Wistar strain albino rats (150-200 g) supplied by National Institute of Nutrition, Hyderabad were used for the present study. The animals were housed under standardized environmental conditions and fed with standard pellet chow feed and water *ad libituim*. The animal experiments conducted as per the protocol approved by Institutional Animal Ethics Committee (IAEC, LS/IAEC/SDT/11/02) followed the guidelines of Commission for Purpose of Controlling and Supervising Experiments on Animals (CPCSEA).

3.11.1. Preparation of liver homogenate of male Wistar rat

Rat liver tissue homogenate was performed with ice-cold 0.15 M potassium chloride (KCl) buffer (pH 7.0) according to the method as described in Ohkawa *et al.*, (1979). Before homogenization, weight of liver was measured and rinsed with pre chilled 0.9 % (w/v) NaCl to remove excess blood. The perfused liver tissue was isolated and 10 % (w/v) homogenate was prepared in KCl buffer using tissue homogenizer under chilled condition followed by centrifugation at 800 g at 4 °C for 15 min, and clear cell-free supernatant was used to study *in vitro* lipid peroxidation. The peroxidation of lipids in liver homogenate was induced by using ferric chloride ($FeCl_3$) according to the method

as described in Gaurav *et al.,* (2007). Varied concentrations of the plant extracts were added to the liver homogenate and incubated for 10 min followed by 100 µL of 10 mM ferric chloride was added to the above reaction mixture and further incubated for 30 min at 37 °C. After incubation the reaction was terminated by adding 1 mL of ice-cold 0.25 N HCl containing 15 % (w/v) trichloro acetic acid (TCA) and 0.38 % (w/v) TBA. The reaction mixture was allowed to heat for 60 min at 80 ºC, cooled to room temperature and centrifuged at 5000 g for 15 minutes. Optical density (O.D) of the supernatant from the each tube was measured at λ 532 nm against blank. The percentage of inhibition of lipid peroxidation was calculated by following formula: % Inhibition = (control-sample/control) × 100. Control is induced reaction mixture without plant extracts.

3.11.2. Preparation of heart homogenate of male Wistar rat

After sacrificing the rat, heart was excised; blood was removed completely by using pre-chilled saline to avoid interference in the reaction. The tissue was immediately transferred to liquid nitrogen to prevent oxidation and stored at -80 °C until use. Further, tissue was homogenized in nitrogen flushed 1.15 % (w/v) KCl buffer and centrifuged at 1500 g for 5 min; the supernatants were collected and used for lipid peroxidation assay. Lipid peroxidation study in heart homogenate was performed according to the method as reported in Meera *et al.,* (2009). The reaction mixture containing heart homogenate and various concentrations of plant extracts were incubated for 5 min and then 10 mM ferric chloride was used as a inducer and incubated the above mixture at 37 ºC for 1 h. After incubation, the reaction was stopped with 500 µL of 70 % (v/v) ethanol, followed by addition of 1 mL of TBA (1 % w/v) and the samples were subjected to boiling in a water bath for 20 min. After incubation, samples were brought down to room temperature and centrifuged at 5000 g for 15 min.

Supernatant of the above mixture was collected and measured at λ 532 nm in a spectrophotometer. Ascorbic acid was used as a reference compound. The effect of various extracts or test/standard compounds against lipid peroxidation were calculated as follows: % Inhibition = (control-sample/control) × 100. Control is induced reaction mixture without plant extracts.

3.12. Cell culture and treatment

Human monocytic (THP1) cells were obtained from National Centre for Cell Science (NCCS Pune, India) and cultured in RPMI 1640 medium containing 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate supplemented with 2 mM l-glutamine, 1xPenStrep (penicillin (100 U/mL) and streptomycin (100 U/mL)) and 10 % (v/v) fetal bovine serum.

3.13. Cell viability assay of human monocytic (THP-1) cells

Cell viability was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann *et al.*, (1983). An aliquot of 500 µL cell suspension (5 x 10^5 cells/mL) was distributed in each well of 24 well plates and then incubated for 24 h in the presence or absence of various concentration of plant extracts and standard compounds. Respective vehicle (alcohol or water) concentration was limited to < 1% (v/v) of the cell culture volume. A quantity of 20 µL of MTT (5 mg/mL in PBS) was added to each well and incubated for an additional 4 h at 37 ◦C. Subsequently, the formed precipitate containing purple-blue formazan was dissolved in 100 µL of DMSO and the optical density was measured at λ 570 nm using a micro plate reader (Tecan, Germany). Media without cells with different concentration of plant extracts or standard compounds were taken as respective blanks. Cell viability at different concentrations of plant extracts and standard compounds were also tested by trypan blue exclusion method proceed by MTT assay. The dye exclusion test was used

to determine the number of viable cells present in a cell suspension treated with plant extracts or standard compounds. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue whereas dead cells do not. In this test, appropriate volume of cell suspension (20 µL) was mixed with 10 µL (0.004 %, w/v) of trypan blue dye and then observed under compound microscope. All viable cells will have the clear cytoplasm whereas nonviable/dead cells will appear in blue colour cells (Strober, 2001).

3.14. Quantification of LPS induced TNF-α and MCP-1 production in THP-1 cells by ELISA

In order to proceed with this experiment, THP-1 cells were seeded in 24-well plates at a density of 5 x 10^5 cells/mL and pretreated with or without plant extracts (1 mg dwt/mL), pure compounds (EUG, RA, LM and β-C) and positive control IMT (5 and 20 µg/mL) at 37 ºC in 5% CO_2 for 12 h. Subsequently the cells were exposed to 0.5 µg/mL of LPS for 3 h. Cells were centrifuged, collected supernatant was stored at -80 ºC until further use. Amount of TNF-α and MCP-1 present in the supernatants were determined using BD OptEIA human TNF-α ELISA and MCP-1 ELISA kits (BD Biosciences) as per the manufacturers protocol.

Preparation of solutions:

Coating buffer (0.1 M sodium carbonate): 7.13 g $NaHCO_3$, 1.59 g Na_2CO_3 were mixed well in 800 mL of water, adjusted pH to 9.5 with 10 N NaOH and made up the final volume to 1 L. Freshly prepared and used within 7 days of preparation, stored at 2-8 °C.

Assay diluent: PBS with 10 % FBS (heat inactivated) pH 7.0.

Wash buffer: PBS with 0.05 % (w/v) Tween-20. used the solution within 3 days of preparation, stored at 2-8 °C.

Stop solution: 1 M H_3PO_4.

Standard preparation (Stock): Lyophilized standard TNF-α was reconstituted in 1 mL of deionized water that yielded a stock of 100 ng/mL and allowed it to equilibrate for at least 15 min, then vortexed and stored at -80 °C and was used within the period of six months. Standard preparation (working): 500 pg/mL TNF- α was prepared on the day of ELISA experiment and prepared various concentration of TNF-α as 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 pg/mL by serial dilution. Blank was run which contained only assay diluents. Similarly commercially available MCP-1 standard was prepared.

Commercially available, BD OptEIA™ test is based on solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). Kit contains human recombinant TNF-α and MCP-1 standards; antihuman monoclonal coating antibody (primary antibody) (biotinylated anti-human TNF-α/MCP-1monoclonal detection antibody) secondary antibody; streptavidin-horseradish peroxidise enzyme; substrate solutions, 3,3',5,5'-tetramethylbenzidine (TMB) reagent. It utilizes a monoclonal antibody specific for TNF-α/MCP-1 coated on a 96-well plate. Standards or plant extract treatment supernatants were added to the wells, TNF-α or MCP-1 present in the samples binds to the immobilized antibody and allowed for incubation. The wells were washed and a streptavidin-horseradish peroxidase conjugate mixed with a biotinylated anti-human TNF-α antibody was added, producing an antibody-antigen-antibody "sandwich". The wells were again washed and TMB substrate solution was added, which produces a blue colour in direct proportion to the amount of TNF-α present in the initial sample. The 50 μL of stop solution (1M H_3PO4) changes the colour from blue to yellow and the microwell absorbances were read at λ 450 nm.

3.15. Phorbol-12-myristate-13-acetate (PMA) induced differentiation of monocytes to macrophages

THP-1 cells were grown in RPMI-1640. In order to stimulate differentiation of THP-1 cells to macrophages, serum free media (2 % FBS, v/v) was used and cells were treated with PMA (5 ng/mL) for 48 h at 37 ºC in 5 % CO_2 according to the method as described in Tsuchiya *et al.,* (1982). The images of the cells were taken under inverted microscope. These experiments were done in the presence or absence of plant extracts or test compounds. THP-1 cells with no PMA were used as control (undifferentiated) cells.

3.16. Treatment of cells and transcript analysis of inflammatory markers

3.16.1. RNA extraction in THP-1 cells

THP-1 cells were grown in T 25 cm^2 vent culture flask and allowed them to grow till they reach the cell density up to 5×10^5/mL. The flasks were pretreated with or without plant extracts or test/reference compounds for 16 h in 5 % CO_2 at 37 °C. After pretreatment, cells were induced with LPS (0.5 µg/mL) for 3 h. After respective treatment, cells were centrifuged at 10,000 rpm, 5 min at 4 °C, supernatants were discarded. Cell pellets were collected in 50 µL of media, 750 µL of trizol reagent was added as per manufacturer's instruction manual, incubated at room temperature for 5 min. After incubation, 150 µL of chloroform was added, the tubes were capped tightly and vortexed vigorously and allowed to incubate at 15-30 °C for 2-3 min, followed by centrifugation at 12,000 × g for 15 min at 2-8 °C. Two phases were formed, among which upper colourless aqueous phase was transferred into sterile, fresh and leveled eppendorf tube, 500 µL of isopropanol was added and incubated at room temperature for 10 min followed by centrifugation at not more than 12,000 × g for 10 min at 2-8 °C, supernatant was discarded and RNA pellet was washed with 1mL of 75 % (v/v)

ethanol and pelleted down by centrifuging at not more than 5000 × g for 5 min at 2-8 °C. RNA pellet was dried in air/vaccum dry for 5-10 min and dissolved in 50 µL of DEPC water followed by incubation at 55-60 °C for 10 min. Total RNA purity was evaluated using the A 260/A 280 ratio. RNA samples were stored at -80°C.

3.16.2. Synthesis of c-DNA

Commercially available iScript™ c-DNA synthesis kit from Bio Rad Laboratories (USA) was used for synthesizing the c-DNA from RNA isolated from cultured THP-1 cells. The total reaction mixture of 20 µL contained 5x iScript reaction mix of 4 µL; iScript reverse transcriptase of 1 µL; Nuclease free water in µL (varies for every treatment) and RNA template of 1 µg. PCR machine was set up for about 30 cycles as per manufacturer's protocol and incubated the complete reaction mixture in cycling reactions: denaturation step at 25 °C for 5 min followed by annealing at 42 °C for 30 min and extension at 72 °C for 5 min. After the synthesis of c-DNA, all samples were stored at -20 °C for further use.

3.16.3. Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR was performed by using an ABI Prism 7500 sequence detector (Applied Biosystems Inc, Foster City, CA) Instrument. Briefly, reverse-transcribed cDNA in duplicate samples were checked for cytokine or chemokine mRNA levels like TNF-α, IL-6, MIP-1α, MCP-1, TLR 2, TLR 4 with SYBR Green PCR master kit (Applied Biosystems, Carlsbad, CA, USA), according to manufacturer's instructions. PCR primers used in this study are listed below (Table 3.1) and were purchased from Eurofin (Bangalore, India). The assays were initiated within 5 min at 95°C, and then 40 cycles of 15 s at 94°C, 1 min at 60°C. It was performed in triplicates. Total reaction mixture was 10 µL. 5 µL contained of 1x SYBR® green mixed with 0.5 µL each of forward and reverse primer, 2 µL of water

and 2 µL of cDNA (1:5). The specificity of the SYBR green fluorescence was tested by plotting fluorescence as a function of temperature to generate the melting curve of the amplicon. The transcript levels of marker genes were normalized with GAPDH mRNA. Relative levels of target mRNA expression were calculated using the 2-ΔΔCT

Table 3.1. Primers used for real time quantitative PCR assay

IL-6	Forward	5´-TGGATTCAATGAGGAGACTTGC-3´
	Reverse	5´-CAGGAACTGGATCAGGACTT-3´
TNF-α	Forward	5´-CCCAGGGACCTCTCTCTAATC-3´
	Reverse	5´-ATGGGCTACAGGCTTGTCACT-3´
MIP-1α	Forward	5´-TTGTGATTGTTTGCTCTGAGAGTTC-3´
	Reverse	5´-CGGTCCTCACCAGACACACT-3´
MCP-1	Forward	5´-GCCAAGGAGATCTGTGCTGAC-3´
	Reverse	5´-CATGGAATCCTGAACCCACTTC-3´
CD14	Forward	5´-TCCGAAGCCTTCCAGTGTGT-3´
	Reverse	5´-ACAGAGAGCCGCCATCAGTC-3´
TLR4	Forward	5´- TGGATACGTTTCCTTATAAG-3´
	Reverse	5´- GAAATGGAGGCACCCCTTC-3´
TLR2	Forward	5´-GCCAAAGTCTTGATTGATTGG-3´
	Reverse	5´-TTGAAGTTCTCCAGCTCCTG-3´
GAPDH	Forward	5´-CACCAACTGCTTAGCACCCC-3´
	Reverse	5´-TGGTCATGAGTCCTTCCACG-3´

3.17. Measurement of ROS generation in THP-1 cells using H_2DCFDA

Intracellular ROS production in control, arachidonic acid and/or plant extracts treated THP- 1 cells was measured using the dye 2, 7-dichloro dihydro fluorescein diacetate (H_2DCFDA) as described in (Kweon *et al.*, 2001). H_2DCFDA, a non-fluorescent cell-permeable dye up on cleavage of acetate group by cytosolic intracellular esterases undergoes oxidation with any oxidizing species inside the cells and becomes the fluorescent compound, 2, 7-dichlorofluorescein (DCF) (Bass *et al.*, 1983 and Carter *et al.*, 1994). THP-1 cells (5×10^5 cell per well) were pre-incubated with or without different concentrations of plant extracts or test/reference compounds for overnight at

37 °C in a humidified incubator containing 5 % CO_2. After preincubation, 5 µM H_2DCFDA was added in dark and further incubated for 30 min at 37 °C. Then the cells were washed twice with growth medium RPMI (FBS free) to wash out excess dye in the medium. After washing, the cells were exposed to arachidonic acid for 5 min to induce intracellular ROS in THP-1 cells. Images were obtained by subjecting the cells to confocal laser scanning electron microscope (Leica DMR). The quantification of fluorescence intensities of cell samples was measured by microplate reader using excitation and emission wavelengths at 488 nm and 525 nm respectively.

3.18. Determination of lipid peroxidation in THP-1 cells

The end product of membrane lipid peroxidation was evaluated. Briefly, 70 µL of double distilled water, 50 µL of 50 mM phosphate buffer, 10 µL of 1 mM Butylated hydroxy toluene (BHT), 75µL of 1.3 % TBA (w/v) was added to 10 µL cell lysate. The end product of reaction were precipitated with 50 µL of 50 % (w/v) TCA. The assay mixture was then incubated at 60 °C for 40 min and further kept in ice for 15 min and 10 µL of 20 % (w/v) sodium dodecyl sulphate (SDS) was added. This assay measures the amount of pink coloured MDA-TBA adduct. The plate was read at λ 530 nm (Tiwari *et al.*, 2010).

3.19. Nuclear translocation of NF-κB in THP-1 cells

Reagents used:

Tris buffer saline (TBS): 20 mM Tris HCl and 150 mM NaCl at pH 7.5.

Tris buffer saline and Tween-20 (TBST): Add 0.05 % (w/v) tween 20 in 100 mL TBS.

4 % (w/v) paraformaldehyde (PFA).

Procedure:

The NF-κB-p65 nuclear localization was detected by indirect immunofluorescence assay using confocal microscopy according to the method as described in Jeong *et al.*,

(2010). THP-1 cells were cultured directly on glass coverslips in 6-well plates for 24 h. After the stimulation with 0.5 μg/mL of LPS, the cells were fixed using 4% paraformaldehyde in PBS, permeabilized with 0.2% triton X-100 (w/v) in PBS, and blocked with 3 % (w/v) bovine serum albumin (Sigma-Aldrch, Germany). Subsequently polyclonal antibodies against NF-κB-p65 (1:250) were applied for 12 h followed by 3 h incubation with anti-rabbit IgG conjugated to Alexa fluro 594 antibody (1:250). Excitation and emission wavelength of Alexa Fluor λ 594 are λ 543 nm and α 569 to λ 677 nm respectively. The nuclei were visualized by DAPI (excitation wavelength 358 nm and emission wavelength λ 400 to λ 500 nm).

3.20. Quantification of eugenol and preparation eugenol standard solution

Eugenol (EUG) a phenyl propanoid was purchased from Sigma aldrich which is available commercially. The total amount of EUG was estimated in both OSAE and OSWE extracts prepared from *O. sanctum* dry leaves according to the method described in Thyagaraj *et al.*, (2013) with minor modifications. HPLC coupled to LC-MS system (Agilent Santa Clara, CA) was used for estimation of EUG. The HPLC column was comprised of Poroshell 120 EC-C 18, 3.0 X 50 mm, 2.7 μM. The mobile phase consisted of solvent A (0.1 % formic acid in water, v/v) and solvent B (acetonitrile, 100 % v/v, LC-MS grade). A controlled flow rate was maintained of 0.55 mL/min for the following linear gradient elution interms of solvent B: (i) 40-90% from 0 to 8 min, (ii) isocratic 90% from 8 to 13 min (iii) 90 % to 40 % from 13 to 15 min. Sample volume used was 1 μL. ESI parameters: positive ion mode; mass range 100-1700; spray voltage 4000v, gas temp. 325 ºC; gas flow 10 L/min, Neublizer 35psi. The run time was set at 18 min. The peaks were identified by comparing the retention time of the reference standard with the extracts and also confirmed by mass fragmentation spectra. Data analysis was done using mass hunter work station software quantitative

analysis system. For calibration purpose, a standard stock solution of eugenol of 40 µg/mL was prepared in methanol and labeled as mother stock. Aliquots of the individual stock solutions were prepared in the concentration range of 4 µg/mL to 0.06 µg/mL. The obtained dilutions were injected into the system. The linearity of the individual standard was plotted by taking mean peak area versus concentration.

3.21. Experimental animal:

Male Wistar albino rats, aged 2 months (body weight: 120-160 g) were used for the present study. Animals were procured from the National Institute of Nutrition, Hyderabad were housed under standardized environmental conditions, fed with standard pellet chow feed and water ad libitium and were housed in acrylic poly cages with not more than two animals per cage at ambient temperature of 18± 20 ºC with 12-h-light/12-h-dark cycle. The experiments were conducted as per the protocol approved by Institutional Animal Ethics Committee (IAEC, LS/IAEC/SDT/11/02). All the animals were acclimatized for a period of 10 days in their new environment before the initiation of experiment.

3.22. Preparation of plant extracts and pure compound eugenol for animal study

Alcoholic and water extracts of HI roots (200 mg/kg body weight/day) were fed to animals for 30 days. Commercially available EUG was procured from Sigma-Aldrich and required concentration of EUG (25 mg/kg body weight/day) was added to high fat diet and fed to the animals for 25 days.

3.23. High fat diet-induced hyperlipidemic study in animal model

The use of high fat diet induced hyperlipidaemia through accelerated cholesterol and other lipid markers was suggested as an important approach in animal model to screen the action of medicinal plants to find out certain therapeutic drugs. Male Wistar rats were randomly divided into 8 groups comprising of 7 rats each: a control group, a high

fat diet group, plant extract-treated group and EUG treated groups and all the groups were labeled as GR-I to GR-VIII.

Group 1: Normal basal diet (ND)

Group II: High fat diet (HFD) (2g/day)

Group III: HIAE (200 mg/kg body wt)

Group IV: HIAE (200 mg/kg body weight/day) + High fat diet

Group V: HIWE (200 mg/kg body weight/day)

Group VI: HIWE (200 mg/kg body weight/day) + High fat diet

Group VII: EUG (25 mg/kg body weight/day)

Group VIII: EUG (25 mg/kg body weight/day) + High fat diet

A high fat diet (Amul butter containing: saturated fat 51%, cholesterol 0.18%, sodium 0.83%, protein 0.5% and vitamin 0.06%) was given to the animal continuously for 30 days. Similarly, HIAE/HIWE extract was also administered orally once daily for 30 days, whereas pure compound eugenol was administrated orally for 25 days.

3.24. Body weight measurement

The initial body weight of each rat from all groups of animals was measured before starts pretreatment with plant extracts/high fat diet. After the end of the dietary treatment of 30 days the final weight of each animal of every group was measured before sacrificing the animals. The difference between initial body weight and final body weight was compared.

3.25. Collection of organs (liver and heart) and lipid peroxidation assay

At the end of the experiment, rats were deprived of food for 12 hours and then anesthetized using diethyl ether. A central longitudinal incision was made into the abdominal wall. Liver and heart organs were excised, rinsed with pre-chilled saline to

removed unwanted blood strain. The removed organs were immediately frozen in liquid nitrogen and stored at -80°C until the analysis. The perfused both liver and heart tissues were homogenated with 10 % (w/v) KCl buffer using tissue homogenizer below 4°C followed by centrifugation at 800 g for 15 min, and clear cell-free supernatant was used to study *in vitro* lipid peroxidation as described in section 3.11.

High fat diet induced peroxidation of lipids in both liver and heart homogenate was measured according to the method as described in Gutierrez *et al.,* (2014). We measured the concentration of protein content of both liver and heart homogenate by Bradford assay of every animals of each group. Further liver homogenate was treated with 1 mL of ice-cold 0.25 N HCl containing 15 % (w/v) trichloro acetic acid (TCA) and 0.38 % (w/v) TBA. The reaction mixture was allowed to heat for 60 min at 80 ºC, cooled to room temperature and centrifuged at 5000 g for 15 min. Optical density (O.D) of the supernatant from the each tube was measured at λ 532 nm against blank. Fold change was calculated on the basis of absorbance value of normal diet fed animals *vs* high fat diet fed animals. Extent of lipid peroxidation was determined in all groups.

3.26. Collection of blood samples, coagulation time period and assay of serum lipid markers

Blood samples were collected via cardiac puncture into tubes without anticoagulant after sacrificing the animals at the end of 30 days from each and every group of animals. The blood samples, which were kept at room temperature for 30 min were centrifuged at a speed of 3500 rpm for 15 min. Serum, which was extracted from the centrifuged samples in the form of supernatant, was used for analysis of lipid profile. The concentrations of total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) and LDL in serum were determined using a microplate

reader (Thermo Scientific) according to the manufacturer's instruction using reagents purchased from Erba Diagnostics Germany. Blood coagulation time period was measured in each animal of all groups. Collected blood sample from each animal after cardiac puncture was immediately transferred to sterile eppendorf tube and measured time taken for blood clotting was recorded in each and every animal of all groups and compared all groups blood clotting time periods.

3.27. Statistical analysis

All the data were subjected to one way analysis of variance (ANOVA) test using Statistical Package for the Life Sciences (SPLS version 11). The values were expressed as mean ± standard deviation (S.D.). Variance at $p < 0.001$ was considered to be highly significant.

Chapter 4

RESULTS and DISCUSSION

Objective 1: Phytochemical profiles and antioxidant activities *(in vitro)* of alcoholic and water extracts of *O. sanctum* (OS) leaves and *H. indicus* (HI) roots

Chapter 4

Results and Discussion

Results

Extraction yield and total phenolic content

Alcoholic (OSAE) and water extracts (OSWE) of *O. sanctum* yielded 4.0 % and 4.5 % (w/w) respectively. In case of HI roots, yields of alcoholic extracts (HIAE) and water extracts were 3.7% and 2.9% respectively based on these extract dry weight obtained from initial dried material taken for extraction procedure in both plants. Phenols are very important plant constituents because of their ROS scavenging ability due to their hydroxyl groups. Some of them are very reactive in neutralizing free radicals by donating a hydrogen atom or an electron. The presence of phenolic compounds may contribute directly to antioxidative action also, so it was reasonable to determine the total amount of phenolic contents in the selected plant materials. The total amount of phenoilc content in alcoholic and water extracts of selected plants were determined by Fiolin-Ciocalteau reagent. Absorbance of samples were measured at λ 760 nm.

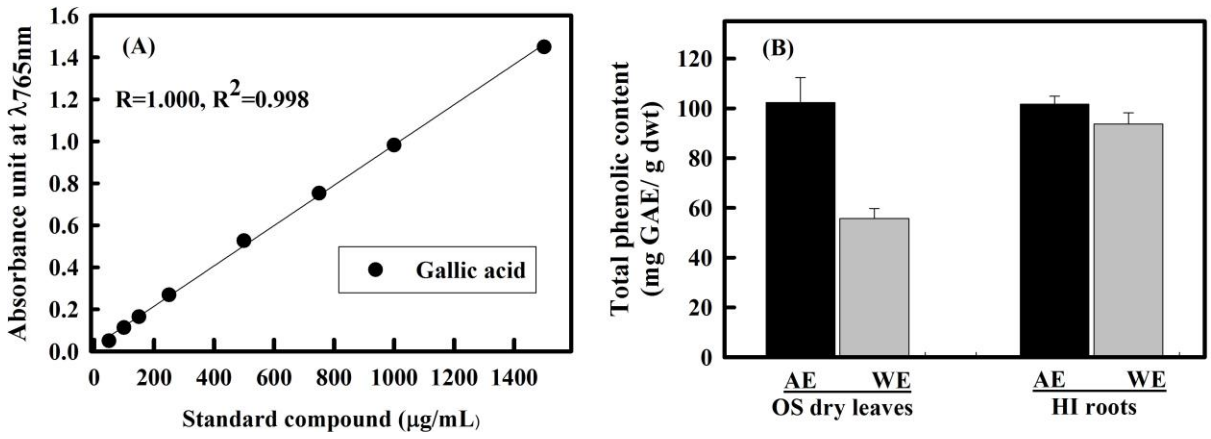


Fig. 4.1. (A) Pure compound gallic acid was used as a standard curves for estimation of total phenolic content in selected plant extracts. (B) Determination of total phenolic content by Folin-Ciocalteau reagent in dry leaf extracts of *O. sanctum* and root extracts of *H. indicus*. Experiments were performed at least in triplicate and the results are expressed as the mean ± S.D**.,** n=6

The polyphenolic content was extrapolated by using different concentrations of gallic acid as standards and are expressed in mg of gallic acid equivalents (Fig. 4.1 A). The total phenolic content of alcoholic extracts of *O. sanctum* (OS) dry leaves and *H. indicus* (HI) roots were about the same (~100 mg GAE/g dwt) (Fig 4.1 B). Water extracts of OS dry leaves contained 55.66 mg GAE/g dwt, whereas water extracts of HI roots contained 93.63 mg GAE/g dwt as shown in (Fig. 4.1B). Observation showed that polyphenolic content in water extracts of HI is higher compared to OS dry leaves.

Total flavonoid content

Flavonoids are considered as diphenylpropanes that are commonly present in plants. More than 4000 flavonoids have been detected in different medicinal plants. They are also considered as frequently found components of the human diet. Most of the flavonoids have been found to possess antioxidant, anti-ischemic and antiinflammatory properties. Therefore, it is important to estimate the total flavonoid content in plant extracts. Here aluminium chloride colorimetric method was used for flavonoid determination in both alcoholic and water extracts of OS dry leaves and HI roots. The flavonoids in the presence of aluminium chloride have intense yellow fluorescence which was observed under UV spectrophotometer at λ 510 nm. Pure compound quercetin with different concentration was used as standard for estimation of total flavonoid content and expressed as mg QE/g dwt (Fig. 4.2 A). The result of the total flavonoid content of alcoholic and water extracts of OS dry leaves and HI roots are shown in Fig. 4.2 B. The total flavonoid content varied from 4 to 7 mg QE/g dwt. Alcoholic extracts of OS dry leaves and HI roots showed 4.25 and 6.98 mg QE/g dwt respectively. Water extracts contained 6.66 and 6.71 mg QE/g dwt in OS dry leaves and HI roots respectively. From the above observation it has been observed that alcoholic extracts of HI roots have higher amount of flavonoid content compared to OS dry leaves, whereas there was no difference observed in water extracts of both the plants.

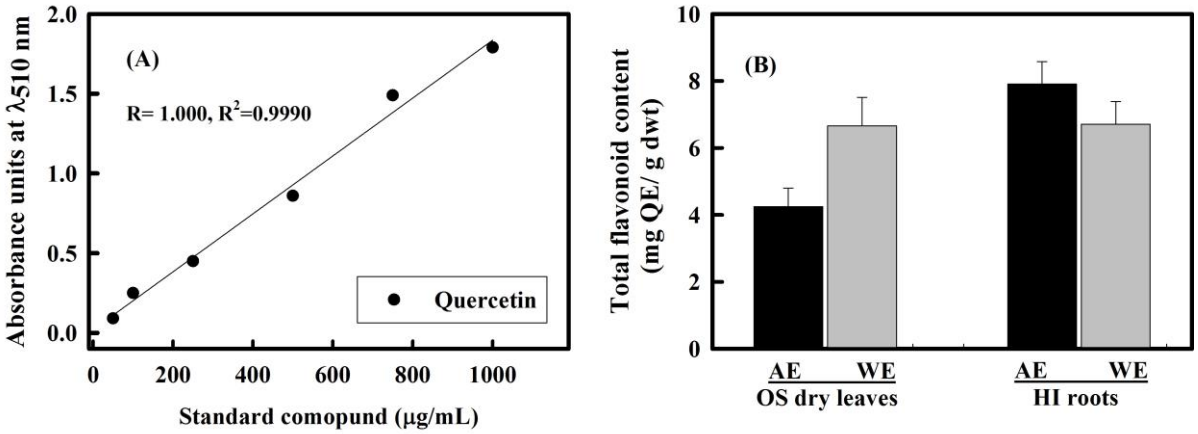


Fig. 4.2. Aluminium chloride colorimetric method was used for determination of total flavonoid content (A) Pure compound gallic acid was used as a standard curves (B) TFC of dry leaf extracts of *O. sanctum* and root extracts of *H. indicus*. Experiments were performed at least in triplicate and the results are expressed as the mean ± S.D**.,** n=6

LC-MS/MS metabolite profiling of OS dry leaves and HI root extracts

LC–MS/MS analysis has been proved to be a powerful and widely accepted technique in metabolite profiling of medicinal plant extracts. This technology is highly sensitive and can also determine low levels of metabolites in the extracts. The accuracy of identification and quantitation can be significantly increased due to coupled diode array detection. Such investigations of metabolite profiling also ensures the authenticity of plant materials. In this study, LC-Q-TOF-MS/MS was employed to analyze the phytoconstituents present in alcoholic (AE) and water extracts (WE) of OS dry leaves and HI roots.

Metabolite profiling of *O. sanctum* dry leaf extracts

OS dry leaf extracts have reported several compounds such as phenolics, flavonoids, phenylpropanoids and terpenoids in both alcoholic and water extracts (OSAE/OSWE). LC-Q-TOF-MS was subjected for the reasons most importantly to (1) authenticate plant samples (2) to distinguish the metabolite profiling in different extracts subjected to different solvents and temperature. Data was acquired in both negative and positive ion modes. Details of the compounds including class of the compound, molecular formula and abundance were in Table 4.1, whereas the mass spectra, retention time and fragmentation patterns obtained in both negative and positive mode of OSAE and OSWE are shown in Fig. 4.3. Negative ion

and positive ion modes of LC-Q-TOF-MS detected 16 and 7 compounds in OSAE; 4 and 6 compounds in OSWE respectively. Majority of the identified compounds belong to either phenolics, or terpenoids. Compounds like vicenin, luteolin-7-O-glucuronide, apigenin-7-O-glucuronide, methyl chavicol, methyl cinnamate, eugenol and a fatty acid, linoleic acid were commonly detected in both OSAE and OSWE, whereas rosmarinic acid, orientin, germacrene D, α-pinene, carvacrol, apigenin, linalool, cirsimaritin, cirsilineol, sinapic acid and ascorbic acid were exclusively detected in OSAE, while methyl eugenol and stearic acid were detected only in OSWE (Table 4.1).

Vicenin, $C_{27}H_{30}O_{15}$ (Fig. 4.3. A). It belongs to flavonoids with defined C-ring fragments. It yielded the peak at RT 14.74 min from OSAE and RT 14.80 min from OSWE. Identification of the compound confirmed by $[M-H]^-$ with m/z 594.14. The mass fragmentation pattern $[M-H]^-$ of the compound were m/z 412, 384, 353, 324, 297 and 191. The compound was identified, confirmed and characterized as per the Jabor *et al.,* (2010).

Luteolin-7-O-glucuronide, $C_{21}H_{17}O_{12}$ (Fig. 4.3 B): It is a flavone and yielded the peak at RT 17.30 min from OSAW and RT 17.71 from OSWE. The identification of compound was confirmed by the mass spectrum exhibiting ions $[M-H]^-$ of m/z 461.06 and [M+H]+ with m/z 463.08. The compound gave MS fragmentation patter $[M-H]^-$ with m/z 431, 358, 133, 175, 151 and 107. The compound was confirmed and characterized according to Lin *et al.,* (2010).

Methyl cinnamate: $C_{10}H_{10}O_2$ (Fig. 4.3 C): It is the methyl ester of cinnamic acid and a white or transparent solid with a strong, aromatic odor. It is naturally found in a variety of plants, including in fruits. Methyl cinnamate is used in the flavor and perfume industries. It was detected both in OSAE and OSWE. It yielded the peak at RT 17.82 min from OSAE and RT 18.83 from OSWE. Identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M-H]^-$ with m/z 161.03 and $[M+H]^-$ with m/z 163.03. The mass

fragmentation spectra $[M - H]^-$ with m/z of this compound are 158, 132, 110, 103. The compound was confirmed and identified from Mass bank record JP008519.

Table. 4.1. Q-TOF profile of positive and negative ion mode of OSAE and OSWE used in LC-MS/MS analysis

Compound name/ class of compound	**Mol formula/ Exact mass**	**Types of extracts**	**Abudance**		**RT min**	**Reference**
			-ESI	**+ESI**		
Stearic acid (Saturated fatty acid)	$C_{18}H_{36}O_2$ 284	OSAE	ND	ND	ND	Mass bank
		OSWE	ND	87950	2.18	
Germacrene D (Sesquiterpenes)	$C_{15}H_{24}$ 204.35	OSAE	13686	ND	10.60	Mass Bank
		OSWE	ND	ND	ND	
Vicenin (Flavonoids)	$C_{27}H_{30}O_{15}$ 594.51	OSAE	7878	23679	14.74	Jabor *et al.*, 2010.
		OSWE	6271	ND	14.8	
α-pinene (Terpene)	$C_{10}H_{16}$ 136.23	OSAE	7379	ND	14.89	Mass Bank
		OSWE	ND	ND	ND	
Luteolin-7-O-glucuronide (Flavonoids)	$C_{21}H_{17}O_{12}$ 462.07	OSAE	10349	53681	17.30	Lin *et al.*, 2010.
		OSWE	6140	ND	17.71	
Methyl cinnamate (Methyl ester of cinnamic acid	$C_{10}H_{10}O_2$ 162.19	OSAE	12549	ND	17.82	Mass Bank
		OSWE	ND	20474	18.83	
Rosmarinic acid (Phenylpropanoids)	$C_{18}H_{16}O_8$ 360.31	OSAE	55621	59927	18.17	Mass bank
		OSWE	ND	ND	ND	
Apigenin-7-O-glucuronide (Flavonoids)	$C_{21}H_{18}O_{11}$ 446.36	OSAE	98423	154399	18.26	Lin *et al.*, 2010.
		OSWE	25169	75816	18.72	
Eugenol (Phenyl propene)	$C_{10}H_{12}O_2$ 164.20	OSAE	15388	ND	20.08	Mass Bank
		OSWE	ND	20474	20.40	
Carvacrol (Monoterpenoid phenol)	$C_{10}H_{14}O$ 150.22	OSAE	8520	ND	20.05	Mass bank
		OSWE	ND	ND	ND	
Methyl eugenol (Penylpropene)	$C_{11}H_{14}O_2$ 178.09	OSAE	ND	ND	ND	Mass bank
		OSWE	ND	23332	20.18	
Linoleic acid (Unsaturated omega-6 fatty acid)	$C_{18}H_{32}O_2$ 280.44	OSAE	9232	ND	20.61	Mass bank
		OSWE	17870	ND	21.92	
Apigenin (Flavonoids)	$C_{15}H_{10}O_5$ 270.24	OSAE	13048	ND	22.09	Mass bank
		OSWE	ND	ND	ND	
Linalool (Terpene alcohol)	$C_{10}H_{18}O$ 154.24	OSAE	8031	ND	23.01	Mass bank
		OSWE	ND	ND	ND	
Methyl chavicol	$C_{10}H_{12}O$	OSAE	ND	68593	23.13	Mass bank

(Phenylpropene)	148.20	OSWE	ND	54927	23.65	
Cirsimaritin (Flavonoid)	$C_{17}H_{14}O_6$ 314.28	OSAE	10349	ND	23.19	Hossain *et al.,* 2010.
		OSWE	ND	ND	ND	
Cirsilineol (Flavonoids)	$C_{18}H_{16}O_7$ 344.32	OSAE	13293	ND	23.22	Sun *et al.,* 2010.
		OSWE	ND	ND	ND	
Orientin (Flavonoids)	$C_{21}H_{20}O_{11}$ 448.09	OSAE	10702	51037	23.25	Mass bank
		OSWE	ND	ND	ND	
L-ascorbic acid (organic compound)	$C_6H_8O_6$ 176.03	OSAE	ND	105361	23.26	Mass bank
		OSWE	ND	ND	ND	
Sinapic acid (Phenylpropanoids)	$C_{11}H_{12}O_5$ 224.21	OSAE	5978	ND	24.32	Mass bank

Apigenin-7-O-glucuronide, $C_{21}H_{18}O_{11}$ **(**Fig. 4.3 D): It belongs to flavonoid group**.** It detected in both OSAE and OSWE. It was eluted at RT 18.26 min from OSAE and RT 18.72 from OSWE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M-H]^-$ with m/z 445.07 and $[M+H]^-$ with m/z 447. MS fragmentation pattern $[M-H]^-$ were m/z 225, 160, 135, 123, 117 and 105. It has been previously reported by Lin *et al.,* (2010).

Eugenol, $C_{10}H_{12}O_2$ **(**Fig. 4.3 E): It is a member of the phenylpropanoid class of chemical compounds. It is a clear to pale yellow oily liquid can be obtained from essential oils of *O. sanctum* leaves and other medicinal plants. It was detected in both OSAE and OSWE. It yielded the peak at RT 20.08 min from OSAE and RT 20.40 min from OSWE. Identification of the compound was confirmed by mass spectra exhibiting ions $[M-H]^-$ with m/z 163.05 $[M+H]^-$ with m/z 165.12. The mass fragmentation pattern $[M-H]^-$ with m/z are 150, 131, 121, 116 and 104. The detailed information of each peak in the compound is similar to Mass bank record JP009332.

Linoleic acid, $C_{18}H_{32}O_2$ **(**Fig. 4.3 F): It is a polyunsaturated omega-6 fatty acid. It is a colorless liquid at room temperature. Chemically, linoleic acid is a carboxylic acid with an 18-carbon chain and two *cis* double bonds; with the first double bond located at the sixth carbon from the methyl end. It is eluted with RT at 20.61 min from OSAE and 21.92 from

OSWE. Identification of the compound was confirmed by the mass spectrum exhibiting the ions [M –H]- with m/z 279.01 and MS fragmentation pattern of the compound $[M –H]^-$ was of m/z 259, 212, 194, 154 and 131. The identification of the compound was confirmed through Mass bank record MT000114.

Methyl chavicol, $C_{10}H_{12}O$ **(**Fig. 4.3 G): It also called as estragole. It is a phenylpropene, a natural organic compound. It is a colorless liquid and major component of various trees and plants, including *Ocimum sanctum*. It was eluted at RT 23.13 min in OSAE and RT 23.65 min from OSWE, its identification was confirmed by $[M–H]^-$ m/z 149.12. The mass fragmentation pattern $[M–H]^-$ of this compounds are 131, 119, 116, 104 and 103.

Germacrene D, $C_{18}H_{36}O_2$ **(**Fig. 4.3 H): It is volatile hydrocarbon and sesquiterpene in nature. Germacrenes are typically produced in a number of plant species. It was detected in only OSAE negative ion mode. It yielded the peak at 10.60 min from OSAE. Identification of the compound was confirmed by their MS fragmentation spectra exhibiting the ions [M –H]- with m/z 203.08. The fragmentation obtained after MS/MS were m/z of 188, 162, 161, 116. The compound was confirmed by mass bank record JP010679 on the basis of their fragment ions.

α-pinene, $C_{10}H_{16}$ **(**Fig. 4.3 I): It is monoterpene in nature. Identification of the compound confirmed by $[M–H]^-$, peak obtained of m/z 135.19 at RT 14.89 min in OSAE with fragmentation $[M–H]^-$ of m/z 127, 123, 119, 108 and 105. The compound was identified and confirmed through Mass bank record JP005733.

Rosmarinic acid, $C_{18}H_{16}O_8$ **(**Fig. 4.3 J): It is a phentylpropanoid, found in a variety of plants. It has antioxidant properties. It yielded the peak at RT 18.17 min from OSAE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions [M –H]- of m/z 359.07 and $[M+H]^+$ with m/z 361.01. MS fragmentation pattern [M –H]- were m/z 161, 136, 133, 122 and 104. It was confirmed from Mass bank record PR040216.

Carvacrol, $C_{10}H_{14}O$ **(**Fig. 4.3 K): It is a monoterpenoid phenol and has a characteristic pungent smell. It yielded the peak at RT 20.05 min from OSAE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions [M –H]- of m/z 149.05. It's MS fragmentation pattern $[M-H]^-$ with m/z 147, 141, 112 and 111 have been identified through Mass bank record JP000909.

Apigenin, $C_{15}H_{10}O_5$ **(**Fig. 4.3 L): It is found in many plants including *O. sanctum* leaves. It is a natural product belonging to the flavone class that is the aglycone of several naturally occurring glycosides. It is a yellow crystalline solid. It yielded the peak at RT 22.09 min from OSAE. Identification of the compound was confirmed by $[M-H]^-$ with m/z 269.04. The mass fragmentation patterns $[M-H]^-$ of the compound with m/z were 242, 231, 117 and 116. This compound was confirmed through Mass bank record PR020018.

Linalool, $C_{10}H_{18}O$ **(**Fig. 4.3 M): It is a naturally occurring terpene alcohol and chemical found in many plant species and having many commercial applications. It was eluted at RT 23.01 min from OSAE only, whereas it was not detected in OSWE. Identification of the compound was confirmed by their MS-fragmentation spectra exhibiting the ions $[M-H]^-$ with m/z 153.12. Its mass fragmentation pattern $[M-H]^-$ of linalool with m/z are 137, 116, 108 and 101. The detailed information of linalool was confirmed by comparing with Mass bank record JP007560.

Cirsimaritin, $C_{17}H_{14}O_6$ **(**Fig. 4.3 N): It is a flavonoid**.** It yielded the peak at RT 23.19 min in OSAE. The identification of the compound confirmed by its mass spectrum exhibiting the ions $[M-H]^-$ of m/z 313.06, mass fragmentation pattern [M –H]- of the compound were m/z 254, 226, 182, 163 and 135. The compound has been previously reported by Hossain *et al.*, (2010).

Cirsilineol, $C_{18}H_{16}O_7$ **(**Fig. 4.3 O): It is a bioactive flavone. It yielded the peak at RT 23.22 min from OSAE. Identification of the compound was confirmed by the mass spectrum

exhibiting the ions $[M –H]^-$ with m/z 343.04. MS fragmentation pattern [M –H]- were of m/z 298, 284, 257,2 40 and 186. The compounded has been previously reported by Sun *et al.*, (2010).

Orientin, $C_{21}H_{20}O_{11}$ **(**Fig. 4.3 P): It is a flavone, a chemical flavonoid like compound. It yielded the peak at RT 23.25 min from OSAE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M –H]^-$ of m/z 447.08 and $[M+H]^+$ of m/z value 449.12. Its MS fragmentation patterns $[M –H]^-$ with m/z 409, 387, 255, 217, 175 and 133. It has been identified through Mass bank record PR020063.

Sinapic acid, $C_{11}H_{12}O_5$ **(**Fig. 4.3 Q): It is a small naturally occurring hydroxycinnamic acid and belongs to phenylpropanoid family. It is a commonly used as matrix in MALDI mass spectrometry. It yielded the peak at RT 24.32 min from OSAE only. The identification of the compound was confirmed by the mass spectrum exhibiting the ions [M –H]- of m/z 223.02. The compound gave the MS fragmentation pattern [M –H]- with m/z 220, 156, 147, 133 and 122. The compound was confirmed through Mass bank record MCH00015.

Ascorbic acid, $C_6H_8O_6$ **(**Fig. 4.3 R): It is a naturally occurring vitamin C with antioxidant properties. It yielded the peak at RT 23.26 min in OSAE. Identification of the compound was confirmed by $[M+H]^-$ with m/z 177.05. The mass fragmentation pattern $[M+H]^-$ with m/z was 163, 136, 134, 121 and 106. The compound was identified through Mass bank records JP006292.

Stearic acid, $C_{18}H_{36}O_2$ **(**Fig. 4.3 S): is a saturated fatty acid with an 18-carbon chain and has the IUPAC name octadecanoic acid. It also found in other medicinal plants. It yielded the peak at 2.18 min from OSWE in positive ion mode. The compound was identified and confirmed by $[M+H]^+$ with m/z 285.07. The mass fragmentation pattern $[M+H]^+$ with m/z 185, 164, 123, 103 The compound was confirmed by mass bank record KZ000270.

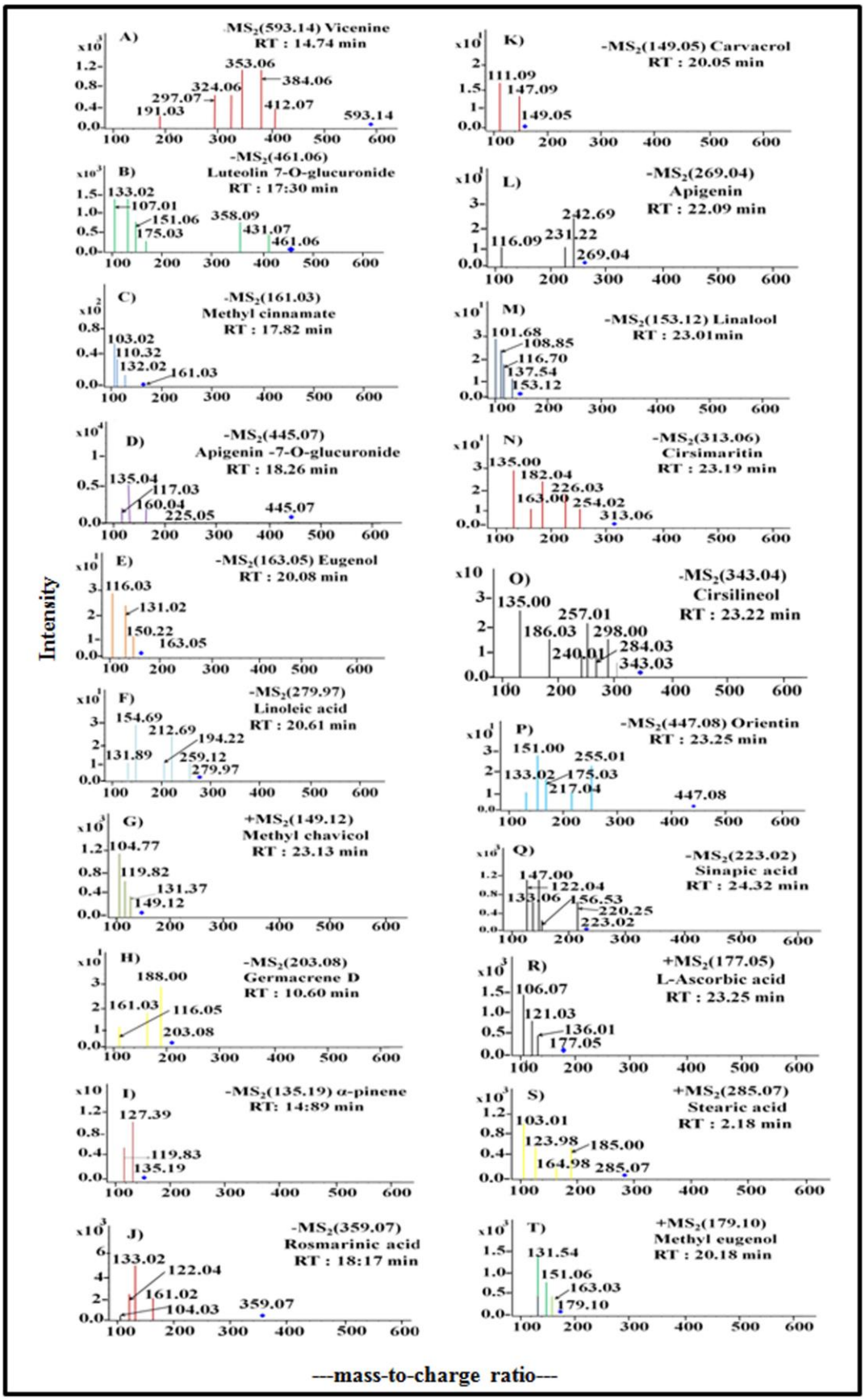


Fig. 4. 3. Negative ion (-) and positive ion (+) ESI/MS/MS of alcoholic and water extracts of OS dry leaves. Labeled peaks are corresponding to the fragments.

Methyl eugenol, $C_{11}H_{14}O_2$ (Fig 4.3 T): a phenylpropene, a type of phenylpropanoid compound, the methyl ether of eugenol. It is found in various essential oils. It yielded the peak at RT 20.18 min in OSWE. Identification of the compound was confirmed by [M –H]- with m/z 179.10. The mass fragmentation pattern is $[M-H]^-$ with m/z 163, 151, 120, 117, 115, 106 and 104. The detailed information of each peak in the compound is similar to Mass bank record JP000747.

LC-Q-TOF-MS-based metabolite profiling of HI root extracts

In the present study, LC-Q-TOF-M/MS was employed for metabolite fingerprinting of the plant extracts. HI root extracts (HIAE/HIWE) have several bioactive compounds like compounds terpenoids, aromatic acids, coumarinolignoids, phytosterols and many others. Data was acquired in both negative and positive ion modes in both type of extracts. Negative ion and positive ion modes of LC-QTOF-MS detected 7 and 12 compounds in HIAE; 4 and 7 compounds in HIWE respectively. Their abundance and RT values in minute are shown in Table 4.2. Both HIAE and HIWE contained some of the compounds in common, like Chlorogenic acid, lupeol acetate, hexatriacontane, nerolidol, hemidesmin 1, hemidesmin 2, hemidesminine, β-sitosterol. 2-hydroxy-4-methoxy benzoic acid, emidine, lupanone, heminine, desinine, rutin, hemidine were exclusively detected in HIAE, while 4-terpineol was detected only in HIWE. MS-MS spectra of the compounds detected in HIAE and/or HIWE are shown in (Figs. 4.4).

Chlorogenic acid, $C_{16}H_{18}O_9$ (Figs. 4.3. A): It is a natural chemical compound which is the ester of caffeic acid. It yielded the peak at RT 9.14 min from HIAE and RT 9.36 min in HIWE. Identification of the compound confirmed by $[M –H]^-$ with m/z 353.10. The mass fragmentation pattern $[M-H]^-$ of the compound were m/z 192, 179, 135, 127. This compound was confirmed through Mass bank record JP000136.

Table 4.2. Q-TOF profile of positive and negative ion mode of HIAE and HIWE used in LC-MS/MS analysis

Compound name/ class of compound	Mol formula/ Exact mass	Types of extracts	Abudance		RT min	Reference
			-ESI	+ESI		
Chlorogenic acid (ester of caffeic acid)	$C_{16}H_{18}O_9$ (354.31)	HIAE	6211	10844	9.14	Mass bank
		HIWE	962	ND	9.36	
Lupeol acetate (Triterpenoid)	$C_{32}H_{52}O_2$ (468.75)	HIAE	13688	48472	9.96	Nasser *et al.*, 2009
		HIWE	5137	15668	9.38	
Hexatriacontane (saturated aliphatic hydrocarbon)	$CH_3(CH_2)_{34}CH_3$ 506.97	HIAE	8638	22761	10.68	Ananthakrishnan *et al.*,1991
		HIWE	9175	ND	10.32	
4-terpineol (monoterpene alcohol)	$C_{10}H_{18}O$ 154.14	HIAE	ND	ND	ND	Mass bank
		HIWE	17206	33185	11.82	
Nerolidol (sesquiterpene)	$C_{15}H_{26}O$ (222.37)	HIAE	ND	45402	12.87	Mass bank
		HIWE	ND	26896	13.11	
2-hydroxy-4-methoxy benzoic acid (aromatic acid)	$C_8H_8O_4$ 168.05	HIAE	150170	ND	12.92	Mass bank
		HIWE	ND	ND	ND	
Hemidesmin 1 (coumarinolignoid)	$C_{21}H_{20}O_9$ (416.11)	HIAE	ND	16715	13.62	Das *et al.*,1992
		HIWE	ND	6937	13.64	
Hemidesmin 2 (coumarinolignoid)	$C_{20}H_{18}O_8$ (386.10	HIAE	ND	19521	13.86	Das *et al.*,1992
		HIWE	19564	ND	13.21	
Hemidesminine (coumarinolignoid)	$C_{23}H_{22}O_8$ (424.12)	HIAE	ND	15676	16.78	Mandal *et al.*, 1991
		HIWE	ND	18386	16.47	
β-sitosterol (phytosterol)	$C_{29}H_{50}O$ (414.71)	HIAE	ND	161233	18.23	Mass bank
		HIWE	ND	144272	17.84	
Emidine (pregnane glycosides)	$C_{39}H_{64}O_{12}$ 724.40	HIAE	4984	ND	18.31	Chandra *et al.*,1994
		HIWE	ND	ND	ND	
Desinine (glycoside)	$C_{37}H_{58}O_{12}$ 694.37	HIAE	ND	13292	19.14	Oberai *et al.*,1985
		HIWE	ND	ND	ND	
Rutin (glycoside)	$C_{27}H_{30}O_{16}$ (610)	HIAE	ND	69084	21.98	Mass bank
		HIWE	ND	ND	ND	
Lupanone (triterpene)	$C_{30}H_{48}O_4$ 472.33	HIAE	14237	ND	22.16	Ashim *et al*., 2013
		HIWE	ND	ND	ND	
Hemidine (pregnane glycoside)	$C_{27}H_{44}O_6$ (464)	HIAE	ND	30174	23.08	Prakash *et al.*,1991
		HIWE	ND	ND	ND	
Heminine (pregnanae glycoside)	$C_{34}H_{56}O_9$ (608.44)	HIAE	16573	20555	25.19	Sigler *et al.* 2000
		HIWE	ND	ND	ND	

Lupeol acetate, $C_{32}H_{52}O_2$ (Figs. 4.3 B): It is terpenoid in nature. It was eluted at RT 9.96 min in HIAE and RT 9.38 min from HIWE. Identification of the compound was confirmed by their the MS fragmentation spectra exhibiting the ions $[M-H]^-$ with m/z 467.09 and the

fragmentation ions $[M-H]^-$ at m/z 192, 179, 135, 127. The compound was identified and confirmed on the basis of fragmentation pattern which has been published by Nasser *et al.,* (2009).

Hexatriacontane, $CH_3(CH_2)_{34}CH_3$ (Fig. 4.4 C): It is a saturated aliphatic hydrocarbon. It yielded the peak at RT 10.68 min in HIAE and RT 10.32 min from HIWE. The identification of the compound confirmed by its mass spectrum exhibiting the ions $[M-H]^-$ with m/z 505.17 and the mass fragmentation patterns $[M-H]^-$ of the compound were m/z 253, 152, 136, 123 and the compound was confirmed from Ananthakrishnan *et al.,*1991.

Nerolidol, $C_{15}H_{26}O$ (Fig. 4.4 D): It is also called as peruviol. It is a naturally occurring sesquiterpene found in the essential oils of many types of plants and flowers including *H. indicus* roots. It yielded the peak at RT 12.87 min from HIAE and RT 13.11 min from HIWE. Identification of the compound was confirmed by the mass spectrum exhibiting the ions [M+H] with m/z 223.06 and of mass fragmentation pattern $[M+H]^-$ with m/z 207, 198, 179 and 137. It is confirmed through Mass bank record JP007724.

Hemidesmin 1, $C_{21}H_{20}O_9$ (Fig. 4.4 E): It is a coumarinolignoid and it yielded the peak at RT 13.62 min from HIAE and RT 13.64 min from HIWE. Identification of the compound is confirmed by the mass spectrum exhibiting the ions [M+H] with m/z 417.12 and mass fragmentation pattern of $[M+H]^+$ with m/z 194, 181, 167 and 158. The compound has been identified by Das *et al.,* 1992.

Hemidesmin 2, $C_{20}H_{18}O_8$ (Fig. 4.4 F): It is a coumarinolignoid. It yielded the peak at RT 13.86 min from HIAE and RT 13.21 min from HIWE. Identification of the compound confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ with m/z 417.12. Mass fragmentation pattern of this compound were $[M+H]^+$ with m/z of 353, 251, 147, 119. The identification of compound was confirmed through Das *et al.,* 1992.

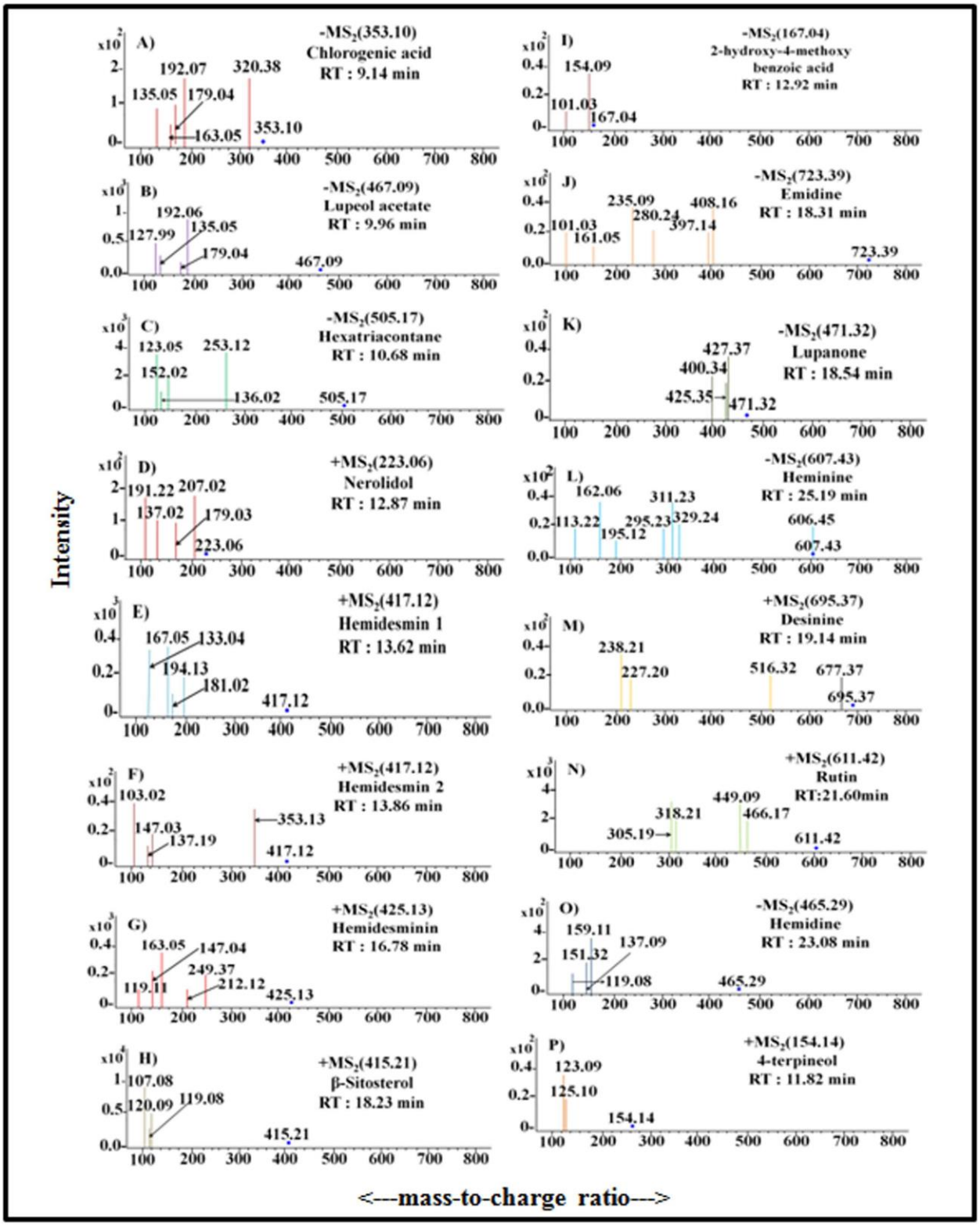


Fig. 4.4. Negative ion (−) and positive ion (+) ESI/MS/MS of alcoholic extracts (HIAE) and water extracts (HIWE) of HI root extracts. Product ion labels correspond to MS fragments

Hemidesminine, $C_{23}H_{22}O_8$ (Fig. 4.4 G): It yielded the peak at RT 16.78 min from HIAE and RT 16.47 min from HIWE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ of m/z 425.13. The compound gave the MS fragmentation pattern $[M+H]^+$ with m/z 249, 212, 163, 147 and 119. It is a coumarinolignoid and has been previously reported by Mandal *et al.,* (1991).

β-sitosterol, $C_{29}H_{50}O$ (Fig. 4.4 H): It is one of several phytosterols (plant sterols). It was eluted at RT 18.23 min from HIAE and RT 17.24 min from HIWE. Identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ with m/z 415.21. MS fragmentation pattern $[M+H]^+$ of the compound were m/z 120, 119, 113, 111and 113. The compound was identified through mass bank record FFF00064.

2-hydroxy-4-methoxy benzoic acid, $C_8H_8O_4$ (Fig. 4.4 I): It is an aromatic acid. It is eluted with RT at 12.92 min from HIAE. Identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M-H]^-$ with m/z 167.04 and MS fragmentation pattern $[M-H]^-$ of the compound were of m/z 154, 143 and 101. The compound was identified and confirmed by Mass bank record JP011443.

Emidine, $C_{39}H_{64}O_{12}$ (Fig. 4.4 J): It s a pregnane glycoside. It yielded the peak at RT 18.31 min from HIAE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M-H]^-$ with m/z 723.29. It's MS fragmentation pattern $[M-H]^-$ with m/z 408, 347, 280, 235, 168 and 101.The compound also has previously been characterised by Chandra *et al.,(*1994).

Lupanone, $C_{30}H_{48}O_4$ (Fig. 4.4 K): It yielded the peak at RT 22.16 min in HIAE. Identification of the compound $[M-H]^-$ was confirmed with m/z 471.32. The mass fragmentation pattern $[M-H]^-$ with m/z were 427, 425 and 400. The compound is triterpene in nature and it was previously been reported by Ashim *et al.,* (2013).

Heminine, $C_{34}H_{56}O_9$ (Fig. 4.4 L): yielded the peak at RT 25.19 min from HIAE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M-H]^-$ of m/z 607.43.The compound gave the MS fragmentation pattern [M–H]- with m/z 606, 329, 311, 295, 195, 162 and 113 and has previously been characterised by Sigler *et al.,* (2000). It is a pregnanae glycoside.

Desinine, $C_{37}H_{58}O_{12}$ (Fig. 4.4 M): It is a glycoside in nature. It yielded the peak at RT 19.14 min from HIAE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ of m/z 695.37. It's MS fragmentation pattern $[M+H]^+$ with m/z were 677, 516, 238 and 227. The compound was confirmed which was previously identified by Oberai *et al*., (1985).

Rutin, $C_{27}H_{30}O_{16}$ (Fig. 4.4 N): It also called rutoside and it is the glycoside between the flavonol quercetin and the disaccharide rutinose. It yielded the peak at RT 21.98 min in HIAE. Identification of the compound was confirmed $M+H]^+$ by m/z value 611.42.The mass fragmentation pattern $M+H]^+$with m/z 466, 449, 318 and 305 the detailed information of mentioned peak in the compound is similar to Mass bank record PR020080.

Hemidine, $C_{27}H_{44}O_6$ (Fig. 4.4 O): It is another pregnane glycoside found in *H. indicus* roots. It yielded the peak at RT 23.08 min from HIAE. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M-H]^-$ of m/z 465.29. MS fragmentation pattern $[M-H]^-$ with m/z 3665,159,151,137 and 119 and has been previously reported by Prakash *et al.*, (1991).

4-terpineol, $C_{10}H_{18}O$ (Fig. 4.4 P): It is a naturally occurring monoterpene alcohol that has been isolated from a variety of medicinal plants including *H. indicus* roots. It was eluted at RT 11.82 min from HIWE. Identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ with m/z 155.14. MS fragmentation patterns $[M+H]^+$

of the compound were m/z138,125, 123 and 108. The compound was confirmed through Mass bank record JP005329.

Total antioxidant capacity

The antioxidant capacity of alcoholic and water extracts of selected plants was influenced by various factors, such as composition of the extract and test system. A reliable standard protocol required to measure the antioxidant property of tested plant material because most secondary metabolites are multifunctional. In this study, ammonium molybdate reagent method was used to measure antioxidant activity. The total antioxidant capacity of the extract was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at λ 695 nm.

Total antioxidant capacity of alcoholic and water extracts (OSAE and OSWE) of OS dry leaves showed to be 262.25 ± 2.1 mg AAE/g dwt and 176.44 ± 3.1 mg AAE/g dwt respectively.While, HIWE showed high antioxidant activity (424 ± 1.6 mg AAE/g dwt) than HIAE (335 ± 1.2 mg AAE/g dwt) as shown in Fig. 4.5 A. Ascorbic acid pure compound was used as a standard (Fig. 4.5 B).

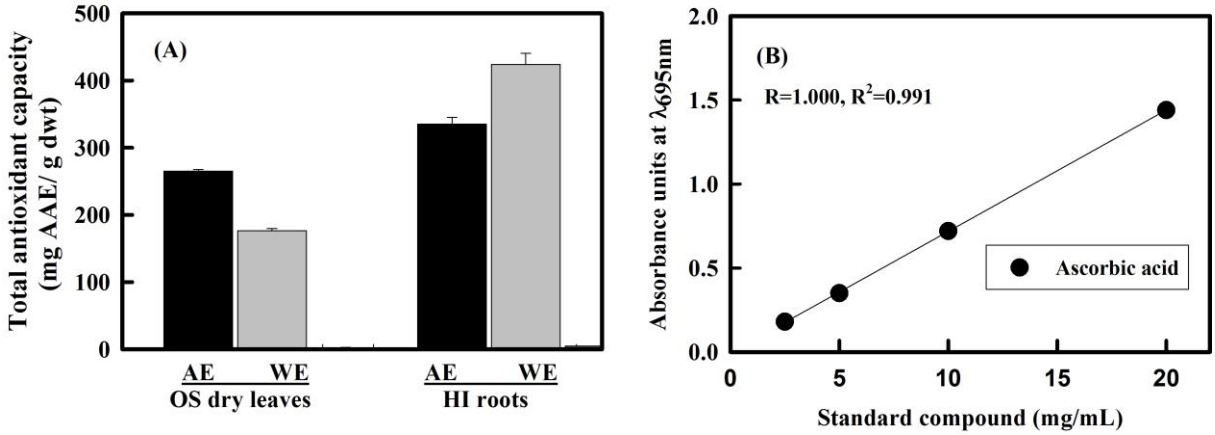


Fig. 4.5. (A) Total antioxidant capacity of alcoholic (AE) and water extracts (WE) of dry leaf of *O. sanctum* and *H. indicus* roots. Data are presented as mean value ± standard deviation SD (n = 6). (B) Pure compound ascorbic acid at varied concentration was used as a standard.

Reducing power activity

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron. It is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which then reacts with ferric chloride to form ferric-ferrous complex (Perl's Prussian blue colored complex) that has an absorption maximum at λ 700 nm. The reducing properties associated with the presence of compounds exert their action by breaking the free radical chain through donating a hydrogen atom. The reductive capabilities of the alcoholic and water extracts of OS dry leaves and HI roots were determined. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity also.

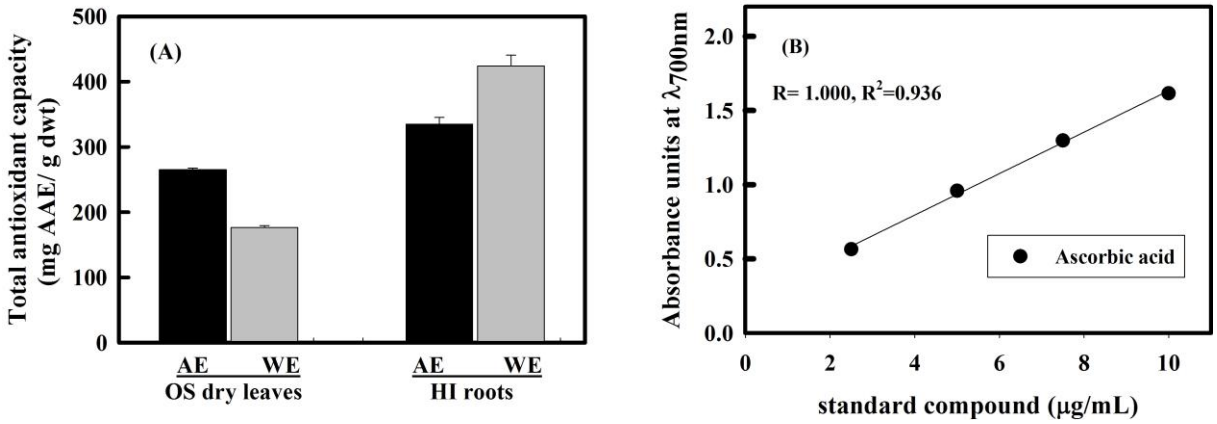


Fig. 4.6. (A) Reducing power activity of different extracts of dry leaf of *O. sanctum* and roots of *H. indicus.* Each value represents a mean ± SD (n=3). AE, Alcoholic extracts; WE, water extracts. (B) Pure compound ascorbic acid was used as standard.

Reducing power activity of alcoholic and water extracts of *O. sanctum* dry leaves are 54 ± 1.5 mg AAE/g dwt and 100.44 ± 3.1 mg AAE/g dwt respectively. However there is no difference observed among alcoholic and water extracts of *H. indicus* roots. HIAE showed 34.23 ± 1.7 mg AAE/g dwt, whereas HIWE expressed 31.89 ± 2.7 mg AAE/g dwt as shown in Fig. 4.6 A. Ascorbic acid at different concentrations was used for preparation of standard

curve (Fig 4.6. B). Among the two selected plants, water extracts (OSWE) of *O. sanctum* showed higher potential of reducing power activity compared to other extracts.

Free radical scavenging assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

It is the best-known, accurate and frequently employed methods for evaluating free radical scavenging properties of plant extracts. DPPH is nitrogen centered free radical that shows strong absorbance at λ 517 nm. It contains an odd electron, which is responsible for the absorbance at λ 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolourised, which can be quantitatively measured from the changes in absorbance. Extent of DPPH radical scavenged was determined by the decrease in intensity of orange color and IC_{50} value for the selected plant extracts. The lower IC_{50} indicates the stronger ability of the extract to act as DPPH radicals scavengers. Both alcoholic and water extracts of OS dry leaves and HI roots scavenged DPPH radical significantly in a dose dependent manner (Fig. 4.7). Among them, OSAE showed much higher activity compared to OSWE in their capacity of scavenging the DPPH radical. OSAE scavenged 95 % of DPPH radical with the IC_{50} being 2.28 µg dwt/mL, where as OSWE also scavenged up to 93% and its IC_{50} values 15 µg dwt/mL (Fig. 4.7 A).

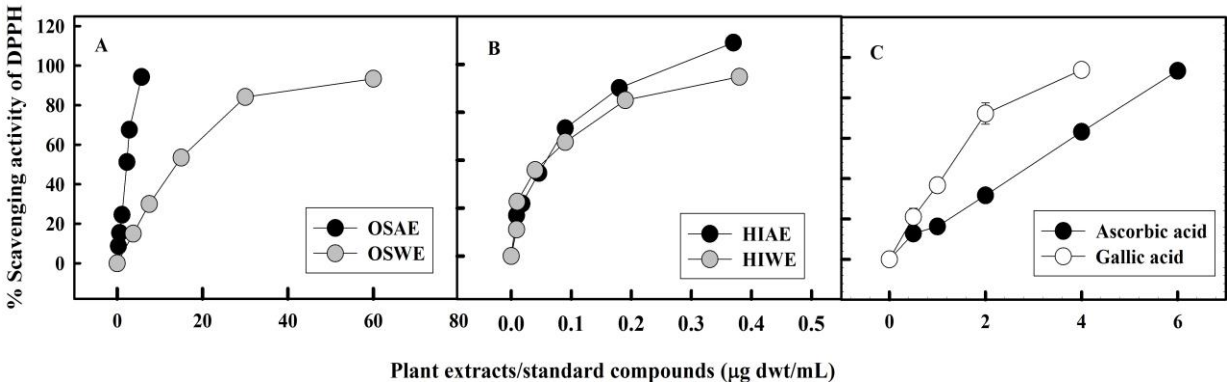


Fig. 4.7. DPPH scavenging activity of alcoholic (AE) and water extracts (WE) of (A) dry leaf of *O. sanctum*; (B) root extracts of *H. indicus* and (C) standard compounds ascorbic acid and gallic acid. Data presented are mean ± S.D., *n=6*. All values are statistically different ($p < 0.001$).

Similarly, HIAE and HIWE also scavenged DPPH radical up to 90 % in a dose dependent manner. IC_{50} values of HIAE and HIWE were 92.5 µg dwt/mL and 96.88 µg dwt/mL respectively (Fig. 4.7 B). Pure compound ascorbic acid was used as a reference and it also showed their inhibition towards DPPH radical in concentration dependent way. The IC_{50} value of ascorbic acid was found to be 3.1 µg/mL (Fig. 4.7 C).

Hydrogen peroxide (H_2O_2) scavenging assay

Hydrogen peroxide (H_2O_2) is a biologically relevant, non-radical reactive oxygen species (ROS). It is inevitably generated as a by-product of normal aerobic metabolism. When present in excess under stress conditions, H_2O_2 could be injurious for cells and furthermore as it can be converted into other ROS, such as hydroxyl radicals. H_2O_2 scavenging activity of the plant extracts was assayed by using Xylenol orange reagent method (FOX reagent) in alcoholic and water extracts of selected medicinal plant extracts. OSAE and OSWE showed biphasic mode in terms of H_2O_2 scavenging activity. OSAE showed maximal scavenging of 80 % with IC_{50} 220 µg dwt/mL and OSWE expressed maximal scavenging activity upto 90 % with IC_{50} of 450 µg dwt/mL (Fig. 4.8 A).

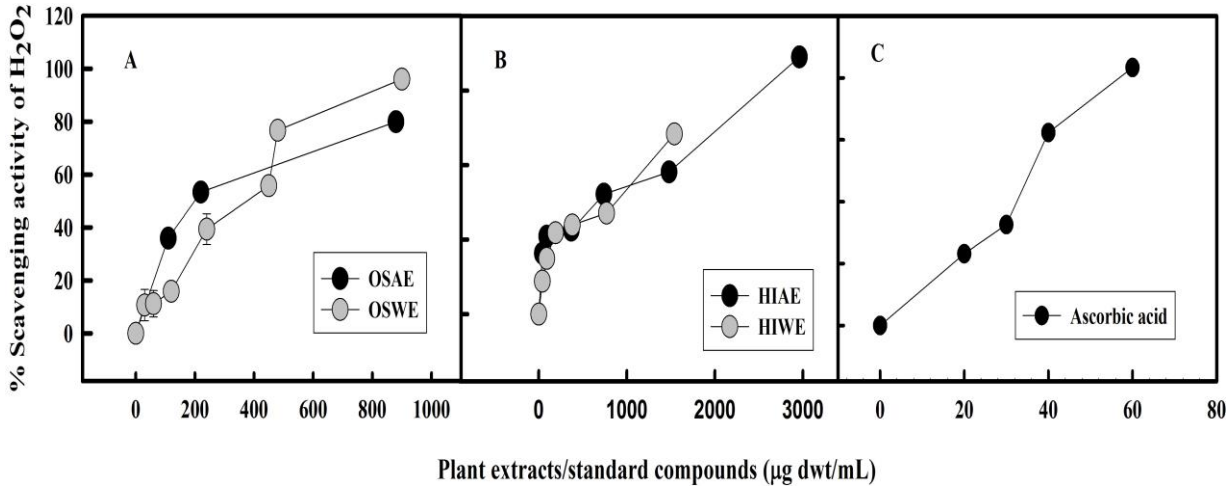


Fig. 4.8. Hydrogen peroxide scavenging activity of alcoholic (AE) and water extracts (WE) of *O. sanctum* dry leaf (A); *H. indicus* rot extracts (B) and standard compound (C). Data presented are mean ± S.D., *n=6*. All values are statistically significant #$p < 0.001$ vs. Control.

Similarly, in case of *H. indicus* roots, both types of extracts showed significant H_2O_2 scavenging activity in dose dependent manner. Alcoholic extract (HIAE) showed maximal inhibition of 70% with IC_{50} 2.1 mg dwt/mL and HIWE showed maximal scavenging capacity of only 50 % and expressed its IC_{50} at 1.5 mg dwt/mL (Fig. 4.8. B). Among the two selected plants, OS dry leaves showed potential effect for scavenging of H_2O_2 compared to HI root extracts. Pure compound ascorbic acid was used as a reference compound. It showed their H_2O_2 scavenging property in a concentration dependent manner. Ascorbic acid showed its maximum scavenging potential 83% and IC_{50} at 32.25 µg/mL (Fig. 4.8 C).

Effect of OS dry leaves and HI-roots on ferric chloride induced lipid peroxidation in rat liver and heart tissue homogenates of male Wistar rats (*in vitro* study)

Biological membranes are a rich source of polyunsaturated fatty acids that are susceptible to lipid peroxidation, in the presence of metal ions and other prooxidants. Lipid peroxidation has been identified as one of the basic reactions involved in free radical induced cellular damages. The inhibitory effect on lipid peroxidation of selected plant species was evaluated using liver and heart homogenate of male Wistar rats which provide an ideal model system. Peroxidation of lipid was stimulated with the ferric chloride ($FeCl_3$) (*in vitro*). Malonaldehyde produced during peroxidation reacts with thiobarbituric acid (TBA) reagent to form a pink colored product which has an absorption maximum at λ 532 nm. The amount of TBARS was found to be very high throughout the experimental period in the control group (without plant extract). A drastic reduction in TBARS was observed in the plant extracts treated groups when compared to control groups.

Lipid peroxidation in liver homogenate

Both alcoholic and water extracts of *O. sanctum* dry leaves (OSAE and OSWE) inhibited $FeCl_3$ induced peroxidation in liver homogenates in a dose dependent manner. OSAE showed maximum inhibition by 90 % at 13.71 µg dwt/mL with an IC_{50} value of 0.5 µg dwt/mL.

Whereas OSWE showed maximum inhibition up to 83 % at 11 µg dwt/mL and expressed IC_{50} values 5 µg dwt/mL as show in Fig. 4.9. A. Similarly, HIAE showed maximum inhibition 91.8% at 0.2 mg dwt/mL with an IC_{50} values 30 µg dwt/mL. However, HIWE (0.01 mg dwt/mL) showed maximum inhibition of 78% with IC_{50} values 270 µg dwt/mL (Fig. 4.9 B). Ascorbic acid and gallic acid was used as positive controls (Fig. 4.9 C). Both the pure compounds exhibited maximum inhibition of 90 % and its IC_{50} values 10 µg/mL and 7.5 µg/mL respectively.

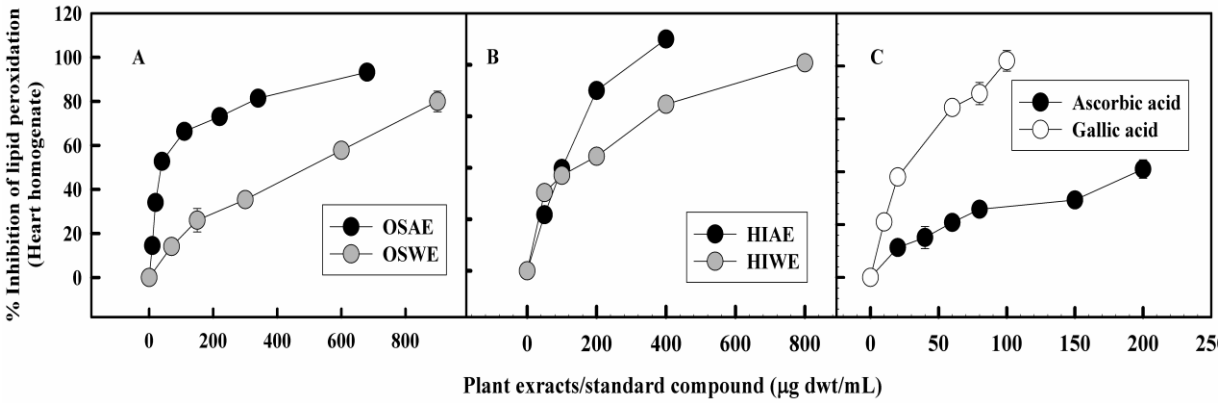


Fig. 4.9. Inhibitory effect of alcoholic (AE) and water extracts (WE) of selected plant extracts or standard compounds on ferric chloride induced lipid peroxidation in rat liver tissue homogenates of male Wistar rats. (A) *O. sanctum* dry leaves (B) *H. indicus* roots nd (C) standard compounds ascorbic acid and gallic acid. Data presented are mean ± S.D., n=6. . All values are statistically significant #p < 0.001 vs. Control.

Lipid peroxidation in heart homogenate

Alcoholic and water extracts of two selected plants inhibited the peroxidation of lipids in heart homogenate of male Wistar rats in a dose dependent manner like liver homogenate (Fig. 4.10). OSAE showed maximum inhibition of 90 %, where as OSWE showed up to 79 % (Fig. 4.10 A). IC_{50} of these extracts were 40 µg dwt/mL and 600 µg dwt/mL respectively. The extracts showed lower effect in inhibiting peroxidation in heart tissue homogenates compared to liver tissue homogenates. Simillarly, alcoholic extracts of HI roots exhibited a maximum inhibitory effect of 90 % at 0.4 mg dwt/mL with IC_{50} values 140 µg dwt/mL, where as HIWE exhibited maximum inhibition up to 80 % at 0.8 mg dwt/mL with IC_{50} values 200 µg/dwt mL (Fig. 4.10 B). Pure compounds, ascorbic acid and gallic acid were used as reference

compounds and inhibit the peroxidation of lipid in heart homogenate in a dose dependent manner and expressed their IC_{50} values 170 µg/mL and 40 µg/mL respectively (Fig. 4.10 C).

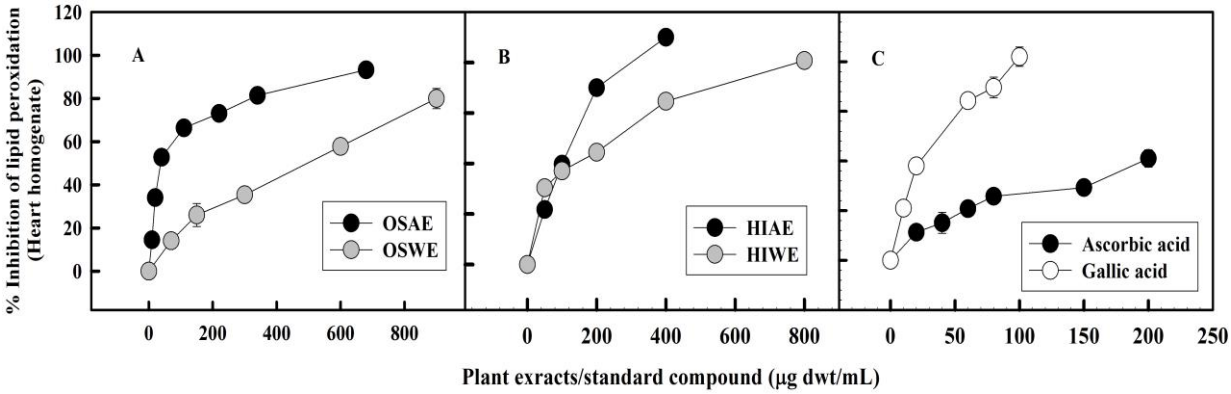


Fig. 4.10. Ferric chloride induced antilipid peroxidation activity of alcoholic (AE) and water extracts (WE) of *O. sanctum* dry leaves (A), *H. indicus roots* (B) and standard compounds (C) in heart homogenate of male Wistar rats. Data presented are mean ± S.D., n=6. All values are statistically significant # $p < 0.001$ vs. Control.

Discussion

As per the scientific literature survey, it has been reported that the interest in the use of different medicinal plant extracts is increasing very rapidly in developing and developed countries for maintaining primary health care, because they are considered as a natural and rich source of antioxidants (Pal and Shukla, 2003). Plant extracts and their isolated and purified constituents have always been an important part of various therapeutic systems in ethanomedicine (Vanitha and Kathiravan, 2006). A part from that, WHO survey also indicated that, about 70 –80% of the world's population totally or partially rely on non conventional medicine, mainly of herbal source (Chan K, 2003). The medicinal plants are rich sources of phenolic, flavonoids and other secondary metabolites. These agents have ability to scavenge free radicals, super oxide and hydroxyl radicals, etc thus automatically they develop strong immunity and antioxidant defense in human body. The therapeutic potential of natural medicinal plants as an antioxidant in reducing such free radical induced tissue injury, suggests that many plants can be therapeutically useful (Kanatt *et al.*, 2007). But for further confirmation and acceptance of any medicinal plant or their constitutents into

scientific medicine, it is highly necessary and mandatory to evaluate the effectiveness and safety of the selected medicinal plant through preclinical and clinical studies. In the present study, two plants were selected on the basis of their wide ethnomedicial uses in India and other countries. Further, we determined the free radical scavenging potential, antioxidant activity and estimate of total phenolic and flavonoid content of selected medicinal plants and construct metabolite profile of the extracts. To maximize the extractive capability of phenolic and flavonoids components from plant material is considerably depended on the type of solvent. Water and alcohol were selected as the extraction solvents since both are commonly used in the food industries in a variety of ways and are more highly stable in the human body than any other solvents.

Phytochemical analysis and antioxidant activity of OS dry leaves and HI roots

In traditional medicine, *O. sanctum* leaves are found to be used for a broad range of health benefits including cardiac health. Commercially available herbal products of *O. sanctum* (e.g.Tulsi by Kirpal Export Overseases, India; Dry herbal extracts by Nikita Extracts, India; *Ocimum sanctum*-Beadlet by Borion Canada Inc., Canada) are known majorly for their cardiac health benefits. Simillarly, a review of prominent literature survey suggests that root extract of *H. indicus* also have wide range of health benefits and it has been used for strong immunity and especially used for blood purification (Nagarajan *et al.,* 2001). Scientific reports clearly reveals that certain polyherbal drugs of HI roots like AROGH, Jwarhar mahakashay are well known for their antiinflammatory, analgesic, antihepatocarcinogenic and antipyretic properties in animal model (Mohan *et al.,* 2009; Gupta *et al.,* 2010; Galhena *et al.,* 2012). In the present study we have compared the antioxidant activity and antilipid peroxidation activity of alcoholic and water extracts and also estimated the secondary metabolites like polypehnols and flavonoids in both modes of extracts of selected plants.

Phenolics are aromatic secondary plant metabolites and major class of bioactive compounds extensively distributed throughout the plant kingdom and acting as primary antioxidants or free radical terminator (Parajuli *et al.,* 2012). A part from that, it also confirmed that, polyphenolics display important role in stabilizing lipid oxidation that is associated with its antioxidant activity because of their hydroxyl group (Gulcin *et al.,* 2005). This phenolic compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are diversified (Naczk and Shahidi, 2004) and they are synthesized by plants due to plant adaptation in response to biotic and abiotic stresses. Plant materials rich in phenolics are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food (Kahkonen *et al.,* 1999). Thus it was reasonable to determine their total amount of phenolic content in selected plant extracts. The phenol content depended on the solvent used and its polarity what we are using. The recovery of polyphenols from plant materials is influenced by their solubility in the extraction solvent, the type of solvent, the degree of polymerization of phenols, the interaction of phenols with other plant constituents and the formation of insoluble complexes. In the present study, we used bio friendly solvents, water and ethanol for preparing medicinal plant extracts. Among the studied plant extracts, there is a variation in total phenolic content ranging from 51 to 100 mg GAE/ dry wt in both alcoholic and water extracts of OS dry leaves and HI roots. In case of OS dry leaves, alcoholic extracts (OSAE) contained higher content of phenolics compared to water extract (Fig. 3.1 A). However, in case of HI roots , both types of extracts (HIAE/HIWE) showed more or less same content of phenol per gram tissue material (Fig. 3.1 B). These plant extracts with higher total phenol content showed higher antioxidant activities which indicated the correlation between total antioxidant activity and total phenolic content present in the plant extracts.

In addition to phenolic compounds of plants fall into various groups, among these flavonoids are well familiar and important phytoconstitutents. Flavoniods are one of the most diverse and widespread subgroup of phenolic compounds are probably the most important natural phenols occurring in plant and are thought to have positive effects on human health. These compounds posses a broad spectrum of chemical and biological activities including radical scavenging properties and antioxidant properties (Nunes *et al.,* 2012). The antioxidant properties of flavonoids depends on their structure, particularly hydroxyl position in the molecule and their ability as a electron donor to a free radical (Parajuli *et al.,* 2012). A part from that, studies on flavonoidic derivatives have shown other pharmacological activiteies in animal model like antiinflammatory, anticancer, and anti allergic activities (Montoro *et al.,* 2005). The review of epidemiological and mechanistic studies supports the role of flavonoids, in protecting the cardiovascular system (Grassi *et al.,* 2009). In this regard, many studies have shown that flavonoids demonstrate protective effects against the initiation and progression of atherosclerosis. In particular, it has been shown that the consumption of flavonoids limits the development of atheromatous lesions and inhibiting the oxidation of low density lipoprotein which is considered a key mechanism in the endothelial lesions occurring in atherosclerosis (Masella *et al.,* 2001). Among the plant extracts in the present study, there is a variation in total flavonoid content in both alcoholic and water extracts of OS dry leaves and HI roots in the range of 4 to 6 mg WE/g dwt. Both the plant materials in different extracts showed more or less same amount of flavonoid contents.

LC-MS/MS analysis revealed the presence of several bioactive compounds like eugenol, rosmarinic acid, vicenin, orientin, apigenin, α-pinene, carvacrol, stearic acid and linoleic acid in both OSAE and OSWE which are mostly, flavonoids, terpenpoids and phenolics in nature (table 3.1). Out of which some compounds like eugenol, methyl eugenol, orientin, are reported to show antioxidant and a free radical scavenging activities (Hou *et al.,*

2001, Wu *et al.*, 2009**)**. LC-MS analysis with HI root extracts showed the presence of several phytoconstitutents in HIAE and HIWE. The selected compounds from the above extracts of HI roots are coumarinolignoids, sesquiterpene, phytosterols in nature. Study shown that, some compounds namely 2-hydroxy-4-methoxy benzoic acid, β-sitosterol from HI roots shown for their different pharmacological activities (Alam and Gomes, 1998; Loizou *et al.*, 2010).

The antioxidant capacity of prepared plant extracts were measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. Antioxidants play very important roles in tissue repair as they significantly prevent tissue damage that stimulates wound healing process. With the increase in evidence that indigenous antioxidants may be useful for the management of disorders due to oxidative stress, there is also increase in interest in natural antioxidants present in herbs and medicinal plants for their biochemical function. Here also we compared the antioxidant activity of both alcoholic and water extracts of selected plants. OSAE showed higher antioxidant activity compared to OSWE as shown in Fig. 3.5 A, whereas HIAE and HIWE of HI roots showed more antioxidant activity compared to OS dry leaf extracts (Fig 3.5 B). These variation of antioxidant expression of two selected plants totally depends up on the type of chemical constituents and their environmental factors. Similarly both selected plants also showed strong reducing power potential in both types of extracts, however, OSWE extracts showed more reducing power activity compared to other types of extracts (Fig. 3.6 A and B). These assays indicate that the selected plant extracts could be significant source of natural antioxidants which might be helpful in preventing the progression of various oxidative stress.

` DPPH is considered as one of the compounds that possess a proton free radical and shows a maximum absorption at λ 517 nm. When DPPH encounters proton radical scavengers, its purple colour fades rapidly. This assay determines the scavenging of stable radical species of DPPH by antioxidants in medicinal plant extracts A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound or plant extracts under test. In the present study both OS dry leaves and HI roots showed 90% scavenging of DPPH radical and showed their effect in a dose dependent manner. However on the basic of IC_{50} values, both extracts of OS dry leaves (OSAE/OSWE) showed more scavenging potential of DPPH compared to HI root extracts (Fig. 3.7 A and B). Generally, hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms and food (Gulcin *et al.*, 2005). H_2O_2 is immediately decomposed into oxygen and water and this may produce hydroxyl radicals (•OH) that can initiate lipid peroxidation and cause DNA damage which leads to several chronic diseases in human body. Here we have tested both alcoholic and water extracts of OS dry leaves and HI roots at different concentrations. Both selected plant extracts showed H_2O_2 scavenging activity in dose dependent manner (Fig, 3.8 A and B). However, OSAE and OSWE showed potent effect in scavenging the H_2O_2 compared to HI root extracts. The IC_{50} values of HI root extracts are expressed in milligram level (mg), whereas OS dry leaves (OSAE/OSWE) expressed their IC_{50} values in micro gram level (µg).

The plant extracts, both OS dry leaves and HI roots inhibited up to 90 % of ferric chloride induced lipid peroxidation in rat liver and heart tissue homogenates of male Wistar rats (Fig. 3.9 and 3.10). In case of inhibiting liver and heart lipid peroxidation, alcoholic extract of both plants are more effective than water extracts. HIAE of HI roots showed more potent compared to HIWE to inhibit the peroxidation of lipid in liver homogenates. Whereas , in case of heart homogenate, both HIAE and HIWE showed equal response in inhibiting lipid

peroxidation (Figs. 3.10. B). Overall, on the basic of calculated IC_{50} values, from the above selected plants, it can be concluded that, OS dry leaves can be considered as best plant parts to inhibit the peroxidation of lipid in liver and heart homogenate of male Wistar rats (*in vitro*) compared to HI roots. The difference can be assumed because of the types and nature of the compounds present in both plant materials.

Chapter 5

RESULTS and DISCUSSION

Objective 2: Evaluation of antiinflammatory and/or antioxidant properties of OS and HI extracts in human monocytic (THP-1) cells

Objective 2

Results and Discussion

Results

Effect of medicinal plant extracts on cell viability

Toxic effect of selected plant extracts can be evaluated using MTT test. MTT is designed to be used for the quantification of both cell proliferation and cell viability in cell population using 96-well plate format. In the present study we applied the MTT test to evaluate the biosafety of the various modes of dry leaf extracts of *O. sanctum* and root extracts of *H. indicus* at varied concentrations in human monocytic (THP-1) cells. Trypan blue exclusion methods and MTT assay at the concentrations of medicinal plant extracts we used for treatments indicated the >95% cell viability. Plant extracts (OSAE, OSWE, HIAE and HIWE) were incubated with THP-1 cells for 24 h at varied concentrations. There was no cytotoxic effect found up to 1mg dwt/mL in both the plant extracts as shown in Fig. 5.1 and 5.2. Imatinib (IMT), a antileukemia drug was also tested for its cytotoxic effect along with plant extracts. Imatinib at 20 µg/mL did not show any toxic effect in THP-1 cells. However 50 µg/mL of IMT showed about 20 % of cell death (Fig. 5.1).

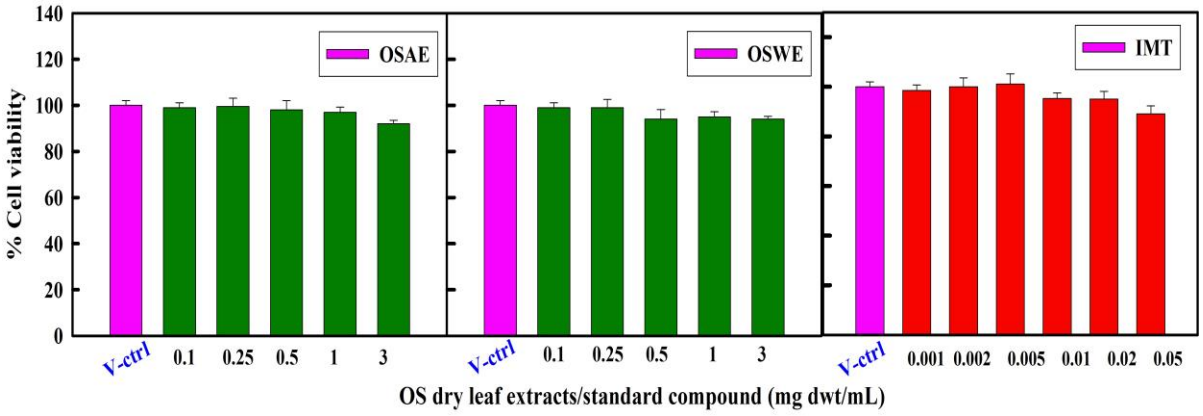


Fig. 5.1. Cell viability study of OSAE, OSWE and IMT in THP- cells. The cells were incubated with different concentrations of plant extracts or IMT for 24 h at 37 ◦C and 5% CO_2. Media without cells but with respective concentrations of plant extracts or IMT were used as appropriate blanks and the cell viability was examined by MTT assay. Vehicle control (alcohol and water) was limited to <1% and DMSO to < 0.5%. Experiments were performed at least in triplicate and the results were expressed as the mean ± S.D. n=6.

In case of HIAE and HIWE, there was no toxic effect seen in THP-1 cells up to 1 mg dwt/mL like OS dry leaf extracts (Fig. 5.2). At least >95 % cells were alive at the used concentrations of both selected plant extracts. Vehicles solvents (alcohol or water of <1%) had no effect on cell viability. Therefore, dosages of both the plant extracts for THP-1 cell treatment were limited to 1 mg dwt/mL.

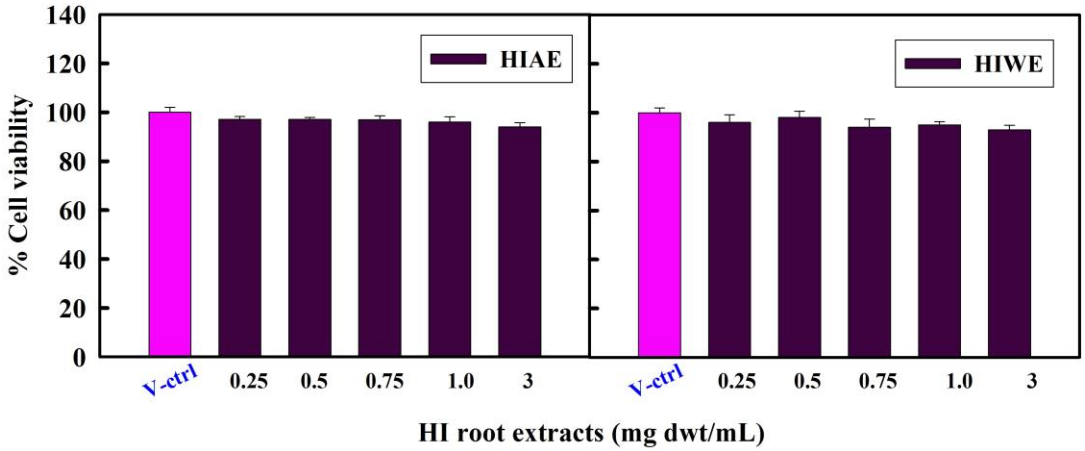


Fig. 5.2. Cytotoxic effect of HI root extracts on human monocytic (THP-1) cells**.** The cells were incubated with different concentrations of HIAE and HIWE for 24 h at 37 ◦C and 5% CO_2. Media without cells but with respective concentrations of plant extracts were used as appropriate blanks and the cell viability was examined by MTT assay. Vehicle control was limited to <1%. Experiments were performed at least in triplicate and the results were expressed as the mean ± S.D, n=6.

Antiinflammatory effect of *O. sanctum* dry leaves and *H. indicus* root extracts in LPS induced THP-1 cells

Lipopolysaccharide (LPS) is a bacterial endotoxin induces inflammation in human monocytes. Therefore, we used LPS as an inflammatory agent to test antiinflammatory activity of the selected medicinal plant extracts. In the present study, we tested the expression of inflammatory cytokines like IL-6, TNF-α, MIP-1α and chemokines like MCP-1 and IL-8 by real time PCR; TNF-α, MCP-1 secretion by ELISA and translocation of NF-κB by immunofluorescence method after the exposure of cells to LPS for 3 h. We also tested the PMA induced macrophage differentiated markers like CD14, TLR2 and TLR4 by real time PCR method.

Effect of OS dry leaves, HI root extracts and IMT on LPS induced TNF-α secretion

TNF-α is a potent proinflammatory cytokine secreted by activated monocytes and macrophages. TNF-α subsequently activate other cell sepsis in the blood vessel and thus initiates progression of atherosclerosis. TNF-α secreted by THP-1 cells (5×10^5 cells/mL) in the presence of LPS was quantitated by performing ELISA assay. Human TNF-α at different concentration was used a s standard to extrapolate the total amount of TNF-α released from THP-1 cells as shown in Fig. 5.3 A. Supernatants of only THP-1 cells (without induction) had negligible amounts of TNF-α (15 pg/mL), whereas quantity of TNF-α released was more by several folds in LPS induced THP-1 cells and accounts to 21 fold induction (435 pg/mL). Pretreatment of cells with *O. sanctum* dry leaf extracts markedly attenuated such LPS induced TNF-α secretion. OSAE with 0.5 mg and 1 mg dwt/mL brought down to 18 (360 pg/mL) and 9 fold (186 pg/mL) whereas OSWE with 0.5 mg and 1 mg dwt/mL reduced to 19 (354 pg/mL) and 6 (120 g/mL) folds respectively as shown in Fig. 5.3 B. Imatinib (IMT) used as a positive drug control, a commercially available drug used for treating cancer. Clinical studies with IMT established that it attenuate proinflammatory gene expression. Cells treated IMT at 5 and 20 µg/mL brought down to 19 (393 pg/mL) and 9 (186 pg/mL) folds respectively in the presence of LPS (Fig. 5.3 B). *H. indicus* root extract showed moderate effect on LPS induced TNF-α secretion in THP-1 cells. Pretreatment of cells with 0.5 mg and 1 mg dwt/mL of HIAE brought down to 20 and 14 folds and HIWE to 20 and 15 folds respectively. (Fig. 5.3 B). From the above selected two plant materials, OS dry leaf extracts showed marked effect in attenuating the secretion of TNF-α in the presence of LPS from THP-1 cells whereas HI root extracts showed only marginal effect. Vehicle solvents (<1 % alcohol and water, v/v; <0.5 % of DMSO, w/v) had no effect on TNF-α secretion.

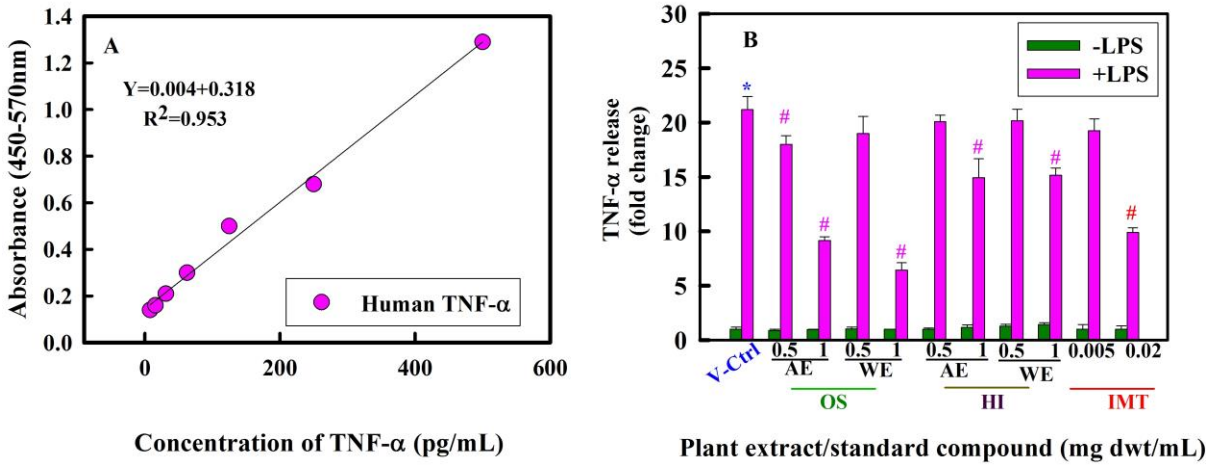


Fig. 5.3. LPS induced TNF-α released from the supernatants of THP-1 cells was measured by ELISA. (A) Human TNF-α standard. Effect of alcoholic (AE) and water extract (WE) of OS dry leaves or HI roots and positive drug control Imatinib (IMT) on LPS induced TNF-α secretion in THP-1 cells (B). Cells were seeded at 5 x 10^5 cells/mL. Data represents fold change in TNF-α release into the media by the cells treated with LPS (0.5 µg/mL for 3 h) compared to untreated cells. Cells were pretreated with selected plant extracts and standard compound was for 12 h prior to incubation with LPS for 3h. Cell supernatants of respective treatments were analysed for TNF-α by ELISA. Mean value was represented as V-control. Data represent n=6 mean of six measurements. *Statistical significance of $p < 0.001$ within the control groups i.e. cells+ LPS *vs* cells − LPS. $^{\#}p<0.001$ compared between cells treated with LPS in the presence of selected plant extracts and positive drug control IMT in their absence.

Effect of OS dry leaves and HI root extracts on LPS induced MCP-1 secretion

Secreted MCP- by THP-1 cells (5 × 105 cells/mL) in the presence of LPS was quantitated by performing ELISA assay. Here also alcoholic and water extract of selected plant species have tested at specific concentration to check inhibitory effect MCP-1 secretion in the presence of LPS. Supernatants of control cells had negligible amounts of MCP-1 (20 pg/mL), whereas substantial increase in MCP-1 release was observed in LPS induced cells i.e 5.8 fold induction (110 pg/mL). Commercially available human MCP-1 at varied concentration was used as standard along with plant extracts to estimate total amount of MCP-1 released during the time of LPS or plant extract treatment (Fig. 5.4 A). Pretreatment of cells with 1 mg dwt/mL of OSAE and OSWE brought down to 5.27 (98 pg/mL) and 4.31 fold (81 pg/mL) respectively as shown in Fig. 5.4 B. Thus OSAE did not show much effect on attenuation of

the MCP-1 secretion, where as OSWE showed significant effect. HIAE and HIWE of *H. indicus* root (1 mg dwt/mL) brought down to 3.54 and 3.87 folds (Fig. 5.4 B). Here HI root extracts showed better responses than OS extracts.

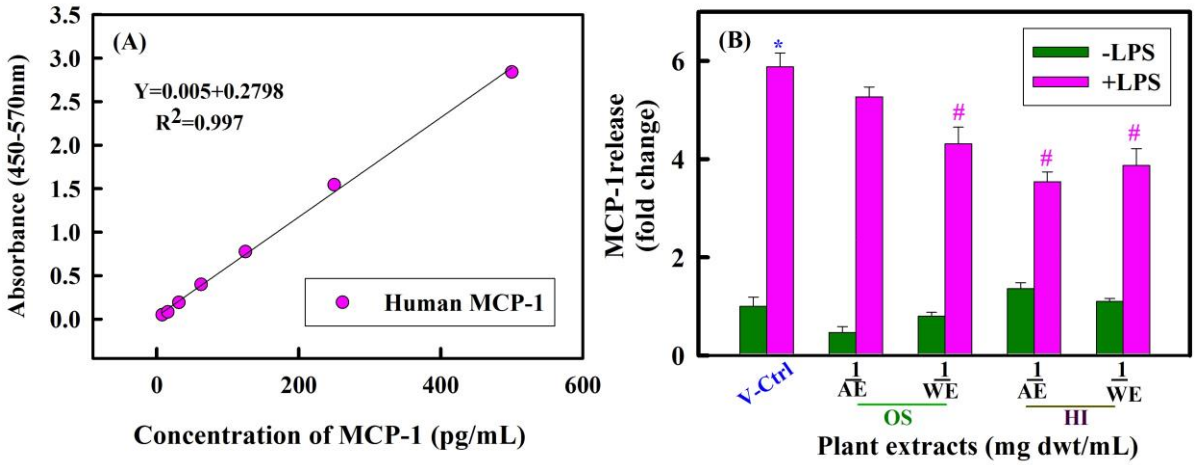


Fig. 5.4. MCP-1 secretion from LPS induced THP-1 cells was measured by ELISA. (A) Human MCP-1 was used as standard.(B) Effect of alcoholic (AE) and water extracts (WE) of OS dry leaves and HI root extracts on LPS-mediated MCP-1 secretion in THP-1 cells. Cells were seeded at $5x10^5$ cells/mL. Data represents fold change in MCP-1 release into the media by the cells treated with LPS (0.5 µg/mL for 3 h) compared to untreated cells. OS dry leaf and HI root extracts were added to the cells 12 h prior to incubation with LPS. Respective vehicle (alcohol or water) concentration was limited to < 1% of the cell culture volume. Data represents n = 6 mean of six measurements. *Statistical significance of $p<0.001$ within the control groups i.e. cells + LPS *vs* cells − LPS. $^{\#}p<0.001$ compared between cells treated with LPS in the presence of plant extracts *vs* in their absence.

Effect of OS dry leaves, HI root extracts and IMT on LPS induced inflammatory markers Antiinflammatory effect of *O. sanctum* dry leaves and *H. indicus* roots was evaluated in THP-1 cells incubated with bacterial endotoxin LPS on gene expression by real-time quantitative PCR. LPS treatment enhanced the expression in THP-1 cells of TNF-α (5 fold), IL-6 (2 fold), macrophage intensity protein (MIP-1α) (2.15 fold) and MCP-1 (36 fold) as shown in Fig. 5.5. Such stimulation of proinflammatory gene expression was markedly inhibited by *O. sanctum* dry leaf extracts (OSAE and OSWE). OSAE at 1 mg dwt/mL attenuated proinflammatory markers of IL-6, TNF-α, MIP-1α expression to 0.90, 1.42 and 0.80 fold respectively. However, it did not show much effect on expression of MCP-1.

OSWE (1 mg dwt/mL) attenuated all the tested markers IL-6 (0.82 fold), TNF-α (1.78 fold), MIP-1α (0.70 fold) and MCP-1 (9.39 fold) as shown in Fig. 5.5 A to E. IMT (20 µg/mL) a reference compound, showed significant inhibition of all the selected markers.

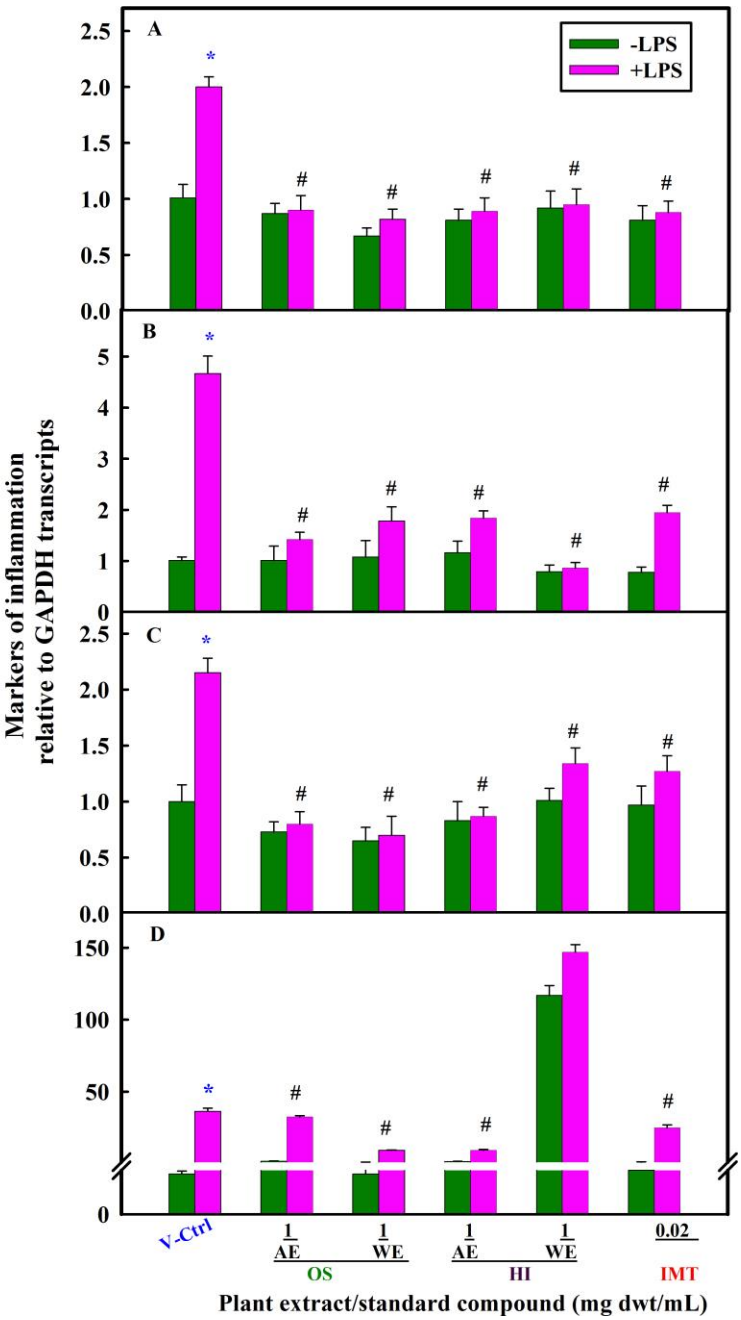


Fig. 5.5. Effect of OS dry leaf extracts (1 mg dwt/mL), HI root extracts (1 mg dwt/mL) and positive drug control IMT (20 µg/mL) attenuated the gene expression of LPS induced IL-6 (A); TNF-α (B); MIP-1α (C) and MCP-1 (D) in THP-1. Vehicle volume (alcohol or water) did not exceed 1% and had no effect. DMSO was 0.2%. Experiments were performed at least in triplicate and the results were expressed as the mean ± S.D. *$p<0.001$ for comparison between Cells+LPS *vs* cells-LPS. $^{\#}p<0.001$ compared between cells treated with LPS in the presence of plant extracts or positive control IMT *vs* in their absence.

In case of HI root extracts, HIAE at 1 mg dwt/mL restricted the expression of IL-6 (0.53 folds); TNF-α (1.85 folds); MIP-1α (0.86 folds) and MCP-1(9.36 folds) whereas HIWE (1 mg dwt/mL) attenuated IL-6, TNF-α, MIP-1 α to 0.94 folds, 0.86 folds and 1.32 folds respectively (Fig 5.5 A to E). However, HIWE did not show much effect on attenuating the expression MCP-1 marker (Fig. 5.5 E).

Effect of plant extracts on PMA induced differentiation of THP-1 cells to macrophage

Human monocytic THP-1 cells are committed to the monocytic cell lineage and are suspension in nature and do not adhere to the plastic surfaces of the culture plates as shown in Fig. 5.6 A. PMA (5 ng/ml) stimulated THP-1 cells differentiated into macrophages as indicated by their adherence to substratum of the culture dish (Fig.5.6 B). The adherent cells showed morphological characteristics similar to macrophages. The effect of alcoholic and water extracts of OS dry leaves and root extracts on such differentiation process was tested by treating the cells with PMA in the presence or absence of plant extracts. Pretreatment with OSAE or OSWE (1 mg dwt/mL) significantly attenuated the process of differentiation by PMA challenged THP-1 cells as shown in Fig. 5.6 C to 5.6 F. IMT (20 µg/mL) also showed similar antiinflammatory effect like plant extracts by inhibiting the differentiation of monocytes to macrophages in the presence of PMA (Fig 5.6 G to 5.6 H). In case of HI root extracts, both HIAE and HIWE (1 mg dwt/mL) inhibited the PMA induced differentiation of monocytes to macrophages (Fig. 5.7 C to 5.7 F).

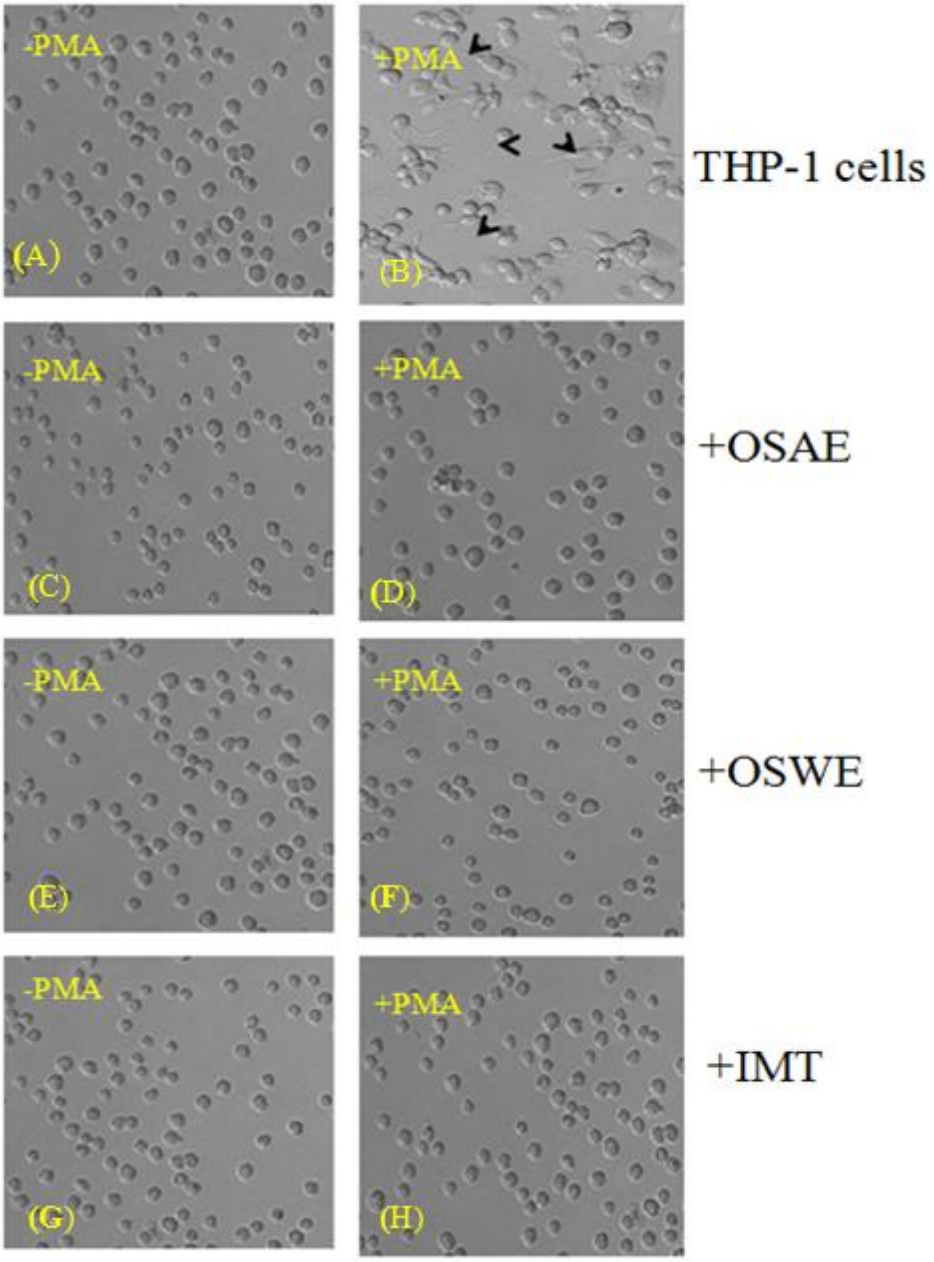


Fig. 5.6. Effect of OS dry leaf extracts and IMT on PMA induced macrophage differentiation on THP-1 cells. Pictures of normal, treated and differentiated THP-1 cell lines were taken under microscope 10 X magnification. Cells were seeded at 5 x 10^5 cells/well and differentiation of monocytes to macrophages was activated by treated the cells with PMA. Cells were pretreated with OSAE/OSWE (1 mg dwt/mL) and IMT (20 µg/mL) for 12 h then challenged with PMA for 48 h. In Cell + PMA treated cells, black colour arrow marks clearly indicates the THP-1 derived elongated, flattened, thread like differentiated macrophage cells in the presence of PMA after 48 h treatment. Vehicle controls concentration of alcohol and water limited to <1% and DMSO 0.2% had no effect on cell differentiation.

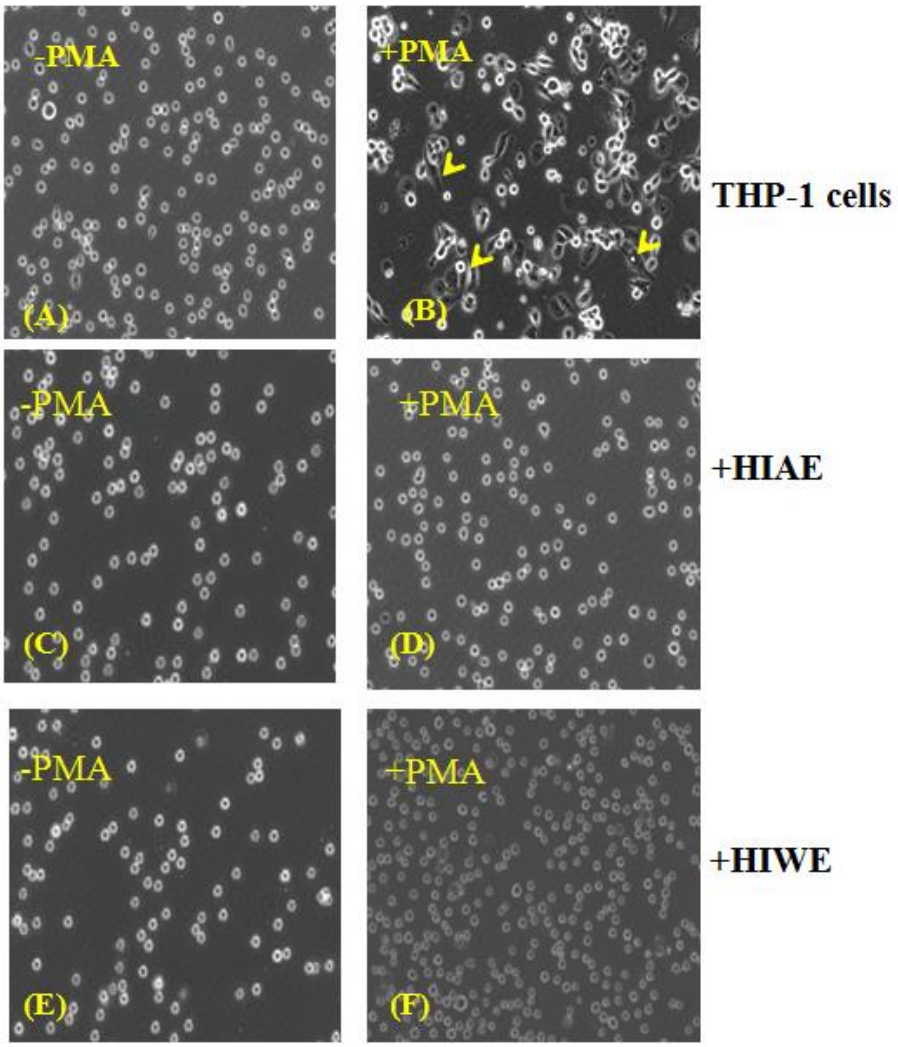


Fig.5.7. Effect of HI root extracts on PMA induced macrophage differentiation on THP-1 cells. Cells were seeded at 5 x 10^5 cells/well. Monocytes were differentiated into macrophages in the presence of PMA (5 ng/mL) for 48 h. HIAE/HIWE (1mg dwt/mL) were pretreated with cells for 12 h then followed by PMA for 48 h. In the presence of PMA, THP-1 cells differentiated, elongated, flattened, thread like macrophage cells which are indicated by arrow marks. Vehicle control was limited to <1%.

Effect plant extracts on PMA induced cell surface markers in THP-1 cells

Literature survey clearly demonstrates that differentiated monocytic cells compared to monocytic cells had an increased expression of CD14, TLR2 and TLR4 which are macrophage-specific antigens (Takashiba *et al.,* 1999). Therefore PMA differentiated THP-1 cells were examined by quantitative real-time PCR to check the expression of selected

genes CD14, TLR2 and TLR4 and compared with untreated cells on the basis of fold changes.

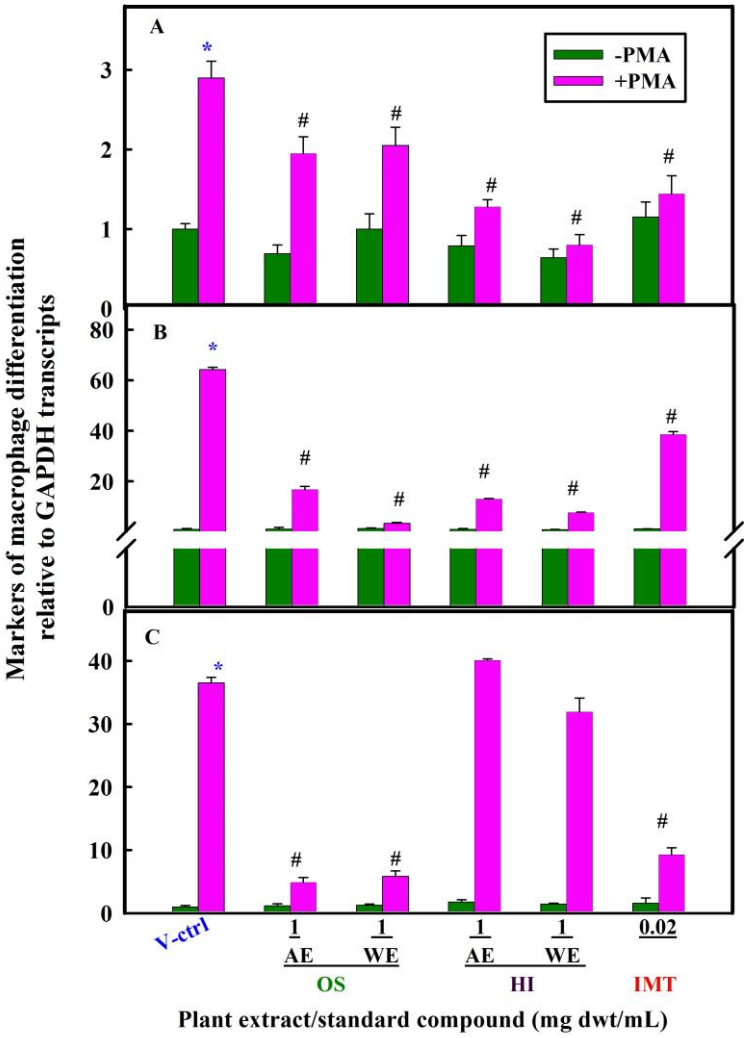


Fig. 5.8. Effect of alcoholic and water extracts of OS dry leaves, HI roots and positive drug control IMT on PMA induced CD14 (A), TLR2 (B) and TLR4 (C) mRNA transcripts in THP-1cells. THP-1 cells pretreated with or without selected plant extracts or Imatinib and incubated with PMA (5 ng/mL) for 48h. After incubations, transcript levels by CD14, TLR2 and TLR4 were determined by RT-PCR as a measure of THP-1 cell differentiation. Data shown are mean value of 3 independent experiments ± S.D. *$p<0.001$, for comparison between treated (cells +PMA) and untreated groups (cells-PMA). $^{\#}p<0.001$ compared between cells treated with PMA in the presence of plant extracts and positive drug control *vs* in the absence of the extracts and positive drug control.

Results showed that PMA upregulated the expression of CD14 by 3 fold and toll like receptors (TLR2 and TLR4) by 64 and 37 fold respectively (Fig. 5.7 A to C). THP-1 cells coincubated with OS dry leaves or HI root extracts along with PMA had much lower levels of

these transcripts. Cells pretreated with OSAE (1 mg dwt/mL) showed the transcript levels of CD14, TLR2 and TLR4 of 2 fold, 17 fold and 5 fold compared to regular THP-1 cells respectively. While in the presence of OSWE (1 mg dwt/mL) and PMA cells had much lower level of all the three transcripts (CD14: 2.05 fold; TLR2: 3.36 fold and TLR4: 6 fold) as shown in Fig. 5.8. Positive drug control Imatinib (20 µg/mL) also attenuated the expression of all three selected markers like plant extracts at their tested concentrations. Similarly, pretreatment of HIAE (1 mg dwt/mL) in the presence of PMA attenuated the expression of CD14 (1.28 folds) and TLR2 (13.01 folds), however, whereas it did not show any effect on the attenuation of TLR4 as shown in the Fig. 5.8 C. HIWE at 1mg dwt/mL attenuated the expression of all the above selected markers (CD14 to 0.80 folds; TLR2 to 7.62 fold; TLR4 to:31.92 folds). Thus HIWE showed only moderate effect on attenuation of TLR4 compared to HIAE significantly attenuated CD14 and TLR2.

Attenuation of arachidonic acid induced ROS by OS extracts

Measurement of intracellular ROS in live cells is highly significant to investigate the status of oxidative stress. A florescence dye 2,7-ichlorodihydrofluorescin diacetate (H_2DCFDA) is most widely used to determine intracellular ROS in various cell lines. Arachidonic acid has been considered as an inducer of reactive oxygen species and it can easily cross the cell membranes and that causes the generation of different types of ROS which are very harmful to the cells. Excess generation of ROS can lead to oxidative stress which leads to the development of different pathophysiological conditions. Therefore antioxidants are considered as potential therapeutic agents including plant extracts. In order to detect intracellular ROS in response to arachidonic acid, human monocytic (THP-1) cells were labeled with a cell permeable ROS sensitive fluorophore named as H_2DCFDA and intensity of fluorescence of dye, which is directly propotional to ROS levels was investigated using confocol microscope. ROS intensity was quantatively measured by fluorescence microplate reader. H_2DCFDA dyed THP-

1 cells were treated with 80 µM arachidonic acid for 5 min showed much brighter fluorescence compared to cells not treated with arachidonic acid as shown in Fig. 5.9 B. Apart from that, we also observed, THP-1 cells treated with arachidonic acid (80 µM) for 5 min increased the level of fluorescence (2 fold) compared to unstimulated cells by several fold (Fig. 5.10). It indicates an enhancement of intracellular ROS inside the cells.

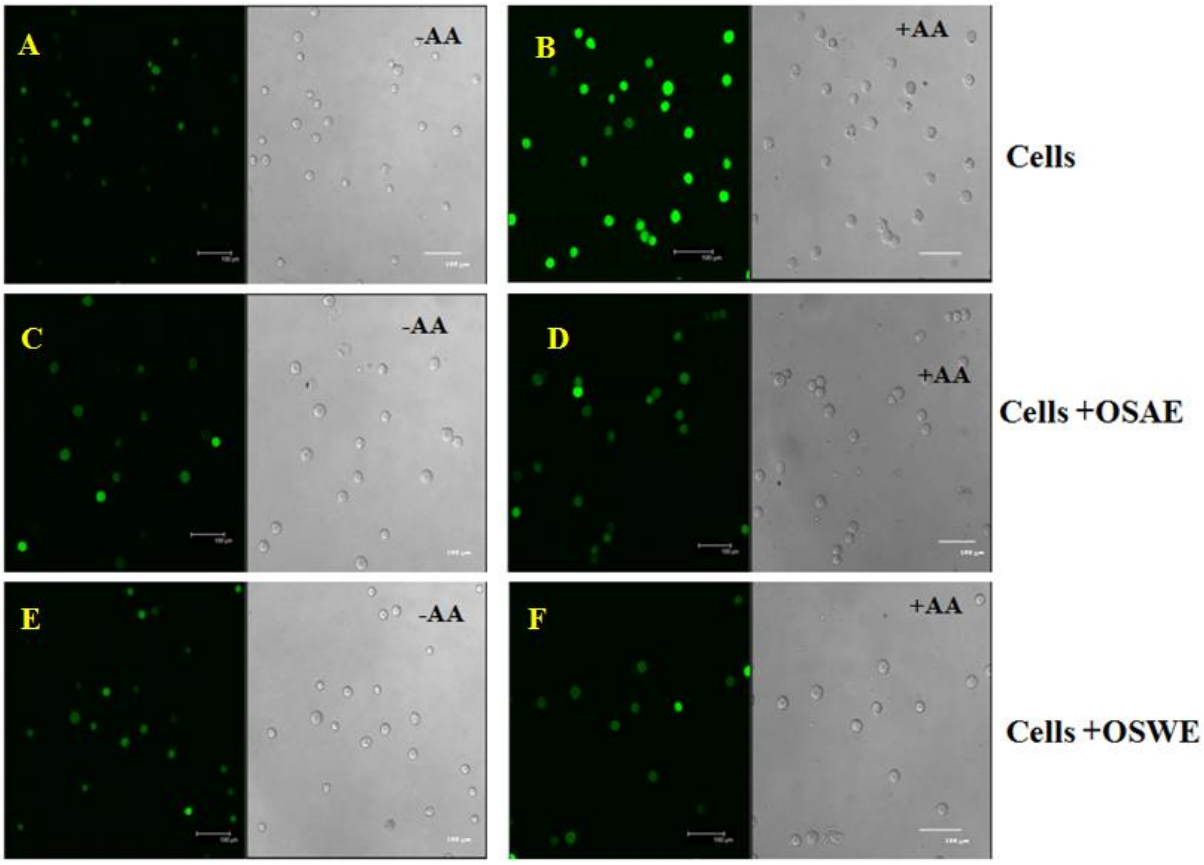


Fig.5.9. Effect of alcoholic (AE) and water extracts (WE) of *O. sanctum* dry leaves on arachidonic acid induced ROS generation in THP-1 cells. ROS generation was detected by H_2DCFDA, a cell permeable dye and cells were observed with a confocol fluorescence microscope. (A) THP-1 cells; (B) THP-1 cells + AA (60 µM); (C) THP-1 cells +OSAE; (D) THP-1 cells + OSAE (1 mg dwt/mL) + AA; (E) THP-1 cells + OSWE (1 mg dwt/mL); (F) THP-1 cells + OSWE (1 mg dwt/mL) + AA.

In the present work, we determined the ROS scavenging potential of OS dry leaf extracts (OSAE/OSWE) in THP-1 cells. Pretreatment of cells with OSAE (Fig, 5.9 C and D) or OSWE (Fig. 5.9 E and F) clearly demonstrated that antioxidant effect of plant extracts at the tested concentrations. We also measured the effect of both OSAE/OSWE at varied

concentration by microplate reader on the basis of H_2DCFDA fluorescence intensity as mentioned above and both OSAE and OSWE markedly attenuated the arachidonic acid induced ROS production in a dose-dependent manner as shown in Fig. 5.10. Thus, our results demonstrate that both OSAE and OSWE possess significant ROS scavenging activity (Fig. 5.9 C and E).

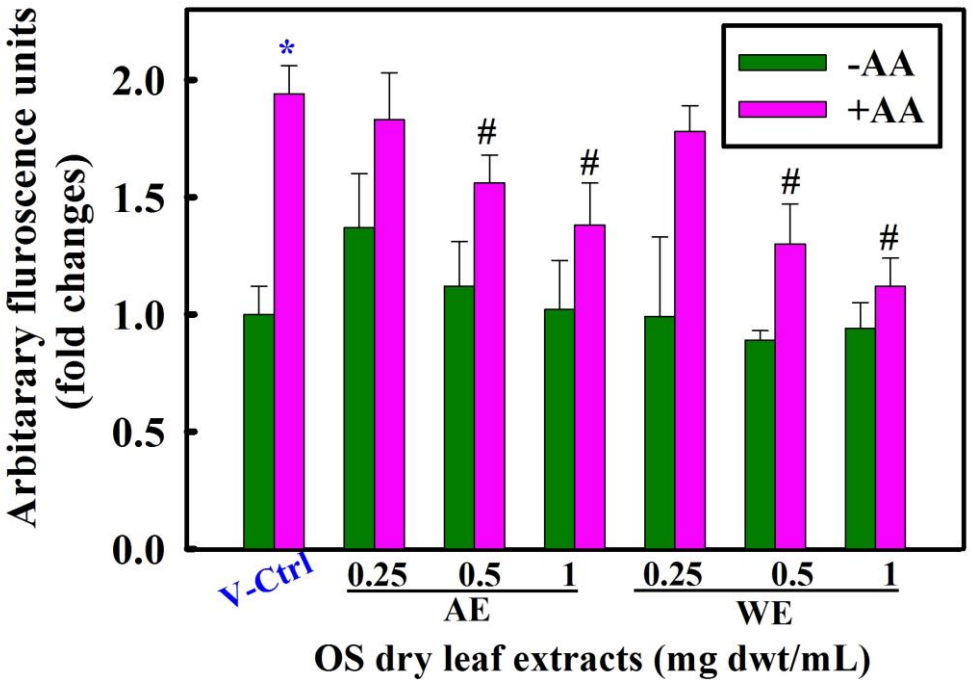


Fig. 5.10. Effect of alcoholic (AE) and water extracts (WE) of *O. sanctum* dry leaves on arachidonic acid induced ROS generation in THP-1 cells. Fluorescence intensity of H_2DCFDA taken by the cells treated as above was measured by microplate reader having fluorescence facility at excitation wavelength of 488 nm and emission wavelength of 525 nm. Data shows mean value of 6 independent experiments ± S.D. (n=6).

Effect of OS extracts on arachidonic acid induced lipid peroxidation in THP-1 cells

Peroxidation of lipids in membrane due to oxidative stress is an important cause of cell damage. So the plant extracts, which show protective effect against lipid peroxidation are of great importance for proper cellular integrity and functions. Arachidonic acid induced peroxidative damage of membrane lipids of human monocytic (THP-1) cells was assessed by level of MDA formation. Here we evaluated the efficacy of OSAE and OSWE in inhibiting

the arachidonoic acid induced lipid peroxidation in monocytic (THP-1) cells. It was observed that, there was an increase in MDA formation in the cells treated with arachidonic acid as compared to untreated control cells (Fig. 5.11). Pretreatment of cells with OSAE or OSWE markedly inhibited the arachidonic acid induced lipid peroxidation in a dose dependent manner as shown in Fig 5.11.. The related IC_{50} values of OSAE and OSWE were 0.34 µg and 0.18 µg dwt/mL respectively.

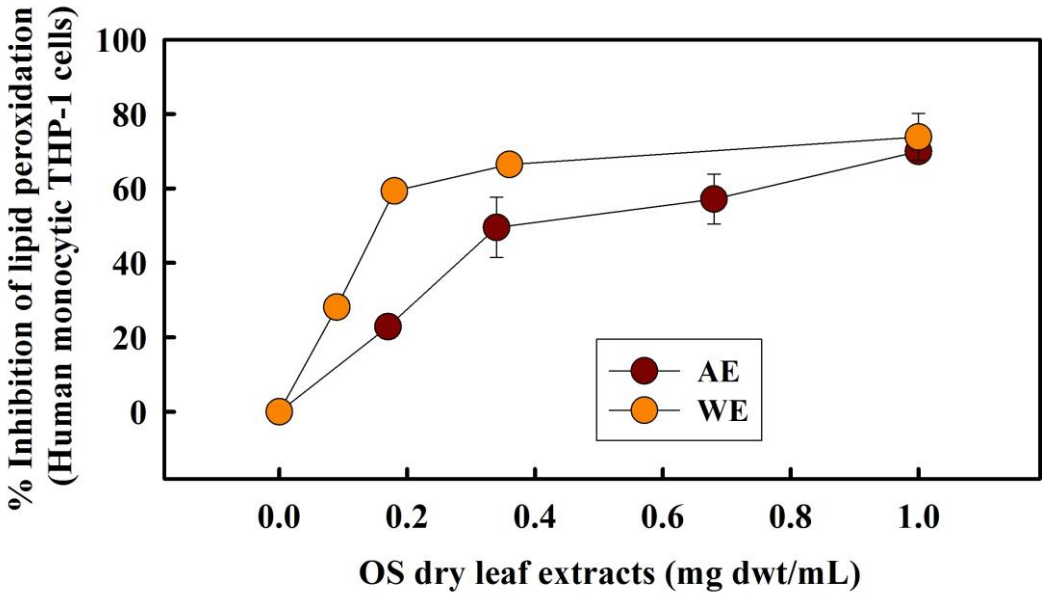


Fig. 5.11. Inhibitory effects of OSAE and OSWE on arachidoinc acid induced lipid peroxidation in THP-1 cells. THP-1 cells pretreated for 12 h with OSAE/OSWE (1 mg dwt/mL) followed by incubation with arachidonic acid for 5 mins. Peroxidation of lipids was measured in THP-1 cells as described in the method. Data shows mean value of 6 independent experiments (n=6). All values are statistically significant # $p<0.001$ vs control. SD not seen is within the symbol of data point.

Thus our results demonstrate that both OSAE and OSWE possess significant inhibition in peroxidation of lipids in THP-1 cells in the presence of arachidonic acid.

Effect of OS and HI extracts in attenuating the translocation of NF-κB p65 into the nuclei of LPS stimulated THP-1 cells

The transcription factor, NF-κB plays a crucial role in the regulation of gene expression of several proinflammatory markers including TNF-α, interlukeins and other chemokines. To investigate further mechanistic on antiinflammatory activity of the OS dry leaves and HI roots,

the protective effect of OSAE, OSWE, IMT (positive control), HIAE and HIWE were examined on translocation of the p65 subunit of NF-κB to the nucleus in LPS stimulated cells. Its activation involves cytoplasmic dissociation of the inhibitor protein IκB and facilitates translocation of the active NF-κB complex into the nucleus. We performed this assay by immunofluorescence method. First THP-1 cells were probed with commercially available p65 antibodies and further stained using secondary antibody conjugated with Alexa Fluor 594 and DAPI was used to stain the nuclei of the cells. In brief, the confocal images quite clearly indicated that NF-κB-p65 was sequestered in the cytoplasm in normal uninduced cells (Figs. 5.12 A to 5.12 C: 1st panel) and its nuclear accumulation was strongly induced after stimulation of THP-1 cells with LPS (0.5 µg/mL) for 3 h (Figs. 5.12 D to 5.9 F: 2nd panel). Such nuclear translocation of NF-kB-p65 was blocked by pretreatment of the cells with OSAE/OSWE at 1 mg dwt/mL as shown in Fig 5.12 G to L. Similarly, positive drug control, IMT (20 µg/mL) also showed similar response and inhibit the translocation p65 subunit of NF-κB into the nucleus in the presence of LPS in THP-1 cells (Fig. 5.12 M to 5.12 O).

In case of HI roots, the extracts inhibited the translocation of NFκB-p65 in THP-1 cells (Fig. 5.13). HIAE at 1mg dw/mL inhibited translocation of NF-κB in the presence of LPS as shown in Fig 5.13 G to I, where as HIWE at the same concentration also showed similar effect like HIAE (Fig. 5.13 J to L). From the above results it can be clearly demonstrated that both extracts of OS dry leaves and HI roots elicit antiinflammatory activity in LPS induced THP-1 cells by modulating subcellular localization of transcriptional factor NFκ-B.

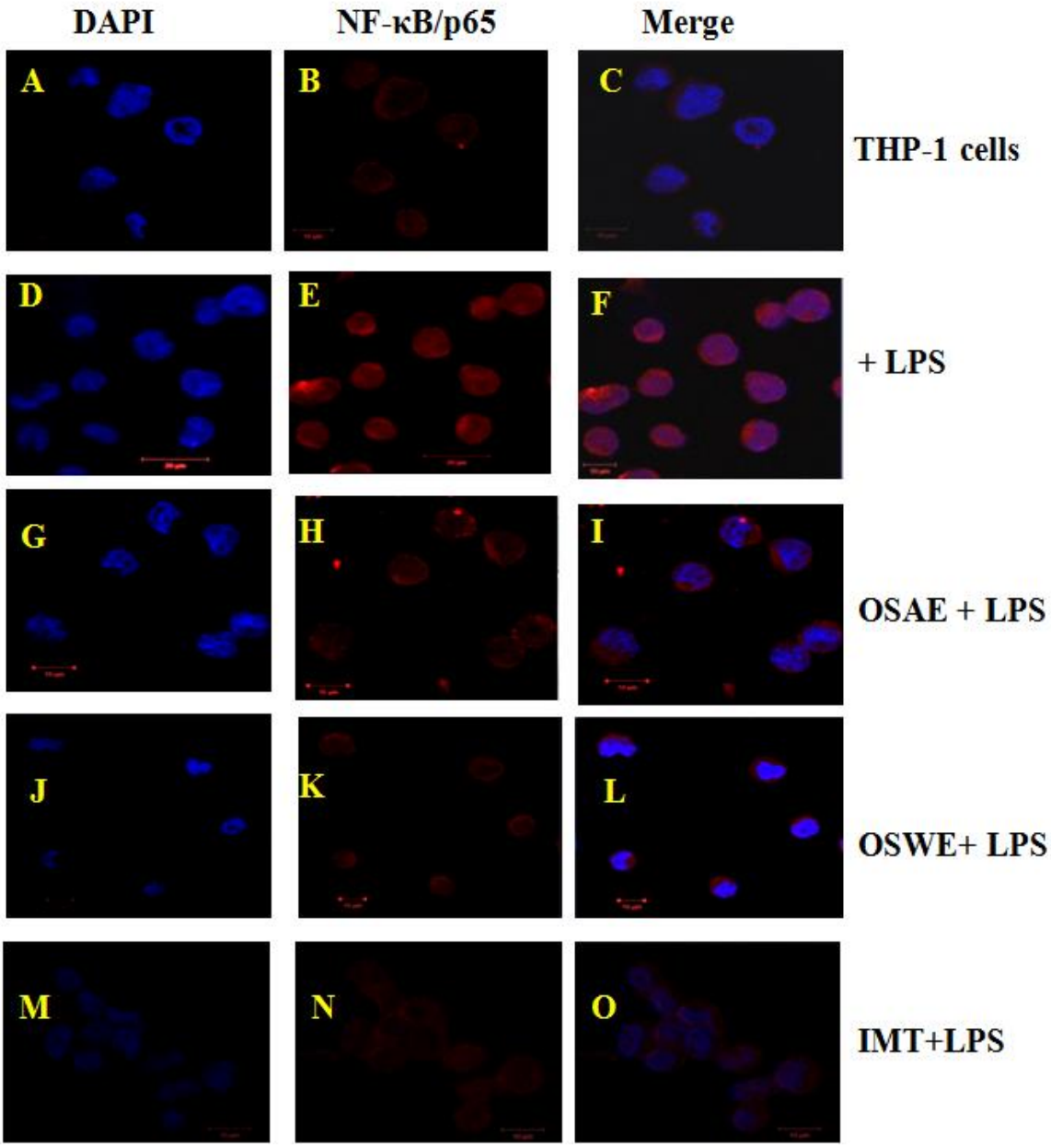


Fig. 5.12. Indirect immunofluorescence based confocal microscopy images showing the effect of OS dry leaf extracts on LPS induced subcellular localization of NF-κB-p65 in THP-1cells. NF-κB translocation was analyzed by staining with NF-κB-p65, red (A); nucleus was stained with DAPI, blue (B); merged image of NF-κB-p65 and nucleus (C). THP-1 cells treated with LPS (0.5 µg/mL) for 3h was seen in panel (D to F); OSAE (1 mg dwt/mL) treated with LPS was visualized in panel (G to I); OSWE (1 mg dwt/ml) with LPS was seen in panel (J to L). Vehicle control was limited to <1%.

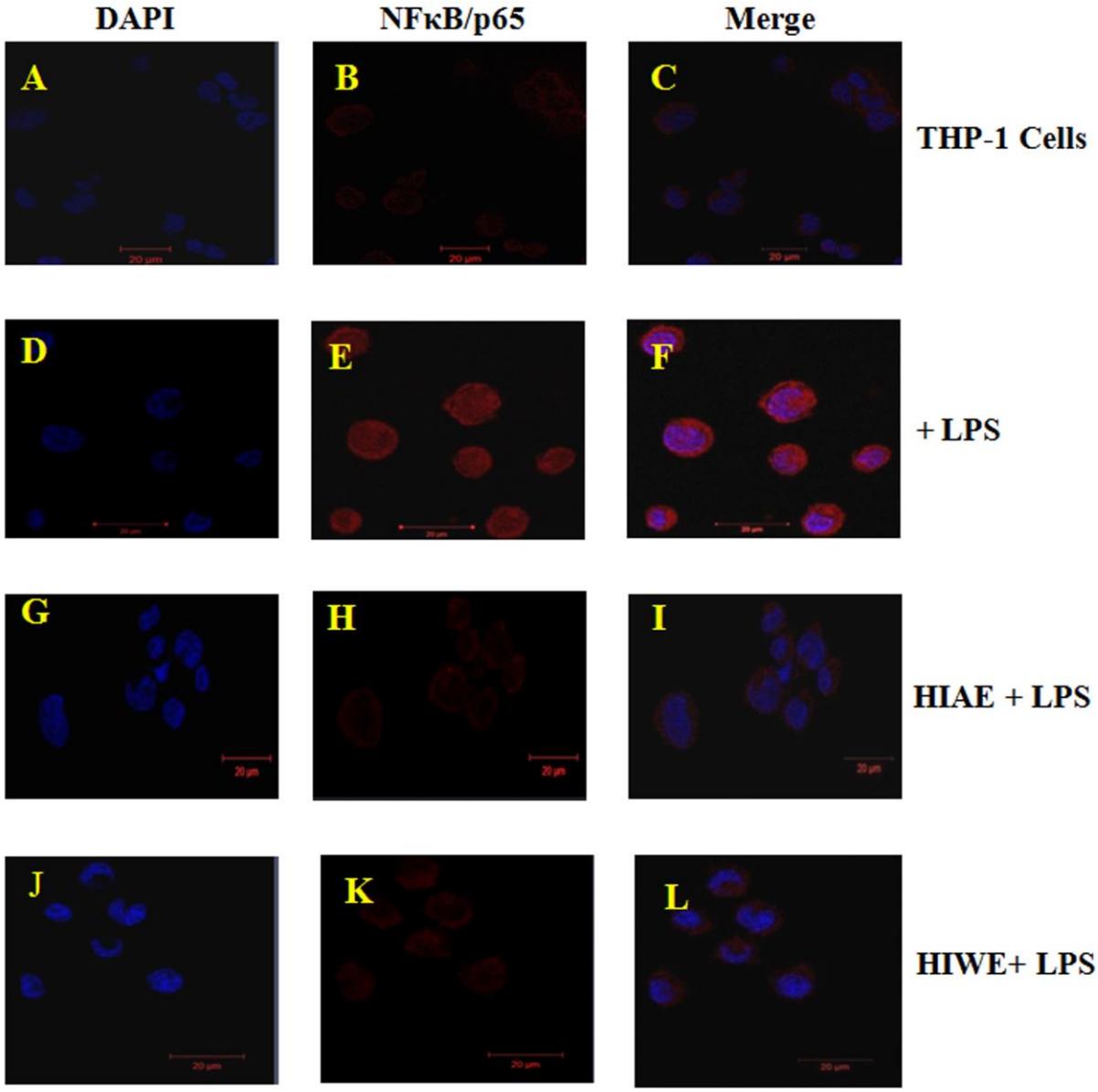


Fig. 5.13. Confocal images showing the effect of alcoholic (AE) and water extracts (WE) of HI roots on inhibition of LPS induced translocation of p65 subunit of NF-κB from cytoplasm to nucleus in THP-1 cells. Images with vehicle control 0.5% (alcohol) cells are shown in panel 1. Vehicle control of water also had no effect (not shown). NF-κB translocation was analyzed by staining with NF-κB-p65, red (A); nucleus with DAPI, blue (B); merged image of NF-κB-p65 and DAPI (C). THP-1 cells treated with LPS (0.5 µg/mL for 3h) shown in 2nd panel (D to F); cells pretreated with HIAE and HIWE (1 mg dwt/mL) and then treated with LPS shown in 3rd (G to I) and 4th panels (J to L) respectively.

Discussion

Scientific studies and several clinical researches clearly mentioned that, the use of medicinal plants and their different herbal formulations showed remarkable effect for maintaining general health especially against several chronic inflammatory conditions. Most of the chronic human diseases are developed due to oxidative and/or inflammatory stress conditions. Among these diseases, atherosclerosis is majorly considered. Atherosclerosis considered as a lipid storage disease, actually involves inflammatory response and is a well known chronic inflammatory blood vessel wall disease (Ross, 1999; Libby, 2002). Several pathophysiological condition including oxidative stress, hypercholesterolemia, hyperglycemia and postpandial lipolysis leads to the activation of monocytes and platelets in the peripheral blood, endothelial dysfunction and vascular permeability (Esper *et al.,* 2006). Development of new drugs those target inflammatory mechanism and their pathways are good candidate for treating several inflammatory disease including cardiovascular diseases (Bailey and Butler, 1973). Traditionally, the leaves of *O. sanctum* have been used as a cardioprotective and its medicinal benefits are well studied it also well studied in animal model (Arya *et al.,* 2006). OS leaves are reportedly found to have broad range of health benefits. Commercially available herbal products of *O. sanctum* (E.g.Tulsi by Kirpal Export Overseases, India; Dry herbal extracts by Nikita Extracts, India; *Ocimum sanctum*-Beadlet by Borion Canada Inc., Canada) are known for their cardiac and/or other general health benefits. Similarly *H. indicus* roots also traditionally used for blood purification and to treat cardiovascular diseases. However, molecular mechanism of action of selected plant materials in offering cardiac health or inhibiting atherogenesis is not clearly understood. In the present study, OS dry leaves and HI root extracts were investigated for their effect on secretion of proinflammatory gene expression like IL-6, MIP-1α, TNF-α and MCP-1 induced by LPS in THP-1 monocytic cells. These cytokines and chemokines shown to be produced by THP-1

cells up on stimulation with LPS (Weiss *et al.,* 2004; Xue *et al.,* 2006). TNF-α has been considered as a potent proinflammatory cytokine secreted by monocytes and macrophages and plays an important role in developing many chronic inflammatory diseases including atherosclerosis and rheumatism (Huo *et al.,* 2012). TNF-α also activates other cell types like endothelial cells (Mackenzie *et al.,* 2002). The activated endothelial cells produce cell surface adhesion molecules and selectins, facilitating rolling, adherence and diapedsis of monocytes into subendothelial space where they differentiate into macrophages (Postea *et al.,* 2006). IL-6 is a proatherogenic cytokine and is associated with fatty lesion development in aorta (Kishikawa *et al.,* 1993). MIP-1α and MCP-1 belong to chemokine family and are also associated with atherosclerotic lesion development. High levels of MIP-1α along with other chemokines like MIP-1β, RANTES and cytokines IFN-γ, IL-2, IL-4, and IL6 are found in blood plasma of atherosclerotic patients (Cagnin *et al.,* 2009). MCP-1 is established as one of the important chemokines involved in the development of atherosclerosis, specifically by playing a role in the monocyte recruitment and infiltration into the arterial wall (Reape and Groot, 1999; Takahashi *et al.,* 1995).

It is traditionally, known that OS and HI are good for the treatment of several inflammatory diseases including cardiovascular diseases. However, its effect on human monocytic cells which are one of the important cell types participating in the pathophysiology of CVD, is not yet known.

Antiinflammatory properties of OS and HI extracts in THP-1 cells

In the present study, both OS leaves and HI root extracts exhibited antiinflammatory activity in THP-1 cells by inhibiting LPS induced proinflammatory gene expression. LPS is a potent bacterial endotoxin, which causes functional changes in monocytes like chemotaxis and maturation of monocyte to macrophages. THP-1 cells treated with LPS secrete much more higher amounts of proinflammatory cytokines particularly TNF-α, MCP-1 and other

chemokines and cytokines compared to unstimulated cells. Pretreatment of cells with OS and HI extracts significantly attenuated LPS induced secretion of selected cytokines in cell supernatant, but OS extracts did not show significant effect on attenuation of MCP-1 compared to HI extracts. However, OSAE and HIWE did not show much effect on MCP-1 attenaution. PMA induced CD14/TLR4/TLR2 complex is most important in the activation of the innate immune system in immune competent cells and macrophages (Shuto *et al.*, 2005). Apart from that, expression of TLR2/TLR on the macrophage cell surface was also recognized by LPS in conjunction with the lipopolysaccharide-binding protein (LBP), and the CD14 co-receptor. Following TLR4 activation by PMA, MAPKs become phosphorylated and the transcription factor NF-κB get activated (Jawan *et al.,* 2008). Our results show that protein expression of the CD14 and TLR2/TLR4 proteins in PMA-stimulated cells was inhibited by selected plant extracts as shown in Fig. 5.8. OS and HI plant extracts at their tested concentration protectively inhibited PMA induced gene expression of CD14, TLR2, TLR4 in PMA induced THP-1 cells and thereby their differentiation into macrophage whereas HIAE did not show any effect on TLR4. Such variation in response could be due to the difference in their metabolite profile. Their results indicated that decreased expression of CD14 and TLR4 could inhibit the initiation of intracellular signaling cascades, which can subsequently suppress the activation of NF-κB and proinflammatory mediators. Studies in *ApoE*$^{-/-}$*/Myd88*$^{-/-}$ double knockout mice also demonstrated that CD14, TLR2 and TLR4 are strongly associated with inflammation and atherosclerosis development (Bjorkbacka *et al.,* 2004).

Arachidonic acid (AA) induces ROS generation in human monocytic (THP-1) cells. In the present work, upon stimulation with arachidonic acid, large amounts of reactive oxygen species were generated in THP-1cells. ROS also plays which play a central role in activation of NF-κB which consequently activates overproduction of inflammatory mediators.

When antioxidant defense is inadequate, it will result in inflammation and tissue damage a condition common for various inflammatory diseases. In this study, the extracts of *O. sanctum* dry leaves (OSAE/OSWE) have been found to reduce the ROS production, thus showing the protective effect of the extracts on ROS production. Both types of extracts of OS dry leaves inhibited the peroxidation of lipid in THP-1 cells induced by arachidonic acid in a dose dependent manner. Regulation of several proinflammatory genes is an ideal step to control most of the inflammatory diseases, the transcription of these inflammatory genes in mammalian cells are under the control of transcription factor NF-κB (Hoareau *et al.,* 2010). Further to know in detail about the mechanistic insight, we investigated the effect of OS and HI extracts on LPS induced nuclear translocation of NF-κB transcription factor. It is well confirmed that in mammalian cells, NF-κB regulates expression of several proinflammatory genes including certain cytokines, cytokine receptors, chemokines and cell adhesion molecules, which drive the inflammatory responses (Ghosh *et al.,* 1998) and also the genes involved in cellular differentiation and cell migration (Karin, 2000; Bindhu *et al.,* 2006). Most of the carcinogens, inflammatory agents and tumor promoters have been observed to activate NF-κB by phosphorylating its p65 subunit. Phosphorylated p65 subunit translocates to nucleus and causes gene expression by binding to promoter element of the genes (Ghosh *et al.*,1998). The plant extracts both OS and HI inhibited LPS induced nuclear translocation of NF-κB-p65 subunit. From our previous study we also confirmed that, OS and HI plant extracts contain several bioactive compounds including different types of phenolics, flavonoids, terpenoids, fatty acids, coumarinolignoids, so the selected plants can be served as a natural source for identifying the compounds with pleiotropic effects (antiinflammatory) which are drugs of interest in treating multifactorial diseases like atherosclerosis. It also strongly recommends further evaluation of medicinal use of OS-leaves and Hi-roots for treating CVD. Future direction of this study is to test the efficacy of selected plants on their

antiatherosclerotic property using chronic inflammatory models, e.g. diabetic or apoE -/- mice or hyperlipidemic rats.

Chapter 6

RESULTS and DISCUSSION

Objective 3: Identification of active metabolite(s) from OS extracts responsible for its antiinflammatory properties in THP-1 cells

Chapter 6

Results and Discussion

Results

Screening and identification of OSAE fractions with highest antilipid peroxidation activity Lipid peroxidation in liver homogenate of male Wistar rats

We performed fractionation of alcoholic extract of OS dry leaves, because of its higher activity with low IC_{50} value in lipid peroxidation study compared to OSWE. Fractionation work was performed by using different organic solvents on the basis of polarity starting from non polar to polar namely ethyl acetate,ethanol and water. E-FR inhibited $FeCl_3$ induced peroxidation in liver homogenates by 80 % at 20 µg dwt/mL with an IC_{50} value of 7 µg dwt/mL. Similarly, ethanol fraction (A-FR) showed maximum inhibition upto 78 % at 20 µg dwt/mL and expressed its IC_{50} value 8 of µg dwt/mL. Both E-FR and A-FR showed similar effect in inhibiting the peroxidation of lipids in liver homogenate. Water fraction (W-FR) showed up to 80 % inhibition at 40 µg dwt/mL with IC_{50} value 20 µg dwt/mL. These results indicated that W-FR showed comparably higher IC_{50} value than E-FR and A-FR as shown in Fig 6.1. E-FR and A-FR showed better effect in inhibiting the peroxidation of lipids in liver homogenate and their calculated IC_{50} values are almost the same.

Lipid peroxidation in heart homogenate (*in vitro*)

Like lipid peroxidation study in liver homogenate, here also all three different fractions of OSAE also were screened against $FeCl_3$ induced lipid peroxidation in heart homogenate of male Wistar rats. Among the following fractions, E-FR had higher activity compared to A-FR and W-FR fractions. E-FR showed reasonable effect compared to A-FR and W-FR as shown in Fig. 6.1B. E-FR showed maximum inhibition of 83% at 250 µg dwt/mL and its IC_{50} value was 119 µg dwt/mL. Whereas A-FR and W-FR showed maximum inhibition of 61 % to 63 % at 250 µg dwt/mL and their IC_{50} values were 204 and 192 µg dwt/mL respectively. Thus the

effect of different fractions on inhibition of peroxidation of lipids in heart homogenate varied depending on the type of phytoconstitutents extracted into the solvent used for extraction. Polarity of the solvent used during the time of extraction plays a major role as the extractions procedure was the same.

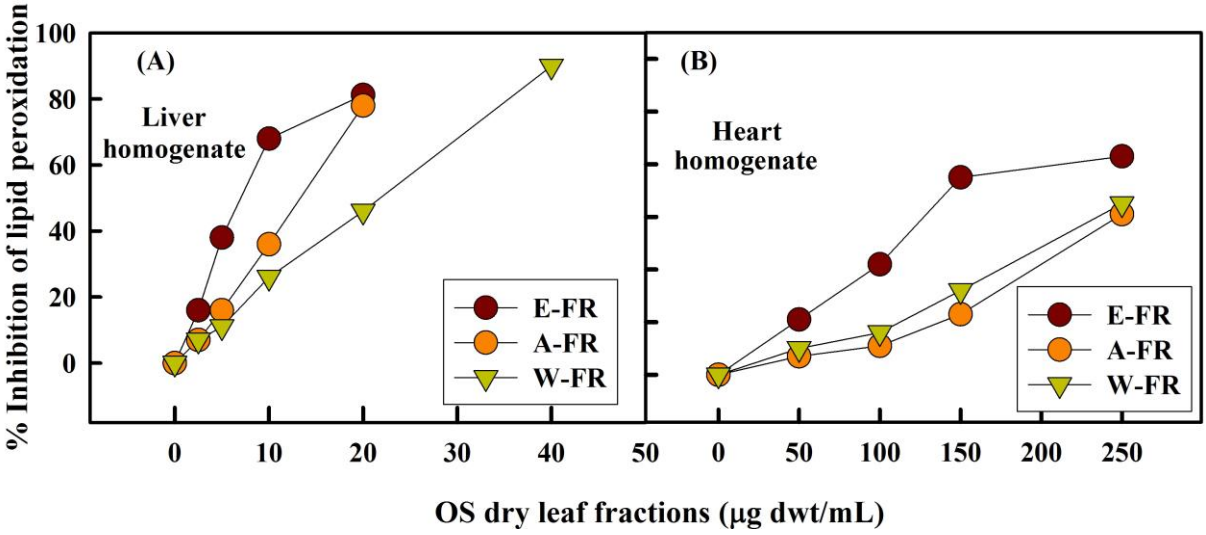


Fig. 6.1. Ferric chloride induced lipid peroxidation activity of ethyl acetate fraction (E-FR), alcoholic fraction (A-FR) and water fraction (W-FR) of alcoholic extracts of OS dry leaf on (A) liver (B) heart homogenates of male Wistar rats. Data presented are mean ± S.D., n=6. All values are statistically significant #$p < 0.001$ vs. Control.

Effect of OSAE fractions on cell viability assay

Varied concentration of different fractions of OS dry leaves (E-FR, A-FR and W-FR) were tested for their toxicity effect on cultured THP-1 cells and dosage determined based on cell viability as shown in Fig. 6.2. At the used concentrations of different fractions for the present study, no cell death was found, >95% cells were alive at 100 µg dwt/mL of all these fractions (Fig 6.2). There was no toxic effect as well as morphological changes occurred at the selected concentration of all three fractions. All THP-1 cells were alive after 24 h of treatment with any of three fractions. Vehicle solvents (alcohol or water of <1%, v/v) had no effect on cell viability. So the rest of the cell based assays were done using pretreatments with 100 µg dwt/mL of any of the three different fractions.

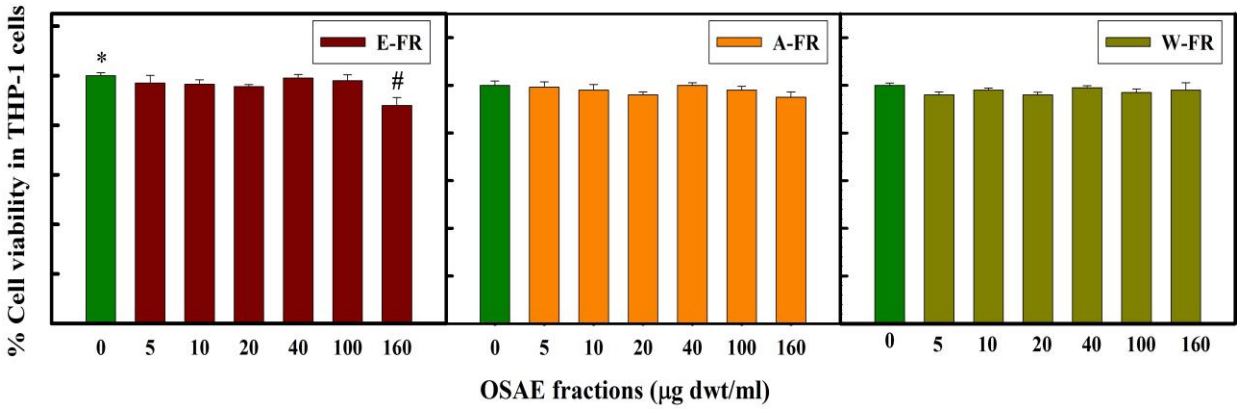


Fig. 6.2. Cell viability of THP-1 cells treated with different fractions of alcoholic extracts of OS dry leaves on THP-1 cells was checked by MTT assay. The cells were incubated with different concentrations of fractions (A) E-FR (B) A-FR; (C) W-FR for 24 h at 37 ◦C and 5% CO_2. Media without cells but with respective concentrations of fractions was used as appropriate blank. Experiments were performed at least in triplicate and the results are expressed as the mean ± S.D., n=6.

Effects of OSAE fractions on LPS induced TNF-α secretion in THP-1 cells

During the time of inflammatory condition or stress, activated monocytes secrete TNF-α, a well known proinflammatory cytokine which plays very important role in pathogenesis of atherosclerosis.

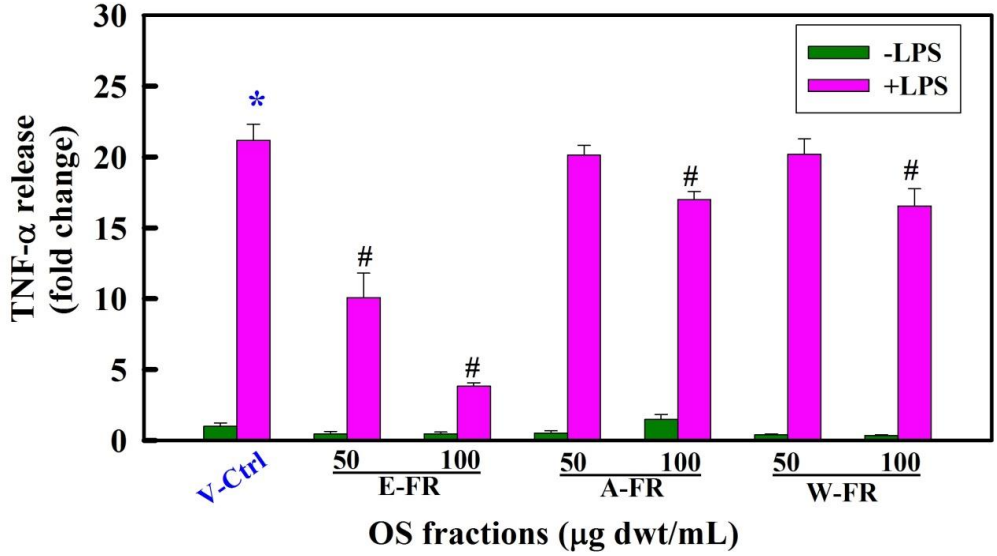


Fig. 6.3. Measurement of TNF-α release from the supernatants of LPS induced THP-1 cells. OSAE different fractions (E-FR; A-FR and W-FR) were used to treatTHP-1 cells for 12 hr prior to incubation with LPS for 3h. The effect of attenuation of TNF-α secretion by OSAE fractions (at 50 and 100 µg dwt/mL) was measured from the supernatant of THP-1 treated cells by ELISA. Data represent n=6 mean of six measurements. *Statistical significance of $p<0.001$ within the control groups i.e. cells+ LPS *vs* cells − LPS. $^{\#}p<0.001$ compared between cells treated with LPS in the presence of OSAE fractions.

Production of TNF-α by cultured THP-1 cells (5 X 10^5/mL) was tested in the culture supernatant using commercial ELISA kit and quantitated the secreted TNF-α from human THP-1 cells in the presence of a bacterial endotoxin, LPS 0.5 µg/mL for 3 h in the medium. As shown in Fig. 6.3, LPS stimulated THP-1 cells for 3 h treatment secreted 22 fold (435 pg/mL) more amount of TNF-α compared to control unstimulated cells. Whereas normal THP-1 cells secreted basal level of TNF-α (25 pg/mL)Pretreatment of LPS stimulated THP-1 cells with OSAE fractions (E-FR, A-FR and W-FR) at 50 µg dwt/mL for 12 h attenuated the secretion of TNF-α (10 fold) 197 pg/mL in case of E-FR, whereas at the same concentration, both A-FR and W-FR pretreated cells secreted (20 folds), 395 pg/mL of TNF-α. But pretreatment with all fraction of OSAE at 100 µg dwt/mL showed significant effect and markedly inhibited the secretion of TNF-α. At 100 µg dwt/mL of E-Fr, A-FR and W-FR treated cells secreted (3.82 fold) 75 pg/mL; (17 fold) 336 pg/mL; and (16 fold) 316 pg/mL respectively (Fig. 6.3). Among the fractions, E-FR showed highest effect compared to A-FR and W-FR. At higher concentration (100 µg dwt/mL), A-FR and W-FR showed significant effect where concentration (50 µg/mL) did not show much effect on secretion of TNF-α in LPS treated THP- 1cells. THP-1 cells incubated with the OSAE fractions (without LPS) secreted basal level of TNF-α production stimulated to control cells which established that fractions did not activate the cells. On the basis of TNF-α screening assay results, rest of the cell based assays including gene expression level by RT-PCR were performed using 100 µg dwt/mL concentration of all fractions of OSAE extract.

Effect of OSAE fractions on LPS induced mRNA transcripts of inflammatory markers in THP-1 cells

Gene expression of MCP-1

Real-time quantitative PCR showed that LPS enhanced the expression of MCP-1 (36 fold) compared to unstimulated THP-1 cells (Fig. 6.4). Such stimulation in proinflammatory gene

expression with LPS was markedly restricted by OSAE fractions. The effect of the OSAE fractions on LPS induced gene expression of inflammatory markers MCP-1 was tested to examine if the effect was at transcriptional or posttranscriptional level. Pretreatment of the THP-1 cells with E-FR (100 µg dwt/mL) showed significant effect in attenuation of LPS induced MCP-1 expression (Fig. 6.4).

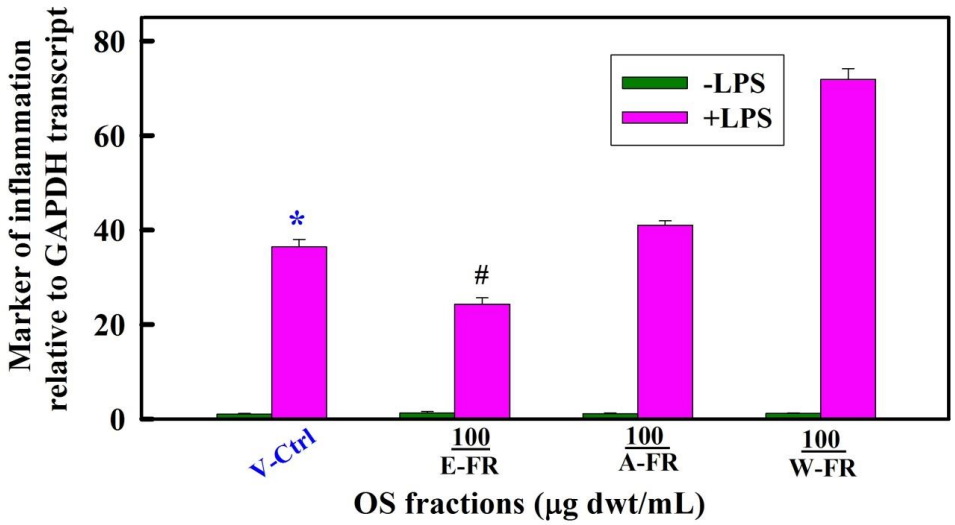


Fig. 6.4. Effect of OSAE fractions on gene expression of LPS induced MCP-1 transcripts in THP-1 cells. Data shown mean value of 3 independent experiments. #$p < 0.001$ and *$p < 0.001$ between the control groups.

E-FR down regulated MCP-1 expression up to 24 fold change. A-FR and W-FR (100 µg dwt/mL) acted as stimulators of MCP-1 expression in LPS induced THP-1 cells (Fig. 6.4). These results suggest hat its activity depends up on the presence of certain metabolites in E-FR but not in A-FR and W-FR. These metabolites have played a major role to attenuate the suppression of cytokines or chemokines at their gene as well as protein level in LPS treated THP-1 cells.

LC-MS/MS metabolite analysis of OSAE fractions

Phytochemical analysis of OSAE fractions with LC-Q-TOF-MS was done to obtain the metabolite profiles and to examine difference among the fractions. Several compounds were detected in both negative and positive ion modes. Compounds in all three different fractions

are mostly phenolic, flavonoids and terpenes in nature as shown in table 6.1. Negative ion and positive ion modes of LC-QTOF-MS detected 8 and 18 compounds in E-FR; 9 and 8 compounds in A-FR and 4 and 9 compounds in W-FR. Rosmarinic acid, orientin, β-sitosterol, Luteolin, apigenin-7-O-glucuronide, eugenol were commonly detected in both E-FR, A-FR and W-FR. Circimartin, palmitic acid, carnosic acid, isothymusin, limonene and β-caryophyllene were exclusively detected in E-FR, whereas apigenin, oleanoilc acid, cirsilineol, methyl eugenol were detected in A-FR. Compounds which were not detected in OSAE and OSWE and not described in chapter 4 are described in the following text.

Table 6.1. Q-TOF profile, negative and positive ion mode of E-FR, A-FR and W-FR used in LC-MS/MS analysis

Compound name/ class of compound	**Mol formula/ Exact mass**	**Types of extracts**	**Abudance**		**Reference**
			-ESI	**+ESI**	
Linoleic acid (Unsaturated omega-6 fatty acid)	$C_{18}H_{32}O_2$ 280.44	E-FR	ND	32569	Mass bank
		A-FR	ND	ND	
		W-FR	ND	19466	
Vicenin (Flavonoids)	$C_{27}H_{30}O_{15}$ 594.51	E-FR	ND	ND	Jabor *et al.*, 2010.
		A-FR	5215	ND	
		W-FR	3065	23348	
Luteolin-7-O-glucuronide (Flavonoids)	$C_{21}H_{17}O_{12}$ 462.07	E-FR	25855	30897	Lin *et al.*, 2010.
		A-FR	16935	ND	
		W-FR	10312	54061	
Rosmarinic acid (Phenylpropanoids)	$C_{18}H_{16}O_8$ 360.31	E-FR	266238	178680	Mass bank
		A-FR	29433	ND	
		W-FR	12500	ND	
Apigenin-7-O-glucuronide (Flavonoids)	$C_{21}H_{18}O_{11}$ 446.36	E-FR	11558	ND	Lin *et al.*, 2010.
		A-FR	8397	ND	
		W-FR	13188	27002	
Eugenol (Phenyl propene)	$C_{10}H_{12}O_2$ 164.20	E-FR	ND	183458	Mass Bank
		A-FR	ND	1024390	
		W-FR	ND	22117	
Palmitic acid (saturated fatty acid)	$C_{16}H_{32}O_2$ 256.42	E-FR	ND	35000	Mass bank
		A-FR	ND	ND	
		W-FR	ND	ND	
Methyl eugenol (Penylpropene)	$C_{11}H_{14}O_2$ 178.09	E-FR	ND	129516	Mass bank
		A-FR	ND	24396	

		W-FR	ND	ND	
Carnosic acid (Diterpene)	$C_{20}H_{28}O_5$ 332.43	E-FR	304667	56564	Hossain *et al.,* 2010
		A-FR	ND	ND	
		W-FR	ND	ND	
Apigenin (Flavonoids)	$C_{15}H_{10}O_5$ 270.24	E-FR	78472	138200	Mass bank
		A-FR	2802	ND	
		W-FR	ND	ND	
Cirsimaritin (Flavonoid)	$C_{17}H_{14}O_6$ 314.28	E-FR	ND	451713	Hossain *et al.,* 2010.
		A-FR	ND	ND	
		W-FR	ND	ND	
Cirsilineol (Flavonoids)	$C_{18}H_{16}O_7$ 344.32	E-FR	3644	807180	Sun *et al.,* 2010.
		A-FR	ND	173372	
		W-FR	ND	ND	
Orientin (Flavonoids)	$C_{21}H_{20}O_{11}$ 448.09	E-FR	30776	56444	Mass bank
		A-FR	37980	584461	
		W-FR	ND	161043	
1,8-cineol (Monoterpenoid)	$C_{10}H_{18}0$ (154.24)	E-FR	ND	ND	Mass bank
		A-FR	40872	ND	
		W-FR	ND	ND	
Luteolin (flavonoid)	$C_{15}H_{10}O_6$ (286.24)	E-FR	ND	279163	Mass bank
		A-FR	4680	298854	
		W-FR	ND	62887	
β-sitosterol (Phytosterol)	$C_{29}H_{50}O$ (414.71)	E-FR	ND	96103	Mass bank
		A-FR	ND	78611	
		W-FR	ND	18488	
Stigmasterol (Phytosterol)	$C_{29}H_{48}O$ (412.69)	E-FR	ND	ND	Mass bank
		A-FR	ND	49644	
		W-FR	ND	ND	
Menthyl acetate (Monoterpene)	$C_{12}H_{22}O_2$ (198.3)	E-FR	ND	136377	Mass bank
		A-FR	ND	ND	
		W-FR	ND	45065	
Oleanolic acid (Triterpenoid)	$C_{30}H_{48}O_3$ (456.70)	E-FR	62127	45315	Mass bank
		A-FR	7310	38023	
		W-FR	ND	ND	
Limolene (Cyclic terpene)	$C_{10}H_{16}$ (136.24)	E-FR	ND	120950	Mass bank
		A-FR	ND	ND	
		W-FR	ND	ND	
β-Caryophyllene (Bicyclic sesquiterpene	$C_{15}H_{24}$ (204.36)	E-FR	ND	115188	Mass bank
		A-FR	ND	ND	
		W-FR	ND	ND	
Isothymusin (flavonoid)	$C_{17}H_{14}O_7$ (330)	E-FR	ND	1453898	Grayer *et al.,* 2000
		A-FR	ND	ND	
		W-FR	ND	ND	

1, 8-cineol, $C_{10}H_{18}O$ (Fig. 6.5. A): It is commonly called as eucalyptol. It is a natural organic compound, colorless liquid. It is a cyclic ether and a monoterpenoid in nature. It yielded the peak at RT 6.33 min from A-FR only. Identification of the compound confirmed by $[M+H]^+$ with m/z 155.09. The mass fragmentation patterns $[M+H]^+$ of the compound were m/z 139, 131, 126, 121, 111, 108 and 107. The compound was identified, confirmed and characterized from Mass bank record JP006595.

Isothymusin, $C_{10}H_{10}O_2$ (Fig. 6.5 B): It is a flavonoid and clear yellow powder. It was detected in E-FR only. It yielded the peak at RT 20.08 min from E-FR. Identification of the compound was confirmed by mass spectra exhibiting ion $[M+H]^+$ with m/z 331.29. The mass fragmentation patterns $[M+H]^+$ with m/z were 157, 155, 142, 127, 121 and 108. The detailed information of this compound was characterized according to Grayer *et al.*, (2000).

Carnosic acid, $C_{20}H_{28}O_4$ (Fig. 6.5 C): It is a natural benzenediol abietane diterpene found in many medicinal plants including *O. sanctum* dry leaves. It represent for its antioxidant properties and it protects skin cells against UV-A radiation. It was eluted at RT 16.33 min from E-FR. The identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ with m/z 333.43. MS fragmentation patterns $[M+H]^+$ were m/z 252, 206, 165, 162, 159, 147, 137 and 128. It has been previously reported by Hossain *et al.*, (2010).

Menthyl acetate, $C_{12}H_{22}O_2$ (Fig. 6.5 D): It is a natural monoterpene. It was eluted at RT 19.96 min in E-FR. Identification of the compound was confirmed by their the MS-fragmentation spectra exhibiting the ions $[M+H]^+$ with m/z 199.31and secondary fragmentation ions at m/z 143, 141, 136, 130, 128 and 117. The compound was confirmed based on Mass bank record JP006483.

Luteolin, $C_{15}H_{10}O_6$ (Fig. 6.5 E): It is a flavonoid and yellow crystalline in appearance. It yielded the peak at RT 20.49 min from E-FR. Identification of the compound was confirmed

by $[M+H]^+$ with m/z 287.06. The mass fragmentation patterns $[M]^+$ of the compound were m/z 241, 161, 154, 137, 135 and 117. This compound was confirmed by the hit from the data bases of Masss bank record PR100231.

Stigmasterol, $C_{29}H_{48}O$ (Fig. 6.5 F): It is a plant sterol or phytosterol. It yielded the peak at RT 21.90 min from A-FR. The identification of the compound confirmed by its mass spectrum exhibiting the ions $[M+H]^+$ of m/z 413.25, mass fragmentation patterns $[M+H]^+$ of the compound were m/z 201, 175, 173, 171, 161, 159, 151, 149 and 147 are comparable with Mass bank record FFF00268.

Limolene $C_{10}H_{16}$ (Fig. 6.5 G): Limonene is a colourless liquid hydrocarbon classified as a cyclic terpene. It was exclusively detected in E-FR, not in other fractions and OSAE/OSWE extracts. It yielded the peak at RT 23.47 from E-FR. Identification of the compound confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ with m/z 137.12 and of mass fragmentation patterns $[M+H]^+$ with m/z 135, 133, 117, 107 and 105. On the basis of mass fragementation, the compound was confirmed through Mass bank record JP006892.

β-caryophyllene, $C_{15}H_{24}$ (Fig. 6.5 H): It is a natural bicyclic sesquiterpene that is a constituent of many essential oils including *O. sanctum* dry leaves. Along with limolene, β-caryophyllene also detected only in E-FR. It was eluted at RT 27.97 min from E-FR. Identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M+H]^+$ with m/z 205.16. MS fragmentation pattern $[M+H]^+$ of the compound were m/z 151, 147, 138, 135, 134, 121 and 118. The compound was confirmed through Mass bank record JP010683 on the basis of MS fragmentation.

Palmitic acid, $C_{16}H_{32}O$ (Fig. 6.5 I): It s the most common fatty acid (saturated) found in several medicinal plants. It was eluted at RT 30.87 min from E-FR. Identification of the compound was confirmed by the mass spectrum exhibiting the ion $[M+H]^+$ with m/z 257.16.

MS fragmentation pattern $[M+H]^+$ of the compound were m/z 150, 149, 145, 135, 131, 129, 121, 115 and 110. The detailed information of each peak in the compound is similar to Mass bank record KZ000259.

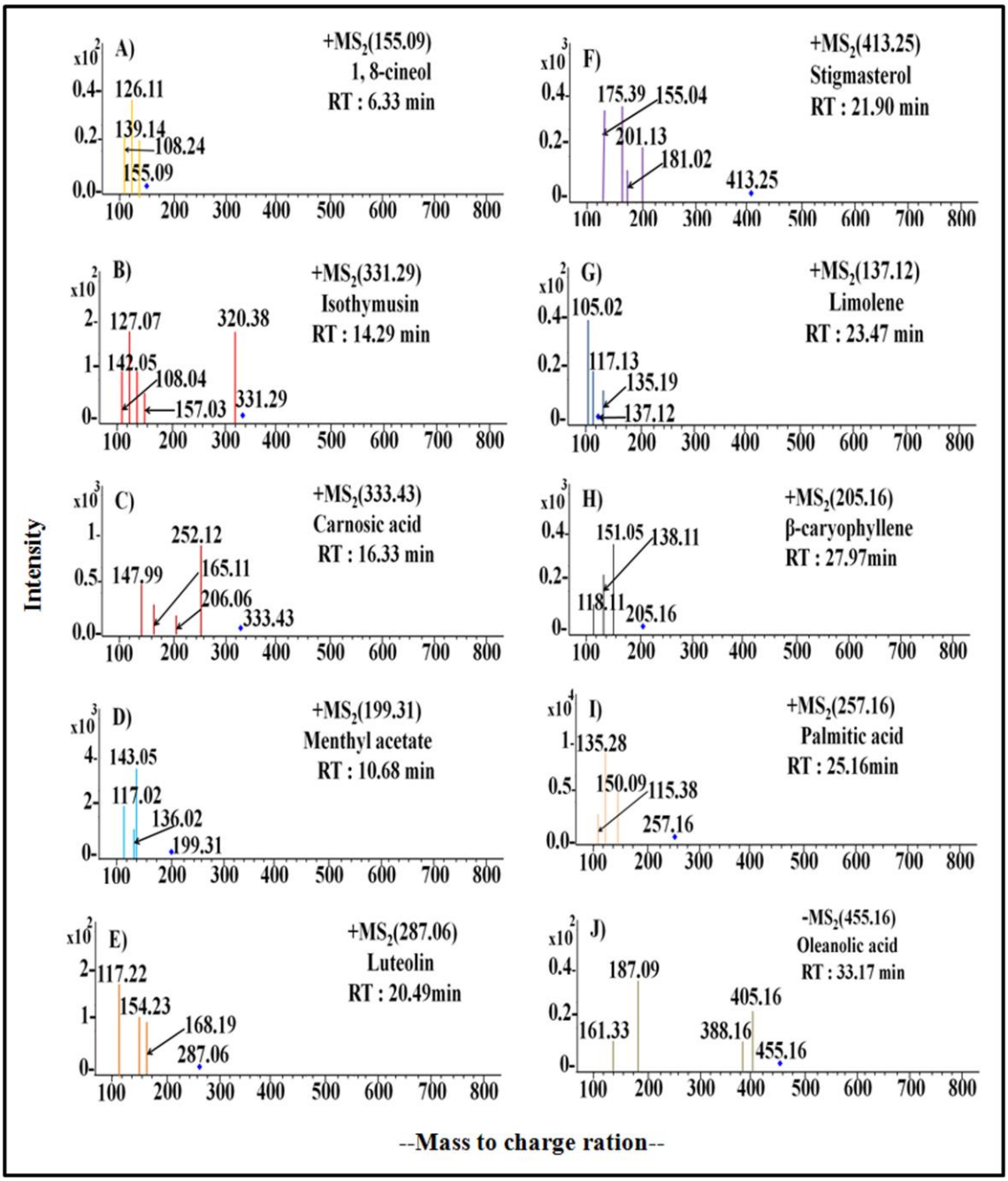


Fig. 6.5. Negative ion (−) ESI/MS/MS of E-FR, A-FR and W-FR of OSAE extracts of *O. sanctum* dry leaves. Product ion labels correspond to MS fragments.

Oleanolic acid, $C_{30}H_{48}O_3$ (Fig. 6.5 J): It is a naturally occurring triterpenoid, widely distributed in food and medicinal plants. It was eluted at RT 33.17 min from E-FR. The

identification of the compound was confirmed by the mass spectrum exhibiting the ions $[M - H]^-$ with m/z 455.16 and $[M + H]^-$ with 457.32. MS fragmentation pattern $[M+H]^+$ were m/z 405, 388, 187, 164 and 161. It has been confirmed from Mass bank record BML0059.

Compounds detected in OS fractions which are commercially available were tested for their antiinflammatory activity in THP-1 cells. Eugenol and rosmarinic acid were detected in all fractions as well as in OSAE/OWE and these two compounds were tested for their antiinflammatory effect in THP-1 cells. Our above results from the different fractions of OSAE in THP-1 cells (*in vitro*) showed that E-FR showed more protective effect compared to A-FR and W-FR to inhibit the expression of certain inflammatory markers in LPS stimulated THP-1 cells. LC-MS/MS analysis confirmed that, limonene and β-caryophyllene were exclusively detected in E-FR and not in other fractions. So these two compounds were selected to check the antiinflammtory activity in THP-1 cells along with eugenol and rosamarinic acid.

Effect of active metabolites of OS dry leaves on cell viability

To elucidate the antiinflammatory properties of selected pure compounds β-C, EUG, LM and RA were tested for their toxic effect on THP-1 cells. We confirmed cytotoxicity effect of all the compounds on THP-1 cells by carrying MTT assay in order to find out nontoxic range of dosage of all the compounds as shown in Fig 6.6. THP-1 Cells were exposed to increasing concentrations of all four compounds for 24 hours. β-C and RA treated cells showed 90 % cell viability at 160 µg/mL, whereas EUG and LM up to 200 µg/mL showed cell viability of 90 %. We limited the concentration of pure compounds to maximum of 60 µg/mL, no toxic effect was observed at this concentration of all the pure compounds and cell death was very negligible. A part from that, at 60 µg/mL concentration, all the compounds showed 100 % viability. Therefore, we restricted our dosage of all compounds at 60 µg/mL to proceed further to prove the antiinflammatory property in THP-1 cells at their gene or protein level.

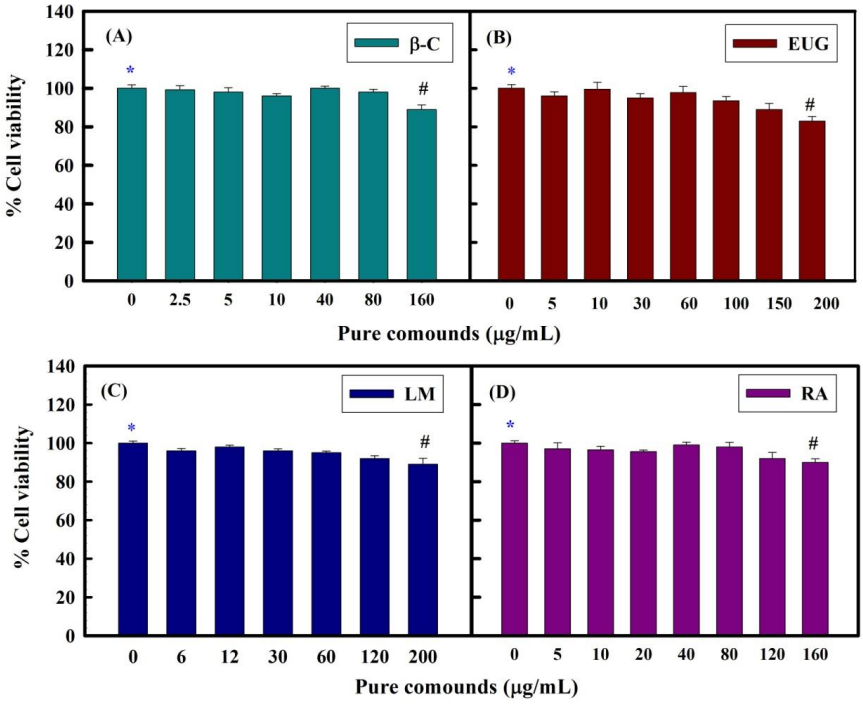


Fig. 6.6. Cell viability was checked by MTT assay after treatment with (A) β-caryophyllene, (B) eugenol, (C) limolene (D) rosarminic acid. All the pure compounds with varied concentration were pretreated with THP-1 cell for 24 h at 37 ◦C and 5% CO_2. Media without cells, but with respective concentrations of respective pure compound were used as appropriate blanks. Data presented is mean±S.D., n = 6.

Effect of selected pure compounds on LPS induced TNF-α secretion in THP-1 cells

In order to carry out and identify the active principle of OS leaf extracts responsible for its antiinflammatory properties, we tested list of four selected metabolites on LPS induced THP-1 cells at proinflammatory protein as well as gene expression level. We investigated the inhibitory effect of EUG, RA β-C and LM on attenuation of TNF-α secretion in LPS induced THP-1 cells by ELISA. Pretreatment of THP-1 cells with varied concentrations of pure compounds for 12 h followed by treatment with LPS for 3 h. The total amount of TNF-α released into the medium was measured by ELISA. Supernatants of control cells had negligible amounts of TNF-α (15 pg/mL), whereas substantial increase in TNF-α release was observed in LPS induced cells i.e., 435 pg/mL and accounts to 22 fold induction (Fig. 6.7). Cells pretreated with pure compounds secreted significantly less amount of TNF-α compared to LPS induced cells. Among the following, EUG at 6 and 60 µg/mL (v/v) suppressed

stimulation of TNF-α secretion to (14.81 fold) 276 pg/mL and (2.21 fold) 43.69 pg/mL respectively.

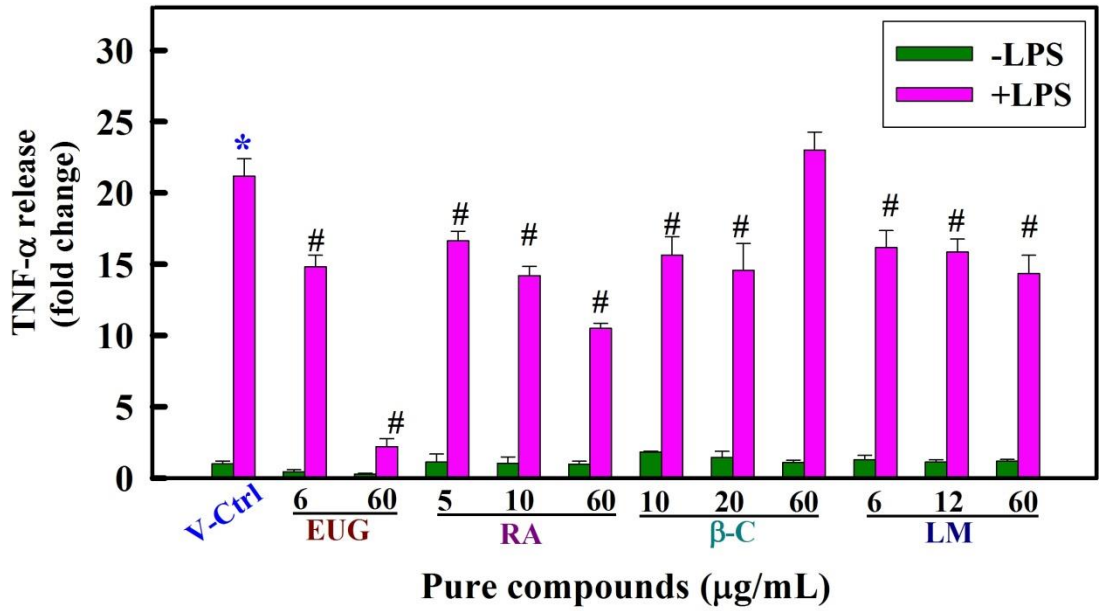


Fig. 6.7. The effect of pure compounds EUG, RA, β-C and LM mediated attenuation of LPS induced TNF-α secretion. THP-1 cells (5 X 10^5 cell/mL) were preincubated with different concentration of pure compounds for 12 h and then further treated with LPS for 3h. Secretory level of TNF-α from the supernatant were measured by ELISA. Data expressed are mean value of three independent experiment ± S.D.*Statistical significance of $p<0.001$ within the control groups i.e. cells+ LPS *vs* cells − LPS. $^{\#}p<0.001$ compared between cells treated with LPS in the presence of pure compounds in their absence.

Whereas. RA at 5, 10 and 60 µg/mL showed (16 fold) 316 pg/mL; (14 folds) 276 pg/mL and (10 fold) 197 pg/mL respectively. β-C and LM did not show significant effect like EUG and RA. LM at 60 µg/mL reduced only to (14 fold) 276 pg/mL where as β-C did not show effect at higher concentration 60 µg/mL (23 fold) 454 pg/mL as shown in Fig. 6.7, but had small effect at lower concentration of 10 and 20 µg/mL of β-C treated cells seen (15.63 folds) 296 pg/mL and (14.56 folds) 280 pg/mL respectively. LM at 6, 12 and 60 µg/mL reduces TNF-α secretion to (16 folds) 316 pg/mL; (15 folds) 296 pg/mL and (14 fold) 270 pg/mL respectively. Among the four compounds, EUG showed higher protective effect at 60 µg/mL. From the above results it may be confirmed that, EUG, a phenyl propanoid could be considered as a one of the most important metabolites that contributed to antiinflammatory activity of OS dry leaves. For

further confirmation of EUG (60 µg/mL) activity was tested for its effect on other chemokines and cytokines at gene expression level.

Effect of eugenol on LPS induced MCP-1 secretion in THP-1 cells

MCP-1 is a potent proinflammatory chemokine secreted by activated monocytes. Secreted MCP-1 subsequently activates other cell sepsis in the blood vessel and thus initiates progression of atherosclerosis. Production of MCP-1 by cultured THP-1 cells was tested in the culture supernatants using commercial ELISA kit.

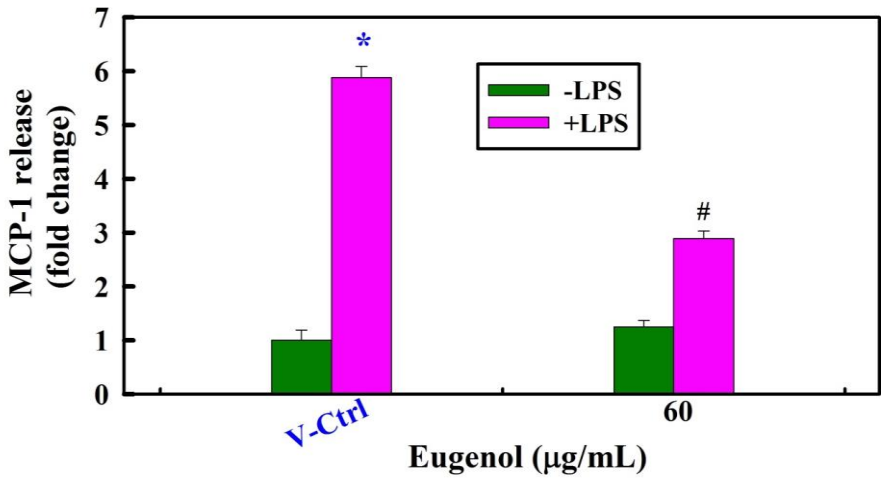


Fig. 6.7. Inhibitory effect of pure compound eugenol on LPS mediated MCP-1 secretion in THP-1 cells by ELISA. Pure compound eugenol was treated with THP-1 (5 X 10^5 cell/mL) for 12 h followed by treatment of LPS for 3 h. Secreted MCP-1 in the supernatant of all treated cells were measured by ELISA. Data shown mean value of three independent experiment ± S.D. (#p<0.001), *p<0.001 between the control groups.

It was found that THP-1 released more amounts of MCP-1 after 3 h stimulation with LPS as shown in Fig. 6.8. LPS stimulated THP-1 cells secreted 5.88 (110 pg/mL) fold more amount of MCP-1 compared to control unstimulated cells (20 pg/mL). Pretreatment of cells with EUG at 60 µg/mL for 12 h brought down to 2.8 fold (52 pg/mL) of MCP-1 release in the presence of LPS (Fig. 6.8).

Effect of eugenol on LPS induced gene expression in THP-1 cells

Cell based (*in vitro)* experiments with human monocytic (THP-1) cell line were conducted to evaluate the antiinflammatory effect of EUG against a bacterial endotoxin, LPS induced inflammation.

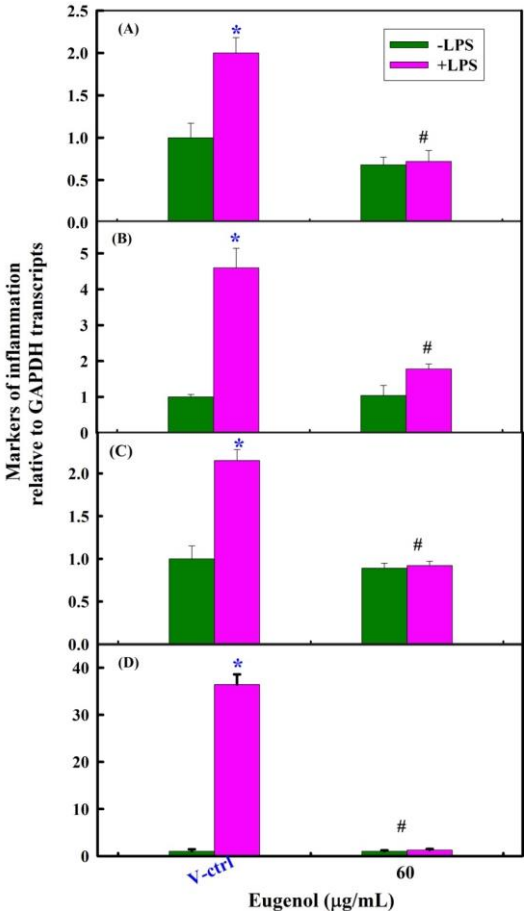


Fig. 6.9. Inhibitory effect of pure compound EUG (60 µg/mL) on gene expression of LPS induced IL-6 (A); TNF-α (B); MIP-1α (C) and MCP-1 (D) transcripts in THP-1. Vehicle Experiments were performed at least in triplicate and the results are expressed as the mean ± S.D. *p<0.001 for comparison between Cells+LPS *vs* cells-LPS. $^{\#}$p<0.001 compared between cells treated with LPS in the presence of eugenol *vs* in their absence.

The effect of pure compound EUG on LPS induced gene expression of certain proinflammatory cytokines and chemokines like IL-6, TNF-α, MIP1α and MCP-1 was tested to examine if the effect was at transcriptional level. Real-time quantitative PCR showed that LPS enhanced the expression of the all the above mentioned markers in THP-1 cell after 3 h treatment. As shown in Fig. 6.9 A to D, the transcript analysis products showed that LPS

stimulated the expression of IL-6 (2 fold), TNF-α (5 fold), MIP-1α (2.15 fold) and MCP-1 (36 fold) by several folds. Such stimulation of proinflammatory gene expression was markedly inhibited by EUG. Pretreatment of THP-1 cells with EUG at 60 µg/mL for 12 h followed by LPS treatment for 3 h caused significant decrease in transcription level of IL-6 (0.72 fold); TNF-α (1.78 fold); MIP-1α (0.48 fold) and MCP-1 (1.26 fold) by several folds.

Effect of EUG on PMA induced differentiation of THP-1 cells

According to several scientific reports, it has been proved that, human monocytic (THP-1) cells are differentiated to macrophages and further leads to foam cell formation and causes atherosclerosis in human body under several stress condition. As mentioned earlier in the previous chapter 5, we used PMA as an inducer to differentiate the monocytic (THP-1) cell in suspension to differentiate into adherent macrophage plate as shown in Fig 6.10 A and B. As shown in our previous results Fig 5.6 and 5.8, we demonstrated that, pretreatment of THP-1 cells with OSAE/OSWE (1 mg dwt/ml) inhibited the differentiation process. In order to find out the active principle, particularly we have tested the inhibitory effect of EUG of PMA induced differentiation. Pretreatment of EUG (60 µg dwt/mL) with THP-1 cells for 12 h significantly attenuated the process of differentiation by PMA challenged THP-1 cell into macrophage as shown in Fig 6.9 C. Further more, we have tested the effect of EUG at their gene expression level of macrophage differentiation markers by RT-PCR.

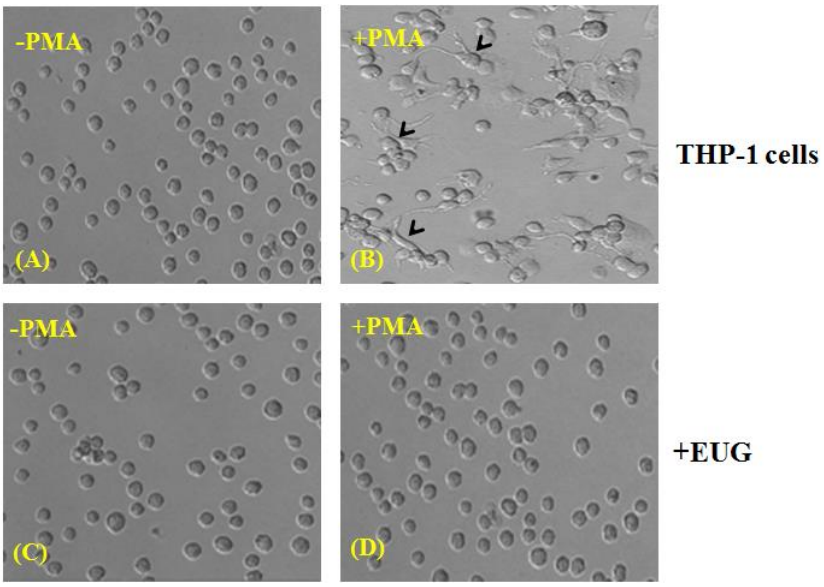


Fig. 6.10. Inhibitory **e**ffect EUG on PMA induced macrophage differentiation on THP-1 cells. Cells were seeded at 5 x 10^5 cells/well for each treatment. Differentiation of monocytes to macrophages was proceeded by stimulating the THP-1 cells with PMA (5 ng/mL). Cells were pretreated with EUG for 12 h then challenged with PMA for 48 h. Photocopies of cells were taken under normal inverted microscope after 48 h treatment. (A) THP-1 cells, (B) THP-1 cells + PMA (5 ng/mL); (C) THP-1 cells + EUG (60 µg/mL) (D) THP-1 cells + EUG (60 µg/mL) +PMA. Black coloured arrow marks indicated in Fig B, are THP-1 derived elongated, flattened, thread like differentiated macrophage cells after PMA treatment. Vehicle controls concentration of alcohol was limited to <1% (v/v) and DMSO to 0.2%, (v/v) and they had no effect on cell differentiation.

Effect of EUG on PMA induced cell differentiation markers

According to literature survey and as per our previous discussion from chapter 5, we demonstrated that, PMA stimulated THP-1 cells show higher expression of certain macrophage differentiation markers on their cell surfaces. Here we showed the effect of EUG to inhibit the expression of PMA induced macrophage differentiated markers by RT PCR method. THP-1 cells treated with PMA upregulated the expression of CD14 (3 fold), toll like receptors, TLR2 and TLR4 by 64 and 37 folds respectively (Fig. 6.11). Pretreatment of EUG with THP-1 cells for 12 h reduced such upregulation of CD14 (1.56 fold), TLR2 (1.61 fold) and TLR 4 (5 fold). Here pure compound EUG showed significant effect in attenuating the expression of above selected macrophage differentiation markers. We can assume that the protective effect of OS dry leaves is due to the presence of EUG only.

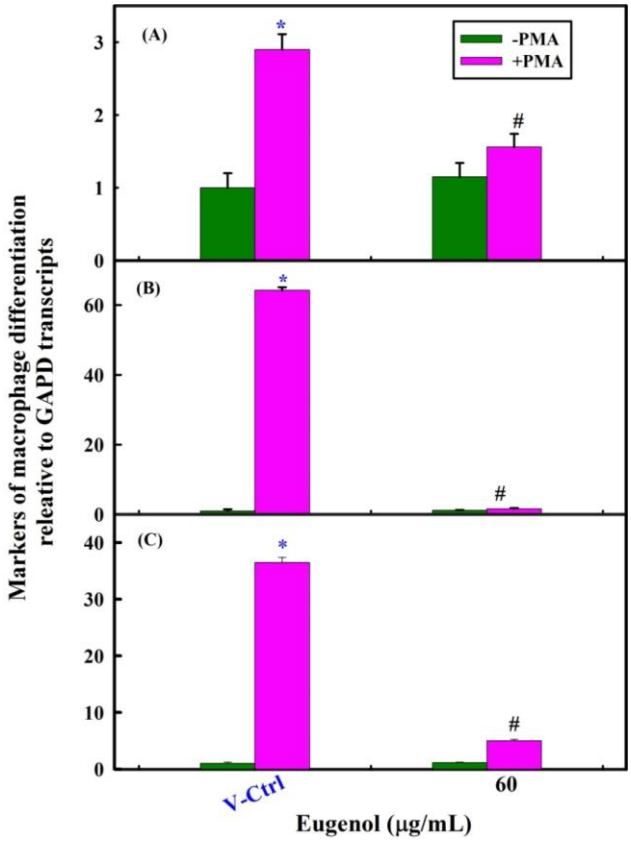


Fig. 6.11. Effect of pure compound EUG (60 µg/mL) on PMA induced macrophage differentiation markers CD14 (A), TLR2 (B) and TLR4 (C) mRNA expression in THP-1cells. Eugenol was preincubated with THP-1 cells for 12 h and then treated with PMA (5 ng/mL) for 48h. After incubations, transcript levels by CD14 (A), TLR2 (B) and TLR4 (C) were determined by RT-PCR as a measure of THP-1 cell differentiation. Data shown are mean value of 6 independent experiments ± S.D. n=6. *$p<0.001$, for comparison between treated (cells + PMA) and untreated groups (cells - PMA). $^{\#}p<0.001$ compared between cells treated with PMA in the presence of eugenol *vs* in their absence.

Effect of EUG on the subcellular localization of NF-κB-p65

The transcription factor NF-κB plays a significant role in different chronic inflammatory diseases, including atherosclerosis. Gene expression of proinflammatory cytokines and chemokines including IL-1, IL-6, TNF-α, MIP-1α, MCP-1 is regulated by nuclear factor NF-κB. We investigated using immunofluorescence microscopy the nuclear translocation of p65 as a surrogate marker for the NF-κB pathway activation. An Alexa fluor 594 conjugated secondary antibody against p65 was used for detection. We examined whether EUG (60 µg/mL) prevents the translocation of the p65 subunit of NF-κB to the nucleus in LPS induced

THP-1 cells. The confocal images revealed that NF-κB p65 was normally sequestered in the cytoplasm of control/unstimulated cells (Fig. 6.12 A to C, 1st panel) and that nuclear accumulation of NF-κB-p65was strongly induced after stimulation of THP-1 cell with LPS (Fig. 6.12 D to F, 2nd panel). The LPS-induced translocation of NF-κB-p65 was completely abolished after pre-treating the cells with EUG (Fig. 612 G to I, 3rd panel).

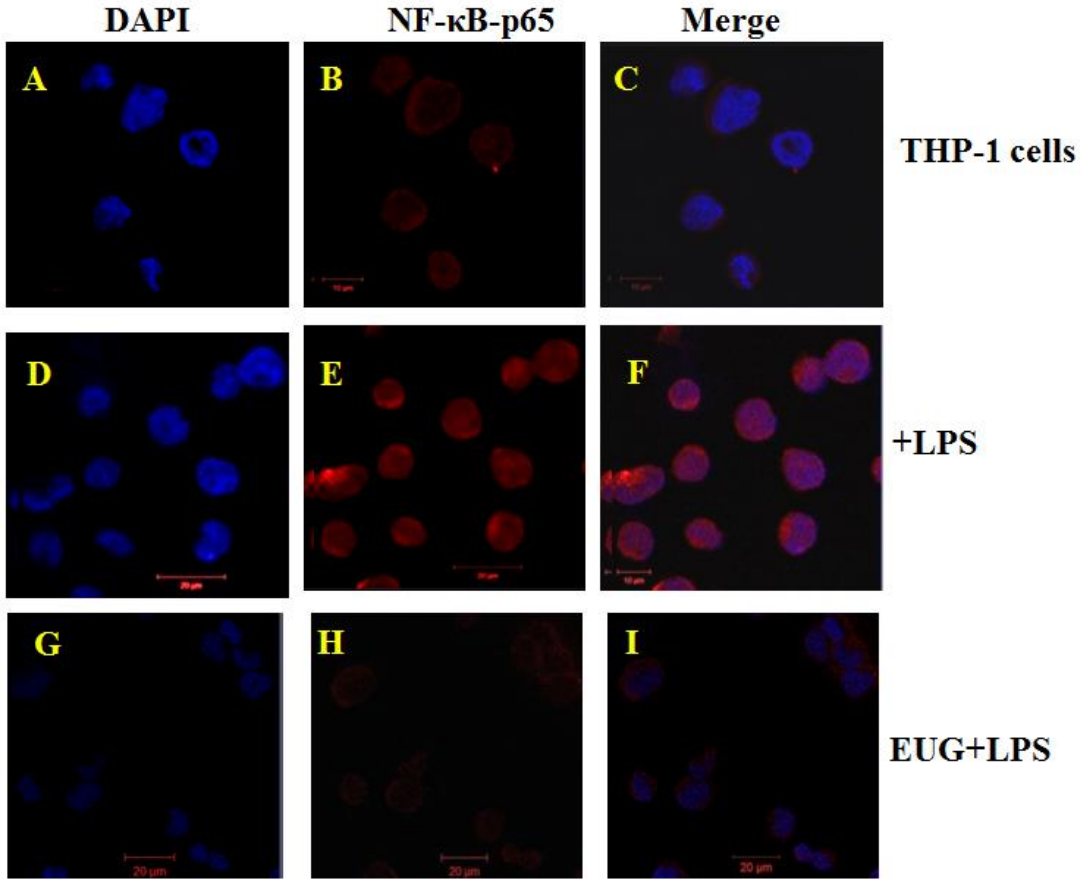


Fig.6.12. Confocal images showing the effect of pure compound EUG (60 µg/mL) on LPS induced subcellular localization of NF-κB-p65 from cytoplasm to nucleus in THP-1cells. Images with vehicle control 0.5%, v/v (alcohol) cells are shown in panel 1. NF-κB translocation was analyzed by staining with NF-κB-p65, red (A); nucleus with DAPI, blue (B); merged image of NF-κB-p65 and DAPI (C). Images A, B, D, E,G, H were taken using Ex 590 nm, Em 617 nm, Ex 345 and Em 455 nm respectively. C, F, I are merged images.

Taken together, the above findings demonstrated that the antiinflammatory effect of EUG in LPS stimulated THP-1 cells is dependent on NF-κB pathway. As per our previous reports of chapter 5, we demonstrated that, both OSAE/OSWE inhibited the translocation of NF-κB in LPS stimulated THP-1 cells. In this chapter we proved EUG also showed similar

effect in inhibiting the translocation of NF-κB. Thus our results suggest that antiinflammatory effect of OS dry leaf extracts could be because of presence of EUG.

Quantitation of eugenol in OS leaf extracts

Eugenol, a natural phenolic compound was detected in both OSAE/OSWE and also in all three fractions fractions of OSAE extracts. Our results showed the antinflammatory properties of EUG in THP-1 cells. In order to investigate if antiinflammatory activity of dry leaf extracts of OS is due to the presence of eugenol alone or its synergetic effect along with other compounds, we quantitated the EUG in the selected extracts.

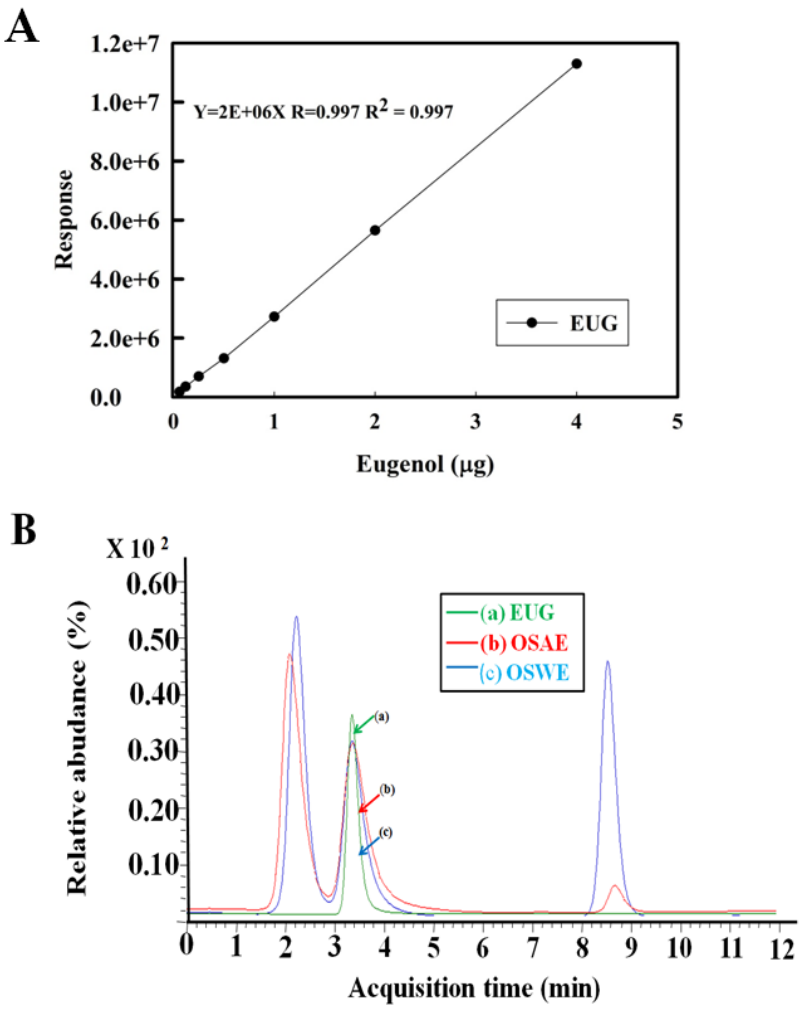


Fig. 6.13. LC-MS analysis for quantification of eugenol in OS dry leaf extracts. (A) Regression plot with varied concentration of eugenol ranging from 0.06 to 4 µg/mL. (B) chromatogram of pure compound (a) eugenol; (b) OSAE; (c) OSWE with marked peak, corresponding to standard eugenol with retention 3.28 and 3.33 min in OSAE and OSWE extracts respectively. Peaks were used to determine the eugenol content in the extracts.

The total amount of eugenol present in both OSAE and OSWE was estimated using HPLC coupled to LC-MS. Titration curve was plotted using commercially available pure compound eugenol. Standard solutions of eugenol in the range from 0.06 µg/mL to 4 µg/mL were prepared to test the linearity (Fig. 6.13 A). After sample analysis through HPLC, we confined pure compound EUG which was eluted at 3.28 min and shown m/z of 165.09. Under the subjected of same MS conditions, eugenol is present in OSAE and OSWE was eluted at 3.28 min and 3.33 min respectively with m/z 165.093 as shown in Fig 6.13 B. Further we also confirmed the presence of EUG by mass fragmentation spectra which was generated in MS/MS analysis. By using titration curve of pure compound EUG, we quantitated the total amount of EUG present in OSAE and OSWE. OSAE and OSWE contained eugenol of 12 ng/mg dwt and 19 ng/mg dwt respectively. Our cell based experiments with pure eugenol compound suggests that at least 60 µg/mL is necessary. Therefore we conclude that the activity of OS extracts is due to synergetic effect of eugenol along with several bioactive compounds present in the extracts.

Discussion

According to different scientific reports, it has been recogonised that, medicinal plants are rich source of diverse kind of phytoconstitutents and are useful in treatment of several health alignments. It is widely accepted that drugs of highly use are herbal origin and are playing essential role in treating diverse diseases. Thus plant kingdom is being considered as gold mine due to existence of many biologically active principles, which have to be investigated thoroughly (Kaladhar *et al.,* 2014). Our understanding of the scientific principles of these herbal drugs is poor.

In living systems of plants as well as animals, free radicals are constantly generated and are over produced during stress and can cause extensive damage to cellular macromolecules leading to the several disease condition. Atherosclerosis and other

inflammatory disorders are developed due to oxidative stress. Many synthetic drugs protect against oxidative damage but they have several adverse side effects. Therefore, an alternative solution to the problem is to consume natural antioxidants from plant materials.

Currently, there has been an increasing interest in the biochemical effects of medicinal plants with antioxidant property as they could be candidates for the prevention of oxidative damage and other inflammatory disorders. Phytomedicines could be in the form of crude preparations containing a wide variety of compounds or pure molecules with a strong and specific activity. Identification of chemical compounds and the molecular targets of these compounds helps to validate the use of these medicines. Plant extracts or their constituents are responsible for the protective effect with powerful antioxidant capacity and protective properties (Shailasree *et al.*, 2012). In Indian traditional system of medicine, *O. sanctum* has been considered as one of the most important medicinal plant for different inflammatory disorders and to treat cardiovascular diseases. OS has shown its potency and can be considered as a therapeutic and safe herb for long term consumption. With reference to our previous discussion in chapter 5, we have confirmed the antioxidant and antiinflammtory properties of OSAE/OSWE, but in order to identify the active principle of the OS extracts, further we have performed the antiinflammatory activity evaluations in different fractions of OSAE. In the present study, we investigated the antilipid peroxidation and antiinflammatory properties of E-FR, A-FR and W-FR of OSAE extracts using human monocytic (THP-1) cells.

Antilipid peroxidation and antiinflammtory activity of OSAE fractions

Lipid peroxidation of biological membranes can cause alterations in fluidity, reductions in membrane potential, increased permeability to H^+ and other ions, and eventual membrane rupture leading to release of cell and organelle contents. Cytotoxic aldehydes resulting from lipid peroxidation can block macrophage action, inhibit protein synthesis, inactivate enzymes, cross-link proteins, and can lead to the generation of thrombin (Halliwell and Gutteridge,

1990). Thus lipid peroxidation can play a crucial role in inflammation and thereby several diseases. Scientific reports suggest that flavonoids are strong metal chelators that inhibit lipid peroxidation and oxidative modification of LDL. OS dry leaves are rich source of flavonoids (Choudhury *et al.*, 2014). Here, different fractions of OSAE were tested for their antilipid peroxidation activity in liver and heart homogenates. The E-FR showed highest lipid peroxidation inhibitory activity (IC_{50} 5.5 and 100 µg dwt/mL compared to A-FR (IC_{50} 15 and 250 µg dwt/mL) and W-FR (IC_{50} 20 and 265 µg dwt/mL) in both liver and heart homogenates as shown in Fig 6.1. This variation could be due to qualitative and quantitative difference in various metabolites extracted into that particular solvent used for it.

Inhibition of cytokines has been considered as very important characteristic of antiinflammatory drugs. Monocytes/macrophages are key mediators of inflammation and widely distributed in the body. LPS, causes several functional changes in monocytes like chemotaxis and maturation of monocytes to macrophages. Since TNF-α is a potent proinflammatory cytokine which plays an important role in developing many chronic inflammatory diseases including atherosclerosis and rheumatism (Branen *et al.*, 2004). Inhibition of TNF-α secretion is important in regular inflammatory diseases. TNF-α activates other cell types like endothelial cell and regulates inflammation-driven atherosclerosis. Studies on knockout mice like ApoE(-/-) clearly demonstrated the role of TNF-α in atherosclerosis. Simillarly chemokine, MCP-1 is a low molecular weight protein playing a major role in monocyte recruitment and infiltration into the arterial wall. Recent studies in hypercholesterolemic mice lacking apo E or the low-density lipoprotein receptor have suggested a role for MCP-1 in monocyte recruitment in early atherosclerotic lesions (Gosling *et al.*, 1999). In the present study, we have showed the inhibitory effect of OSAE fractions on LPS induced secretion of TNF-α and expression of MCP-1 markers at gene level. E-FR showed significant effect in attenuating the secretion of TNF-α in THP-1 cells as compared to

other fractions at the same concentration (Fig. 6.3). Pretreatment E-FR also inhibited the gene expression of MCP-1chemokines in LPS stimulated THP-1 cells (Fig. 6.4). Whereas other fractions did not show much effect at the same concentration.

Phytochemical analysis of OSAE fraction

The HPLC coupled to mass profile of OS fractions provided means for characterization of compound identity in a mixture of plant compounds. Because of higher antinflammtory effect of E-FR compared to A-FR and W-FR, the metabolite profiling of E-FR compared to A-FR and W-FR. Many of the compounds present in all three fractions of OSAE are phenolic, flavonoid and terpenoid in nature. Eugenol, rosmarinic acid, linoleic acid, apigenin-7-o-glucuronide were commonly detected in all three different fractions. Whereas β-caryophyllene and limonene were exclusively detected in E-FR, but not in other fractions. For further confirmation, two common compounds present in all three fractions namely EUG and RA and two unique compounds limonene and β-caryophyllene of E-FR were checked for presence of antiinflammatory activity. Abudance of EUG and RA were detected in various fractions are shown in Table 6.1.

Antiinflammatory activity of EUG and other metabolites

In the present study, we confirmed the EUG and RA inhibited the expression of LPS induced TNF-α secretion in LPS stimulated THP-1 cells in a dose dependent manner. Whereas β-C and LM did not show effective inhibition of TNF-α secretion. EUG at 60 µg/mL also inhibited the other proinflammatory markers like IL-6, TNF-α, MIP-1α, MCP-1 at their gene transcriptional level in LPS stimulated THP-1 cells. Earlier, Mahapatra *et al.,* (2011) showed inhibitory activity of eugenol on NF-κB activation in murine macrophages as well as the protective effect of EUG in COX-2 expression and other inflammatory cytokines in animal models (Jaganathan and Supriyanto, 2012). Studies in *Apoe*$^{-/-}$*/Myd88*$^{-/-}$ double knockout mice demonstrated that certain macrophage differentiation markers like CD14, TLR2 and TLR4 are

strongly associated with inflammation and atherosclerosis development (Bjorkbacka *et al.*, 2004). The pretreatment of EUG (60 µg/mL) inhibited the expression of all the selected markers (CD14, TLR2/TLR4) in PMA induced THP-1 cell. However, LC-MS study revealed that EUG content in both OSAE and OSWE are very minimal (12 ng/mg dwt of OSAE and 19 ng/mg dwt of OSWE) suggesting that antiinflammatory effect of the extracts could be due to synergistic/cumulative activity of EUG along with other metabolites present in the extracts. The present study demonstrated that bioactive compounds such as eugenol, which can attenuate monocytic activation, a key step in the atherogenesis, can be extracted easily from the leaves of *O. sanctum* using nontoxic solvents like ethanol and water.

Chapter 7

RESULTS and DISCUSSION

Objective 4: Evaluation of antilipidperoxidation and antihyperlipidemic effect of HI roots and pure compound eugenol in male Wistar rats (*in vivo* study)

Chapter 7

Results and Discussion

Results

Effect of HI root extracts and eugenol on high fat diet induced body weight in male Wistar rats

The body weight gain of the animal of all groups at the initial and at the end 30 days of the study was presented in table 7.1. A significant additional weight gain was observed in rats fed with high fat diet compared to normal saline treated group of animals (negative control). After 30 days, final and average weight of all animals fed normal diet and saline (GR-I) were 190 g, whereas the rats fed with HFD (GR-II) continuously for 30 days showed an average body weight 225 g as shown in table 7.1. Such additional body weight gain was reduced in HFD fed rats co treated with HI root extracts (HIAE/HIWE) at 200 mg/kg/day. Rats fed with HFD along with HIAE (200 mg/kg/day) (GR-IV) showed an average body weight 188 g. Similarly, HIWE along with HFD (GR-VI) also showed positive response and the average body weight of this group of animals was 189 g (Table 7.1). Continuous feeding HIAE or HIWE (GR-III and GR-V) for 30 days to the animals fed with normal diet did not show any side effect and their average body weight was 180 and 185 g respectively. These observation also indicated that treatment of the HI root extracts did not show toxic effect to the animals.

Apart from that, oral administration of pure compound eugenol, a phenylpropene at a dose of 25 mg/kg body weight/day for 25 days also showed small significant effect against the reduction in body weight compared to hyperlipidemic rats as shown in Table 7.1. From the above results, among the HI root extracts and pure compound EUG, HI root extracts were more effective on the reduction of body weight compared to EUG in HFD fed male Wistar rats.

Table. 7.1. Effect of *H. indicus* root extracts and pure compound eugenol on high fat diet fed changes in body weight in male Wistar rats.

S.No	Group	Treatment	Body weight (g)	
			1st day	30th day
1	I	NC	126 ± 2.81	190.83 ± 21.31
2	II	+HFD	132.85 ± 4.06	225.71 ± 2.90
3	III	+HIAE	122.5 ± 4.15	180.83 ± 9.17
4	IV	+HIAE+HFD	135 ± 3.54	188.57 ± 3.75
5	V	+HIWE	132.8 ± 6.98	185 ± 5.49
6	VI	+HIWE+HFD	142.5 ± 1.42	189.16 ± 3.12
7	VII	+EUG	140.7 ± 1.92	180 ± 2.60
8	VIII	+EUG+HFD	157.85 ± 2.53	205 ± 6.12

Each value represents mean ± S.E.M. (n = 7 animals in each group. NC: normal control; HFD: high fat diet; HIAE: alcoholic extracts; HIWE: water extract of *H. indicus* roots; EUG: eugenol.

Effect of HI root extracts and eugenol on HFD induced blood clotting time period

It has been confirmed that, feeding of high fat diet for 30 days reduced the blood coagulation time period compared to normal diet feed animals as shown in Fig. 7.1. In normal diet fed animals (GR-1), the average blood clotting time was recorded as 2 min 25 sec, whereas regular HFD fed animals (GR-II) after 30 days, the blood clotting time period was reduced to 1 min 46 sec as shown in Fig. 7.1. However, co-treatment of the HFD fed animals with HIWE at 200 mg/kg body weight/day for 30 days showed normal clotting time periods 2 min 02 sec like control groups of animals. HIWE treated group of normal diet fed animals (GR-V) showed the coagulation time period of 2 min 05 sec. However, treatment of HIAE with and without HFD (GR-III and GR-IV) showed remarkable change and significantly prolonged coagulation time periods (3 min 12 sec) compared to normal group of rats as shown in Fig. 7.1. Feeding of a pure compound EUG at 25 mg/kg body weight/day (GR-VIIII) for 25 days showed normal blood clotting time 2 min 08 sec as compared to normal diet induced animals

(GR-I) (Fig 7.1). Co-treatment of EUG along with HFD did not show any side effect and alteration of blood clotting time.

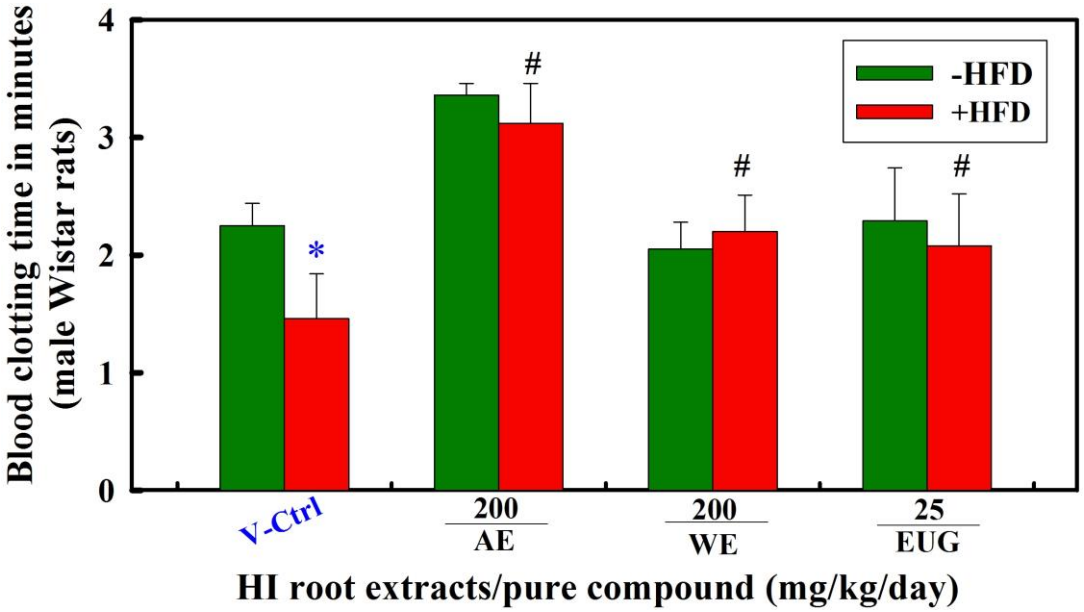


Fig. 7.1. Effect of HI root extracts and pure compound eugenol on alteration of blood clotting time in high fat diet fed male Wistar rats. Plant extracts HIAE and HIWE (200 mg/kg body weight/day) for 30 days and EUG (25 mg/kg body weight/day) for 25 days fed along with HFD to the all animals of respective groups. At the end of treatment periods blood clotting time period was recorded in each and every animal of all tested and controlled groups and compared with HFD fed blood clotting time period group. Data shown are mean value of 7 animals in each group ± S.D.

Effect of HI and eugenol on HFD induced lipid peroxidation in male Wistar rats

Liver and heart tissues

In the present study, HI root extracts and pure compound eugenol were evaluated for their ability to inhibit HFD induced lipid peroxidation in liver and heart tissues (*in vivo*). Lipid peroxidation, particularly, of unsaturated fatty acid in membranes causes various pathophysiological changes. Feeding of animals with high fat diet for 30 days resulted in increase in peroxidation of lipids in liver tissues (2.1 folds) as compared to control group of diet fed animals (Fig. 7.2 A). Co treatment of HIAE/HIWE (200 mg/kg body weight/day) for 30 days reduced such increase in peroxidation of lipids in liver tissues (Fig. 7.2 A). Similarly, peroxidation of lipids has also increased significantly in heart tissues up to 2.4 fold in high fat

diet fed animals compared to control group of animals (Fig. 7.2 B), whereas co-treatment with HIAE/HIWE at 200 mg/kg body weight/day along with high fat diet reduced the lipid peroxidation and showed normal levels of control group of animals (Fig. 7.2 B). Pure compound eugenol at 25 mg/kg body weight/day for 25 days also reduced lipid peroxidation in both liver and heart homogenates, they showed 0.89 and 1.18 fold of control group of rats respectively (Fig. 7.2 A and B).

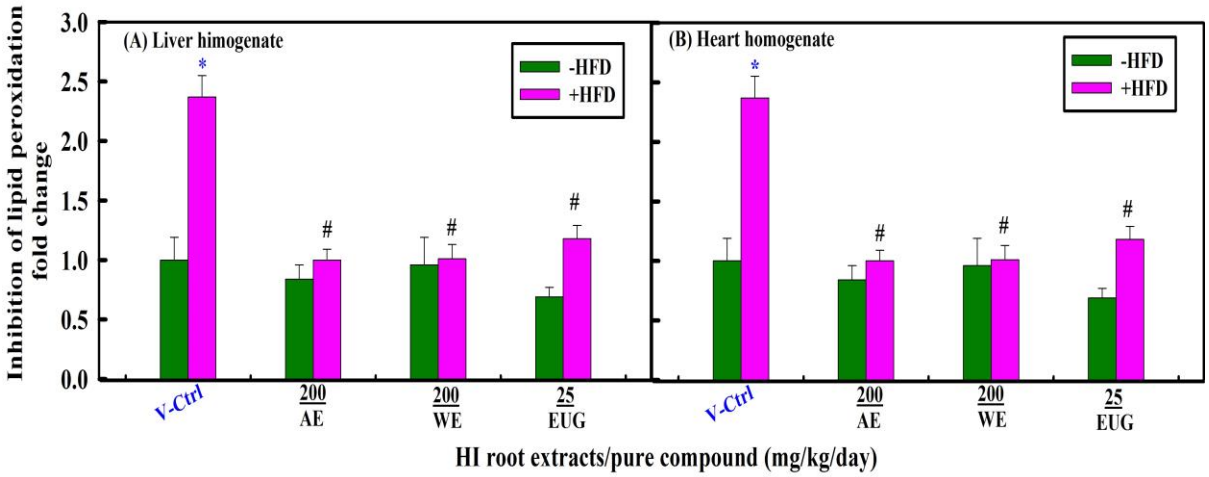


Fig. 7.2. Effect of HI root extracts and pure compound EUG on high fat diet (HFD) induced lipid peroxidation on (A) liver and (B) heart tissue homogenates of male Wistar rats. Data presented are mean ± S.D., n = 7.

Hypolipidemic activity of HI root extracts and pure compound eugenol in male Wistar rats

High cholesterol diet supplemented with HI root extracts or pure compound eugenol was studied and identified that significant reduction of the lipid profile level in the rat serum. No rat death was found throughout the feeding experiments of test diets under study. Animals did not show any toxic symptoms, animal aspects and behavior were normal in all groups during the test period. The systematic administration of the high fat diet for 30 days to Wistar rats results in an enormous elevation of serum cholesterol level (18 5%), triglycerides (187 %),

LDL-cholesterol (240 %) whereas serum HDL-cholesterol has significantly reduced to (77 %) with respect to the baseline value of normal group of rats as shown in Fig.7.3 A to D.

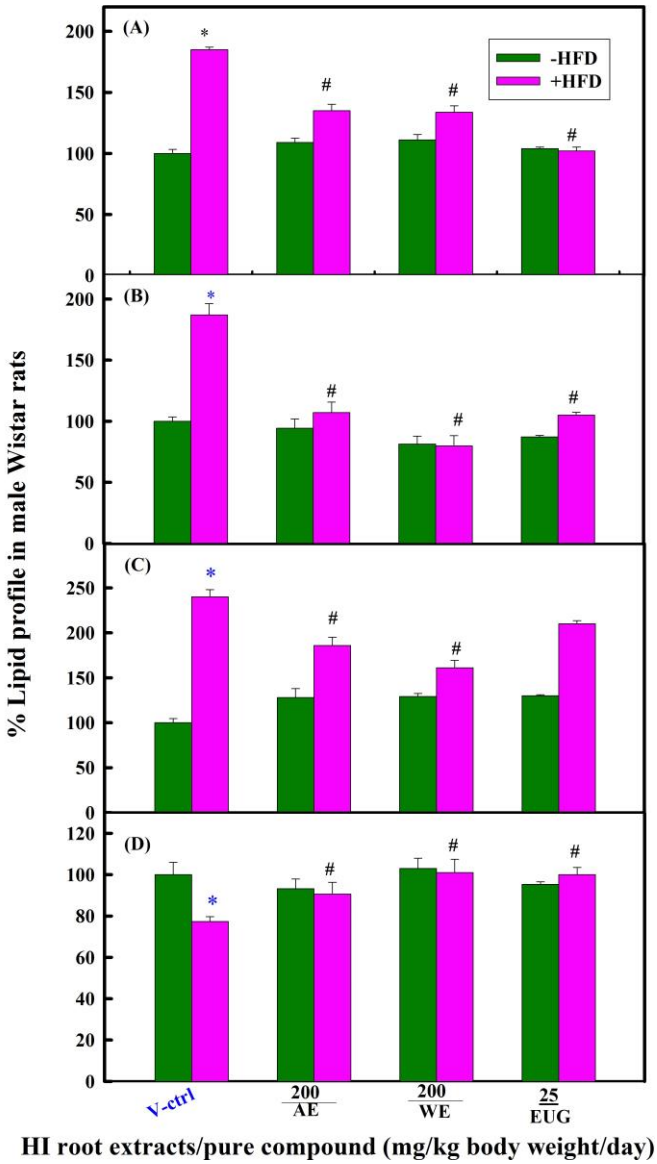


Fig. 7.3. Effects of HI root extracts (HIAE/HIWE) and pure compound EUG in HFD fed hyperlipidemic male Wistar rats on the lipid profile markers on total cholesterol (A); triglycerides (B), LDL-cholesterol (C) and HDL-cholesterol (D). Each bar represents the mean ± S.D from 7 animals of each group. *$p<0.001$, for comparison between (–HFD) group *vs* (+HFD) group. $^{\#}p<0.001$ compared between animals fed with HFD in the presence or absence of plant extracts and pure compound. Group-I: Normal control, Group-II: hyperlipidemic group (High fat diet (2 mL/kg body weight/day, for 30 days), Group-III: HIAE (200 mg/kg body weight/day p.o, for 30 days), Group-IV: HIAE (200 mg/kg body weight/day p.o, for 30 days) + High fat diet (2 mL/kg body weight/day), Group-V: HIWE (200 mg/kg/day p.o, for 30 days), Group-VI: HIWE (200 mg/kg body weight/day p.o. for 30 days)+ High fat diet (2 mL/kg body weight/day), Group-VII: EUG (205 mg/kg body weight/day p.o, for 25 days), Group-VIII: EUG (25 mg/kg body weight/day p.o, for 25 days)+ High fat diet (2 mL/kg body weight/day).

Conversely, in the present study, co-treatment of alcoholic extracts of HI-roots (HIAE) at 200 mg/kg body weight/day for 30 days prevented such high fat diet induced increase in serum total cholesterol (135 %), triglycerides (107 %), LDL-cholesterol (186 %). HIAE also inhibited HFD induced decrease in HDL-cholesterol (90.64 %). Similarly, HIWE (200 mg/kg body weight/day) inhibited changes in all the HFD induced selected serum lipid profile markers, total cholesterol (133%), triglycerides (79 %), LDL-cholesterol (161 %) and HDL-cholesterol (101 %) (Fig. 7.3 A to D). The lipid lowering activity of *H. indicus* root extracts may be attributed to the phytoconstitutents present such as coumarinolignoids, phytosterol along with other secondary metabolites. Further studies with oure compounds would help in identifying the active metabolite.

Similarly, feeding of EUG (25 mg/kg/body weight/day) a phenolic propanoid supplemented with high fat diet for 25 days also yielded similar effect, it changes in HFD induced serum lipid markers: cholesterol (102 %), triglycerides (104 %) and HDL-cholesterol (100 %), except on LDL-cholesterol as shown in Fig. 7.3 A to D.

Discussion

In recent years, high fat diet fed animal model has been considered as good model to study antihyperlipidemic effect of herbal extracts. HFD diet also induces weight gain similar to in humans (Onyeike *et al.,* 2012). Human studies have clearly demonstrated that increased fat intake is associated with body weight gain, which can lead to cardiovascular diseases (CVD) and other metabolic disorders (Lee *et al.,* 2008). This study has confirmed that male Wistar rats fed with HFD for 30 days causes a significant increase in body weight (Table 7.1). There was a significant difference in the body weight gain between the high fat diet and normal diet group, although there was no significant difference observed in the amount of food intake of animals. This observation provides us with the fact that an increase in body weight is not

necessarily depends on the amount of food consumed by the animals and can also depend on quality food and amount of calories present in food. The treatment of the HFD groups with HI root extracts showed significant reduced weight gain at the 30 days in this study when compared to the HFD group. Our work can be confirmed that treatment with HI root extracts or pure compound EUG along with HFD reduced weight gain, thus this study demonstrated that selected plant or EUG can be used for weight management by obese people.

Published literature strongly indicates that on HFD effects on coagulation factors or coagulation potency in rats (Levendal and Frost, 2006). We noticed a significant reduction in the blood clotting time period of animals those were fed with HFD, which is rich source of unsaturated fatty acids (Fig. 7.2). There was significant reduction in the blood clotting time in between HFD fed experimental group relative to the normal diet fed control group. Our data suggest that HFD led to a hypocoagulable state in rat plasma. Further investigation is necessary to understand the underlining mechanism for such response. The results indicate that regular treatment of alcoholic extracts of *H. indicus* roots (HIAE) has prolonged the blood clotting compared to normal control group, whereas HIWE and pure compound EUG maintained normal blood clotting time as control group. Hypolipidemic activity of HIAE compared to HIWE can be due to type of phytoconstituents present in the extracts. HIAE can be considered as an anticoagulant agent, however, its long term usage needs to be thoroughly investigated since it prolonged coagulation time much long then normal which may be associated with bleeding disorders.

Oxidative stress in human body causes major damage to macromolecules like lipids, protein and DNA. Lipid peroxidation, particularly polyunsaturated fatty acids in cell membrane have been considered as a major biomarker for increased oxidative stress. Inhibition of peroxidation of lipids by antioxidants has been considered as a promising approach to combat oxidative stress (Prokai *et al.,* 2013). Literature survey clearly confirmed

that studies on apolipoprotein E-deficient mice demonstrated that high fat diet causes peroxidation of lipids in different cell membrane. Lipid peroxidation is well studied in animal model, which may contribute to free radical induced process leading to modification of polyunsaturated fatty acids (Anitha and Karuppasamy, 2011). It is reported in the literature that elevated level of lipid peroxides in the liver and heart of HFD fed hyperlipidemic rats (Mehra *et al.,* 2013) and lipid peroxidation is one of the characteristic features of chronic inflammaotory disorders. In the present study LPO end products measured as TBARS and LOOH were found to be increased in the liver and heart tissues of HFD fed rats. We examined its antilipid peroxidation properties in HFD fed animal model, leading to demonstration of its protective effects against cardiovascular risks. In the present study, high fat diet fed rats showed significant rise in peroxidation of lipids in liver and heart tissue homogenates. The oral administration of HI root extracts and pure compound EUG markedly prevented the high fat diet induced elevation of MDA and resulted in a significantly decreased lipid peroxidation in liver and heart tissues (Fig. 7.2 A and B).

Hyperlipidemia is a major and important risk factor for the development of atherosclerosis and progression of coronary artery disease (Prasad and Kalra, 1993; Deepa and Varalakshmi, 2005). In hyperlipidemia, there is an increase in serum markers like TC, TG and LDL-C levels, which results in an increased risk for the development of atherosclerosis. According to different scientific studies, it is widely accepted that the high amount of saturated fat and cholesterol in the diet increase the level of serum cholesterol when compared to low fat diet (Salam *et al.,* 2013). Elevated levels of serum cholesterol, TG and LDL-C that are accompanied by reduced HDL-C levels are often associated with an increased risk of coronary heart disease (Smith *et al*., 2004). According to many scientific studies, it has confirmed that, LDL-C is considered the most dangerous lipid marker among the serum lipids, and the oxidation of LDL-C leads to its increased penetration of arterial

walls (Steinberg *et al.,* 1989; Aviram, 1993). Moreover, elevated LDL-C levels play a crucial role in the development of atherosclerotic lesions that progress from fatty streaks to ulcerated plaques (Schaefer *et al.,* 1995; Ross, 1993). Thus, serum LDL-C levels are used as the basis for initiating and monitoring the treatment of patients with elevated blood cholesterol levels (Grundy, 1993). Regulating serum cholesterol level is highly essential for prevention of atherosclerosis and other coronary artery diseases (CAD), as it has been shown that atherosclerosis can be suppressed by controlling the levels of serum cholesterol (Hor *et al*., 2011). Identification of certain therapeutic drugs from medicinal plant extracts that are without side effects have been considered as a major challenge in today's scientific research field (Yokozawa *et al.,* 2003). A HFD is frequently used to increase serum lipid profile level to evaluate the effectiveness of the plant extracts on hyperlipidemia in animal model. In Wistar rats, the HFD results in marked alterations in the type and distribution of the plasma lipoproteins and their apoproteins (Mahley and Holcombe 1977). In this present scenario, hyperlipidemia was induced in Wistar rats by regular feeding of HFD (Amul butter, 2g/kg/day) for 30 days. The rats that were fed HFD had higher concentration of serum TC, TG, LDL-C levels and decreases the level of HDL-C than those fed a normal diet (control group) which clearly confirmed that hyperlipidemia was successfully established in our rat model. In our present study, the administration of HI root extracts and pure compound EUG in rats fed a HFD caused a reduction in the levels of TC, TG and LDL-C and increase in HDL-C as compared to control group. Hence, these results indicate that HI root extracts along with pure compound EUG can prevent hyperlipidemia in HFD fed rats, whereas EUG did not show much effect on LDL-C. HI root extracts consists of a high amount of phytosterols. β-sitosterol which is being reported as useful in the treatment of hyperlipoproteinemia (Lees A and Lees M, 1976). It is also reported that β-sitosterol inhibits cholesterol absorption (Lees A and Lees M, 1976). β-sitosterol present in HI root extracts

may be responsible for the hypolipidemic effect. It can be confirmed from the above data that the levels of total serum cholesterol, triglycerides and LDL cholesterol which are actually raised in HFD diet can be lowered significantly with concurrent feeding of HI root extracts and pure compound EUG. We are reporting for the first time the effect of Hi roots extracts and EUG in HFD fed hyperlipidemia rats. Thus our result suggests that HI root and pure compound EUG can be used for the development of hypolipidemic drug in the prevention of atherosclerosis.

Chapter 8

SUMMARY AND CONCLUSIONS

Chapter 8

Summary and Conclusions

Pharmacognosy bridges the system of traditional herbal medicine and modern medicine. Indian medicinal plants are rich sources of substances that have several therapeutic properties. In Indian traditional system of medicine, Ayurveda, several plants have been identified and used for general health and cardioprotective effects. Scientific studies over the period of time supported medicinal value of natural plant products. According to WHO reports, around eighty percent of the global population still relies on botanical drugs. Origin and progression of several diseases occurs due to oxidative damage. Lipid peroxidation occurs during oxidative stress as the main molecular mechanism and complex process that involves in the formation and propagation of lipid radicals, eventually destruction of membrane lipids with generation of a variety of breakdown products including hydroxyl radical, hydrogen peroxide, ketones and alcohol that causes oxidative damage to the cell structure which can eventually lead to cell death. Oxidative stress is responsible for human diseases including cardiovascular diseases (CVD), Alzheimer's disease, Parkinson's disease, diabetes, cancer etc. Apart from oxidative stress several other conditions including postprandial lipemia, hypercholesterolemia, hyperglycemia, hyperuricemia and microbial infections activate peripheral blood cells like monocytes and platelets. Cells like monocytes and macrophages under stress conditions release several cytokines and chemokines in the blood or vessel wall, which leads to the activation of endothelial cells. Endothelial cell dysfunction causes vascular wall permeability leading to the development of atherosclerotic lesions in the blood vessel wall. Natural plant products posses antioxidant property, which alleviate oxidative stress, and thus can help in preventing many of the human diseases.

Based on the literature survey, we have designed the present study to evaluate and establish the biochemical and molecular basis for cardioprotective properties of *O. sanctum* dry leaves and *H. indicus* roots.

Ocimum santum

Ocimum sanctum (OS), (family Lamiaceae) commonly known as Holy basil/Tulsi. The plant grows widely in all dried parts and subtropical regions in India. Scientific investigations have found that, it has a wide range of pharmacological benefits such as hepatoprotective neuroprotective, hypolipidemic, immunomodulatory and anticoagulant properties.

Hemidesmus indicus

Hemidesmus indicus (HI) is commonly called as Sarsaparilla. The dried roots of these plants are well known in Indian pharmacopoeia as the drug 'Anantmul', a common tonic prescribed for blood purification and other physiological disorders. It is a twinging, laticiferous and slender shrub found in India, Sri Lanka, Pakistan and Iran. Its roots are woody with a diameter of 0.5–1.5 cm, slender brownish or purple in colour, aromatic odour, sweet in taste and are used for preparation of soft drinks. Scientific reports also suggests that *H. indicus* roots have several pharmacological effects, such as hypoglycemic antioxidant and antiinflammatory effects in animal model.

Objectives of the present study:

1. Phytochemical profile and antioxidant activities (*in vitro*) of alcoholic and water extracts of *O. sanctum* (OS) leaves and *H. indicus* (HI) roots.
2. Evaluation of antiinflammatory and/or antioxidant properties of O*S and* HI extracts in human monocytic (THP-1) cells.
3. Identification of active metabolite(s) from OS extracts responsible for its antiinflammatory property in THP-1 cells.

4. Evaluation of anti-lipidperoxidation and antihyperlipidemic effect of HI and pure compound, eugenol, of OS in male Wistar rats (*in vivo* study).

Objective 1: Phytochemical profile and antioxidant activities (*in vitro*) of alcoholic and water extracts of OS leaves and HI roots

Phytochemical analysis of OS and HI extracts by LC-Q-TOF-MS/MS revealed the presence of several bioactive compounds. Commonly identified compounds in both OSAE and OSWE are vicenin, luteolin-7-O-glucuronide, apigenin-7-O-glucuronide, methyl chavicol, methyl cinnamate, eugenol and linoleic acid. The compounds, which were exclusively detected in OSAE are rosmarinic acid, orientin, germacrene D, α-pinene, carvacrol, apigenin, linalool, cirsimaritin, cirsilineol, sinapic acid and ascorbic acid, whereas methyl eugenol and stearic acid were detected only in OSWE. Similarly, in case of HI root, metabolite profiling analysis revealed the presence of several bioactive compounds which belong to pregnane glycosides, terpenoids or coumarino-lignoids. Negative ion and positive ion modes of LC-QTOF-MS detected 7 and 12 compounds in HIAE; 4 and 7 compounds in HIWE respectively. Chlorogenic acid, lupeol acetate, hexatriacontane, nerolidol, hemidesmin 1, hemidesmin 2, hemidesminine, β-sitosterol were commonly detected in both HIAE and HIWE. 2-hydroxy-4-methoxy benzoic acid, emidine, lupanone, heminine, desinine, rutin, hemidine were exclusively detected in HIAE, while 4-terpineol was detected only in HIWE.

The antioxidant capacity and reducing power activity of both OS and HI extracts were significantly high. Among them, OSAE showed much higher antioxidant activity than OSWE, but OSWE showed higher reducing power activity than OSAE. In case of HI root extracts, HIWE showed stronger antioxidant activity than HIAE, whereas both alcoholic and water extracts of HI root showed similar levels of reducing power capacity.

Both OS leaf and HI root extracts showed concentration dependent inhibition for scavenging the free radicals like DPPH and H_2O_2. OSAE showed significantly much higher antioxidant activity than OSWE on the basis of IC_{50} value. OSAE and OSWE showed about maximum activity of 90% inhibition with IC_{50} value of 2.28 and 15 µg dwt/mL respectively. In case of *H. indicus*, both extracts HIAE and HIWE showed 90% inhibitory activity with 92.5 and 96.88 µg dwt/mL respectively. Thus both extracts showed almost the same potential. In case of H_2O_2 radical scavenging activity, both OSAE and OSWE showed maximum of 95 % activity with IC_{50} values of 0.22 and 0.45 mg dwt/mL respectively. HIAE showed upto 70 % scavenging activity with IC_{50} of 2.14 mg dwt/mL. HIWE showed lower IC_{50} value (1.54 mg dwt/mL) than HIAE, however maximal inhibitory activity did not exceed 55 %.

Antilipid peroxidation activity of OS and HI extracts were determined on $FeCl_3$ induced lipid peroxidation in rat liver and heart tissue homogenates of male Wistar rats *(in vitro*). OSAE and OSWE inhibited about 90% of lipid peroxidation in both types of selected tissue homogenates. However, OSAE showed much lower IC_{50} value compared to OSWE. The IC_{50} value of OSAE and OSWE for liver homogenates were 0.5 and 5 µg dwt/mL and for heart homogenates were 40 and 600 µg dwt/mL respectively. In case of HI root, both HIAE and HIWE inhibited about 80% lipid peroxidation in both types of tissue homogenates. The IC_{50} value for inhibition of HIAE and HIWE were 30 and 270 µg dwt/mL for liver homogenates, 140 and 200 µg dwt /mL for heart homogenates. IC_{50} value of standard compound ascorbate was found to be 10 and 200 µg/mL for inhibition of lipid peroxidation in liver and heart tissues.

Objective 2: Evaluation of antiinflammatory and/or antioxidant properties of OS *and* HI extracts in human monocytic (THP-1) cells.

Dosage of plant extracts like OS leaf, HI root and standard compound, IMT in THP-1 cell treatment were determined based on MTT assay and trypan blue exclusion method. At least 95 % cells were alive at used concentrations of tested plant extracts or standard compound. Arachidonic acid induced ROS in THP-1 cells is increased by 2 fold. Pretreatment with OSAE and OSWE attenuated arachidoinc acid induced ROS generation. TNF-α and MCP-1 was considered as a potent proinflammatory cytokine and chemokines secreted by activated monocytes. They play a significant role in development and progression of lesions in vascular wall. LPS stimulated THP-1cells secreted 22 folds more amount of TNF-α (435 pg/mL) compared to control unstimulated cells (15 pg/mL). Pretreatment of cells with 0.5 mg and 1 mg dwt/mL of OSAE brought down to 360 pg/mL (18 folds) and 186 pg/mL (9 folds); and OSWE to 354 pg/mL (19 folds) and 120 g/mL (6 folds) respectively. Imatinib (IMT) used as a positive drug control, which is a commercially available drug used for treating cancer clinical studies established that it alternates proinflammatory gene expression. Cells treated with positive drug control (IMT) at 5 and 20 µg/mL brought down to 393 pg/mL (19 folds) and 186 pg/mL (9 folds) respectively in the presence of LPS. However, pretreatment of cells with 0.5 mg and 1 mg dwt/mL of HIAE brought down to 395 pg/mL (20 folds) and 276 pg/mL (14 folds); HIWE brought to 395 pg/mL (20 folds) and 296 pg/mL (15 folds) fold respectively. Similarly LPS stimulated THP-1cells secreted 110 pg/mL (5.88 folds) more amount of MCP-1 compared to control unstimulated cells 20 pg/mL. Pretreatment of OSAE and OSWE (1 mg dwt/mL) did not show much effect, they were 98 pg/mL (5.27 folds) and 81 pg/mL (4.31 folds) respectively. Pretreatment of cells with 1 mg dwt/mL of HIAE and HIWE brought down to 66 pg/mL (3.54 folds) and 72 pg/mL (3.87 folds) respectively.

Activated monocytes and their retention within the atherosclerotic lesion contribute to the progression of plaque development. The effect of OS and HI extracts and positive drug control IMT tested on LPS induced gene expression of inflammatory markers. The transcript analysis by real-time quantitative PCR showed that LPS stimulated the expression of IL-6 (2-fold increase), TNF-α (5 foldd), macrophage inflammatory protein (MIP-1α) (2.15 folds) and MCP-1 (36 folds). Such stimulation of proinflammatory gene expression was markedly inhibited in the cells pretreated with OS and HI root extracts at 1 mg dwt/mL. OSWE showed stronger effect than OSAE in attenuating the MCP-1 marker. However, HIWE did not show much effect in attenuating MCP-1 marker. A positive control, IMT (20 µg/mL) also showed significant effect on inhibition of all the selected markers.

Human monocytic (THP-1 cells) are suspension in nature and do not adhere to the plastic surfaces of the culture dishes. PMA (5 ng/mL) stimulates differentiation of THP-1 cells to macrophages and such PMA induced differentiation process was inhibited by the plant extracts. Pretreatment with OS and HI extracts at 1 mg dwt/mL significantly attenuated the process of PMA induced differentiation of THP-1 cells. THP-1 derived macrophages showed upregulation in the expression of CD14 and toll like receptors (TLR2 and TLR4) by 3, 64 and 37 folds respectively. Such upregulation of the markers was markedly attenuated by OS and HI extracts at 1 mg dwt/mL. IMT (20 µg/mL) also showed the inhibition of PMA induced differentiation.

In mammalian cells, expression of several proinflammatory markers including cytokines and chemokines is regulated by NF-κB transcription factor. Therefore, further elucidation of mechanism of antiinflammatory activity of selected medicinal plant extracts is necessary. Effect of extracts on LPS induced translocation of p65 subunit of NF-κB was studied in THP-1 cells by confocal microscopy. Cells were probed with antibody of NF-κB subunit p65 and stained using secondary antibody conjugated to Alexa Fluor 594. Nuclei

were stained with DAPI. THP-1 cells induced with LPS showed translocation of p65 from cytoplasm to nucleus and such translocation was inhibited by OS, HI extracts and IMT at their selected concentrations.

Objective 3. Identification of active metabolite(s) from OS extracts responsible for its antiinflammatory property in THP-1 cells.

All different fractions of OSAE (E-FR, A-FR and W-FR) inhibited $FeCl_3$ induced lipid peroxidation in liver and heart homogenates of male Wistar rats. In case of liver lipid peroxidation, IC_{50} value of E-FR, A-FR and W-FR were 5.5, 15 and 20 µg dwt/mL respectively and in heart homogenates, IC_{50} value of E-FR, A-FR and W-FR were 100, 250 and 259 µg dwt/mL respectively. The results indicated that E-FR fraction of OS leaves were more potential than other fractions in suppressing lipid peroxidation of both rat liver and heart tissue homogenates.

Activated monocytes secrete TNF-α, a proinflammatory cytokine has been considered as most important cytokine which plays an important role in pathogenesis of atherosclerosis. ELISA was performed using cell supernatants to quantitate the secreted TNF-α by human THP-1 cells in the presence of a bacterial endotoxin, LPS (0.5 µg/mL) for 3 h. Normal THP-1 cells secreted basal level of TNF-α (25 pg/mL) whereas LPS treated cells showed 22 fold increase in TNF-α secretion (435 pg/mL). Pretreatment with different fractions of OSAE (E-FR, A-FR and W-FR) at 100 µg dwt/mL attenuated the secretion of TNF-α 75 pg/mL (3.82 folds); 336 pg/mL (17 folds) and 316 pg/mL (16 folds) respectively. The results indicate that among the fractions, E-FR showed highest effect in attenuating the secretion of TNF-α from LPS treated THP-1 cells. A-FR and W-FR marginally inhibited the secretion. The effect of the OSAE fractions (100 µg dwt/mL) tested on LPS induced gene expression of chemokines. The transcript analysis by real-time quantitative PCR showed that LPS stimulated significantly the expression of MCP-1 (37 fold increase). Such stimulation of

proinflammatory gene expression was markedly inhibited in the cells pretreated with E-FR (100 µg dwt/mL), whereas A-FR and W-FR did not show much effect.

In the present study, LC-Q-TOF-MS/MS was employed for metabolite fingerprinting of the E-FR, A-FR and W-FR fractions of OSAE. Several compounds were detected in both negative and positive ion modes. Compounds in all three different fractions are phenolic, flavonoids and terpenes in nature. Eugenol, rosmarinic acid, linoleic acid, apigenin-7-o-glucuronide were commonly detected in all three different fractions. β-caryophyllene and limonene were exclusively detected in E-FR. Eugenol, β-caryophyllene and limonene were procured and tested for their antiinflammatory effect. In order to identify the active principle of OS leaf extracts responsible for its antiinflammatory property. On the basis of LC-MS based metabolite profiling, we tested antiiflammaoty activity of eugenol, rosmarinic acid, limonene and β-caryophyllene in human monocyitc (THP-1) cells. LPS stimulated THP-1 cells secreted (22 folds) 435 pg/mL more amount of TNF-α compared to control unstimulated cells 15 pg/mL. Pretreatment of cells with eugenol (60 µg/mL) suppressed stimulation of TNF-α secretion to (2.21) 43.69 pg/mL, whereas rosmarinic acid (60 µg/mL) and limonene (60 µg/mL) reduced to (10 folds) 197 pg/mL and (14 folds) 276 pg/mL respectively. β-caryophyllene (60 µg/mL) did not show much effect on attenuation of TNF-α secretion. Based on these results, eugenol was selected to test its targets in inflammatory cascade in THP-1 cells.

MCP-1 a low molecular weight proinflammatory chemokine secreted by activated monocytes has been considered to play an important role in the pathogenesis of atherosclerosis. LPS stimulated THP-1 cells secreted (5.88 fold) 110 pg/mL fold more amount of MCP-1 compared to control unstimulated cells (20 pg/mL). Pretreatment of cells with eugenol (60 µg/mL) brought down to (2.8 folds) 52 pg/mL of MCP-1 release in the presence of LPS. The inhibitory effect of eugenol on LPS induced gene expression of

selected proinflammatory markers was tested to examine if the compound can modulate gene expression at transcriptional level. The gene expression analysis by real-time quantitative PCR showed that LPS stimulated the expression of IL-6 (2 folds), TNF-α (5 folds), macrophage inflammatory protein (MIP-1α) (3 folds) and MCP-1 (36 folds). Such stimulation of proinflammatory gene expression was markedly inhibited by pretreatment of eugenol at 60 µg/mL. Pretreatment of THP-1 cells with EUG (60 µg/mL) significantly attenuated the process of differentiation by PMA challenged THP-1 cells. PMA induced upregulation of CD14 (3 folds) and toll like receptors TLR2 and TLR4 (64 and 37 folds respectively) in THP-1 cells was attenuated by EUG (60 µg/mL) to respective folds of 1.56, 1.61 and 5. In the present study, the effect of eugenol on transcriptional factor NF-κB was investigated since several genes which are regulated by NF-κB were attenuated. Pure compound EUG at 60 µg/mL inhibited LPS induced translocation of NF-κB-p65 from cytoplasm to nucleus in THP-1 cells.

The total amount of eugenol present in both OSAE and OSWE was quantified using titration curve generated using pure compound eugenol. We used standard solutions of eugenol in the range from 0.06 µg/mL to 4 µg/mL to test the linearity. Pure compound eluted at 3.28 min and shown m/z of 165.09. Under the subjected MS conditions, eugenol from OSAE and OSWE was also eluted at 3.28 min and 3.33 min respectively with m/z 165.093 and authenticated by mass fragment spectra generated in MS/MS analysis. OSAE and OSWE contained eugenol of 12 ng/mg dwt and 19 ng/mg dwt respectively. However eugenol was effective in THP-1 cells at 60 µg/mL. Therefore we conclude that activity of OS extracts is due to cumulative effects of several bioactive compounds present in the extracts.

Objective 4. Evaluation of anti-lipidperoxidation and antihyperlipidemic effect of HI and pure compound eugenol in male Wistar rats (*in vivo* study)

In the present study, high fat diet (HFD, Amul butter) fed rats for a period of 30 days showed increased body weight compared with the normal saline treated group animals. Treatment with HIAE and HIWE at their selected concentration (200 mg/kg body weight/day) reduced such increase in body weight. Eugenol (25 mg/kg body weight/day) treated high fat diet group also showed less increase in body weight. As indicated in the literature, it has been observed that, feeding of high fat diet for 30 days reduces the blood coagulation time period (1min 46 sec) than compared to normal group animals (2 min 25 sec). Co-treatment of the animals with HIWE or pure compound eugenol and HFD showed normal clotting time periods as control groups of rat like 2 min 2 sec and 2 min 08 sec) respectively. The alcoholic extracts of HI roots (HIAE) co-treated animal had much higher blood clotting time period (3 min 12 sec) compared to control animals. Feeding of animals with high fat diet for 30 days resulted in increase in peroxidation of lipids in liver and heart tissues, whereas co-treatment with the HIAE, HIWE or pure compound eugenol prevented such peroxidation of lipids in both liver and heart tissues.

High fat diet fed animals showed a significant increase in their serum total cholesterol (185 %), triglyceride (187 %) and LDL (240 %) levels with respect to the baseline value of normal rats, whereas serum HDL-cholesterol level was reduced to 77 %. In present study, co-treatment with HIAE or HIWE prevented such HFD induced increase in serum total cholesterol, LDL-cholesterol, triglycerides and increase in HDL-cholesterol. Eugenol also yielded similar results in all the markers except for LDL-cholesterol.

Sailent findings of the work done

- Dry leaf extracts of OS and root extracts of HI showed remarked antioxidant and anti-lipid peroxidation activity in liver and heart tissues of male Wistar rats (*in vitro* study).

- LC-Q-TOF-MS/MS based metabolite profiling revealed that OS dry leaf and HI root extracts indicated the presence of several bioactive compounds like phenolic, terpenoids, flavonoids, and coumarino lignoids.
- Both selected plant extracts attenuated LPS induced proinflammatory gene expression in THP-1 cells and also inhibited nuclear translocation of NF-κB.
- The tested plant materials inhibited PMA induced monocytes to macrophage differentiation.
- Pure compound eugenol detected in OS showed antiinflammatory effect in LPS/PMA induced THP-1 cells.
- High fat diet fed male Wistar rats showed increase in body weight and decrease in blood clotting time period. HI extracts and pure compound eugenol reduced the increase in body weight of HFD fed animalsand also restricted blood clotting time periods. However, HIAE prolonged the blood clotting time period by (1.38 fold) compared to that of normal control group.
- HIAE/HIWE and eugenol prevents the peroxidation of lipids in liver and heart tissues of HFD fed animals.
- HI extracts and eugenol showed hypolipidemic effect in HFD fed animal.

Concluding remarks

Dry leaf extracts of *O. sanctum* and root extracts of *H. indicus* showed their antioxidant and antiinflammatory activity in human monocytic (THP-1) cells. In addition, eugenol, one of the bioactive metabolite of OS extracts and root extract of *H. indicus* showed significant antihyperlipidemic activity in HFD fed male Wistar rats. Phytochemical analyses have suggested that the selected medicinal plant extracts are enriched with several bioactive

compounds. Therefore, the study supports traditional use of *O. sanctum* and *H. indicus* in treating cardiovascular diseases.

Chapter 9

LITERATURE CITED

Chapter 9

Literature cited

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Annexure I

RESEARCH PAPERS

Sudhansu S Choudhury, Leena Bashyam, Nalini Manthapuram, Prasanth Bitla, Padmasree Kollipara, Sarada D. Tetali (2013). *Ocimum sanctum* leaf extracts attenuate human monocytic (THP-1) cells activation. Journal of Ethnopharmacology 156, 148-155.

POSTERS PRESENTED

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ATTENDED CONFERENCES

International Conference on Novel Updates In Reproductive Biology and Comparative Endocrinology and the 27th Annual Meeting of The Society For Reproductive Biology And Comparative Endocrinology (SRBCE-XXVII), JANUARY, 19-21, 2009. University of Hyderabad, Hyderabad, India.

International Conference on Genomes: mechanism and Function and the 82nd Annual meeting of the Society of Biological Chemists (India), DECEMBER 2-5, 2013. School of Life Sciences, University of Hyderabad, Hyderabad, India.

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Research Paper

Ocimum sanctum leaf extracts attenuate human monocytic (THP-1) cell activation

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Ethnopharmacological relevance: Ocimum sanctum (OS), commonly known as Holy basil/Tulsi, has been traditionally used to treat cardiovascular diseases (CVD) and manage general cardiac health. The present study is designed to evaluate the antiinflammatory effect of O. sanctum and its phenolic compound and eugenol (EUG) in human monocytic (THP-1) cells and validate its traditional use for treating cardiovascular diseases.

Materials and methods: The phytochemical analysis of alcoholic and water extracts of OS-dry leaves (OSAE and OSWE) was done using LC–QTOF–MS. A phenolic compound, EUG was quantified in both OSAE and OSWE by an LC–MS technique using a mass hunter work station software quantitative analysis system. The effect of both OSAE, OSWE, pure compound EUG and positive control imatinib (IMT) was investigated in THP-1 cells by studying the following markers: lipopolysaccharide (LPS) induced tumor necrosis factor alpha (TNF-α) secretion by ELISA, gene expression of inflammatory markers (TNF-α, IL-6, MIP-1α and MCP-1) by real time PCR and translocation of nuclear factor kappa B (NF-κB) by confocol microscopy. Furthermore, the effect of the extracts, EUG and IMT, was studied on phorbol-12-myristate-13-acetate (PMA) induced monocyte to macrophage differentiation and gene expression of CD14, TLR2 and TLR4.

Results: The LC–MS analysis of OSAE and OSWE revealed the presence of several bioactive compounds including eugenol. Quantitative analysis revealed that OSAE and OSWE had EUG of 12 ng/mg dwt and 19 ng/mg dwt respectively. OSAE, OSWE (1 mg dwt/mL) pure compound EUG (60 mg/mL) and positive control IMT (20 mg/mL) showed marked inhibition on LPS induced TNF-α secretion by THP-1 cells. At the selected concentration, the plant extracts, EUG and IMT inhibited gene expression of cytokines and chemokines (IL-6, TNF-α, MIP-1α, MCP-1) and translocation of NF-κB-p65 to the nuclei. In addition, they showed significant inhibition on PMA induced monocyte to macrophage differentiation and the gene expression of CD14, TLR2 and TLR4 markers.

Conclusion: The result of the present study validated traditional use of Ocimum sanctum for treating cardiovascular disease for the first time by testing antiinflammatory activity of Ocimum sanctum in acute inflammatory model, LPS induced THP-1 cells. The plant extracts showed significant antiinflammatory activity, however, further to be evaluated using chronic inflammatory animal models like diabetic or apolipoprotein E-deficient mice to make it evidence based medicine. The phenolic compound eugenol (60 mg/mL) showed significant antiinflammatory activity. However the amount of eugenol present in 1 mg of OSAE and OSWE (12 ng/mg and 19 ng/mg dwt respectively) used for cell based assays was very low. It suggests that several other metabolites along with eugenol are responsible for the efficacy of the extracts.

1. Introduction

Ocimum sanctum Linn. (Lamiaceae) widely known as 'Holy basil/Tulsi' is a herbaceous sacred plant found across India. Extracts of this plant, particularly of leaves, have been traditionally used for their cardioprotective purposes (Joseph and Nair, 2013; Kumar et al., 2012;

Abbreviations: AE, Alcoholic extract; EUG, Eugenol; IMT, Imatinib; LPS, Lipopolysaccharides; PMA, Phorbol-12-myristate-13-acetate; WE, Water extract

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Pandey and Madhuri, 2010; Pattanayak et al., 2010; Rashid et al., 2013). Of recently, scientific investigations have found out that Ocimum sanctum (OS) possesses wide range of pharmacological benefits such as hypolipidemic (Hussain et al., 2001), hepatoprotective (Akilavalli et al., 2011) and neuroprotective (Yanpallewar et al., 2004) properties. Its anticoagulant (Khan et al., 2011), antioxidant, antiinflammatory (Kalabharathi et al., 2011; Kath and Gupta, 2006) and immunomodulatory (Mediratta et al., 2002) effects have been reported using animal models. All these pharmacological activities of OS are attributed to its phytoconstituents, one such compound which has been comprehensively investigated is eugenol. Eugenol is a phenolic compound which is a major constituent of Ocimum sanctum (Singh et al., 2013) and other aromatic plants, Eugenia aromaticum (Raghavendra and Naidu, 2011), Cinnamomum verum, Pimenta racemosa, etc. (Jaganathan et al., 2011). In last few decades several studies have been carried out globally by scientific community to suggest the role of eugenol in therapeutic potentials of Ocimum sanctum (Prakash and Gupta, 2005). Antioxidant (Nagababu et al., 2010), antiinflammatory (Daniel et al., 2009) and antiplatelet aggregatory (Raghavendra and Naidu, 2011) effects of eugenol have been demonstrated using the animal model. Further, its vasorelaxing action suggested its therapeutic importance as a vasodilator (Nishijima et al., 1999).

Cardioprotective property of OS and its metabolite EUG against isoproterenol induced myocardial infarction in rats has been demonstrated (Choudhary et al., 2006; Sharma et al., 2001). However, pharmacological action of OS as well as EUG against atherosclerosis is poorly addressed so far. Atherosclerosis, a type of cardiovascular disease (CVD) develops in the large arteries due to complexed interaction between activated peripheral blood monocytes and vascular endothelium (Boos and Lip, 2006; Libby, 2002; Ross, 1999). In view of validating traditional use of OS-leaves, the present study was designed to investigate the antiinflammatory effect of alcoholic and water extracts of OS-leaves and pure compound EUG on lipopolysaccharide (LPS) and/or phorbol-12-myristate-13-acetate (PMA) induced activation of human monocytic (THP-1) cells.

2. Materials and methods

2.1. Chemicals

Power SYBR Green PCR Master Mix was obtained from Applied Biosystems (Carlsbad, CA, USA), BD OptEIA human TNF-α ELISA kit was purchased from BD Biosciences (USA), iScript cDNA synthesis kit was from Bio-Rad (Hercules, CA), Alexa Fluor 594 goat anti-rabbit IgG (H+L), fetal bovine serum (FBS), L-glutamine, pen-strep, prolonged gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI), RPMI 1640, and trizol reagent were purchased from Invitrogen. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide (MTT) was obtained from Merck, imatinib (IMT) from Natco Pharma Limited, eugenol, lipopolysaccharide-Escherchia coli (LPS), phorbol-12-myristate-13-acetate (PMA), trypan blue, and DEPC treated water were purchased from Sigma-Aldrich (Germany). NF-KB-p65 polycolonal rabbit antibody was obtained from Thermo Scientific (USA).

2.2. Plant material collection and preparation of OS dry leaf extracts

The seeds of the Ocimum sanctum plants (voucher #46803) were obtained from CIMAP, Hyderabad and authenticated by Prof. B. R. Rao, Scientist in charge, CIMAP. The plants were grown in plant house located in UoH campus. The leaves were collected from one year old plants and shade dried for at least 7 to 10 days and stored at room temperature in dark until further use.

2.3. Preparation of OS dry leaf extracts

The protocol followed for the preparation of alcoholic and water extracts from Ocimum sanctum dried leaves was quite similar with the one as mentioned by Kokkiripati et al. (2011). Alcoholic extract was prepared by soaking the fine powder obtained from 190 mg of leaves in 20 mL of 80% (v/v) ethanol. This process was followed by continuous stirring at 40 °C for 5–6 h until the volume reached approximately to 1/5th of the initial volume. Water extract (OSWE) was prepared by soaking the same amount of the powder in 4 mL of distilled water for 12–16 h at room temperature. Later on, the extracts were centrifuged at 10,000 rpm for 10 min and supernatants were collected and stored until further use at 4 °C and used within 3 days. The respective extracts were dried completely under the vacuum to determine the yields of OSAE and OSWE. The alcoholic and water extracts yielded 4% (w/w) and 4.5% (w/w) based on the amount of dried leaves taken to prepare the extracts. Commercially available EUG was procured from Sigma-Aldrich and stock solution was prepared (60 mg/mL) in absolute alcohol and stored at −20 °C until further use. IMT of 10 mg/mL was solubilized in DMSO and subsequently diluted in a cell growth medium. Vehicle concentration, in case of OSAE, OSWE, EUG and IMT did not exceed to 1% of cell culture volume in all cell based experiments. The effect of respective vehicle volume was tested in each and every set of experiment.

2.4. ESI–LC–MS/MS analysis

Phytochemical analysis of the OSAE and OSWE was done using a 6520 Accurate Q-TOF (Agilent Santa Clara, CA) mass spectrometer coupled to HPLC. Metabolites of the extracts were separated using HPLC according to Dutta et al. (2007) on reverse phase column (Zorbax Eclipse XDB-C_{18}, 4.6×50 mm, 1.8 μm). Solvent A and solvent B of mobile phase were water with acetic acid (0.1% v/v) and methanol with acetic acid (0.1%) respectively. A controlled flow rate of 0.2 mL/min was maintained for the following linear gradient elution in terms of solvent B: (i) 20–55% from 0 to 15 min, (ii) 55–90% from 15 to 20 min, (iii) 90% isocratic from 20 to 23 min, (iv) 90–20% from 23 to 26 min, and (v) isocratic 20% from 26 to 35 min. Sample volume injected was 2 μL. ESI parameters: both on negative and positive ion modes; mass range 100–1700; spray voltage 4 kV; gas temperature 325 °C; gas flow 10 L/min; Nebulizer 40 psi. Quantification of eugenol in OSAE and OSWE was done and details are given in Supplementary materials.

2.5. Cell culture and treatments

Human monocytic (THP-1) cell line was obtained from National Centre for Cell Sciences, Pune, India. The cells were cultured at 37 °C in 5% CO_2 in RPMI media supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL pen-strep. Thereafter the experiments were conducted at a cell density of 5×10^5 cells/mL. An acute inflammatory stimulus was given to the cells by incubating them with 0.5 μg/mL of LPS for 3 h at 37 °C in 5% CO_2 (Kokkiripati et al., 2011). In order to stimulate the differentiation of THP-1 cells to macrophages, the cells were treated with 5 ng/mL of PMA for 48 h at 37 °C in 5% CO_2 as described in Tsuchiya et al. (1982). The images of the cells were taken under an inverted microscope.

2.6. Plant extracts dosage determination

Cell viability of THP-1 cells in the presence of various amounts of OSAE/OSWE, EUG and IMT was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann (1983). An aliquot of 500 mL cell suspension (5×10^5 cells/mL) was distributed in each well of 24 well plates and then incubated for 24 h in the presence or absence of various concentrations of OSAE, OSWE, pure compound EUG, and positive control IMT. Effect of respective vehicle solvents was tested. Vehicle concentration was limited to ≤1% of cell culture volume. MTT (5 mg/mL in PBS) reagent of 20 mL was added to each well and incubated for an additional 4 h at 37 °C. Subsequently, the formed precipitate containing purple-blue formazan was dissolved in 100 ml of DMSO and the optical density was measured at 570 nm using a micro-plate reader (Tecan, Germany).

2.7. Quantification of secreted TNF-α

In order to carry out this experiment, cells were seeded in 24-well plates at a density of 5×10^5 cells/mL and pretreated with or without plant extracts (1 mg dwt/mL), EUG (6 and 60 mg/mL) and IMT (5 and 20 mg/mL) at 37 °C in 5% CO_2 for 12 h. Subsequently the cells were exposed to 0.5 mg/mL of LPS for 3 h. Cells were centrifuged, collected supernatant stored at −80 °C until further use. TNF-α present in the supernatants was determined using BD OptEIA human TNF-α ELISA kit (BD Biosciences) as per the manufacturers protocol.

2.8. Transcript analysis of inflammatory markers

After required treatments with LPS or PMA in the presence or absence of plant extracts (1 mg dwt/mL), EUG (60 mg/mL) and IMT (20 mg/mL), total RNA from the cells was isolated using trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Subsequently, 1 μg of RNA was used for c-DNA synthesis (Bio-Rad synthesis kits). Then the transcripts were quantitated by real time PCR using specific set of primers which are listed in Supplementary Table 1.

2.9. Nuclear translocation of NF-KB

The NF-KB-p65 in THP-1 cells was detected by indirect immunofluorescence assay using confocal microscopy (Jeong et al., 2010). THP-1 cells were cultured directly on glass coverslips. After the respective treatments of plant extracts (1 mg dwt/mL), EUG (60 mg/mL) and IMT (20 mg/mL), the cells were fixed using 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (w/v), and blocked with 3% bovine serum albumin (Sigma-Aldrich, Germany). Subsequently polyclonal antibodies against NF-KB-p65 (1:250) were applied for 12 h followed by 3 h incubation with anti-rabbit IgG conjugated to Alexa fluor 594 antibody (1:250). Excitation and emission maxima used were 543 nm 617 nm respectively. The nuclei were visualized by staining with DAPI (excitation wavelength, λ_{ex} 358 nm and emission wavelength, λ_{em} 400 to 500 nm).

2.10. Statistical analysis

To analyze the obtained data, Sigma Plot 11 software was used. All the data obtained were analyzed by one way analysis of variance (ANOVA) test using statistical package for the Life Sciences (SPLS version 11). Data obtained from all the experiments were expressed as mean ± S.D of three individual experiments. p-values lesser than 0.001 were considered as statistically significant.

3. Results

3.1. Metabolite profiling of OSAE and OSWE

Negative ion and positive ion modes of LC–QTOF–MS detected 16 and 7 compounds in OSAE; 4 and 6 compounds in OSWE. MS–MS fragmentation spectra of the compounds are shown in Fig. 1 and other details including m/z values, retention time, etc. are shown in Supplementary Tables 2 and 3. Majority of the identified compounds belong to either phenolics or terpenoids. Vicenin, luteolin-7-O-glucuronide, apigenin-7-O-glucuronide, methyl chavicol, methyl cinnamate, eugenol, fatty acid, and linoleic acid were commonly detected in both OSAE and OSWE. Rosmarinic acid, orientin, germacrene D, α-pinene, carvacrol, apigenin, linalool, cirsimaritin, cirsilineol, sinapic acid and ascorbic acid were exclusively detected in OSAE, while methyl eugenol and stearic acid were detected from OSWE. Eugenol, which has been detected in both modes of extracts, was investigated for its antiinflammatory activity in THP-1 cells. Quantitative analysis of EUG in plant extracts revealed that OSAE and OSWE contained 12 ng/mg dwt and 19 ng/mg dwt respectively.

3.2. Effect of OSAE, OSWE and EUG on LPS induced TNF-α secretion

TNF-α is a potent proinflammatory cytokine secreted by activated monocytes, subsequently activate other cell sepsis in the blood vessel and thus initiates/promotes the progression of atherosclerosis. As shown in Fig. 2, LPS stimulates THP-1cells to secrete 22 fold more amount of TNF-α compared to control unstimulated cells. Pretreatment of cells with 0.5 mg and 1 mg dwt/mL of OSAE brought down to 18 and 9 fold and OSWE to 19 and 6 folds respectively. EUG at 6 mg/mL reduced to 14 fold, whereas 60 mg/mL completely attenuated LPS induced stimulation. The cells secreted similar to unstimulated control cells (Fig. 2). Pretreatment of cells with positive drug control IMT at 5 and 20 mg/mL brought down to 19 and 9 fold respectively in the presence of LPS (Fig. 2). The rest of the cell based assays were done using pretreatments with 1 mg dwt/mL of the plant extracts, 60 mg/mL of EUG and 20 mg/mL of IMT.

Toxicity of plant extracts or pure compound EUG and positive control IMT, if any, on THP-1 cells was determined based on MTT assay. No cell death was observed up to 3 mg dwt/mL of OSAE and OSWE and the data is shown up to 1 mg dwt/mL in Supplementary Fig. 2. Similarly cell viability in the treatments with EUG (up to 60 mg/mL) and IMT (up to 20 mg/mL) was ≥95% (Supplementary Fig. 2). Vehicles solvents (alcohol or water of ≤1%) had no effect on cell viability as well as TNF-α secretion. Volume of DMSO did not exceed 0.5% and had no effect on cell viability.

3.3. Effect of OSAE, OSWE and EUG on LPS induced inflammatory markers

The effect of the plant extracts, EUG and positive drug control IMT on LPS induced gene expression of inflammatory markers including TNF-α was tested to examine if the effect was at transcriptional or posttranscriptional level. The transcript analysis by real-time quantitative PCR showed that LPS stimulated the expression of IL-6 (2-fold increase), TNF-α (5-fold increase), macrophage inflammatory protein (MIP-1α) (2.15-fold increase) and MCP-1 (36-fold increase) as shown in Fig. 3. Here it is noteworthy that such stimulation of proinflammatory gene expression was markedly inhibited in the cells pretreated with OSAE/OSWE (1 mg dwt/mL). OSWE showed stronger effect than OSAE in attenuating the MCP-1 marker. EUG (60 mg/mL) and IMT (20 mg/mL) showed significant inhibition of all the selected markers. At these concentrations, EUG showed higher inhibitory effect

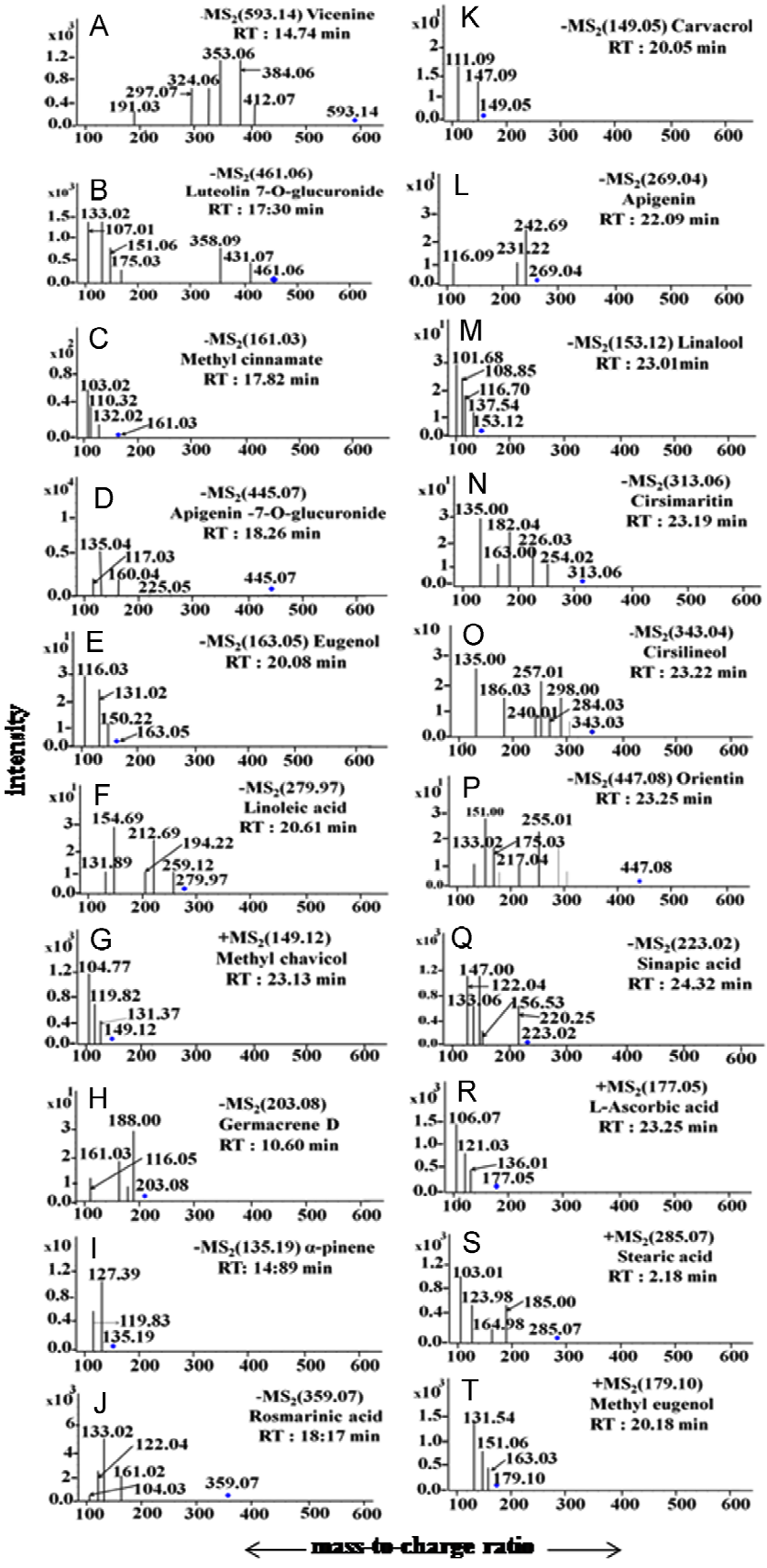

Fig. 1. Negative ion (_) and positive ion (þ) ESI/MS/MS of alcoholic and water extracts of OS-dry leaves. Labeled peaks are corresponding to the fragments.

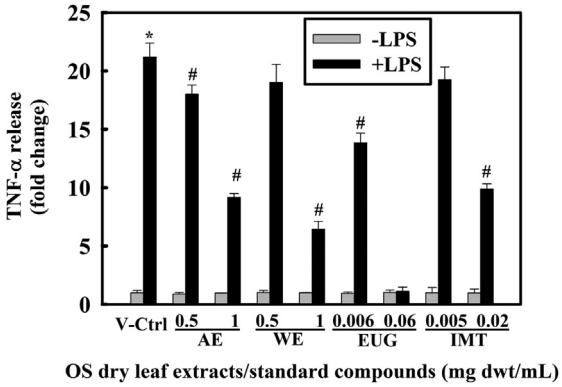

Fig. 2. Effect of OS-dry leaf extracts, EUG and IMT on LPS-mediated TNF-α secretion in THP-1 cells. Cells were seeded at 5 _ 10^5 cells/mL. Data represents fold change in TNF-α release into the media by the cells treated with LPS (0.5 mg/mL for 3 h) compared to untreated cells. OSAE, OSWE, EUG and IMT added to the cells 12 h prior to incubation with LPS. Vehicle solvents of alcohol/water (was limited to ○1%) and of DMSO used (was limited to○0.5%) had no effect. Both types of solvents had no effect on TNF-α secretion; mean value is represented as V-control. Data represent n¼6 mean of six measurements. *Statistical significance of p○0.001 within the control groups i.e. cells þLPS vs cells_LPS. #p○0.001 compared between cells treated with LPS in the presence of OSAE/OSWE, pure compound EUG and positive control IMT in their absence.

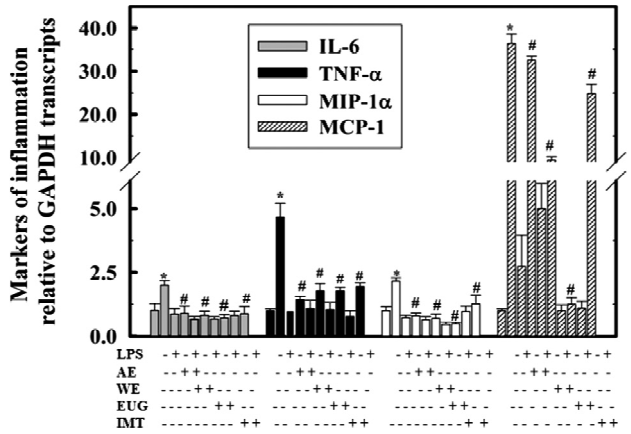

Fig. 3. Effect of OS-dry leaf extracts (1 mg dwt/mL), EUG (60 mg/mL) and IMT (20 mg/mL) on gene expression of IL-6; TNF-α; MIP-1α and MCP-1 in control vs LPS induced THP-1. Vehicle volume of alcohol or water (did not exceed 1%) and DMSO (○0.2%) had no effect. Experiments were performed at least in triplicate and the results are expressed as the mean7S.D. *p○0.001 for comparison between cells þLPS vs cells-LPS. #p○0.001 compared between cells treated with LPS in the presence of plant extracts, pure compound and positive control IMT vs in their absence.

than IMT on MIP-1α and MCP-1. IMT at 50 mg/mL stimulated proinflammatory effect in LPS unstimulated cells (data not shown).

3.4. Effect of OSAE, OSWE and EUG on the subcellular localization of NF-KB-p65

In order to further elucidate, the molecular mechanism by which the plant extracts modulate gene expression in THP-1 cells, the effect of OSAE/OSWE and EUG on transcriptional factor NF-KB was investigated. The transcription factor NF-KB plays a crucial role in the regulation of gene expression of several proinflammatory markers including TNF-α, interleukins and chemokines. Results presented in Fig. 4 clearly indicate that the plant extracts and pure compound EUG inhibit LPS induced translocation of NF-KB in THP-1 cells. The confocol images of Fig. 4A–C (1st panel) showed that NF-KB-p65 was mostly sequestered in the cytoplasm of normal uninduced cells, whereas it was predominately located in the nuclei of the LPS stimulated cells (Fig. 4D–F). Such LPS dependent nuclear translocation of NF-KB-p65 was blocked in the cells pretreated with OSAE (Fig. 4G–I), OSWE (Fig. 4J–L) or EUG (60 mg/mL) (Fig. 4M–O). These results demonstrated that OSAE, OSWE or EUG elicited antiinflammatory activity in THP-1 cells by modulating subcellular localization of transcriptional factor NF-KB at their tested concentration. Positive drug control, IMT (20 mg/mL) showed similar response (Fig. 4P–R).

3.5. Effect of OSAE, OSWE and EUG on PMA induced cell differentiation markers

Human monocytic THP-1 cells committed to the monocytic cell lineage are suspension in nature and do not adhere to the plastic surfaces of the culture plates as shown in Supplementary Fig. 2A. PMA (5 ng/mL) stimulated differentiation of THP-1 cells to macrophages as indicated by their adherence to substratum of the culture dish (Supplementary Fig. 2B). The adhered cells showed morphological characteristics similar to macrophages. Such PMA induced differentiation process was inhibited by the plant extracts, pure compound EUG and positive control, IMT. As shown in Supplementary Fig. 3C–E, pretreatment with OSAE/OSWE (1 mg dwt/mL) and EUG (60 mg/mL) significantly attenuated the process of differentiation by PMA challenged THP-1 cells. In agreement with the literature, differentiated macrophages (PMA challenged THP-1 cells) showed upregulation in the expression of CD14 and toll like receptors (TLR2 and TLR4) by 3, 64 and 37 folds respectively (Fig. 5). Such upregulation of the markers was markedly attenuated by OSAE/OSWE (1 mg dwt/mL) and EUG (60 mg/mL) in PMA treated monocytes. Results are able to replicated by IMT (Supplementary Fig. 3F and Fig. 5).

4. Discussion

In recent years, the use of herbal products raised steeply due to increased interest in the developed and developing countries for their use as antioxidants and good health supplements (Mukherjee et al., 2010). In traditional medicine, Ocimum sanctum leaves are found to be used for a broad range of health benefits including cardiac health. Commercially available herbal products of Ocimum sanctum (e.g. Tulsi by Kirpal Export Overseases, India; Dry herbal extracts by Nikita Extracts, India; Ocimum sanctum-Beadlet by Borion Canada Inc., Canada) are known majorly for their cardiac health benefits. Eugenol has been extensively investigated while addressing the therapeutic potentials of Ocimum sanctum. However, molecular mechanism of action of OS-leaves as well as eugenol in offering cardiac health, specifically inhibiting atherogenesis, is not clearly understood. The present study investigated the antiinflammatory properties of Ocimum sanctum dry leaf extracts and its pure compound EUG using human monocytic (THP-1) cells. To the best of our knowledge, this is the first attempt that has been made to understand the cellular and molecular basis of atheroprotective activity of OS-dry leaf extracts using human monocytic (THP-1) cells.

In this study, the effect of Ocimum sanctum on the LPS induced secretion of TNF-α and gene expression of cytokines and chemokines like IL-6, TNF-α, MIP-1α and MCP-1 in THP-1 cells was tested because of their significance in inflammation. These cytokines and chemokines are shown to be produced by THP-1 cells up on stimulation with LPS (Weiss et al., 2004; Xue et al., 2006). TNF-α is a potent proinflammatory cytokine, and plays an important role in developing many chronic inflammatory diseases including atherosclerosis and rheumatism (Huo et al., 2012). TNF-α produced by

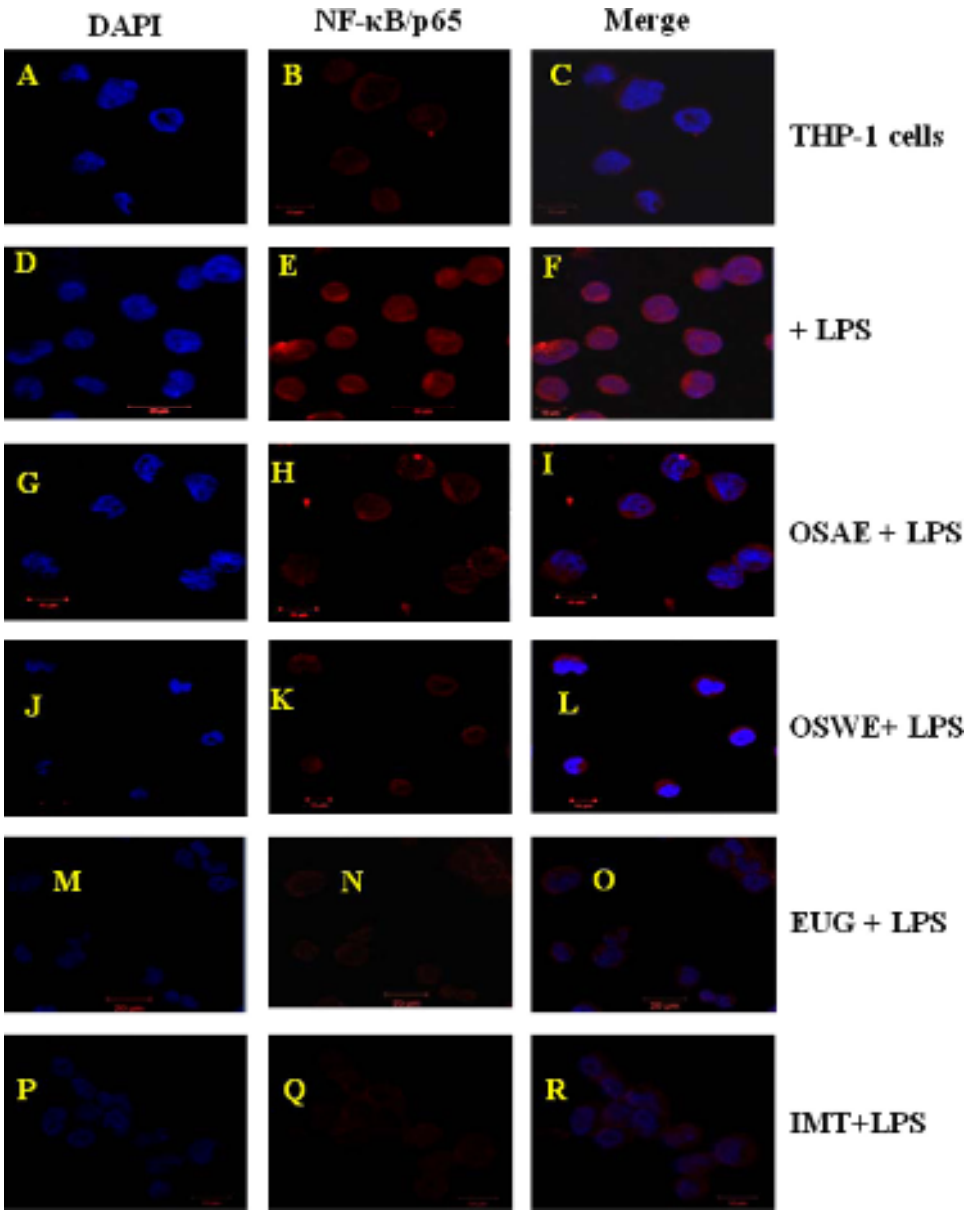

Fig. 4. Confocal microscopic images demonstrating the effect of OS-dry leaf extracts, pure compound EUG and positive control IMT on LPS-induced subcellular localization of NF-κB-p65 in THP-1cells. Images with vehicle control 0.5% (alcohol) cells are shown in panel 1. Vehicle controls of water (ʘ 1%) and DMSO (0.2%) also had no effect (not shown). NF-κB translocation was analyzed by staining with NF-κB-p65, red (A); nucleus with DAPI, blue (B); merged image of NF-κB-p65 and DAPI (C). THP-1 cells treated with LPS (0.5 mg/mL for 3 h) shown in 2nd panel (D–F); cells pretreated with OSAE or OSWE (1 mg dwt/mL) and then treated with LPS shown in 3rd (G–I) and 4th panels (J–L). Panel 5 (M–O) and panel 6 (P–R) represent cells preincubated with EUG (60 mg/mL) and IMT (20 mg/mL) respectively, then challenged with LPS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

monocytes and macrophages (Mackenzie et al., 2002) activates other cell types like endothelial cells. Endothelial cells up on activation produce cell surface adhesion molecules and selectins, facilitating rolling, adherence and diapedsis of monocytes into subendothelial space where they differentiate into macrophages (Postea et al., 2006). IL-6 is a proatherogenic cytokine and is associated with fatty lesion development in aorta (Kishikawa et al., 1993). MIP-1α and MCP-1 belong to chemokine family and are also associated with atherosclerotic lesion development. High levels of MIP-1α along with other chemokines like MIP-1β, RANTES and cytokines IFN-γ, IL-2, IL-4, and IL6 are found in blood plasma of atherosclerotic patients (Cagnin et al., 2009). MCP-1 is established as one of the important chemokines involved in the development of atherosclerosis, specifically by playing a role in the monocyte recruitment and infiltration into the arterial wall (Reape and Groot, 1999; Takahashi et al., 1995). The present study showed inhibitory effect of Ocimum sanctum dry leaf extracts on LPS induced production of TNF-α and expression of some of the above mentioned inflammatory markers, IL6, MIP-1α and MCP-1. LPS activates expression of these markers via NF-κB pathway (Ci et al., 2010). In our study, Ocimum sanctum leaf extracts showed significant inhibition of nuclear translocation of NF-κB in LPS stimulated THP-1 cells. These results suggest that the plant extracts may have the potential to inhibit several other genes regulated by this pathway. In addition to the effects on LPS induced cytokine and chemokine expression, the extracts inhibited the expression of PMA induced cell differentiation markers CD14, TLR2 and TLR4 as well as morphological characteristics of macrophages. All these three markers are strongly associated with inflammation and atherosclerosis plaque development (Schlitt et al., 2004). All the inhibitory effects showed by plant extracts are comparable to the positive drug control IMT.

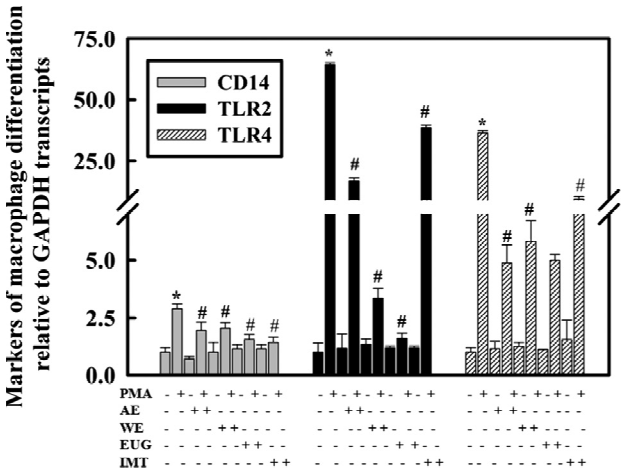

Fig. 5. Effect of OS-dry leaf extracts, pure compound EUG and IMT on PMA induced CD14, TLR2 and TLR4 mRNA transcripts in THP-1cells. THP-1 cells pretreated for 12 h with or without OSAE (1mg dwt/mL), OSWE (1mg dwt/mL), EUG (60 mg/mL) and IMT (20 mg/mL) and then incubated with PMA (5 ng/mL) for 48 h. After incubations, transcript levels by CD14, TLR2 and TLR4 were determined by RT-PCR as a measure of THP-1 cell differentiation. Vehicle control solvents, alcohol/water (were limited to ≤1%) and DMSO (0.2%) had no effect. Data shown are mean value of 3 independent experiments ± S.D. *$p \leq 0.001$, for comparison between treated (cells + PMA) and untreated groups (cells-PMA). #$p \leq 0.001$ compared between cells treated with PMA in the presence of OSAE, OSWE,EUG and IMT vs in their absence.

LC–MS analysis showed the presence of eugenol in both OSAE and OSWE along with many other bioactive compounds like rosmarinic acid, vicenin, orientin, apigenin, α-pinene, carvacrol, stearic acid and linoleic acid (Supplementary Tables 2 and 3). Some of these compounds like linoleic acid and α-pinene are shown for their antiinflammatory property in THP-1 cells (Zhao et al., 2005; Zhou et al., 2004). In the present study, we tested the effect of eugenol in LPS/PMA stimulated THP-1 cells, EUG at 60 mg/mL inhibited the expression of both LPS/PMA induced inflammatory cytokines, chemokines (IL-6, TNF-α, MIP-1α, MCP-1); macrophage differentiated markers (CD14, TLR2 and TLR4) and translocation of NF-KB protein into the nucleus in (THP-1) cells. However, LC–MS based quantification of EUG revealed for its presence in minute quantity in both the plant extracts (12 ng/mg dwt of OSAE and 19 ng/mg dwt of OSWE) suggesting that the antiinflammatory effect of the extracts could be due to synergistic/cumulative activity of several metabolites an addition to EUG present in the extracts. The efficacy of the plant extracts cannot be attributed to EUG alone. Earlier, Mahapatra et al. (2011) showed inhibitory activity of eugenol on NF-KB activation in murine macrophages. The present study suggests that bioactive compounds such as eugenol and others, which can attenuate monocytic activation, a key step in the atherogenesis, can be extracted easily from the leaves of Ocimum sanctum using nontoxic solvents like water, and it is commonly used for preparation of herbal extracts in traditional medicine. Therefore, Ocimum sanctum can be considered as a rich natural source for isolating drug molecules, which can be of use in treating inflammatory diseases like atherosclerosis, as these plants can be grown widely in tropical and subtropical regions of the world. With respect to traditional use of OS-leaves for treating cardiovascular diseases, the present study raised the question on dosage requirement. The amount of OS extracts (1 mg dwt/mL) required to elicit antiinflammatory in LPS/PMA stimulated THP-1 cells is towards on higher side, though the dosage was per se not cytotoxic to THP-1 cells. Thus the study strongly recommends further evaluation of medicinal use of OS-leaves for treating CVD. Future direction of this study is to test the efficacy of OS-leaves on their antiatherosclerotic property using chronic inflammatory models, e.g. diabetic or apoE-/-mice.

5. Concluding remarks

Critical cellular and biochemical investigation of the present study demonstrated antiinflammatory activity of alcoholic and water extracts of OS-dry leaves: (i) attenuated LPS induced proinflammatory gene expression in THP-1 cells by inhibiting nuclear translocation of NF-KB; (ii) inhibited PMA induced monocyte to macrophage differentiation and (iii) had bioactive compounds like phenolics, flavonoids, terpenoids and fatty acids. Pure compound, eugenol (60 mg/mL) a phenylpropanoid showed remarkable antiinflammatory activity in LPS/PMA induced THP-1 cells. LC–MS quantification of EUG from the plant extracts suggested that the efficacy of OSAE and OSWE is due to their richness with several bioactive compounds along with EUG. Thus the study supports the traditional use of OS-dry leaves for treating cardiovascular diseases, with leaving questions on dosage requirement for clinical use. It suggests, further testing the extracts, using chronic inflammatory models to make it evidence based on traditional use of Ocimum sanctum for treating cardiovascular diseases.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.03.049.

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