Immunomodulatory effect of *Tinospora cordifolia* and its metabolites in activated human monocytic (THP-1) cells

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By

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Immonomodulatory effect of *Tinospora cordifolia* and its metabolites in activated human monocytic (THP-1) cells",** has been carried out by me under the supervision of Dr. Sarada D. Tetali in the Dept. of Plant Sciences, School of Life sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute. The work is plagiarism free.

Reddi Kiran Kumar **Dr. Sarada D. Tetali**

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CERTIFICATE

This is to certify that this thesis entitled "**Immonomodulatory effect of *Tinospora cordifolia* and its metabolites in activated human monocytic (THP-1) cells**" is a record of bonafide work done by R. Kiran Kumar, a research scholar for Ph.D programme in Department of Plant sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. The work presented in the thesis is original and plagiarism free. We recommend his thesis for submission for the degree of Doctor of Philosophy of the University.

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Abbreviations

MCP-1: Monocyte chemotactic protein-1

IL-6: Interleukin-6

IL-8: Interleukin-8

I- κB: Inhibitor kappa B

P- IκB: Phosporylated Inhibitor kappa B

ICAM-1: Intracellular adhesion molecule-1

VCAM-1: Vascular cell adhesion molecule -1

E-selectin1: Endothelial selectin1

NF-κB: Nuclear factor-kappa B

H_2 DCF-DA: 2', 7'-dichlorodihydrofluorescein diacetate

ROS: Reactive oxygen species

TCAE: *Tinospora cordifolia* alcohol extract

TCWE: *Tinospora cordifolia* water extract

TNF-α: Tumor necrosis factor – alpha

TLR-4: Toll like receptor-4

20-HOD: 20-Hydroxyecdysone

BBR: Berberine

PMA: Phorbol-12-Myristate-13-Acetate

CAT: Catalase

GPx: Glutathione peroxidase

SOD: Sodium oxide dismutase

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

DCF: Dichlorofluorescein

THP-1: Human monocytic leukemia cell line

HAECs: Human aortic endothelial cells

FITC: Fluorescein isothiocyanate

PE: Phycoerythrin

SGY: Syringin

GAE: Gallic acid equivalents

QE: Quercetin equivalents

ASE: Ascorbic acid equivalents

DPPH: 2,2-diphenyl-1-picrylhydrazyl

ELISA: Enzyme linked immunosorbent assay

qRT-PCR: Quantitative Real time polymerase chain reaction

FACS: Flow activated cell sorter

LPS: Lipopolysaccharide

LC-MS: Liquid chromatography mass spectrometry

RT: Retention time

SD: Standard deviation

WHO: World health organisation

GAPDH: glyceraldehyde 3- phosphate dehydrogenase

MS/MS: Tandem Mass spectrometry

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

Ox-LDL: Oxidised low density lipoprotein

CVD: Cardiovascular disease

COX-2: Cyclooxygenase-2

AA: Arachidonic acid

NE-PER: Cytosol-nuclear extraction buffer

TBS: Tris buffered saline

IMMUNOMODULATORY EFFECTS OF TINOSPORA

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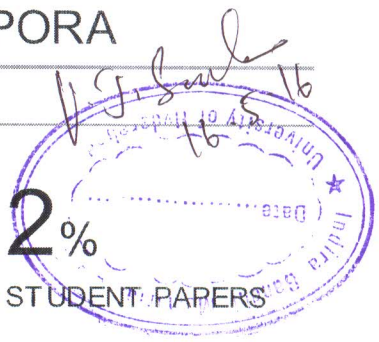

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Chapter - 1

Introduction

Chapter -1

Introduction

Herbal medicine

Herbal medicine is defined as usage of plant derived natural products valued for their general health and therapeutic properties. Medicinal plants are rich sources of bioactive compounds (secondary metabolites) possessing various pharmacological properties, which can be exploited for human benefits (Kennedy and Wightman 2011; Hussain et al., 20 12; Rogerio et al., 2010). Herbal medicine has been used by all the cultures throughout history for treating various health ailments (Pan et al., 2014; Shaw et al., 2012; Barnes et al., 2008).

According to World Health Organisation (WHO), 80 % of total population size chiefly rely on herbal medicine for maintaining good health (Ekor, 2014; Bag et al., 2013; Kunle et al., 2012). WHO defined 'health' stating as perfect physical, mental and social wellbeing and not merely the absence of disease or infirmity (Hoareau and DaSilva, 1999).

Medicinal plants are enriched with secondary metabolites which play significant role in their survival against abiotic and biotic stresses (Pandey and Rizvi). Many of these metabolites are beneficial to human beings as antioxidants and anti-inflammatory agents. Many pharmacy companies are investing in isolating bioactive metabolites from medicinal plants and investigating their molecular mechanism of action in treating human diseases (Vaidya and Devasagayam 2007), thus medicinal plants are continued to be resources for therapeutic agents for alleviating health ailments of mankind. WHO reported that 80 % of total pharmaceutical medicines (119 medicines) used in today's medication for treating various heart diseases, diabetes, hypertension, asthma, and other diseases are of herbal origin. It clearly demonstrates that usage of natural products is integrated into the modern medicine. It is highly encouraging to note the importance and recognition given to the traditional medicine by Nobel committee consisting of 50 professors at Karolinska Institute, the committee had given 2015 Nobel Prize

in the field of 'Physiology and Medicine' for discovering antimalarial drug artemisinin to a Chinese pharmacologist Youyou Tu **(Fig. 1.1)**. Ms. Youyou Tu shared the 2015 Nobel prize with other scientists, Prof. William C. Campbell and Satoshi Omura. Ms. Youyou Tu and her team screened several medicinal plant extracts against malaria and found *Artemisia annua* extracts are highly effective in curing malaria and later identified the active principal artemisinin responsible for its antimalarial activity.

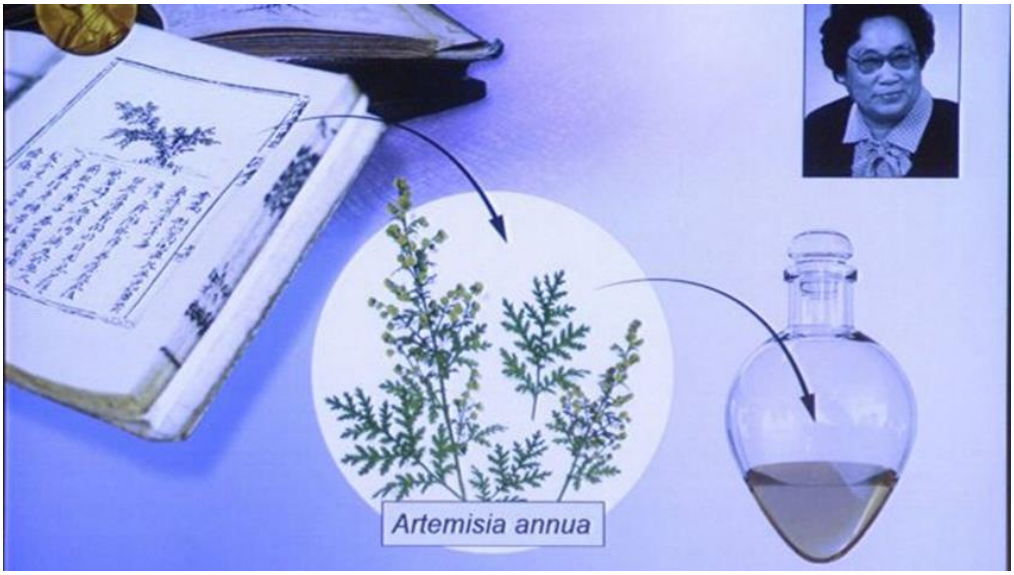


Fig. 1.1. Youyou Tu, 2015 Nobel prize winner in medicine for discovering antimalarial drug artemisinin from *Artemisia annua* extracts.

Indian biodiversity

India is considered to be one of the leading biodiversity centres enriched with 45,000 plant species out of which 15,000 plant species are identified to possess medicinal properties and about 7,500 species are exploited by traditional medicine practitioners for curing diseases (Parasuraman et al., 2014). Ayurveda and Unani use 700 medicinal plants each whereas Siddha uses around 600 plants in their medicinal products (Parasuraman et al., 2014). India has a great role to play in exporting herbal products and has advantage in generating huge international

trade revenues. Herbal industry is being projected next to information technology in generating huge revenues to India (Wakdikar, 2004).

Role of World Health Organisation in phytomedicine

WHO showed interest in documenting the usage of medicinal plants by tribals from different parts of the world. It encourages herbal medicine because natural products are readily available at less cost and free of harmful side effects. WHO demonstrated 80 % of the world population to rely on natural products for the health care due to high cost and side effects of modern medicine (Muthu et al., 2006; Parasuraman et al., 2014). WHO framed the following guidelines to assess the efficacy of herbal drugs (Calixto, 2000) :

- Quality
- Safety
- Stability
- Efficacy

Polyherbal formulations

Usage of multi herbs for effective therapeutic applications is considered to be one of the major principle in Ayurvedic traditional medicine. Cumulative action of components of multi herbs for the therapeutic treatment of human ailments or diseases is known as polyherbalism. In case of formulations the concentration of the metabolites from each individual medicinal plants are at minimum level and their accumulation in the body is unlikely, hence herbal formulations are preferred highly in Ayurveda. Further, scientific studies proved promising results of several herbal formulations. Global acceptance of herbal formulations is increasing day by day due to the fact that they are natural products, cheap and eco-friendly (Parasuraman et al., 2014). In

spite, of availability of synthetic drugs in the market, public have been preferring herbal formulations as latter are natural.

Few examples of herbal formulation's which are used in Ayurvedic traditional medicine are cited below **(Table. 1.1)**.

1. Combination of *Tinospora cordifolia* and turmeric works very effectively as immune booster formulations (Parasuraman et al., 2014).

2. Ginger and black pepper in combination works better in reducing mucous problems (Parasuraman et al., 2014).

3. Cumin and black pepper are used in combinations in traditional medicine to overcome digestion problems (Parasuraman et al., 2014).

Herbal medicine and its association with conventional drugs

Herbal medicine has been in use since ancient times for the treatment of various human diseases. Herbal medicine was employed in different practices like Ayurveda, Unani and Siddha in India. General interest and demand for herbal products is been increasing day by day and its annual global market at present amounts to 60 $ billion. Export of herbal drugs from India has been increased substantially in the past few years with an annual global market of about $1 billion of which $80 million has been exported. Approximately there were 25,000 licensed pharma companies, which registered 1000 herbal drugs and 3000 formulations using 8000 medicinal plants. The turnover rate of herbal medicine industry per year is about Rs. 2,300 crores with 15 % growth rate (Sheetal Verma, 2008; Vaidya and Devasagayam, 2007).

Table. 1.1. Example list of scientifically validated Ayurvedic polyherbal formulations and their medicinal properties

S.NO	Formulation	Medicinal plants	Therapeutic property	References
1.	Diabet	*Coscinium fenestratum, Curcuma longa, Phyllanthus reticulatus, Strychnos potatorum, Tamarindus indica and Tribulus terrestris*	Antidiabetic	Parasuraman et al., 2014
2.	Dihar	*Azadirachta indiaca, Curcuma longa Emblica officinalis, Emblica officinalis, Enicostemma littorale Gymnema sylvestre, Momordica charantia, Syzygium cumini* and *Tinospora cordifolia*	Antihyperlipidemia	Parasuraman et al., 2014
3.	Liv 52	*Cichorium intybus, Chillea millefolium, Cassia occidentalis, Capparis spinosa, Terminalia arjuna, Solanum nigrum* and *Tamarix gallica*	Hepatoprotective	Girish et al., 2009
4.	Octogen	*Phyllanthus niruri, Arogyavardhini rasa*	Hepatoprotective	Girish et al., 2009
5.	Vedic guard	*Asphaltum, Bacopa monnieri, Curcuma longa, Commiphora mukul, Eclipta alba, Emblica officinalis, Tribulus, Glycyrrhiza glabra, Mesua ferrea Piper longum, Puereria tuberose, Sida cordifolia, Terminalia arjuna, Terminalia chebula, Tinosporia cordifolia, Tribulus terrestris* and *Withania somnifera,*	Cardioprotective	Koti et al., 2013

Herbal medicines, prepared by following good practise conditions, can be more trusted over the conventional drugs since they not only have long history of therapeutic effectiveness but also their compatibility/non-toxicity with human subjects is practically been demonstrated over the period of time. Safety, eco-friendly, good quality and having long history of therapeutic effectiveness makes herbal medicine superior than some of the synthetic drugs (Firenzuoli and Gori, 2007). In fact, many of the conventional drugs have originated from herbal medicines, several synthetic drugs are chemically synthesized pharmaceuticals based on the structure of chemical constituents of herbal medicine. Unlike herbal medicinal products, which are complexed and heterogenous mixtures of several plant compounds, synthetic drugs comprise mostly of single active compound. Thus conventional drugs are enriched with single individual ingredient which acts on specific target in the body in curing particular disease whereas multicomponent herbal medicine works in line with our human body design which works on thousands of mini relationships. Few examples of herbal origin conventional drugs are cited in **Fig. 1.2.** However, quality control and reproducibility in the composition of herbal medicines is been a daunting task, particularly while scaling up in order to meet today's market demand.

Maintenance of standard quality and controlled measures in collection or plant material, harvesting season and toxicity studies not only improve the trust of the market and thereby revenue generation but also toxicity, if any, would be revealed since toxicity should not be undermined. Hence proper steps need to be taken to develop standard quality control methods to ensure the safety and efficacy of herbal drugs, (Sharma et al., 2010).

Clinical trials with herbal drugs: In order to promote evidence based medicine, clinical trials with herbal drugs should be testing the following:

1. Mechanism of action

2. Testing the safety and efficacy of drug with human subjects (Calixto, 2000).

3. Determining the dosage and route of administration of drug after validating the efficacy (Calixto, 2000).

List of allopathic drugs derived from plants used by conventional medical practioners:

Digitalis purpurea

Digoxin

Cardiac diseases

Salix alba

Aspirin

Pain reliever

Taxus species

Taxol

Cancer

Lythospermum erythrorhizon

Shikonin

Cancer

Fig. 1.2. List of some herbal derived drugs and their medical applications.

Simlar to synthetic drugs, clinical trials with herbal drugs are consists of the following three phases: Phase-I: Herbal drugs should be tested on cell lines and animal models with respect to establishment of mechanism of action and toxicity

Phase-II: After proving in cellular and animal models, drugs should further be tested on small batch of human subjects.

Phase-III: Additional appropriate and extensive clinical testing should be extended to more number of human subjects to confirm the safety, efficacy, dosage and route of administration.

Rational selection of medicinal plant for our study

According to WHO statistics coronary heart diseases (CDH), cancer, diabetes and obesity are majorly responsible for 60 % death and disability throughout the world. Among these burden of diseases, CHD considered to be the primary contributor of mortality and morbidity in the world. Hence, it is essential to focus research in screening medicinal plants having cardio protective properties for reducing the prevalence and mortality of CHD. There were numerous medicinal plants used in traditional medicine for treating heart diseases but only few of them are scientifically investigated. *Tinospora cordifolia* is commonly known as Giloya and its selection is purely based on its widely usage in folk/ Auyurvedic medicine for the treating various cardiac diseases and some of the scientific reports further proved its cardioprotectve properties. *Tinospora cordifolia* dry leaves were screened in the current study to prove their immunomodulatory effects in relevance to atheroprotection.

Tinospora cordifolia

Pharmacological relevance: *Tinospora cordifolia* is a herbacious wine that is native to India is known for its various medicinal properties in folk medicine. Its root, stems and leaves are used in Ayurvedic medicine for the treatment of high cholesterol, allergic rhinitis (hay fever), diabetes and etc. *Tinospora cordifolia* belongs to Menispermaceae family **(Table 1.2.)** and it is commonly known by various names such as Amrita, Guduchi and so on. In Ayurvedic traditional medicine it is used for the therapeutic treatments of diabetes, rheumatoid arthritis, jaundice and cardiac diseases. Scientific studies of *Tinospora cordifolia* extracts were shown as anti-diabetic (Rajalakshmi et al., 2009), antineoplastic activity (Jagetia et al., 1998), antioxidant (Sivakumar and Rajan 2010) anti-ulcer (Bafna and Balaraman 2005), cardioprotective (Rao et al., 2005), neuroprotective (Rawal et al., 2004), immune stimulating

(Nair et al., 2004) and anti-angiogenic activity (Leyon and Kuttan 2004) especially with stem, leaves and root parts of *Tinospora cordifolia* in animal models. Most of its therapeutic properties are attributed due to phytochemicals of various classes belonging to alkaloids, phytosteroids, sesquiterpenes and diterpene-glucosides and flavonoids etc.

Table 1.2. Taxonomy of *Tinospora cordifolia*

Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Ranunculales*
Family	*Menispermaceae*
Genus	*Tinospora*
Species	*cordifolia*

Fig. 1.3. Aerial parts of *Tinospora cordifolia*

Macroscopic study

Tinospora cordifolia possess glabrous succulent stem with white to grey coloured papery bark. Leaves are simple heart-shaped, pulvinate, alternate, existipulate and petiolate. Lamina broadly ovate with 10-12 cm in length and 8-15 cm in breadth. The flowers are yellow axillary stalked racemes and fruits are pea-sized red coloured drupes **Fig. 1.3**.

Phytochemistry

Phytochemical profiling of *T. cordifolia* reported its enrichment with metabolites belonging to various classes like alkaloids, phytosteroids, terpenoids, lignans and others till date. Some of the phytochemicals present in different parts of *T. cordifolia* are listed in **Table. 1.3**.

Herbal products of *Tinospora cordifolia* and their biological applications

1. Guduchi 60 capsules - Immunomodulator - Himalaya

A

2. SAFI- Blood purifying agent – Hamdard laboratories

B

3. Brave heart capsule – Hypolipidaemic - Vee Excel Drugs and Pharmaceuticals Private Limited

C

4. Tonplex – boosts immune response - Angel Drugs & Pharmaceuticals

D

5. Rebuild – Antioxidant - Herbal life nutrition

E

Fig. 1.4. Commercial products of *Tinospora cordifolia* (A) Guduchi, (B) SAFI, (C) Brave heart capsules, (D) Tonplex (E) Rebuild.

Table 1.3. Phytochemicals of various classes reported in the literature from different parts of *T. cordifolia*

S.No	Group	Part	Active principles	References
1.	Alkaloids	Stem/Root	Berberine. Palmatine Tembetarine Magnoflorine Tinosporin Isocolumbin, Tetrahydropalmatine choline.	Upadhyay et al., 2010 Saha and Ghosh 2012 Rout, 2012 Patel and Mishra 2011
2.	Gycosides	Stem	Furanoid diterpene glucoside 18-norclerodane glucoside Syringin, Palmatosides Tinocordioside Tinocordiofolioside Cordioside Cordiofolioside A-E.	Kapil and Sharma 1997 Saha and Ghosh 2012 Jahfar and Azadi 2004
3.	Steroids	Aerial parts and stem	β-sitosterol 20-Hydroxyecdysone Ecdysterone Makisterone Giloinsterol.	Saha and Ghosh 2012
4.	Lignans	Whole plant	3(a,4-dihydroxy-3-methoxybenzyl)-4-(4-hydroxy-3-methoxybenzyl)	Saha and Ghosh 2012
5.	Ditepene lactones	Whole plant	Furanolactone Clerodanederivatives Tinosporon Tinosporides Jateorine	Saha and Ghosh 2012 Swaminathan et al. 1989 Dhanasekaran et al. 2009
6.	Sesquiterpenoid	Stem	Tinocordifolin	Saha and Ghosh 2012
7.	Aliphatic compounds	Whole plant	Octacosanol Heptacosanol Nonacosan-15-one.	Saha and Ghosh 2012
8.	Other compounds	Root/whole plant	Giloin, Giloinin Tinosporic acid Tinosporidine Cordifol Cordifelone and Jatrorrhizine.	Saha and Ghosh 2012

Table. 1.4. Experimental validation of various pharmacological activities of different parts of *Tinospora cordifolia* using cellular and animal models.

Part of the plant and mode of extraction	Pharmacological activity	Test model	Concentrations	References
Stem: Methanol, aqueous and methylene chloride	Anticancer/ Antitumour	1.HELA cells 2.Balb C /mice	0, 5, 10,25,50 and 100 g/mL 200 mg/kg/day	Jagetia and Rao 2006 Mathew and Kuttan 1999
Leaves: Aqueous / alcohol / chloroform	Ant diabetic Hypoglycaemic Hypoglycaemic	1.Rabbits 2.Pancreatic islet beta-cells 3. Rats/ Rabbits	50, 100, 200 mg/kg/day 400 mg/kg/day	Wadood et al., 1992 Raghnathan.. and Sharma 1969
Roots: Aqueous	Hypolipidaemic	1.Rats	2.5 and 5 mg/kg/day	Stanely Mainzen Prince et al., 1999
Stem: Aqueous	Antinflammatory	1.Carrageen induced Rats 2.Croton oil induced Rats 3.Cotton pellet induced granuloma 4. Formalin induced Rats	50 mg/kg/day 50 mg/kg/day 1250 and 500 mg/kg/day 1mg/kg/day	Patgiri et al., 2014 Pendse 1977 Gulati OD, Pandey DC. 1982
Whole plant: Ethanol Stem: Aqueous	Immuno-modulatory	1.Ochratoxin induced Rats 2.cyclophospamide induced mice	100mg/kg/day 50,100mg/kg/day	Dhuley, 1997 Mathew and Kuttan 1999
Roots: Aqueous	Antioxidant	Alloxan diabetic rats	2.5-5mg/kg/day	Prince and Menon 1999

Stem: ethanol Whole plant: water	Radio protective	1. Mice exposure to gamma radiation 2. Mice exposure to gamma radiation	200mg/kg/day 5,10,20mg/kg/ day	Goel et al., 2004 Pahadiya and Sharma 2003
Roots: Extract suspensions (petroleum ether,ethane,acetone and water)	Antistress	mice	100mg/kg/day	Patil M. et. al., 1997
Roots: ethanol	Antiulcer	Male rats	100mg/kg/day	Sarma et al., 1995
Aerial parts: Ethanol	Neuroprotective	Male rats	100 mg/kg/day	Kosaraju, 2014
Whole plant:Formulation in ghee Whole plant: water soluble fraction / ethanol	Antipyretic	1.Yeast induced albino rats 2. Yeast induced Albino rats	900 and 1800 mg/kg/day 500 mg/kg/day	Ashok et al., 2010 Vedavathy and Rao 1991
Stem: Ethanol /aqueous and chloroform	Antibacterial	*E. coli, S. aureus, S. marcescens, E. faecalis, P. vulgaris and S. typhi*	100mg/mL	Jeyachandran et al., 2003
Stem: ethanol	Antiviral	Ranikhet disease virus	100µg/mL	Dhar et al., 1968
Aerial parts: Ether	Antitubercolosis	*Mycobacterium tubercolosis*	100mg/mL	Gupta and Viswanathan 1956
Stem Aqueous	Hepatoprotective Hepatoprotective	CCL_4 intoxicated rats Horse serum injected liver damage in rats	100 mg/kg 100 mg/kg	Bishayi et al., 2002 Nagarkatti et al., 1994

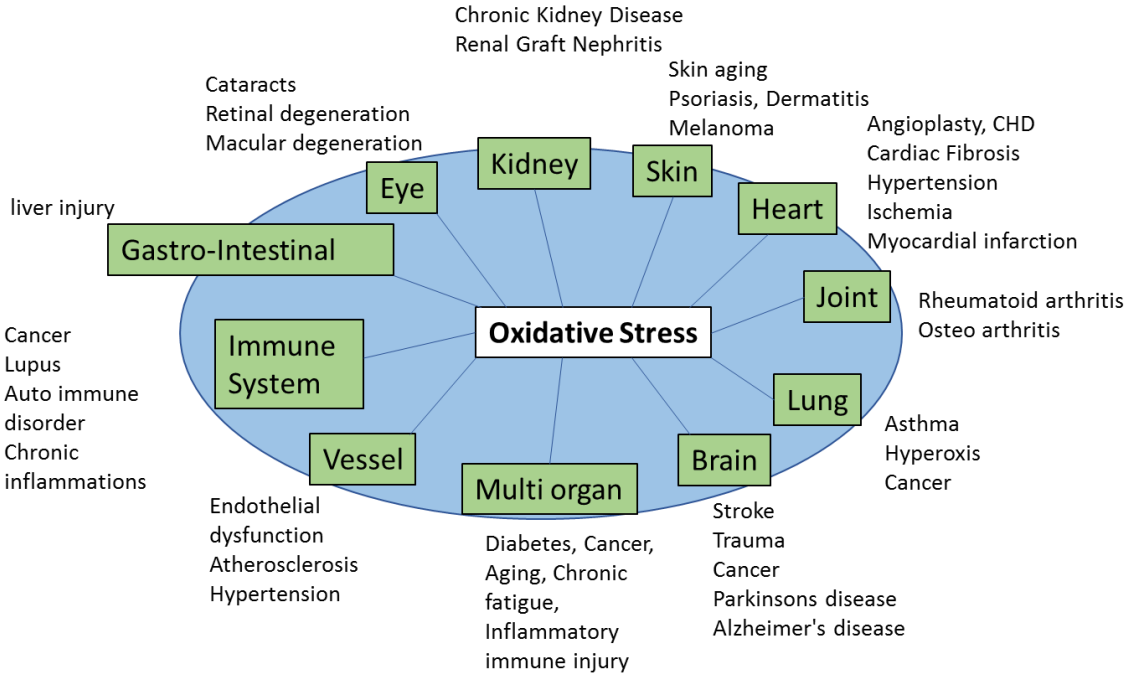
Oxidative stress and human diseases

Oxidative stress is a natural and harmful phenomenon developed in the body due to the imbalance between reactive oxygen species (ROS) generation and the ability of biological

system to detoxify their damaging effect (Aprioku, 2013; Chen et al., 2012). Oxidation of cellular macromolecules is promoted by ROS production when the effectiveness of antioxidant defense is compromised. Reactive oxygen species (ROS) belong to chemically a class of unstable molecule comprised of free radicles and peroxides which are predominantly generated by cellular metabolism occurs in all aerobic microorganisms (Rahman, 2007). Free radicles and peroxides attack macromolecules of cells such as lipids, proteins and DNA and cause hazardous effect such as lipid-peroxidation. Oxidation of DNA results in end products like hydro peroxides, isoprostane, 8-hydroxyguanine and ubiquine-10 (Styskal et al., 2012; Johri and Beal 2012), which are mutagenic in function. Oxidative stress takes part in the development of various chronic diseases like atherosclerosis, Parkinson's disease, diabetes mellitus, hypertension, Alzheimer's disease, ischemic heart malignancies and etc (Pham-Huy et al., 2008; Uttara et al., 2009; Kim and Park 2012). However ROS have been defined in beneficial way in other way as are used by immune system to kill pathogens during their invasion and maintains intracellular redox signaling. All forms of life maintain a redox environment within the cells. Fluctuations in the normal redox state can cause damaging effects through the production of free radicals that degrade macromolecules of the cell including lipids, proteins, and DNA.

Oxidative stress is induced by both endogenous and various exogenous factors such as mitochondrial metabolism for energy production, liver detoxification reactions involving cytochrome-P450 enzyme systems and exposure to cigarette smoking, alcohol consumption ionising radiations, infections by viruses, bacteria, fungi and etc (Saikat Sen and Raja Chakraborty 2011; Sainz et al., 2012). In a normal healthy human, ROS/RNS production are kept in check by antioxidant enzymes. However, when the humans get exposed to excessive doses of physiochemical, environmental or pathological agents, the balance will be shifted towards pro-oxidants leading to oxidative stress. Cellular damage caused due to oxidative stress

is responsible for the pathogenesis of human diseases (**Fig. 1.5.)** and ageing process (Rahman et al., 2012).

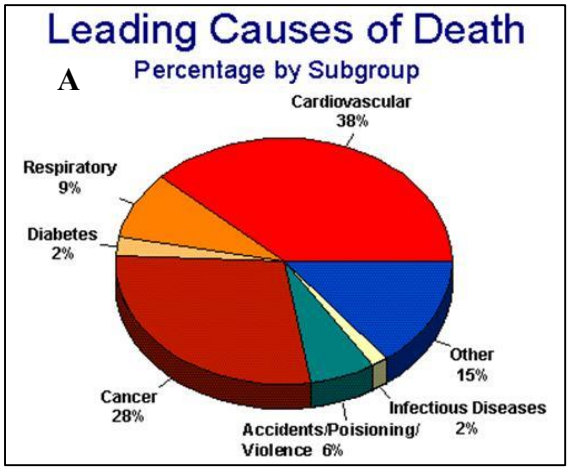


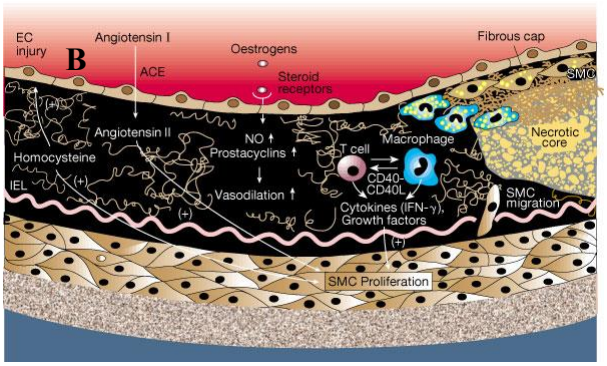
(Galli et al., 2005)

Fig. 1.5. Generation of free radicals responsible for Oxidative stress and diseases

Atherosclerosis

It is a chronic inflammatory vascular disease associated with blood vessels. Atherosclerosis is associated with the development of lipid enriched plaque **Fig. 1.6. (B)** in the artery wall (Insull, 2009; Galkina and Ley 2009). It is responsible for the actual causes of angina (chest pain), stroke and other vascular diseases which are collectively known as cardiovascular diseases (CVD). Arteries are lined by layer of cells known as endothelium which ensure proper blood flow within arteries.



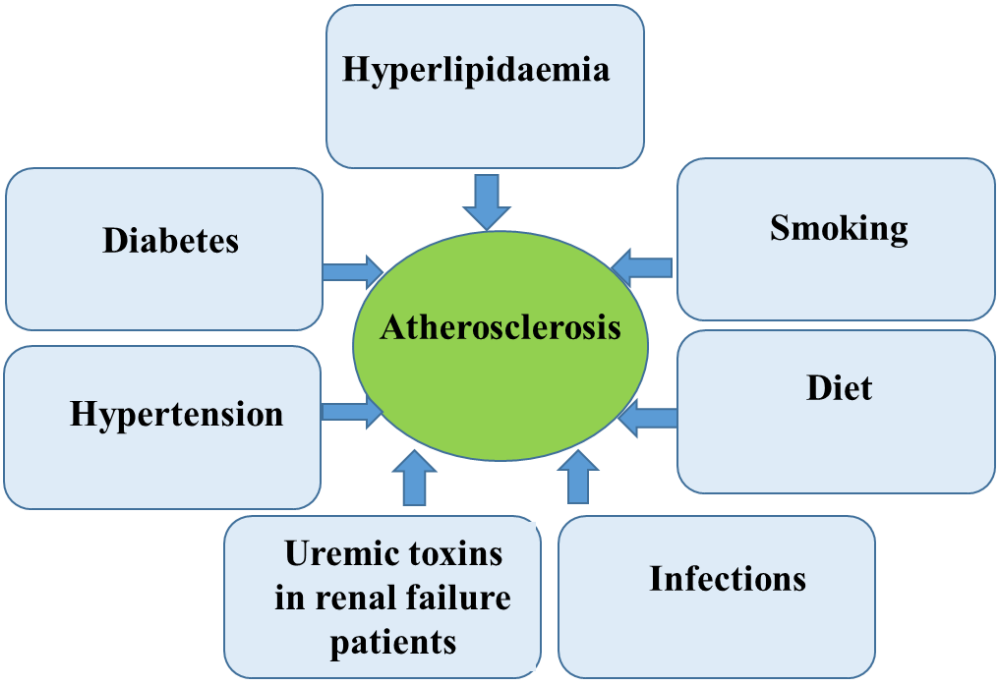


(Lusis, 2000)

Fig. 1.6. (A) Shows the statistics of various chronic inflammatory diseases leading to mortality and morbidity throughout the world and **(B)** Atherosclerosis is identified by the presence of lipid enriched plaque in the artery wall.

Atherogenic risk factors and their effects (Singh et al., 2002)

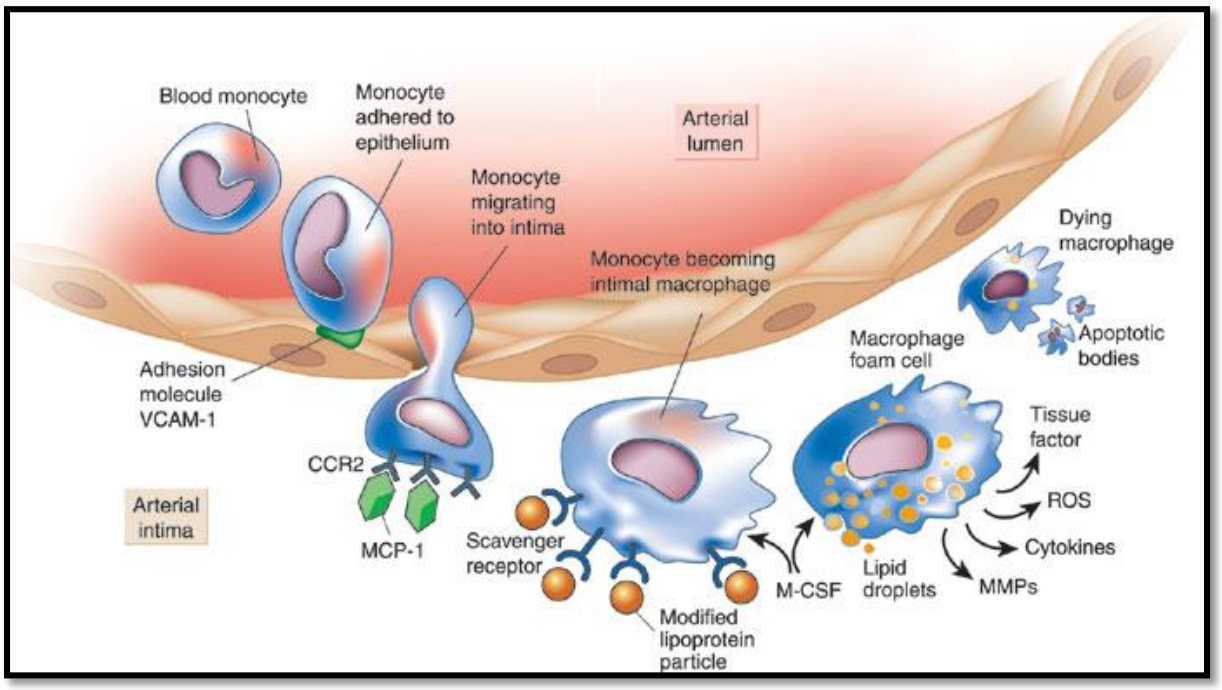
Elevated low density lipoprotein (LDL) cholesterol - foam cell development.
Elevated triglycerides - foam cell formation
Low high density lipoprotein (HDL) cholesterol – cholesterol transport to liver gets affected
Oxidised LDL- foam cell formation
Hypertension –Endothelial dysfunction
C - reactive protein (CRP) – causes inflammation
Lp-PLA_2 – Vascular inflammation
Infection bacterial endotoxin- oxidative stress and inflammation
Elevated homocysteine - Endothelial dysfunction
Elevated fibrinogen – Thrombosis



(Fruchart et al., 2004)

Fig. 1.7. Various risk factors leading to atherosclerosis. Risk factors like hypertension, hyperlipidemia, diabetes, smoking, uremic toxins in renal failure patients, infections and diet causes inflammation, thrombosis, foam cell formation and endothelial damage which ultimately leads to atherosclerosis.

Atherosclerosis is generally accelerated due to the above mentioned risk factors (**Fig. 1.7)**, a part from genetic, gender and ageing factors, which damage endothelium leading to deposition of cholesterol and LDL (Low-density particles) into sub endothelial space. Latter white blood cells present in the endothelium in take lipid particles through scavenging receptors and converted into lipid loaded foam cells (**Fig. 1.8)**. Accumulation of these foam cells over years forms plaque in the artery wall which gradually grows into bump and then converts to advanced/vulnerable plaque. Plaque undergoes rupture and releases blood into artery wall, forms clots with the inflowing blood (Thrombosis) subsequently leads to stroke (Szmitko et al., 2003).



(Libby et al., 2002)

Fig. 1.8. This figure represents the steps involved in the pathogenesis of atherosclerosis. Blood cells get activated on exposure to various adverse factors and secretes various inflammatory mediators. Under inflammatory conditions, blood cells like monocytes adhere to endothelium surface *via* adhesion markers like VCAM-1, ICAM-1 and E-selectin (Libby, 2002). Monocytes enters into intima of endothelium and converts to macrophages, in take lipid molecules through scavenging receptors and forms foam cells (X.-H. Yu et al., 2013; Bobryshev, 2006). Foam cells secret proinflammatory cytokines, ROS and MMPs which play a key role in thrombotic complications of atherosclerosis producing atherosclerotic lesion.

Oxidative stress and Inflammation roles in the development of atherosclerosis

Pathophysiology of atherosclerosis remains to be complicated till date, but it is widely proved that atherosclerosis is an inflammatory disease which is strongly promoted by oxidative stress (Bonomini et al., 2008; Li et al., 2014; Madamanchi et al., 2005). Risk factors like Diabetes mellitus, hyperlipidaemia, smoking, infections and hypertension stimulate ROS generation which in turn cause endothelial dysfunction. ROS penetrate into sub endothelial space and

cause LDL oxidation which are engulfed by macrophages *via* scavenging receptors present on their surface and turn into lipid loaded foam cells. ROS being secondary messenger also involved in the activation of various vascular inflammatory markers right from the formation of initial fatty streak to the rupture of plaque (Libby et al., 2002). Oxidative stress by H_2O_2 activates several tyrosine kinases and causes binding of neutrophils to endothelium leading to endothelial dysfunction. Oxidative stress also translocate transcription factors NF-κB (nuclear factor- κB) and AP-1 (activator protein-1) into the nucleus and activates proinflammatory cytokines (TNF-α, MCP-1, IL-6, IL-8) and adhesion molecules (Morgan and Liu, 2011). These activated molecules accelerates atherosclerosis by stimulating adhesion of neutrophils to endothelium *via* endothelial dysfunction. ROS also limits the NO (Nitric oxide) availability inside the endothelium synthesised by nitric oxide synthase (NOS) and this leads to vasoconstriction, platelet aggregation and adhesion of neutrophils to surface of endothelium and thereby promotes atherosclerosis.

Chapter - 2

Objectives and Study Design

Chapter -2

Objectives and Study Design

Objectives of the present study

Following objectives were designed for the present study with *Tinospora cordifolia* dry leaves based on its therapeutic applications in Indian traditional medicinal system and its scientific validations.

1. Evaluation of antioxidant and anti-inflammatory activities of *Tinospora cordifolia* dry leaves in activated human monocytic (THP-1) cells.
2. Metabolite profiling of dry leaf extracts of *Tinospora cordifolia* and identification of metabolites responsible for their effects in activated human monocytes (THP-1).
3. Molecular basis for antioxidant and anti-inflammatory activities of berberine (BBR), an alkaloid of *T. cordifolia* in activated human monocytes (THP-1).

Study design

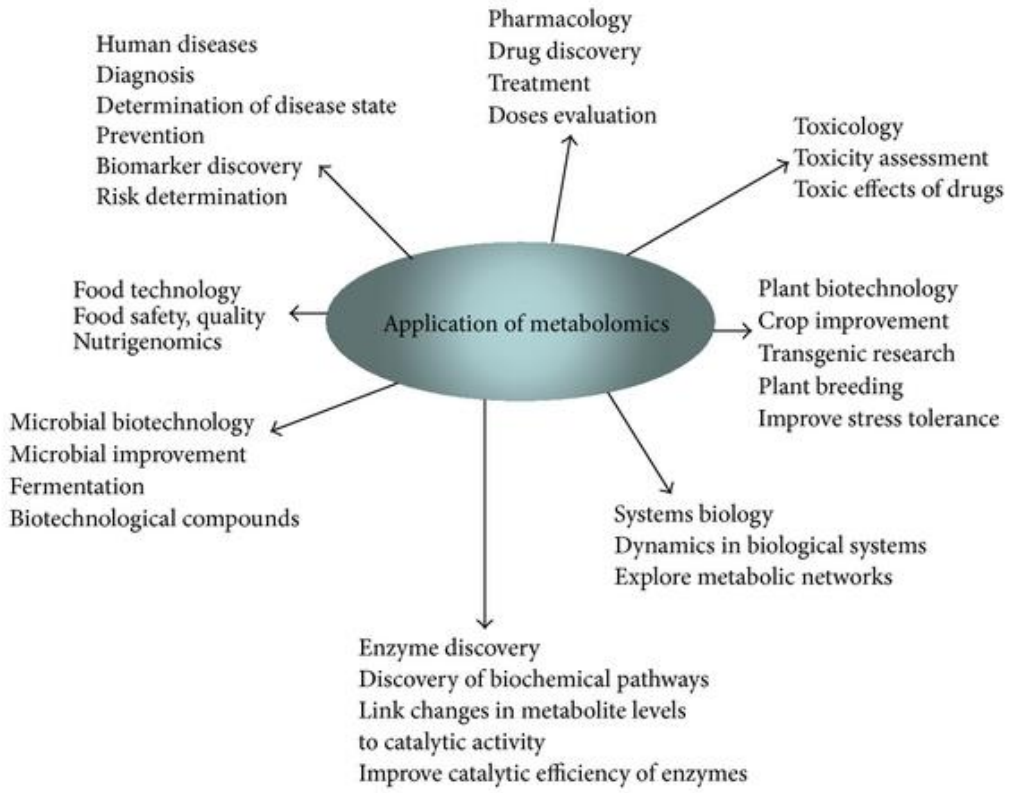
Selected medicinal plant for the study: *T. cordifolia*

Scientific studies demonstrated antiinflammatory activity of various parts like stem, leaves and roots of *Tinospora cordifolia* using animal models, however no studies are reported with human cell culture models in relevance to its therapeutic effects towards atherosclerosis, a cardiovascular disease.

Metabolite profiling of medicinal plant extracts using LC-MS

Metabolomics is a systematic study which deals with the identification and quantification of chemical process known as metabolites using highly sophisticated analytical techniques. Metabolomics mainly aims in the qualitative and quantitative analysis of both targeted and un targeted molecules in the biological system (Patti et al., 2012). This approach has been

employed in various field of scientific studies, e.g. biomarker discovery, food technology, microbial technology, toxicology, systems biology and etc (**Fig. 2.1).** Metabolomics acts as bridging gap between genotype and phenotype. Plant metabolism contains numerous complex compounds with varying properties like solubility, size, polarity, volatility, quantity and stability. Metabolomics employs powerful sensitive techniques like GC-MS, ESI-LC-MS and NMR to identify and quantify both known and unknown metabolites in the sample. Application of these powerful sensitive metabolic techniques in the field of phytomedicine provides information of various metabolites having pharmacological significance (Fiehn et al., 2000; Ulrich-Merzenich et al., 2007; Yang et al., 2008).



(Gomez-Casati et al., 2013)

Fig. 2.1. Applications of metabolomics in various fields of scientific studies

Liquid chromatography -mass spectrometry (LC-MS) is a highly powerful sensitive technique. LC -MS involves the physical separation of metabolites in liquid mobile phase and MS involves analysis of metabolite mass using mass spectrometry. In the present study LC – MS is employed to detect metabolites extracted into hydroalcoholic and water extracts of *T. cordifolia* dry leaves. Mass spectrometry works on the principal of ionising the chemical compounds and separates the charged ions according to mass to charge ratio. The ions are detected and processed to mass analyser to detect the mass of the ion. LC-MS is most widely used analytical tool in the field of metabolomics, which is mainly projected for the separation, general detection and identification of particular metabolite of specific mass in the mixture i.e. natural products from herbal extracts. Electron spray ionisation (ESI)-LC/ MS is an ionisation analytical tool which transfers heat labile molecules from condensed phase into the gaseous phase and produces ions without fragmentation pattern and latter analyse the ion mass using mass spectrometry. In recent past, ESI-LC-MS has become a regular tool in identifying the secondary metabolites in the plant extracts. Mobile phase selection plays a vital role in the separation and detection of molecules in LC-MS. Generally solvents like methanol, ethanol, water, acetonitrile, ammonium formate and formic acid are used as mobile phases in LC-MS for the separation and detection of compounds. Some of the metabolites detected by LC –MS from the dry leaf extracts of *T. cordifolia,* which are commercially available, namely, 20 – hydroxyecdysone, syringin along with TC's root alkaloid berberine were investigated in the present study for their antioxidant and antiinflammatory properties.

Human monocytic (THP-1) cells

THP-1 cells are most widely used cell line to investigate the role and regulation of monocytes in atherosclerosis for the following reasons

1. Key innate immune cells isolated from the blood of acute leukaemia patients and mimics the functions of primary monocytes and macrophages of healthy blood donors.

2. Monocyte cells interaction with vascular cells like endothelial and smooth muscle cells plays a key role in initiating atherosclerosis.
3. Can be easily differentiated to macrophages and foam cells (**Fig. 2.2**).
4. Reported to secrete various inflammatory mediators similar to peripheral blood monocytes when activated with LPS and foam cell formation on exposure to Ox-LDL. Hence THP-1 cells serves as a good cellular model to investigate potent antiinflammatory properties of plant extracts and their active metabolites.

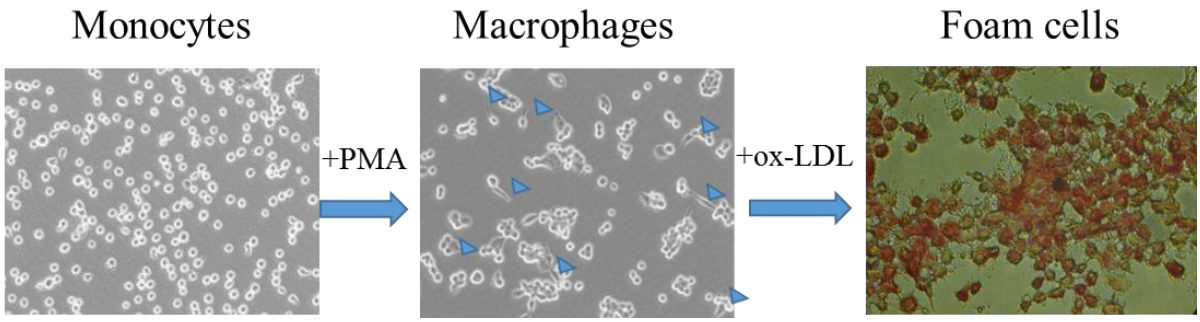


Fig. 2.2. Images of 10X magnification of human monocytic THP-1 cells captured under inverted microscope.

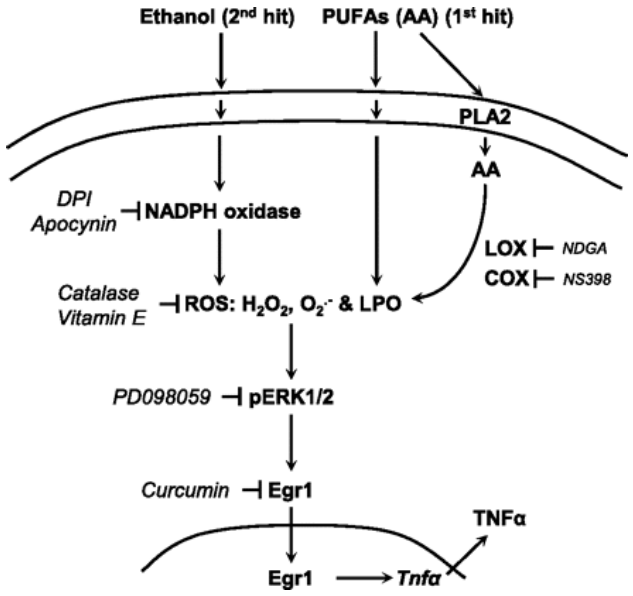
In the current work, we have utilised THP-1 cells to prove the antioxidant and antiinflammatory studies of *Tinospora cordifolia* dry leaf extracts and its active metabolites.

Oxidative stress and biological markers

Oxidative stress, which is generally caused due to imbalance between ROS production and scavenging systems during metabolisms in cells. Oxidative stress takes part in pathogenesis by damaging macromolecules of the cell leading to cell death (Uy et al., 2011; Vladykovskaya et al., 2012 and C.-H. Yu et al., 2013).

Arachidonic acid is a polyunsaturated fatty-acid which was shown inducing inflammation *via* oxidative burst in previous scientific studies **(Fig. 2.2)**. Arachidonic acid metabolism stimulates

ROS production which in turn activates downstream signaling molecules and secretes proinflammatory cytokines leading to inflammation (Cubero and Nieto 2012). Arachidonic acid metabolism causes inflammation by secreting proinflammatory mediator (TNF-α) *via* oxidative burst releasing ROS (Shin and Kim 2009). Oxidative degradation of proteins due to stress generated by ROS produces adducts like nitrotyrosine and S-glutathionylation. Scientific studies have shown the levels of these protein adducts correlates in CAD patients.



Cubero and Nieto 2012

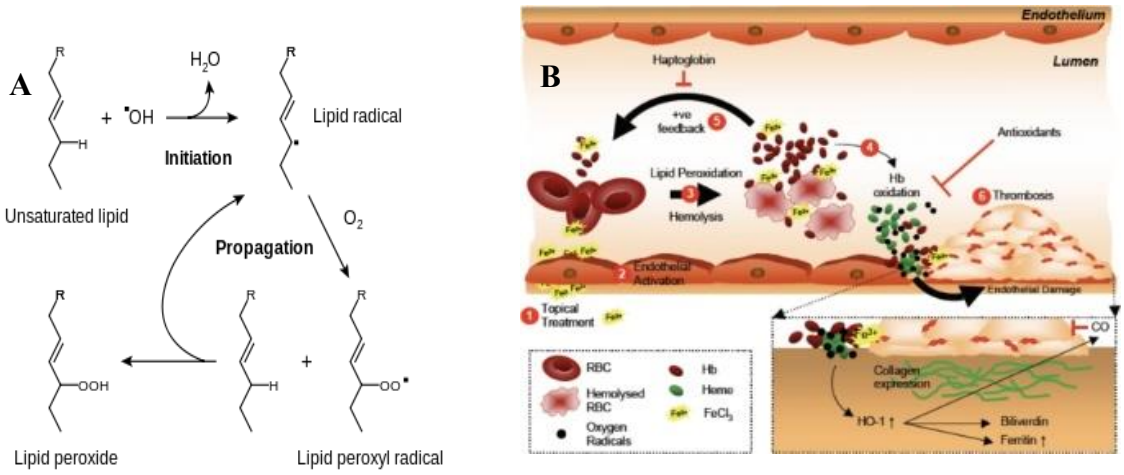
Fig. 2.3. Extracellular arachidonic acid induced oxidative stress mediated inflammation. Arachidonic acid stimulates the secretion of proinflammatory cytokine TNF-α through ROS generation. ROS in turn activates downstream signalling molecules pERK and Egr1 transcription factor. Egr1 binds to the TNF- α gene promoter, undergoes transcription and translation leading to inflammation.

Isoprostanes are prostaglandin like compounds formed due to arachidonic acid oxidation present in the cell membranes. Isoprostanes are mutagenic in function and forms nucleic acid adducts with DNA. Isoprostane related oxidative stress is involved in the pathogensis of atherosclerosis and elevated levels of 8-isoprostane was shown in

atherosclerotic patients (Basarici et al., 2007). Arachidonic acid is also involved in the synthesis of eicosanoids through COX-2 enzyme, which are considered to be important contributors in the pathogenesis of atherosclerosis. Eicosanoids activate and recruit monocytes to the site of inflammation and alters the gene expression pattern of many proinflammatory markers in vascular endothelial and smooth muscle cells, promoting inflammatory signalling. In view of atherosclerosis, it is very much essential to protect monocytes from being exposed to COX-2 products i.e. eicosanoids. Arachidonic acid also produces oxidative stress non-enzymatically through isoprostanes production and enzymatically by inhibiting antioxidant enzymes (CAT, GPx and SOD). Therefore, we used arachidonic acid induced THP-1 cells as a model system to investigate antioxidant activity of *T. cordifolia* leaf extracts and their metabolites in pure form.

Lipidperoxidation: Oxidative modification of membrane lipids is known as lipidperoxidation. It affects polyunsaturated fatty acids present in the membranes due to the presence of double bonds in between the methylene groups. It is free radical chain mechanised reaction which is initiated by radicals produced during oxidative stress **(Fig. 2.4 A)**. Unstable free radicals abstracts the electrons from the lipids present in the membranes and forms lipid peroxides like malonaldehyde (MDA) and 4-hydroxyneonal (4-HNE) as end products. Lipid peroxides are involved in the initiation of vascular diseases **(Fig. 2.4 B)** especially cardiovascular diseases (Woollard et al., 2009). Malonaldehyde is formed due to oxidation of polyunsaturated fatty acids (PUF) in the membranes. MDA interacts with protein cellular components and forms protein adducts, mostly attacks lysine residues to form lys-lys cross links. MDA impairs Ox-LDL and macrophage interaction and there by promotes atherosclerosis. MDA was generally quantified by thiobarbituric acid assay (TBARS). Lipids present in the membranes reacts with

thiobarbituric acid and forms pink coloured MDA measured spectrophotometrically at 532nm (Ho et al., 2013).



Woollard et al., 2009

Fig. 2.4. Free radical mechanism of lipidperoxidation (A). Lipid peroxidation role in leading to vascular injury and thrombosis through endothelial dysfunction and platelet aggregation (B).

ROS scavenging enzymes

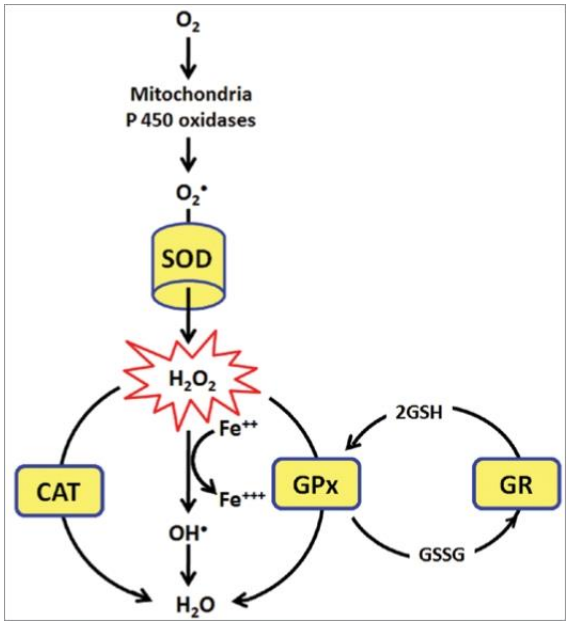
Enzymatic components like catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) comprise antioxidant defence mechanisms in the cell. Antioxidant enzymes gets operated under various stressful conditions in various compartments of the cell. Therefore we analysed the effect of *T. cordifolia* leaf extracts and their metabolites on ROS scavenging enzymes.

Catalase (CAT): It is a tetramer heme containing enzyme which catalyses the conversion of two molecules of H_2O_2 into H_2O and O_2 (Matés et al., 1999). It was abundantly present in peroxisomes and has high affinity towards H_2O_2. Catalase is the highest turnover enzyme which can convert 6 million molecules of H_2O_2 to H_2O and O_2. Under stress conditions H_2O_2 gets released continuously and in turn degraded efficiently by catalase in an energy dependent

manner. Scientific studies have shown the alterations in the catalase activity under stress are associated with physiology / pathophysiology conditions.

Superoxide dismutase (SOD): SOD is a metalloenzyme which catalyses the conversion of superoxide anion to hydrogen peroxide and molecular oxygen (Fernandez et al., 2009). Metabolism of oxygen in turn produces superoxide which causes cellular damaging effects along with H_2O_2 produced. Active sites of SOD are occupied by metals like copper, zinc, manganese and iron. SOD generally exists in three forms SOD-1, SOD-2 and SOD-3. Copper and zinc are occupied in the active sites of SOD-1 and 3 whereas SOD-2 contains manganese in the active site (Perry et al., 2010). SOD knock-out and expression in mice resulted in cardiomyopathy and neonatal lethality (Li et al., 1995). Similarly SOD expression in transgenic mice resulted in protection towards cardiac toxicity induced by Adriamycin (Chaiswing et al., 2005) and oxygen induced pulmonary injury (Gao et al., 2008). Similarly, elimination of SOD gene increased oxygen sensitivity in *Saccharomyces* (Strain et al., 1998).

Gluthione peroxidase (GPx): It is an endogenous selenocystein containing enzyme found in both cytosol and mitochondria, catalyses the decomposition of H_2O_2 and lipid hydro peroxides thereby provides protection from oxidative damage (Zhang et al., 2014). It converts lipid and organic hydro peroxides to their alcohols utilizing GSH (Pandey and Rizvi). Gpx also shares H_2O_2 as a substrate along with CAT but it is more effective in decomposing H_2O_2 only under low oxidative stress conditions. GPx exists in four isoenzyme forms GPx-1, GPx-2, GPx-3 and GPx-4 (Paukert et al., 2011). GPx -1 and GPx-2 detoxifies H_2O_2 and organic peroxides like cholesterol and fatty acid peroxides but not phospholipid hydroperoxides, GPx-3 occurs in plasma and epithelial cells of kidney has an extracellular function distinct from GPx-1. GPx-4 localised in peroxisomes exists as monomer and detoxifies phospholipid hydroperoxides. Impairment of GPx-1 was observed in myocardial infarction and atherosclerotic patients. GPx along with homocysteine serves as best markers for predicting CVD (Zhang et al., 2014).



(Matés et al., 1999; Fernandez et al., 2009; Zhang et al., 2014)

Fig. 2.5. Above diagram represents the mechanism of ROS scavenging enzymes such as CAT, GPx and SOD responsible for the decomposition of hydrogen peroxide thereby providing protection against oxidative damage. Both CAT and GPx decompose H_2O_2 whereas SOD decomposes superoxide anion. SOD converts superoxide anion to molecular oxygen and H_2O_2 and latter H_2O_2 was subsequently degraded by CAT and GPx to H_2O and O_2.

Inflammation

It was defined as first line of biological defence of host in response to harmful stimuli such as infectious bacteria, virus, fungi and injured tissues for healing process. Inflammation is a natural phenomenon developed in the body and was tightly regulated.

Acute inflammation develops immediately (rapid onset) and persists only few days to weeks which converts to chronic in latter stages. It leads to diseases like appendicitis, tonsillitis, bronchitis, sore throat and meningitis etc.

Chronic inflammation is a long term inflammation which lasts for longer periods of months to years. Chronic inflammation leads to diseases like cardiovascular diseases, diabetes, rheumatoid arthritis, tuberculosis, asthma, and peptic ulcers etc.

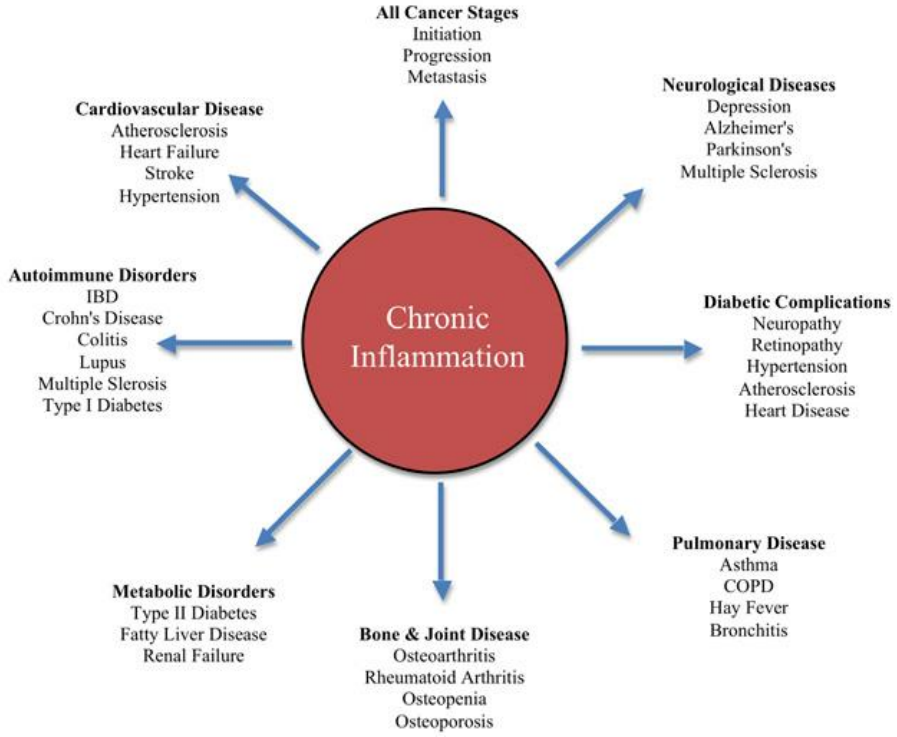


Chart ©2015 Avalon Yoga International, Inc.

Fig. 2.6. Represents various chronic inflammatory diseases e.g. cardiovascular, cancer, neurological, diabetes, pulmonary, autoimmune, metabolic, bone and joint etc leading to death.

Mediators of Inflammation

Lipopolysachcaride (LPS): Gram negative bacterial endotoxin which acts as ligand to TLR-4 and activates NF-κB inflammatory signalling cascade.

Phorbol myristate acetate (PMA): PMA is a specific activator of Protein Kinase-C (PKC) and NF-κB which differentiates monocytes to macrophages leading to secretion of proinflammatory cytokines.

Oxidised lowdensity lipoproteins (ox-LDL): ox-LDL converts differentiated macrophages to foam cells which plays a pivotal role in the progression of atherosclerosis.

Biomarkers of inflammation

Several of various proinflammatory markers used for the diagnosis of various inflammatory diseases are described as below and soe of which are assayedto define the activation/ inflammatory state of THP-1 cells.

Tumor necrosis factor alpha (TNF-α):

It is an intracellular pleotropic (multifunction) cytokine, secreted by various activated immune cells during injury, infection or stress which activates others inflammatory mediators during the process leading to chronic inflammation (Thomas, 2001; Mula and Shashidharamurthy, 2013; Bruunsgaard et al., 2000; Wang et al., 2012; Parameswaran and Patial 2010). On positive note, it can enhance the bactericidal activity of neutrophils (Rainard et al., 2000; Kenny et al., 1993) but excess secretion stimulates chronic inflammation leading to CVD *via* blood clotting (Ferrari, 1999; Berg and Scherer, 2005; Vaddi et al., 1994; Stangl et al., 2001; Popa et al., 2007; Feldman et al., 2000) and cancer through tumour formation (Wang and Lin 2008). TNF-α, being one of the earliest proinflammatory secretions from the activated monocytes and macrophages is involved in the progression of many inflammatory diseases. Therefore TNF-α has become a major target for various pharma companies to generate inhibitors specifically for the treatment of diseases. Cells on activation with LPS induces the transcription and translation of TNF- α gene and produces 26KD protein which activates endothelium dysfunction. Therefore in our study we tested the inhibitory effect of *Tinospora cordifolia* dry leaf extracts and its active metabolites in LPS activated THP-1 cells.

In our study, we also tested the inhibitory activity of *T. cordifolia* extracts and its compounds against chemokine MCP-1 and Interleukins IL-6 and IL-8.

Interleukins

Interleukins (IL) helps in the adhesion and recruitment of neutrophils to site of inflammation. IL-1β, IL-8 and IL-6 are the most important cytokines which are generally studied. IL-1β helps in the movement of neutrophils out of the vascular blood vessels into the damaged tissue whereas IL-6 and IL-8 aids in the resolution of acute inflammation by recruiting immune cells to the site of injury (Arango Duque and Descoteaux 2014; von der Thüsen et al., 2003; Ait-Oufella et al., 2011; Merhi-Soussi et al., 2005). IL-6 and IL-8 have significant roles in inflammation, they causes rolling of monocytes to firmly adhere to endothelial monolayer expressing VCAM-1, ICAM-1 and E-selectin.

Monocyte chemotactic protein (MCP-1)

MCP-1 is a chemokine released by monocytes and endothelial cells upon activation by adverse factors promotes the transmigration and recruitment of monocytes and macrophages to the site of inflammation. Proinflammatory chemokine like MCP-1 is found abundantly in atherogenic lesions (Tedgui and Mallat 2006). MCP-1 aids in the chemotaxis, recruitment, rolling and adhesion of monocytes during the pathogenesis (Gerszten et al., 1999).

Transcription factor - nuclear factor kappa-B (NF-κB)

NF-κB is a redox sensitive transcription factor, which gets activated by various external factors (oxidative stress, infection, stimulus by TNF-α) and translocate into the nucleus (Piette et al., 1997; Christman et al., 2006) where binds to the promoters of various proinflammtory genes i.e. approximately 400 including various cytokines, chemokines and enzymes like cyclooxygenase (COX-2), lipoxygenase (LOX-2) involved in the synthesis of leukotrienes and prostaglandins. These activated molecules show cumulative effect leading to inflammation

(Kempe et al., 2005; Hoesel and Schmid 2013; Yuan et al., 2013 and Ben-Neriah and Karin 2011).

Foam cells

Foam cells are lipid engulfed (cholesterol) monocyte differentiated macrophages found in the in the lumen of artery. Differentiated macrophages migrate to the fatty material deposited site on the wall of the artery. Macrophages wraps around the fatty material and makes an attempt to destroy bad lipids, but gets filled with fats and converts to foam cells. Foam cells which take part in the pathogenesis of atherosclerosis can be reduced by increasing the uptake of HDL and avoiding LDL. Activation of PMA differentiated THP-1 cells i.e. macrophages with oxidised LDL, intake lipid molecules through scavenging receptors SR-B1 and coverts to foam cells. Identifying and developing inhibitors towards foam cell formation would become a novel therapy for the treatment of atherosclerosis. Therefore in our study we tested the attenuation effects of *TC* metabolite on Ox-LDL induced foam cells using oil red staining method.

Chapter - 3

Materials and Methods

Chapter-3

Materials and Methods

Collection of *T. cordifolia* leaves

Fresh and healthy leaves of *Tinospora cordifolia* (**Fig. 3.1.**) were collected in the morning time of sunny days from the surroundings of University of Hyderabad and authenticated by Dr. K. Venkata Ratnam, Post-doctoral fellow in UoH (voucher no # 46806). Collected leaves were thoroughly washed with double distilled water and subsequently weighted for 1g, followed by air drying for 1 week. The dried leaf material was ground to fine powder using mortar and pestle and later used for extraction.

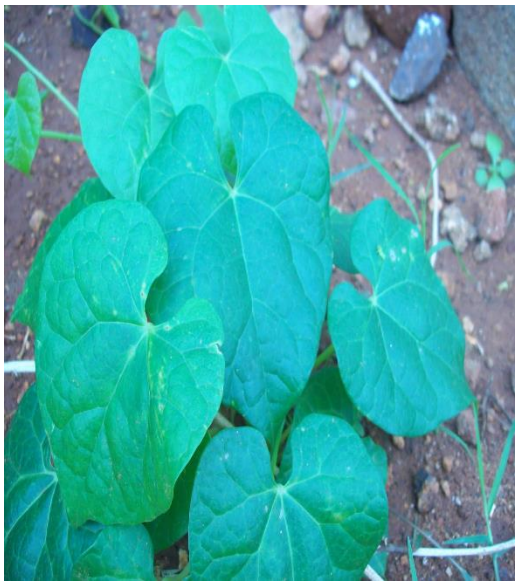


Fig. 3.1. *T. cordifolia* leaves

Chemicals

Chemicals used in this study were Xylenol orange, Lipopolysaccharide (LPS), 2-thiobarbituric acid (TBA), trypan blue, PMA, Isopropanol, Paraformaldehyde, DPPH, Oil Red stain and DEPC treated water were purchased from Sigma–Aldrich (Germany). potassium di-hydrogen

phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), ferrous ammonium sulphate (SRL), hydrogen peroxide, ammonium molybedate, sodium hydrogen phosphate, 2-Thiobarbituric acid (TBA), ferric chloride, trichloroaceticacid (TCA), ascorbic acid (Qualigens), Gallic acid (SRL), Folin-ciocalteu reagent (Merck), sodium nitroprusside (SNP), sulphanilamide, N-1-naphthylethylenediamine dihydrochloride (NED, Hi-media), Quercetin, Aluminium chloride etc. ELISA kits were purchased from BD bioscience (USA), iScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA), Power SYBR GREEN real time master mix was purchased from Applied Biosystem (USA). RPMI 1640 medium, L-glutamine, penstrep, fetal bovine serum (FBS), Alexa flour 594 goat anti-rabbit IgG, antifade 4', 6-diamidino-2-phenylindole (DAPI) and Trizol reagent were obtained from Invitrogen. NF-κB p65 subunit polyclonal rabbit antibody, I-κB monoclonal rabbit antibody, P-IκB monoclonal mouse antibody, anti-rabbit HRP, NE-PER buffer were obtained from Pierce (Thermo scientific, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrezolium bromide (MTT) was obtained from Merck. $NaHCO_3$ and $FeCl_3$ (merck), Triton X-100 (Hi-media), ox-LDL (Kalen). All other reagents used were of analytical grade.

Methods

Preparation of dry leaf extracts of *T. cordifolia*

Dry leaf extracts TC were prepared according to the protocol described elsewhere (Choudhury et al., 2014; Kokkiripati et al., 2011). Hydro-alcoholic extract (TCAE) and water extract (TCWE) of dry leaves were prepared by dissolving 180mg of dry leaf powder in 20 mL of 80 % ethanol. Hydro-alcoholic (TCAE) extract was further subjected to continuous stirring at 40 °C for 5–6 h until it gets concentrated to $1/5^{th}$ of its original volume whereas Water extract (TCWE) was prepared by dissolving the same amount of powder in 4 mL of water incubated for overnight at room temperature.

The extracts were than centrifuged at 10,000 rpm for 10 min and subjected to various studies. TCAE or TCWE obtained from 1 g of leaf material were completely dried under vacuum. Dry weight of the extract was determined to calculate the yield.

Phytochemical estimations in *T. cordifolia* leaf extracts

Estimation of total phenolic content

Total polyphenolic content in *T. cordifolia* leaf extracts was estimated spectrophotometrically using FC (Folins-ciocalteu) reagent as suggested in Folin-ciocalteu method (Singleton et al., 1999). FC reagent is a mixture of phospotungestic and phospomolybdic acids which undergoes reduction and forms blue coloured complex in alkaline solution.

Preperation of reagents

Gallic- acid stock: 500 mg of gallic acid was solubilised in 1 mL of 80 % ethanol and made the volume to 10 mL with double distilled water and stored the solution at 4 °C.

Sodium carbonate solution: 200 g of sodium carbonate (Na_2CO_3) was dissolved in distilled water (800 mL) and heated at room temperature on magnetic stirrer for complete solubilisation. After dissolving, the solution was left undisturbed at room temperature for 24 h, filtered the solution using whatman filter paper and made up the volume to 1L. Solution was stored at room temperature till further use.

Gallic acid standards: 0, 50, 100, 150, 250 and 500 µg/mL of working standards of gallic acid were prepared from the stock of 500 mg/L. 20 µL gallic aicd standard solutions and different concentrations of plant extracts were diluted to 1.58 mL with distilled water and 100 µL of FC reagent was added, mixed well and incubated for a period of maximum 8 min, then 300 µL of the sodium carbonate solution was added to stop the reaction, and again incubated at 20 °C for 2 h and determined the absorbance of blue coloured mixture at λ 765 nm against the blank and

plotted the absorbance vs. concentration. The amount of total phenols were calculated as GAE mg/g dw from the calibration curve of gallic acid standard solution.

Determination of total flavonoid content

Total flavonoid content in TC extracts was determined by using Aluminium chloride ($AlCl_3$) method (Sultana et al., 2012). Different aliquots of plant extracts were placed in a 10 mL volumetric flask glass tubes and distilled water of 5 mL was added followed by 300 µL of 5% $NaNO_2$ and incubated for 5 min. After incubation, 600 µL of $AlCl_3$ (10 %) and 2 mL of 1 M NaOH was added to the reaction mixture and made up the remaining volume with distilled water. The solution was mixed and measured the absorbance at λ 510 nm using Shimadzu spectrophotometer (Kyoto,Japan). Quercitin was used as a standard reference and different concentrations ranging from 0.1- 1.2 µg/mL were used to construct a a standard plot and total flavonoids present in plant extracts were expressed as quercetin equivalents per g dw. All the reactions were run at least in triplicates.

Metabolite profiling by LC-QTOFMS:

The compounds of both TCAE and TCWE were separated using 6520 Accurate Q-TOF (Agilent Santa Clara, CA) mass spectrometer coupled to HPLC according to (Ahmed et al., 2006). The samples were analysed at 30 °C on a reverse phase column ((Zorbax Eclipse XDB-C_{18}, 4.6 X 50 mm, 1.8 µ)). UV–DAD detection was performed at 215 nm. The analysis was carried out using a gradient mobile phase of water–acetonitrile (initially at 90:10, changing to 0:100 in 40 min, followed by isocratic elution (0:100) for 5 min and then changed 90:10 in 10 min and finally, it was an isocratic elution at 90:10 for 5 min). The total analysis time was 60 min. Mobile phase was delivered at a flow rate of 1mL/min. 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.

Quantitative determination of 20-HOD in TCAE by LC-MS

Construction of 20-Hydroxyecdysone (20-HOD) standard calibration curve

The marker compound 20-Hydroxyecdysone (20-HOD) in TCAE was quantitated by external standard method using LC-MS. 5mg/mL stock of standard 20-HOD was prepared in HPLC grade ethanol and further diluted in ethanol to prepare working stocks ranging from 0.1 to 0.5 ng/µL for the construction of calibration curve. Sample volume of 2µL of each working stock was injected and constructed the curve based on the peak areas.

Quantification of 20-HOD

Peak areas of injected 2 and 4 µL of TCAE was extrapolated using the standard curve for calculating the concentration of 20-HOD in cells treated 0.4 and 0.8 mg dwt/mL extract dry weight concentrations.

Pharmacological assays

Determination of total antioxidant capacity

Antioxidant capacity in TC extracts was measured by phosphomolybdenum method (Govindarajan et al., 2003). The phosphomolybdenum method is based on the reduction of Mo (VI) to green coloured Mo (V) by the antioxidant with a maximal absorption at 695 nm. Increase in the absorbance indicated the increase in total antioxidant capacity of the test sample. Briefly, various aliquots of standard ascorbic acid (2.5-25 µg) or plant extracts were added to 1 mL of reagent solution (0.6 M sulphuric acid + 4 mM ammonium molybdate and 28 mM sodium phosphate) and incubated at 95 °C for 90 min. The absorbance of the solution was measured at λ 695 nm against blank after cooling to room temperature. The amount of total antioxidant capacity was calculated as ASE mg/g dw from the calibration curve of ascorbic acid standard solution.

Free radical scavenging studies

DPPH anti-radical scavenging activity

DPPH radical scavenging activity was determined according to Dasgupta and De 2007. Different concentrations of plant samples were mixed with 1ml of 0.004 % (w/v) methanolic DPPH solution. The mixture was subjected to vigorous shaking and then kept in the dark for 30 min at room temperature. The optical density (O.D.) of the reaction mixture was measured at λ 517 nm using spectrophotometer (Shimadzu UV-VIS). DPPH solution was freshly prepared prior to use. Gallic acid and ascorbic acid were tested and used as reference standards. The inhibition percentage (% I) of the DPPH radical was calculated according to the following formula:

[A λ517 nm of control– A λ517 nm of sample / A λ517 nm of control] x 100

Where $A_{control}$ was the absorbance without plant extract/ test compound and A_{sample} was the absorbance with plant extract/ test compound. The extract concentration required to obtain (IC_{50}) was obtained by interpolation of concentration−inhibition curves.

Hydrogen peroxide scavenging activity

Xylenol orange reagent: 100 mM sorbitol, 25 mM H_2SO4, 250 mM ammonium iron (II) sulfate, and 125 mM xylenol orange were prepared. Thus prepared xylenol orange solution was stored in amber bottle at 4 °C. The scavenging of hydrogen peroxide by plant extracts was measured according to Pedraza-Chaverrí et al., (2006). Different concentrations of plant samples were treated with 10 µl of 40 mM hydrogen peroxide in dark for 5 min and 400 µL of xylenol orange was added and finally made up the samples to 1mL with water. After 30 min of incubation, the decrease in absorbance of the resulting solution was measured at 584 nm with a spectrophotometer (Shimadzu UV-VIS). Ascorbic acid and gallic acid were tested and used as reference standards. The inhibition percentage of the hydrogen peroxide$^{\bullet}$ radical was calculated

according to the following formula: [A λ517 nm of control– A λ517 nm of sample / A λ517 nm of control] x 100

A_0 is the absorbance of the control solution and $A_1$is the absorbance after addition of test samples. The extract concentration required to obtain (IC_{50}) was obtained by interpolation of concentration−inhibition curves.

Ferric chloride induced lipid peroxidation assay using rat liver and heart tissue homogenates

Lipid peroxidation refers to oxidative damage of lipids by various oxidizing agents. Thiobarbituric acid reactive species (TBARS) assay is the most commonly used test to study lipid peroxidation in which the TBA reagents reacts with lipid peroxides and produces pink coloured fluorescent end product. Unsaturated fatty-acids present in the membranes generally undergoes lipid-peroxidation.

Liver tissue homogenate

Male Wistar rats were used to study lipid peroxidation, were fed with a standard chow and water in wire-bottom cages. Animals were subjected to cervical dislocation and organs were immediately collected and washed with 0.9 % saline to completely remove the blood. Liver tissue was suspended in 0.15 M KCL and homogenised using teflon homogeniser and collected a clear supernatant by centrifugation at 800 g for 15 min.

This assay was carried out according to method described by (Harlalka et al., 2007) by measuring the pink colour of TBA-MDA complex formed at the end of the reaction. The rat liver homogenate was pretreated with different concentrations of TCAE/TCWE and latter induced with 100 µl of 1 mM ferric chloride, incubated for 30 min at 37 °C. After incubation the reaction was stopped by the addition of 2 mL of ice cold 250 mM HCl containing trichloroacetic acid (15 % TCA), 0.38 % TBA to the reaction mixture followed by heating at

80 °C for 60 min. The samples were cooled and centrifuged at 5000 g for 15 min, absorbance of samples was measured at λ 532 nm. Inhibitory concentrations of plant extracts required to produce 50 % inhibition of lipid peroxidation, i.e. IC_{50} value, were calculated. Gallic acid was used as a reference compound. The protective effects of different concentrations of TC extracts against lipid peroxidation were calculated as follows: control – test sample/control × 100.

Heart tissue homogenate

Rat heart tissue was washed with 0.9 % saline and immediately frozen in liquid nitrogen after collecting through cervical dislocation. Frozen tissue was suspended in 0.15 M KCl, flushed with nitrogen gas and homogenized in the ratio 10 % (w/v). Homogenized sample was centrifuged at 1500 g for 15 min to obtain cell free clear supernatant and performed the assay. Anti-heart lipid peroxidation of TCWE/TCWE was measured by using modified TBARS method as described by Meera et al., (2009). An aliquot of 50 µL of heart tissue homogenate was incubated with various concentrations of TCAE/TCWE. Lipid peroxidation was induced by adding 10 µM ferric chloride to tissue homogenate and incubated at 37 °C for 1 h. After induction, reaction was terminated by adding 500 µL of 70 % ethanol. TBA (1 %) of 1 mL was added to the reaction mixture and boiled in water bath for 20 minutes. The tubes were centrifuged and collected the supernatants. To the supernatants, 50 µL of acetone was added and optical density of pink coloured TBARS formed was measured at λ 532 nm using a spectrophotometer. An assay medium containing tissue homogenate, ferric chloride without TCAE and TCWE extracts was considered to be 100 % oxidation. Ascorbic acid was used as a reference compound.

Cell Culture

Human monocytic (THP-1) cell lines were obtained from NCCS (National Centre for Cell Science) Pune, India. Cells were cultured in RPMI-1640 growth medium containing 10% (v/v) fetal bovine serum supplemented with PenStrep (1 %) and cultures were maintained in

incubator at 37 °C containing 5 % CO_2. Latter, cells were subjected to oxidative stress by inducing with AA (100 µM) for 10 min and acute inflammation by LPS (0.5 µg /mL) for 3 h at 37 °C (Kokkiripati et al., 2013). For differentiation studies cells were treated with 25 ng/ mL of PMA for 48 h at 37 °C as reported by Choudhury et al., (2014)and images were captured under inverted microscope.s

Cell viability

MTT assay:

The effect of dosage concentrations of plant extracts and pure compounds on THP-1 cells were tested by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide (MTT) assay as described by Kokkiripati et al., (2011). THP-1 cells were plated at a density of 0.5×10^6 cells / mL in 24 well plates and treated with varying concentrations of plant extracts/ test compounds and incubated at 37 °C overnight. After overnight incubation cells were harvested and the cell pellet was washed three times with RPMI media. 20µl of MTT (5mg/ml) dissolved in media was added and incubated for 4 h. Cells were pelleted by low speed centrifugation and incubated for 15 min with 100 µL DMSO to dissolve the insoluble purple coloured formazan crystals. The absorbance of MTT formazan was determined at λ 570 nm with reference λ 690 nm using a multimode plate reader (Tecan, Germany). Plain media with tested extracts or pure compounds were taken as blanks. The percentage of viable cells from each well after incubation with plant material extracts was obtained by using the following equation:

% viability = (O.D. of treated cells / O.D. of untreated cells) X 100.

Cell viability was further confirmed by Trypan blue exclusion method by measuring the number of viable cells under inverted microscope (Leica DMR) using hemocytometer. Trypan blue method is based on the integrity of cell membranes which takes up the dye in case of dead cells as a result cytoplasm appears blue in colour whereas live cells shows clear cytoplasm

(Strober, 2001). 20 µL of cell suspension was mixed with 10 µL trypan blue (0.004 %) and observed the cells under microscope.

Cell based assays

PMA (phorbol-12-myristate-13-acetate) stimulated monocyte derived macrophage differentiation

THP-1 cells grown in serum free media (2 % v/v) were pre-treated with plant extracts/test compounds and stimulated with PMA (25 ng/mL) for 48 h at 37 °C in 5 % CO_2 as described by Choudhury et al., 2014. Phenotype of THP-1 cells were observed under inverted microscope. THP-1 cells without PMA were used as control cells (undifferentiated).

Foam cell formation

THP-1 cells differentiated to macrophages with PMA (25 ng/mL), pre-treated with tested compound overnight and induced with 50 µg/mL ox-LDL for 24 h. The cells were fixed using 4 % Paraformaldehyde for 20 min and washed gently with TBS twice for 5min on a rocker. Rinsed the cells with iso-propanol (60 %) and stained with of Oil Red working stock for 30 min in dark at room temperature. After 30 min of incubation, cells were washed thrice with TBS at 15 min interval to remove unbound stain and observed lipid staining under inverted microscope (Dong et al., 2014). Oil Red stain was eluted with isopropanol and measured the absorbance at 492 nm in 96 well plate using microplate reader.

Measurement of intracellular ROS

Intracellular ROS in cells were generally measured by using a fluorescent probe 2', 7'-dichlorofluorescein-diacetate (H_2DCF-DA) dye **(Fig. 3.2)**. H_2DCF-DA generally exists either in acetate or ester forms which undergoes cleavage inside the cell by the cellular esterases present in the cytosol and forms non fluorescent intermediate dihydrochlorofluorescein

(H_2DCF) and undergoes oxidation in the presence of ROS and forms highly fluorescent 2',7'-dichlorofluorescein (DCF) (Bass et al., 1983).

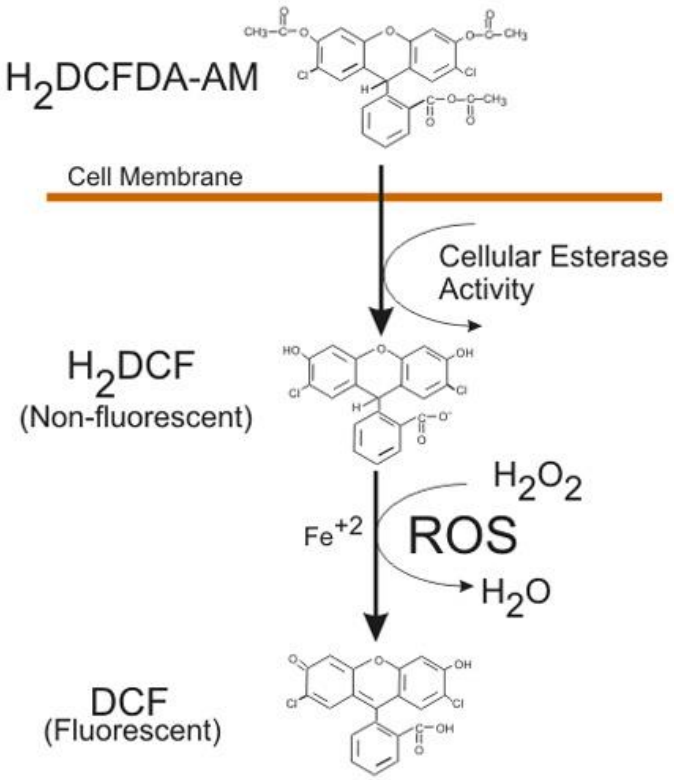


Fig. 3.2. Conversion of H_2DCF-DA to fluorescent DCF product by the cellular esterase's in presence of ROS.

Cells seeded at a density of 5×10^5 were pre-treated with tested concentrations of plant extracts or test compounds for overnight and then induced with 100 µM arachidonic acid (Kweon et al., 2001) for 10 min on the next day. 5 µM H_2DCF-DA was loaded to the cells for 15 min following induction and cells were washed with serum free medium (RPMI) twice to remove the plant extracts, compounds and unbound dye. DCF images were obtained using confocal microscope (Lieca DMR). In addition to confocal studies, ROS levels were further quantified by spectrofluorometric analysis using excitation and emission wavelengths of λ 488 nm and λ 525 nm respectively. Cells treated with plant extracts and test compounds alone were used as respective blanks.

ROS scavenging assay in THP-1 cells – Catalase

ROS scavenging potential of TC extracts and tested metabolites were done (Kokkiripati et al., 2011) in THP-1 cell lysates. THP-1 pretreated with plant extracts or tested compounds and induced with AA (100 µM) for 10 min. Cells latter washed with growth media plant extracts or tested compounds. After washings, cells were sonicated for 3 min on ice and centrifuged at 10000 rpm for 3 min to collect the cell lysates. Cell lysates were subjected to catalase enzyme assay.

Catalase enzyme assay

Catalase is antioxidant enzyme which catalyses the decomposition of hydrogen peroxide (H_2O_2). Catalase is involved in detoxification of H_2O_2 to molecular oxygen and two molecules of water. Intracellular catalase activity was measure by the degradation rate of H_2O_2 in the form of decrease of absorbance at λ 240 nm. 7.5 mM of H_2O_2 was added extracellularly as a substrate to 200 µL of AA induced cell lysate and measured the consumption of H_2O_2 or decrease in optical density (O.D) at λ 240 nm for 5 min at an interval of every 10 sec at 37 °C using multi-mode plate reader. CAT activity was expressed as fold change which reduces 1 µM H_2O_2 / min at 25 °C.

Quantification of secreted pro-inflammatory markers by ELISA Reagents

1. Coating buffer- 0.1 M sodium carbonate, p^H 9.5.

2. Assay diluent - PBS with 10 % FBS. p^H- 7.0.

3. Wash buffer- PBS with 0.05 % Tween-20. Freshly prepared and used within 3days of preparation, stored at 2-8 ^OC.

4. Substrate solution – Hydrogen peroxide and TMB (Tetramethylbenzidine)

5. Stop solution – 1M phosphoric acid (H_3PO_4).

Principle / Methodology

BD ELISA kits employed for the detection of cytokines in cell culture supernatants were based on sandwich principle which contains recombinant human standards, monoclonal coating antibodies/capture antibodies, biotinylated detection antibodies, TMB substrate (3,3',5,5'-tetramethylbenzidine) and streptavidin-HRP conjugate. Cytokines secreted after LPS stimulation possess two antigenic epitopes capable of binding to coating antibody in the first step followed by blocking with assay diluent and washings with PBS buffer. Enzyme conjugated detection antibody was added to each well which reacts with antigen-antibody complex resulting in sandwich Ab-Ag-Ab reaction. TMB substrate was added to each well after PBS washings which produced blue colour. Addition of phosphoric acid changed the intensity of blue colour to yellow colour and measured at λ 450 nm with wavelength and reference at λ 570 nm within 30 min. Intensity of yellow colour generated is directly proportional to the amount of cytokine secreted in the culture supernatants.

Release of TNF-α, IL-6, IL-8 and MCP-1 from THP-1cells were measured by BD OptEIA ™ Set Human ELISA kits (BD Biosciences). THP-1cells at a density of 5×10^5 cells/ml were incubated with and without different concentrations of plant extracts or test compounds overnight and induced with LPS (0.5µg/mL) on the next day for 3 h. After induction, cells were spun and collected the supernatants for the quantification of the above marker levels by ELISA kits (BD-Biosciences). The colour generated was determined by measuring the absorbance at λ 450 nm using micro-plate reader with the wavelength correction set at λ 570 nm. Secretory levels in culture supernatants were determined by using a standard curve which was constructed using serial dilutions of respective standard markers (TNF-α, MCP-1, IL-6, IL-8) provided with the kit.

Transcript profiling of antioxidant enzymes and inflammatory markers

RNA isolation from THP-1 cells

THP-1 cells (5×10^5) were seeded in 25 cm^2 tissue culture flasks and incubated with and without plant extracts or test compounds in presence or absence of inducer compounds, namely AA (100 μm), LPS (0.5 μg/mL) or PMA (25 ng/mL) for specific time period of 10min for AA, 3h for LPS and 48h in case of PMA treatments. After discarding the growth medium, total RNA was isolated by adding 750 μL of Trizol reagent to 250 μL of cell suspension and incubated at room temperature by mixing properly for lysis. 200 μL of chloroform was added following lysis, capped tightly and shaken vigorously for 15 seconds and incubated at room temperature for 3 min. After incubation samples were spun at 12000 g for 15min at 4 °C to separate into 3 layers (upper aqueous, middle phenol and lower organic layer). Aqueous layer which contains RNA was transferred without disturbing the middle phenol layer and precipitated with 500 μL of 100 % isopropanol at room temperature for 10 min and centrifuged at 12000 g for 10min at 4 °C. RNA pelleted at the bottom of the tube was washed with 75% ethanol by vortexing gently and centrifuged at 7500 g for 5min at 4 °C to pellet down the pure RNA. RNA pellet was air-dried for 5-10 min at room temperature and suspended in 20-50 μL of DEPC treated water. Quality and quantity of RNA was checked at $A_{260/280}$ using nano-drop and stored in -80 ^{O}C for further use.

cDNA synthesis

cDNA was synthesized using cDNA Synthesis Kit (Iscript-Bio-Rad laboratories, USA) using total RNA isolated from the samples. 20 μL reaction mixture was prepared which consists of 4 μL 5x reaction mix, 1 μL reverse transcriptase enzyme, 1 μg of RNA template and nuclease

free water (made upto 20 µL). 30 cycles of cDNA amplification was carried out as per manufacturer's instructions with the following cycling conditions

Denaturation: 25 °C for 5 min

Annealing: 42 °C for 30 min

Extension: 85 °C for 5 min

After the synthesis, cDNA samples were stored at -20 °C for further gene expression studies.

Real-time PCR (q-RT-PCR)

It is a standard biochemical PCR, which amplifies specific DNA sequences from a small amount of starting material using fluorescent dyes. Syber green is the most commonly used fluorophore which intercalates and binds to specific DNA sequences and emits fluorescence, which is directly proportional to the amount of DNA amplified after each cycle. The specificity of the SYBR green fluorescence was tested by plotting fluorescence as a function of temperature

The gene expression levels of antioxidant enzymes and inflammatory markers were analysed by RT-qPCR using SYBR green. The 10 µl reaction mixture consisted of 2 µl of cDNA template and 8 µl of cocktail mix (5 µl of 1x SYBR green, 0.5 µl of 1x forward and reverse primers, 2 µl of water). The transcripts were normalized using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal control. Negative controls contain 8 µl of cocktail mix and 2 µl of water. The primer sequences of tested transcripts were given in **Table 3.1.**

Table 3.1. Primer sequences of proinflammatory cytokines for gene expression studies

S.no	Genes		Sequence
1.	**TNF-α**	**Forward**	5´-CCCAGGGACCTCTCTCTAATC-3´
		Reverse	5´-ATGGGCTACAGGCTTGTCACT-3´
2.	**MCP-1**	**Forward**	5´-GCCAAGGAGATCTGTGCTGAC-3´
		Reverse	5´-CATGGAATCCTGAACCCACTTC-3´
3.	**IL-6**	**Forward**	5´-TGGATTCAATGAGGAGACTTGC-3´
		Reverse	5´-CAGGAACTGGATCAGGACTT-3´
4.	**IL-8**	**Forward**	5´-GTGTAAACATGACTTCCAAGCTGG-3´
		Reverse	5´-GCACCTTCACACAGAGCTGC-3´
5.	**GAPDH**	**Forward**	5´-CACCAACTGCTTAGCACCCC-3´
		Reverse	5´-TGGTCATGAGTCCTTCCACG-3´

Immunofluorescence – Confocal microscopy

NF- κB translocation in THP-1 cells was further studied by immunofluorescence method using confocal microscope (Zhou et al., 2004). THP-1 cells were grown at a confluence of 50×10^4 cells /mL, pre-incubated with respective concentrations of TCAE, TCWE and pure metabolites induced with LPS (0.5µg/mL) for 3 h on the next day. After induction, the cells were fixed with para-formaldehyde (4 %) for 15 min and treated with 0.5 % Triton X-100 for 15 min for permeabilisation. After 1h blocking with BSA (Sigma- Aldrich, Germany), cells were incubated with rabbit anti-NF-κB p65 (Pierce) diluted at 1:50 in TBS for 2 h, washed and then incubated with Alexa- fluor 594 dye diluted at 1:100 in TBS for 1 h. Excitation and emission maxima used were 543 nm, 617 nm respectively. To identify the nuclei, the Alexa- flour labelled samples were counterstained with DAPI for 15 min.

NF-κB translocation/ I- κB degradation

THP- cells (15×10^6) were pre-treated with and without TC extracts, test compounds or mixture metabolites which are abundant in TCAE (mimicking TCAE) overnight in presence or absence of LPS for 3 h. After LPS induction, cells were then lysed in nuclear extraction (NE-PER) buffer in order to obtain cytosol and nuclear fractions.

Isolation of nuclear and cytosolic extraction

1. Harvested THP-1 cells were centrifuged at 500 × g for 5 minutes and washed cells by suspending the cell pellet with PBS.

2. To the cell pellets CER-I was added.

3. Vortexed the tube vigorously on the highest setting for 15 seconds to suspend the cell pellet completely and incubated the tubes on ice for 10 minutes.

4. Added 13.25 µL ice-cold CER II to the tube and vortexed the tube vigorously on the highest setting speed for 5 seconds. Incubate tubes on ice for 1 minute.

5. Again vortexed the tube vigorously on the highest setting for 5 seconds and centrifuged the tubes for 5 minutes at maximum speed (~16,000 × g).

6. After the centrifugation, transferred the cytoplasmic extract and stored at 80 °C until further use.

7. Suspended the nuclear pelleted fraction in 125 µL of ice-cold NER.

8. Vortexed on the highest setting for 15 seconds. Place the samples on ice and continued vortexing for 15 seconds at an interval of every 10 minutes, for a total of 40 minutes.

9. After vortexing, centrifuge the tubes at maximum speed (~16,000 × g) for 15 minutes to collected the nuclear extract.

10. After centrifugation, transfered the nuclear extract fraction to a clean pre-chilled tube and store at -80 °C until use.

Immunoblotting

Total protein in the both the fractions was measured by Bradford's method. Proteins (50µg/ lane) were subjected to SDS-PAGE and then wet transferred onto nitrocellulose membrane (NCM). NCM was blocked with 15 % milk powder in TBS at room temperature for 1 h and then probed with the primary antibodies (rabbit anti-NF-κB p65, mouse anti-I-κB, rabbit anti-mouse P- κB) (1:1000, Pierce,USA) overnight at 4 °C. After incubation, NCM was washed with TBST for 1hr and incubated with anti-rabbit and anti-mouse HRP secondary antibodies. Proteins of equal amount were loaded to detect the expression of β-actin, histone and MTA-1 (Metastasis tumour antigen-1), internal references for cytosol and nuclear extracts. Finally, an enhanced ECL reagent was used for visualization.

Chapter – 4

Results and Discussion

Objective 1: Evaluation of antioxidant and antiinflammatory activities of dry leaf extracts of *T. cordifolia* (TC) in activated human monocytic (THP-1) cells

Chapter-4

Results and Discussion

Results:

Phytochemical analysis

Estimation of total phenolic and flavonoid content in TC extracts

Water and hydroalcoholic extracts of dry leaves of *Tinospora cordifolia* (TCWE and TCAE) showed positive for phenolics and flavonoids upon estimation using FC reagent and aluminium chloride ($AlCl_3$) methods. Total phenolic and flavonoid content of both the extracts were obtained by extrapolating standard curves of gallic acid and quercetin respectively. TCWE showed total phenolic and flavonoid content of 3.1 ± 0.3 mg GAE/g dw and 0.178 ± 0.05 mg QE/g dw whereas TCAE exhibited 3.56 ± 0.5 mg GAE/g dw and 0.307 ± 0.1 mg qE/g dw. In brief both modes of extracts showed higher phenolic content but TCAE had higher flavonoid content when compared to TCWE **(Table. 4.1)**.

Table. 4.1. Total polyphenols and flavonoid content in dry leaf extracts of *T. cordifolia*

S. NO.	Mode of extract	Total phenol content (mg GAE/g dw)	Total flavonoid content (mg QE/g dw)
1.	TCWE	3.1 ± 0.3	0.178 ± 0.05
2.	TCAE	3.56 ± 0.5	0.307 ± 0.1

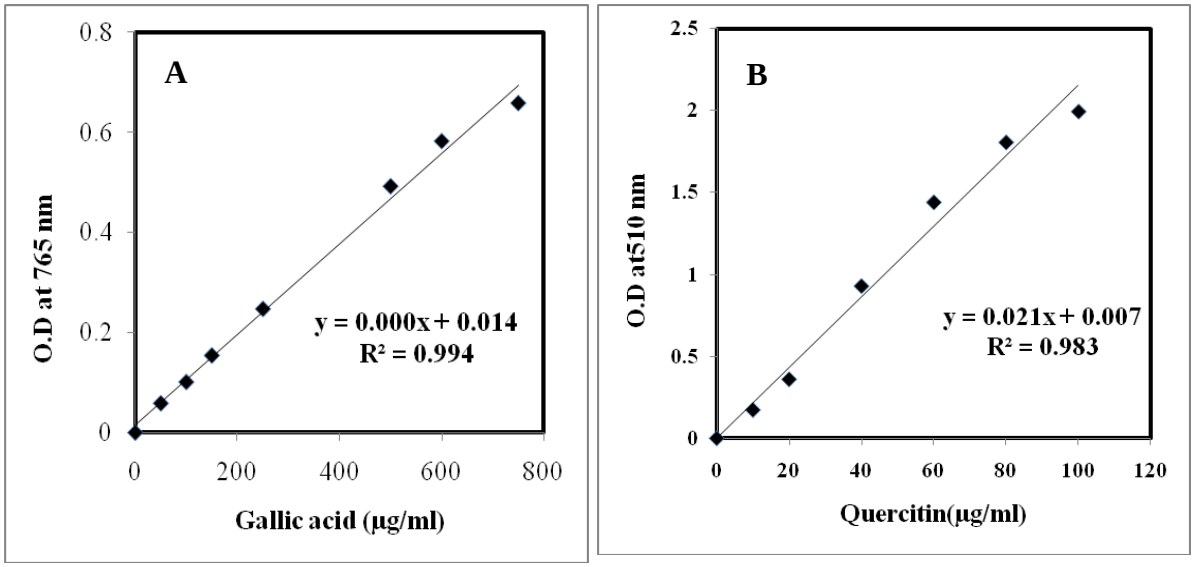


Fig. 4.1. Standard calibration curves for total phenolic and flavonoids in dry leaf extracts of *T. cordifolia.*

Total antioxidant activity

Antioxidant activity was evaluated by ammonium molybdate reagent and revealed that TCAE had higher antioxidant activity when compared to TCWE. TCWE showed an antioxidant activity of 3.55 ± 0.2 mg ASE /g dw whereas TCAE showed 4.0 ± 0.3 mg ASE /g dw (**Table. 4.2).**

Table. 4.2. Total Antioxidant activity in dry leaf extracts of *T. cordifolia*

s.no	Mode of extract	Total Antioxidant activity (mg ASE /g dw)
1.	TCWE	3.55 ± 0.2
2.	TCAE	4.0 ± 0.3

Free radical scavenging studies

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

Both TCAE and TCWE effectively scavenged DPPH in a concentration dependant manner. Both TCWE and TCAE scavenged 90% of DPPH radical with an IC_{50} values of 3.8 ± 0.4 µg dw/mL and 11.9 ± 2.2 µg dw/mL **(Fig. 4.2.A)** respectively. When compared to IC_{50} values, TCAE is more effective in scavenging DPPH than TCWE. Gallic acid is used as a positive reference which scavenged DPPH radical effectively in a concentration dependent manner with IC_{50} being 1.32µg/mL **(Fig. 4.2.B)**.

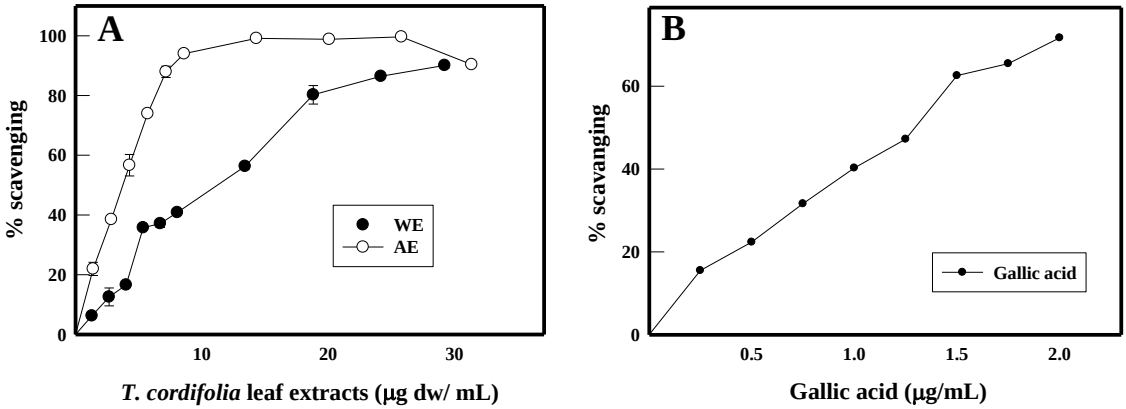


Fig. 4.2. DPPH radical scavenging studies of dry leaf extracts of *T. cordifolia* (A); Gallic acid standard curve (B)**.** Data represented are mean of standard deviation of three independent experiments (n=3). All values are statistically significant ($P < 0.001$).

Hydrogen peroxide (H_2O_2) scavenging assay:

Dry leaf extracts of *T. cordifolia* tested for its *in vitro* radical scavenging potential towards hydrogen peroxide. Both TCWE and TCAE showed efficient scavenging towards H_2O_2 with the increase in concentrations. TCWE and TCAE scavenged 90 % of H_2O_2 with an IC_{50} Values of 18 ± 2.7 µg dw/mL and 5.4 ± 0.9 µg dw/mL **(Fig. 4.3A)**. Standard metabolite gallic acid scavenged H_2O_2 in a concentrated dependent manner with 50 % scavenging at 4.4 µg/mL **(Fig. 4.3.B)**.

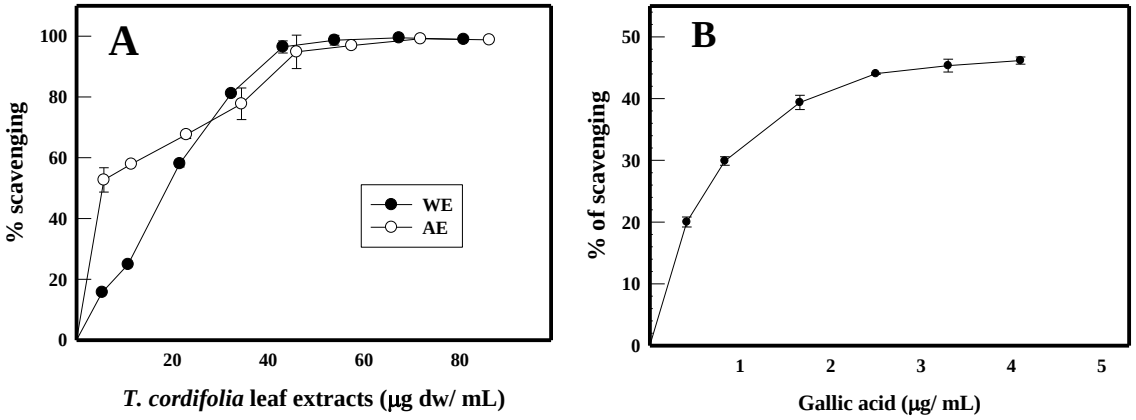
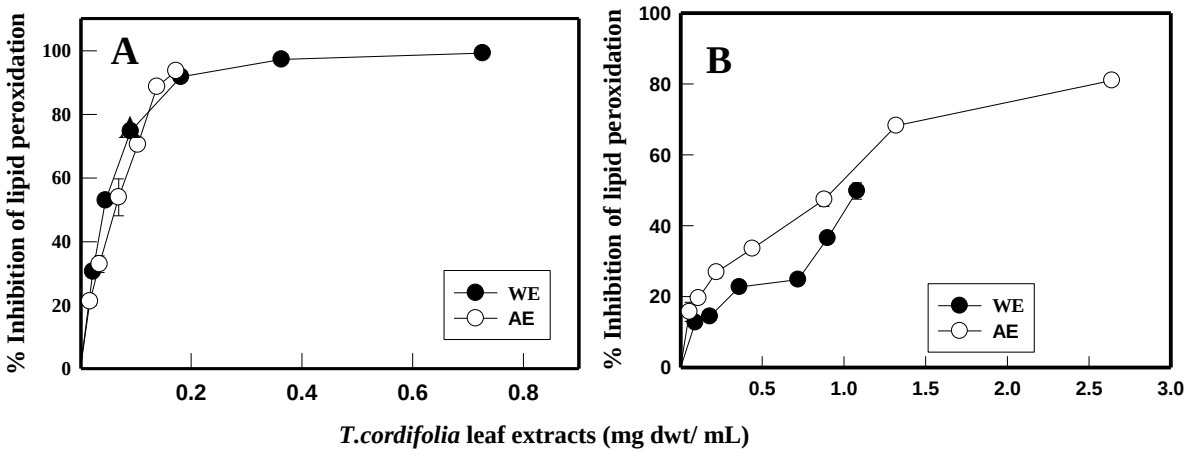


Fig. 4.3. H_2O_2 radical scavenging studies of dry leaf extracts of *T. cordifolia* (A); Gallic acid standard curve (B)**.** Data represented are mean of standard deviation, n=3. All values are statistically significant (P<0.001).

Effect of *T. cordifolia* dry leaf extracts on $Fecl_3$ induced lipid peroxidation in rat liver and heart homogenates

Oxidative stress can be assessed with the changes of markers like MDA. Generally MDA levels increase during oxidative stress conditions. Anti-lipid peroxidation activity of TC dry leaf extracts were tested (*in vitro*) in rat`s liver and heart homogenates stimulated with $Fecl_3$. Results in **Fig. 4.4** showed antilipid peroxidation activity of TC extracts in rat's liver and heart homogenate. TC extracts exhibited potent anti-liver and heart lipid peroxidation against $Fecl_3$ stimulation in a concentrated dependent manner. TC extracts exhibited potent anti-liver and heart lipid peroxidation against $Fecl_3$ stimulation in a concentrated dependent manner. Both TCWE and TCAE exhibited inhibition of lipid peroxidation in liver homogenate with an IC_{50} values of 37 µg dw/ and 56 µg dw/mL respectively **(Fig. 4.4A)**. TCWE and TCAE also scavenged lipid peroxide formation in heart homogenate effectively with 50 % inhibition at 1.1 and 0.64 mg dw/mL **(Fig. 4.4B)**. These results showed the efficacy of TC extracts in

suppressing the levels of lipid peroxides in rat liver and heart homogenates. Reference compounds like gallic acid and ascorbic acid are used as positive controls for liver and heart lipid peroxidation. Gallic acid exhibited 90 % antillipidperoxidation activity in liver tissue with an IC_{50} value of 6 µg/mL whereas ascorbic acid exhibited 80 % antilipidperoxidation in heart tissue with an IC_{50} value of 6.97 µg/mL.



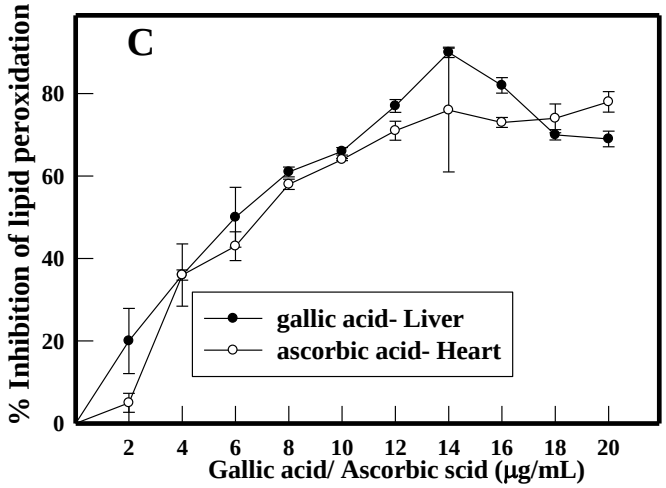


Fig. 4.4. Antilipidperoxidation of (A, B) *Tinospora cordifolia* dry leaf extracts; (C) Reference compounds gallic acid and ascorbic acid. Data represented are mean of standard deviation, n=3. All values are statistically significant ($P<0.001$).

Effect of *T. cordifolia* on cell viability- MTT assay

Cytotoxicity of TC extracts on THP-1 cells was determined by MTT assay. In this study, dosage of the extracts for cell treatments were determined, that up to 0.8 mg dwt/mL of both TCWE and TCAE showed no significant toxic effect on THP-1 cells, cell viability was more or less equal to the control cells **(Fig. 4.5)**. Hence for subsequent experiments, dosage of plant extracts for cellular treatments did not exceed 0.8 mg dwt/mL.

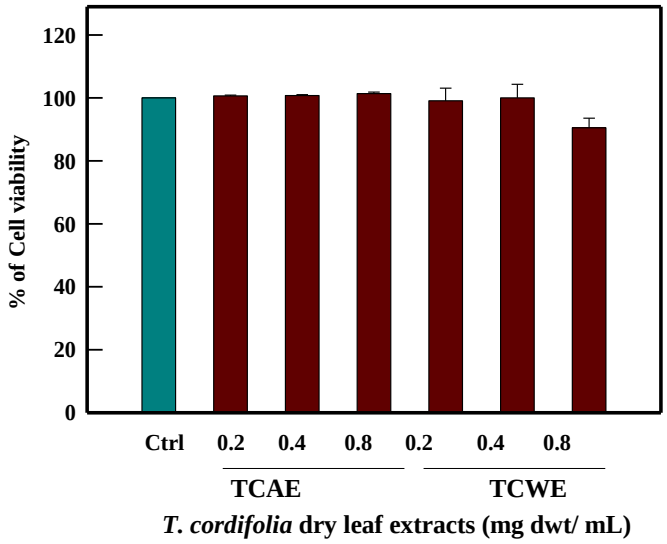


Fig. 4.5. MTT assay of *T. cordifolia* extracts in THP-1. Cell viability after treating with different concentrations of TC extracts ranging from 0.2 to 0.8 mg dwt/mL for 16 h at 37 °C and 5 % CO_2 was checked by MTT assay. Media alone treated with TC extracts were used as respective blanks. Data represent mean ± SD of 3 independent experiments.

Effect of *T. cordifolia* dry leaf extracts on arachidonic acid stimulated ROS and antioxidant enzymes in THP-1 cells

Arachidonic acid is one of the main source of H_2O_2 production which can rapidly cross the cell membranes and generate different types of hydroxyl radicals which causes cellular damage. In

spite of antioxidant enzymes in cells, excess ROS can lead to oxidative stress mediated diseases. Therefore, we tested the effects of TCWE and TCAE in attenuating arachidonic acid induced ROS production in a human monocytic (THP-1) cell line. Upon exposure to 100 µM arachidonic acid significantly increased ROS levels (1.6 folds) in THP-1 cells **(Fig. 4.6 C)**, compared to the unexposed cells **(Fig. 4.6 B)**. Pretreatment of cells with TCAE or TCWE markedly attenuated the arachidonic acid induced ROS production **(Fig. 4.6 D to K)**. Spectrofluoremetric data represented quantitative aspect of radical scavenging activity. TCAE (0.4 and 0.8 mg dwt/mL) inhibited AA dependent ROS generation to 0.96 and 0.7 folds whereas TCWE (0.4 and 0.8 mg dwt/mL) inhibited to 0.71 and 0.66 folds respectively **(Fig. 4.6 A)**. Thus, our results demonstrate that both extracts possess significant ROS scavenging activity. TC extracts has effectively increased the transcripts of antioxidant enzymes **(Fig. 4.7)**. Cells induced with arachidonic acid alone has little or no effect on the above transcripts when compared to normal control cells. TCAE (0.4 and 0.8 mg dwt/mL) significantly upregulated the expression of CAT (3 folds), SOD (6 folds) and GPx enzymes (4 folds). However TCWE (0.4 and 0.8 mg dwt/mL) upregulated only CAT (3 folds) and GPx (6 folds) and has no effect on SOD enzyme. Transcript analysis with 3 genes CAT, SOD and GPx suggested that CAT upregulation plays important role in scavenging AA induced ROS generation. Therefore activity of CAT enzyme was tested. Catalase protein activity was increased by both extracts in THP-1 cells which correlates with CAT transcript analysis **(Fig. 4.8)**. TCAE increased CAT activity to 9.63 and 15.21 folds whereas TCWE increased CAT activity to 22.54 and 17.58 folds. Arachidonic acid treated cells did not show any effect on catalase.

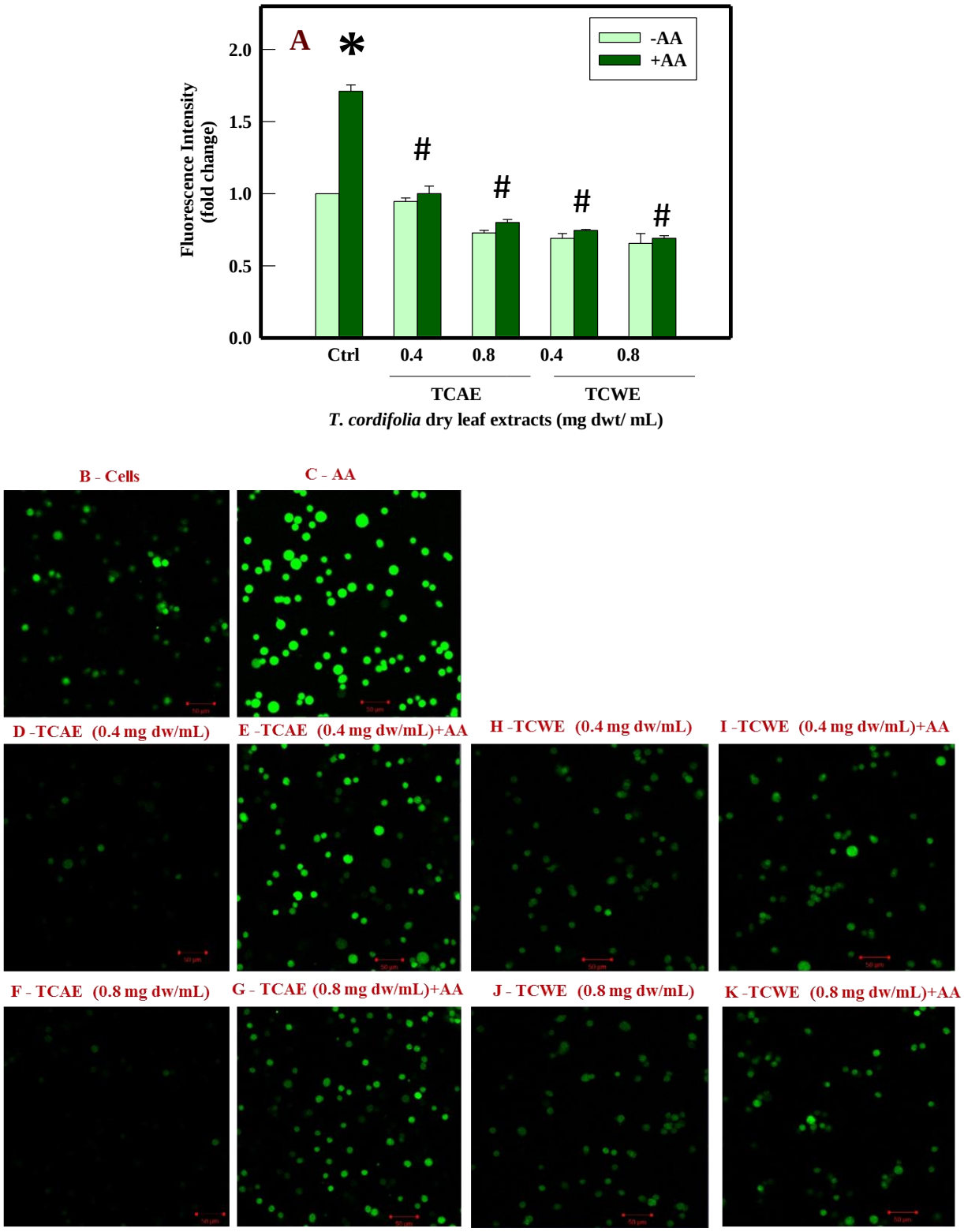


Fig. 4.6. Measurement of arachidonic acid induced ROS. THP-1 cells were pretreated with TC extracts prior to induction with arachidonic acid (100 µM) for 10 minutes followed by

incubation with 5μM H_2DCF dye for 15 min. After incubation, cells were washed twice with growth medium. Fluorescence intensity was measured by spectrofluorimetry (Fig. A) and further fluorescence images were captured by confocal microscope (Fig. B-K). Data represent mean ± SD of 3 independent experiments and # $p<0.001$ compared between treated and untreated with the controls whereas *$p<0.001$ represents significance between the control groups.

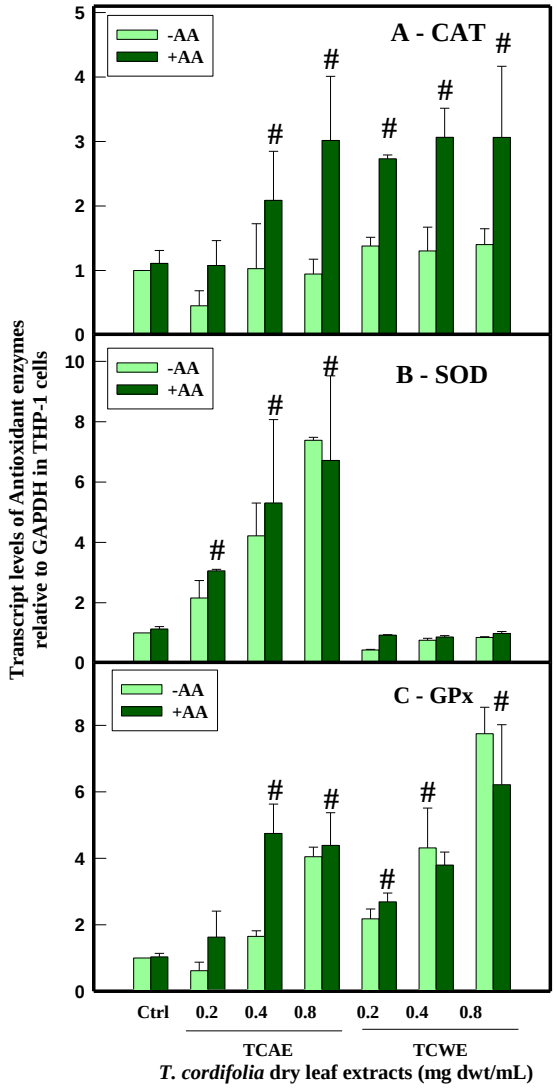


Fig. 4.7. Real time measurement of gene transcripts of antioxidant enzymes (CAT, SOD and GPx). THP-1 cells were treated with TC extracts and induced with arachidonic acid (100

μM) for ten minutes. At the end of the treatment, RNA was isolated using TRIzol reagent. Gene expressions of CAT (A), SOD (B) and GPx (C) were studied by quantitative real time PCR. Data obtained represent mean of ± SD of 3 independent experiments. # $p<0.001$ signifies the comparison of treated and untreated with controls.

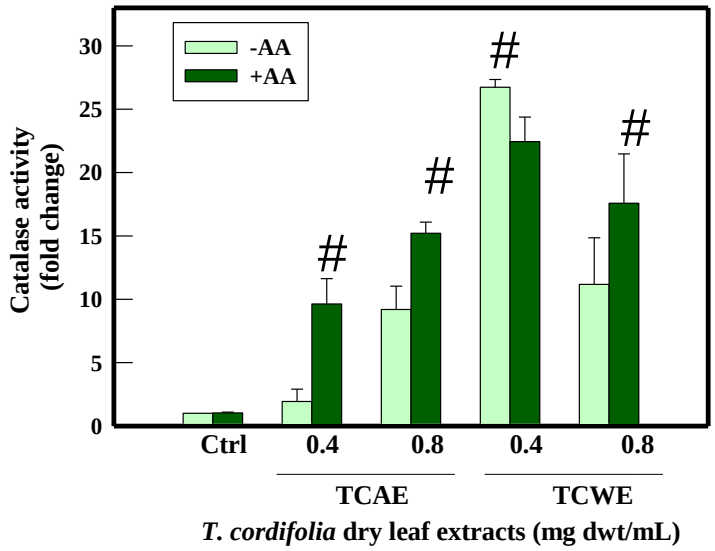


Fig. 4.8. Effect of TC extracts on catalase activity in THP-1 cells exposed to arachidonic acid. THP-1 cells were treated with TC- extracts and induced with arachidonic acid (100 μM) for ten minutes. At the end of the treatment, cell lysates were tested for catalase activity on exposure to 7.5 mM H_2O_2 using multimode microplate reader. Rate of consumption of H_2O_2 i.e. decrease in absorbance at λ 240nm was measured. Data represent mean of ± SD of 3 independent experiments. # $p<0.001$ compared between treated and untreated with the controls whereas *$p<0.001$ refers to the significance between induced and non-induced controls.

Effect of dry leaf extracts of *T. cordifolia* on LPS induced proinflammatory markers in THP cells:

Proinflammatory markers like TNF-α, MCP-1, IL-6 and IL-8 were secreted by activated monocytes which initiates the formation of atherogenic lesions in vascular wall. Proinflammatory cytokines /chemokines in pretreated cell culture supernatants were measured by ELISA as shown in **Fig. 4.9**. Uninduced cells released basal levels of all four markers TNF-α (6.87 pg/mL), MCP-1 (38.9 pg/mL), IL-6 (5.8 pg/mL) and IL-8 (12.1 pg/mL). Induction of THP-1 cells with LPS for respective time points significantly enhanced the release of the proinflammatory mediators like TNF-α (2111.46 pg/mL: 283.24 ± 31 fold change), MCP-1 (1031 pg/mL: 26.5 ± 0.1 fold change), IL-6 (30.42 pg/mL: 5.25 ± 0.9 fold change) and IL-8 (1693.18 pg/mL: 145 ± 49 fold change). Such LPS dependent induction in TNF-α secretion was significantly inhibited by TCWE and TCAE pretreatments, however, did not show similar effect on MCP-1, IL-6 and IL-8 release. LPS induced TCWE treated cells released 1075 pg/mL (156.5 ± 5.41fold change), 1199 pg/mL (174.58 ± 19.441fold change) of TNF-α at concentrations of 0.4 and 0.8 mg dwt /mL whereas TCAE pretreated cells secreted 1424 pg/mL (177 ± 6.8 fold change) and 1332 pg/mL (152 ± 11.5 fold change).

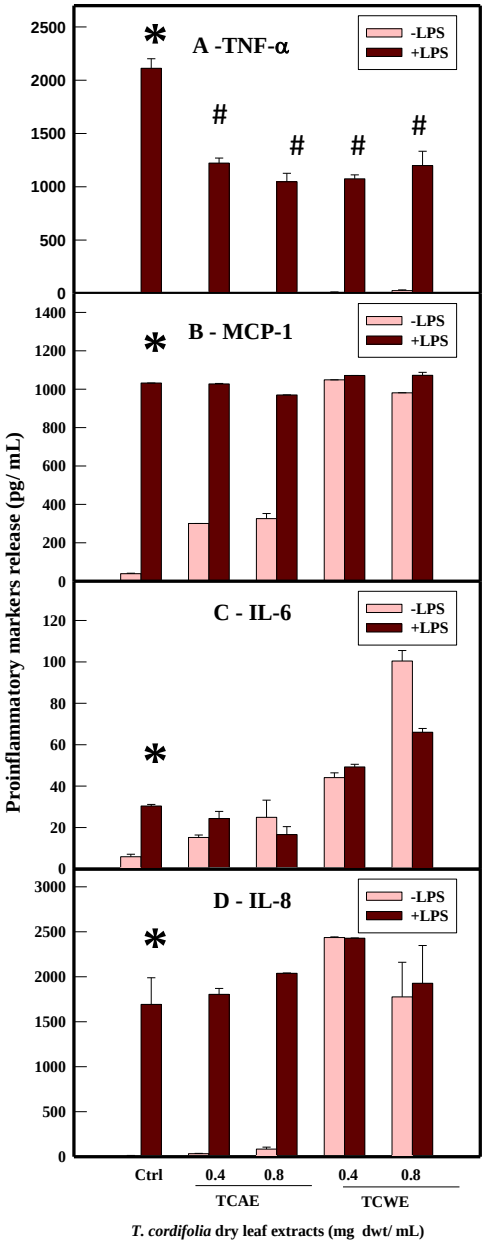


Fig. 4.9. Effect of *T. cordifolia* extracts on LPS induced proinflammatory markers by ELISA. THP-1cells pre-treated with TC dry leaf extracts and stimulated with LPS for 3 h, 6 h and 12 h. At the end of the treatment, cells were spun and collected the culture supernatant. Protein levels of the markers TNF-α (A), MCP-1 (B), IL-6 (C) and IL-8 (D) were quantified by ELISA. Data represent mean ± SD of 3 independent experiments and # $p<0.001$ compared between treated and untreated with the controls whereas *$p<0.001$ represents the significance between the control groups.

Effect of *T. cordifolia* dry leaf extracts on LPS stimulated proinflammatory transcripts in THP-1 cells:

Cells induced with LPS for 3 h enhanced the transcripts of TNF-α (4.8 folds), MCP-1(8.4 folds, IL-6 (2.43 folds) and IL-8 (148.47 folds) genes significantly when compared to normal control cells. Both TCWE and TCAE pretreatments decreased the expression of TNF-α gene transcript (1.99, 1.33 folds) significantly whereas MCP-1, IL-6 and IL-8 transcripts were unaffected by plant extracts in the presence of LPS. Both extracts showed more than 50 % of inhibition for TNF-α gene transcript at their respective concentrations of 0.4 mg dwt /mL for TCWE and TCAE **(Fig. 4.10)**.

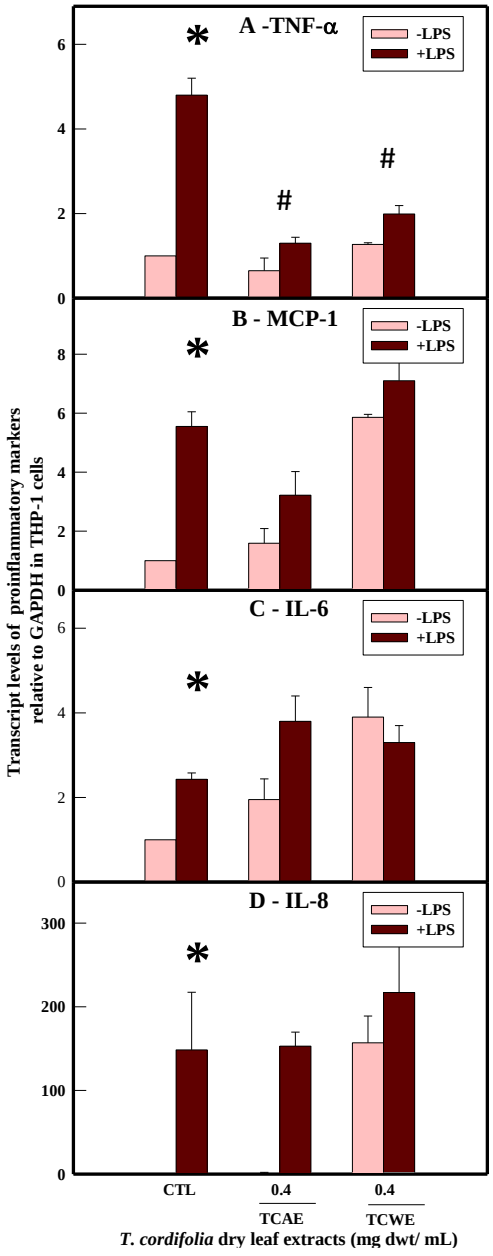


Fig. 4.10. Effect of *T. cordifolia* extracts on LPS induced proinflammatory gene transcripts. THP-1 cells pre-treated with ***T. cordifolia*** **extracts** and induced with LPS for 3 h. At the end of the treatment, RNA was isolated using TRIzol reagent. Gene transcripts normalized with GAPDH of TNF-α (A), MCP-1 (B), IL-6 (C) and IL-8 (D) were quantified by real time PCR. The data obtained represents mean ± SD of 3 independent experiments and # $p<0.001$ compared between treated and untreated with controls, *$p<0.001$ between the control groups.

Effect of TCWE/ TCAE on LPS induced NF-κB Translocation/I- κB degradation in THP-1 cells:

Expression of many proinflammatory genes were controlled by NF-κB signalling pathway. We tested the effect of plant extracts on NF-κB targeting into the nucleus by Western blotting and immuno-fluorescence methods **Fig. 4.11 and 4.12**. As expected, stimulation of THP-1 cells with LPS translocated the p65 subunit of heteromeric NF-κB protein into the nucleus whereas uninduced cells retained the p65 protein in the cytosol. Pretreatment of cells with TCAE (0.4 mg dwt / mL) completely suppressed the p65 translocation, which is confirmed by the presence of clear intensified band in the cytosol and its absence in the nucleus but TCWE (0.4 mg dwt / mL) activated the LPS targeting p65 protein into the nucleus indicated by the presence of clear bands in the nucleus. Further tested the effect of TC extracts on LPS induced phosphorylation and degradation of I-κB and found that TCAE significantly inhibited the degradation of I- κB whereas TCWE activated I- κB degradation.

Further, immunofluorescence studies has shown that TCAE clearly pretreatment strongly sequestered the inactive p65 in the cytosol **(Fig. 4.12 G to L),** which is unlike in LPS treated cells in which most of p65 protein is localised in nucleus **(Fig. 4.12 D to F)**. Uninduced cells retained p65 protein in an inactive state in cytosol (**Fig. 4.12 A to C)**. Based on these results, it is demonstrated that TCAE possess anti-inflammatory activities *via* attenuating the translocation of p65 in THP-1 cells but not TCWE.

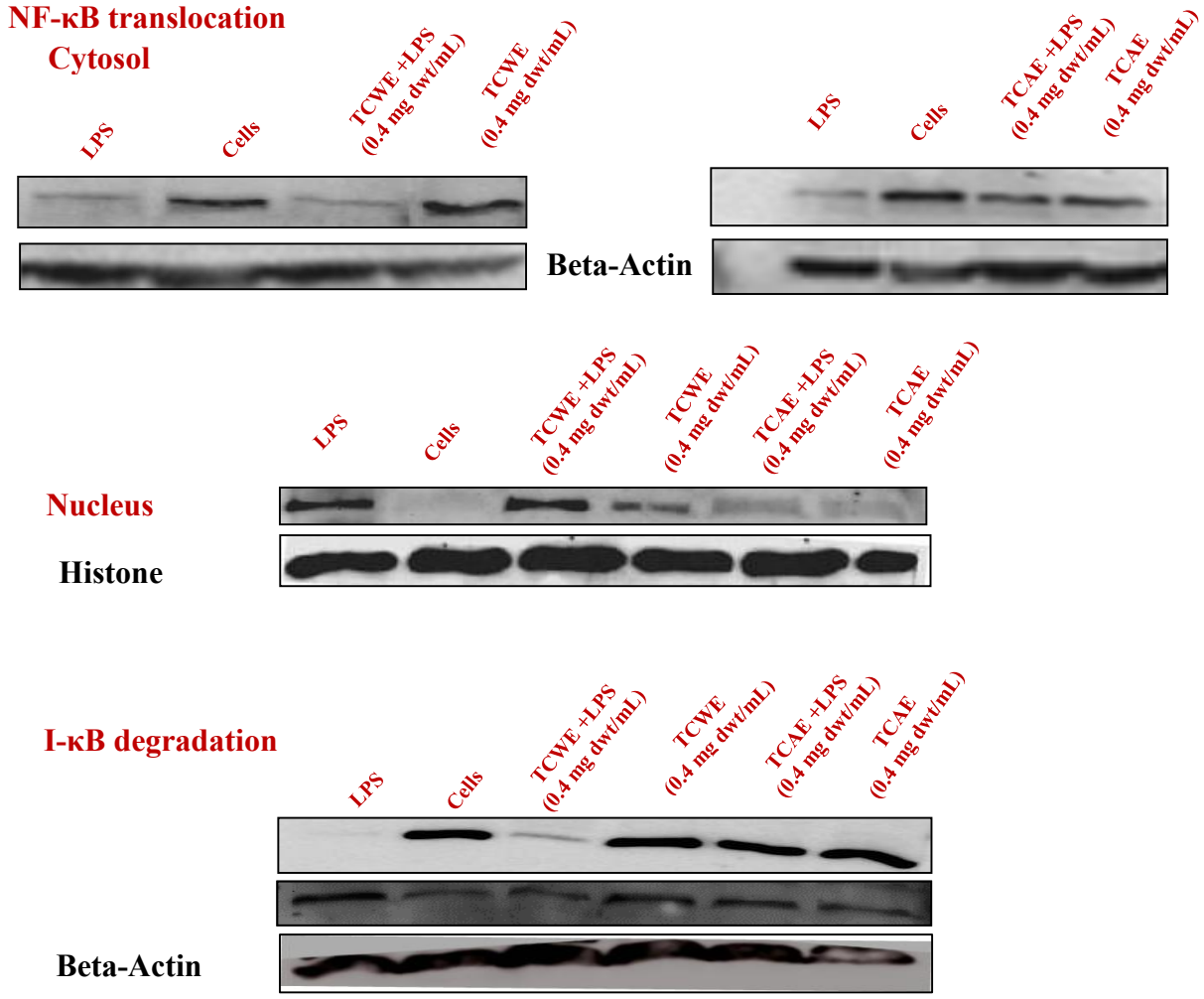


Fig. 4.11. Effect of *T. cordifolia* extracts on LPS induced NF-κB P-65 subunit translocation and I-κB degradation. THP-1 cells stimulated with LPS (0.5µg/mL) in presence of TCWE and TCAE and sub cellular localisation of p-65 subunit and I-κB degradation were detected by Western blotting analysis. THP-1 cells pretreated with TC extracts were induced with LPS (0.5µg/mL) and nuclear and cytosolic isolated protein fractions using NE-PER buffer. Lysate was loaded onto 10 % SDS PAGE and transferred to blotting membrane, probed with primary antibodies of NF-κB, I-κB and p-IκB. After primary antibody incubation, membrane was washed with TBST for 1 h and incubated with anti-rabbit and anti-mouse HRP secondary antibodies. Finally NF-κB, I-κB and P- IκB levels were detected by ECL reagents using versadoc.

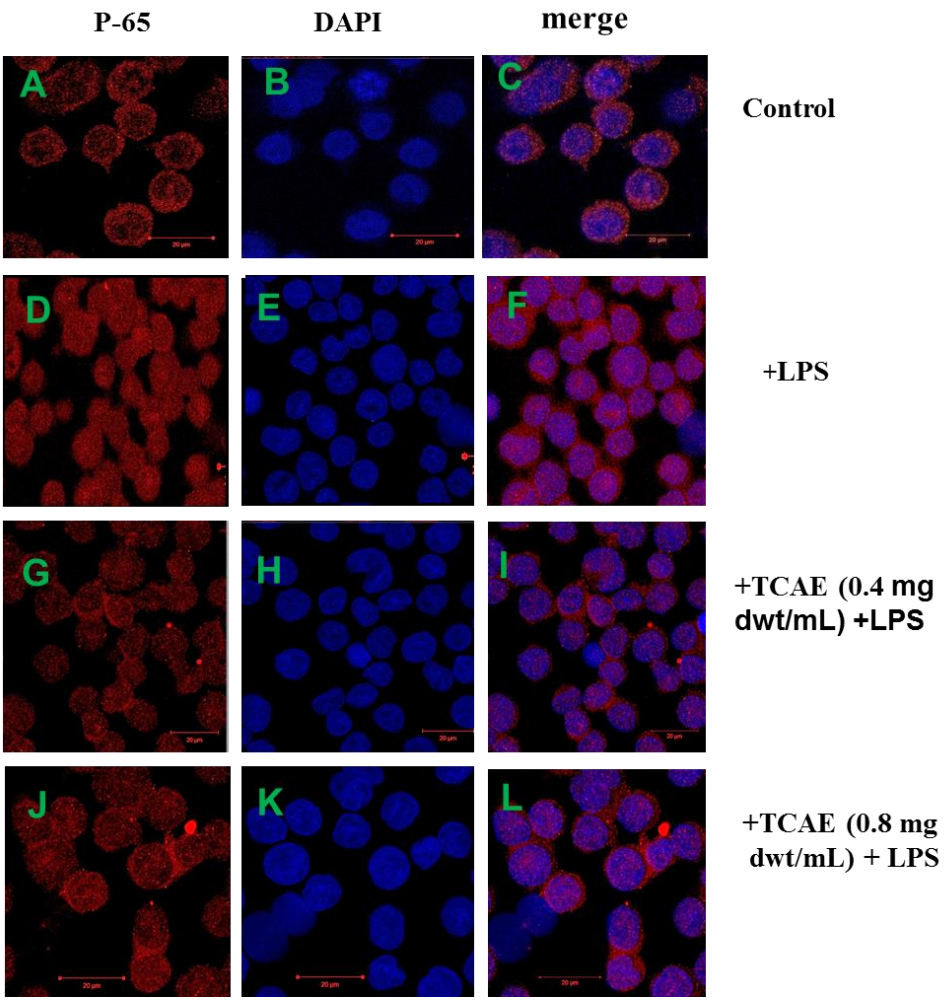


Fig. 4.12. Effect of *T. cordifolia* extracts on LPS induced NF-κB P-65 subunit translocation. Cells stimulated with LPS (0.5µg/mL) in presence of TCAE (0.4 and 0.8 mg dwt/mL) and sub cellular localisation of p-65 subunit was determined by immunofluorescence technique. NF-κB p-65 subunit was stained with alexa-fluor 594 conjugated secondary antibody and nucleus was counterstained with DAPI. Images were captured under Ziess confocal microscope. A, D, G, J are Alexa fluor labelled cells; B, E, H, K are DAPI stained cells and C, F, I, L are merged images of respective Alexa fluor p-65 and DAPI stained cells.

Effect of TCWE/ TCAE on PMA induced monocyte to macrophage differentiation:

Human monocytic THP-1 cells are spherical and suspension in nature which do not adhere to the plastic surfaces of the culture plates as shown in **Fig. 4.13 A**. THP-1 on stimulation with PMA (25 ng/mL) for 48 h, monocytic cells were differentiated into macrophage like cells which adhered to substratum of the culture dish (**Fig. 4.13 B**). Such PMA induced differentiation process was not inhibited by the plant extracts of TCAE or TCWE (0.4 and 0.8 mg dwt/mL) shown in **Fig. 4.13 C to 3.13 F**. Cell differentiation markers, toll- like receptors-4 (TLR 4) was upregulated when treated with TC extracts in the presence of PMA **Fig. 4.13 E**. Therefore, further studies were not focused on this phenomenon.

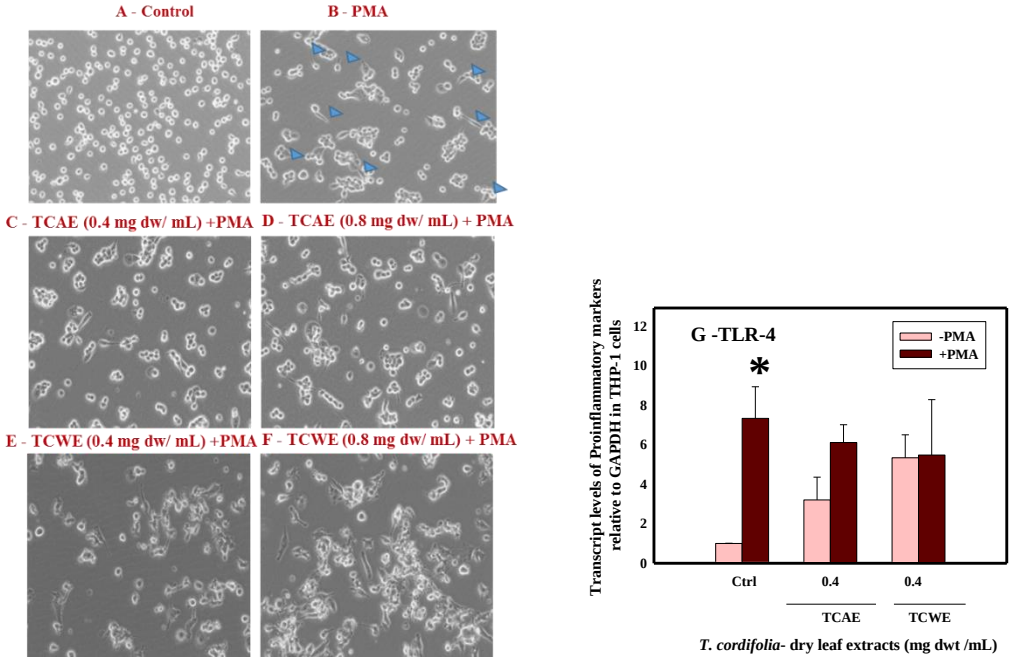


Fig. 4.13. Effect of *T. cordifolia* extracts on PMA induced macrophage differentiation of THP-1 cells. THP-1 cells seeded at a density of 5× 10^5 were pre-treated with TC extracts (04. mg dw/mL) and stimulated with PMA of concentration 25 ng/mL for 48 h to obtain macrophage differentiation **(B)**. Effect of plant extracts on differentiation of THP-1 cells to macrophages were observed under inverted microscope **(C, D, E and F)**. PMA stimulated cells for 48 h were used as positive control. RNA was isolated using TRIzol reagent. Gene expressions of cell differentiation markers like TLR-4 **(G)** were quantified using real time PCR.

Data represent mean ± SD of 3 independent experiments and *p<0.001 compared between treated and untreated.

Discussion

Public interest in usage of herbal medicine as health remedies has been increased day to day, since medicinal plants are known for richness in diversity of secondary metabolites. Phenolic and flavonoid compounds are the major abundant secondary metabolites known for their effective antioxidant properties due to their complex structure (Rice-Evans et al., 1997). Antioxidants are compounds that protect the oxidation of biological macromolecules by inhibiting the propagation of the oxidizing chain reaction. Consumption of dietary foods rich in antioxidants promotes proper health by scavenging ROS due to oxidative stress (Lobo et al., 2010). In the living system, oxidative stress is initiated by free radicals like ROS/ RNS which are generated constantly and seek electron pairing stability with biological macromolecules such as DNA, proteins and lipids of human cells under physiological conditions and can cause various diseases. Oxidative stress generated by ROS/ RNS is generally kept under check by antioxidant enzymes like catalase, glutathione peroxidase and sodium oxide dismutase present in the body (Halliwell, 2006). However, when free radical generation exceeds the cellular capacity of scavenging results in pathophysiology. In such situations supplementary natural antioxidants are very effective in scavenging oxidative stress and thereby prevent the initiation of various diseases. According to antioxidant hypothesis "intake of antioxidant supplements enhances human health by reducing various chronic inflammatory vascular diseases" (Stanner et al., 2007). Oxidative stress and inflammation interactions play key role in the pathogenesis of vascular diseases (Hajjar and Gotto 2013). Hence, the present study mainly focused on antioxidant and antiinflammatory activities of dry leaf extracts of *Tinospora cordifolia* and phytochemical analysis of the extracts possessing high biological activity.

Phenolic compounds being good reducing agents and stable metal chelators are considered to be very important plant constituents **(**Rice-Evans., 1995**)**. They also have scavenging ability due to their hydroxyl groups. Phenolic content in TC extracts was quantified by FC (Folin- ciocalteaue) reagent, phenols reacts with FC reagent and produced blue coloured complex measured spectrophotometrically at 765nm. Flavonoids, which constitute major part of polyphenolic compounds known for promoting various health benefits through their antioxidant properties. Total Flavonoid content in TC extracts was estimated by $AlCl_3$ method which forms orange coloured acid labile complexes on conjugation with hydroxyl groups present in flavonoids, measured spectrophotometrically at 417 nm. Total phenol and flavonoid content of TCWE and TCAE was estimated from the standard gallic acid and quercetin curves and found that both modes of extracts exhibited higher phenol and flavonoid content.

Total antioxidant activity in both extracts was estimated on reducing phospomolybdenum (VI) to phospomolybdenum (V) and calculated reducing equivalents using the calibration curve of ascorbic acid. Antioxidant activity was found to be higher in both the extracts which correlates with total phenol content. With reference to total antioxidant activity TC extracts were further tested for their reducing ability towards free radicals like DPPH and H_2O_2. DPPH was used to evaluate the hydrogen donating ability of medicinal plant extracts. Antioxidants reduces purple coloured DDPH to colourless reducing form which can be measured at 517 nm (Kedare and Singh 2011). Various H_2O_2 mediated pathways have been linked to inflammatory diseases like atherosclerosis, osteoporosis, arthritis, asthma and neurodegenerative and so on. Hydrogen peroxide is a weak oxidizing agent that inactivates certain enzymes, permeates cell membrane directly and thus leads to toxic effects. Hydrogen peroxide scavenging was measured by using Xylene orange dye which reacts with hydroperoxides in acidic solution and forms blue coloured product proportional to the H_2O_2 concentration in the sample. Percentage of scavenging increased with the increase in free

radical inhibition, both extracts of TC showed antiradical activity in scavenging both DDPH and H_2O_2 in a concentration dependent manner effectively (**Fig. 4.1 and 4.2)**.

Inhibition of lipid peroxidation establishes the antioxidant nature of a medicinal plant extracts. Fe^{3+}/Fe^{2+} catalysis produces ferry-perferryl complex or hydroxyl radicals that decomposes lipid hydro-peroxides. These radicals damage biological molecules by reacting with unsaturated fatty acids (PUF) in the cell membranes that eventually yield pink coloured carbonyl product like malondialdehyde (MDA) on reaction with TBA. TCWE and TCAE showed equal response in inhibiting lipid peroxide formation induced by ferric chloride in liver tissue homogenate but in case of heart lipid peroxidation TCAE was more efficient than TCWE (**Fig. 4.3 and 4.4)**.

Atherosclerosis is disease of blood vessel wall supplying the heart muscle, characterized by the formation of vulnerable plaque in arterial lumen and responsible for 30 % mortality and morbidity throughout the world. Various risk factors like hyperlipidemia, infections, oxidative stress, hypertension, diabetes, smoking and diet etc are associated in accelerating atherosclerosis directly by causing endothelial dysfunction (Hadi et al., 2005). Oxidative stress and Inflammation set stage in the blood vessel wall for various vascular diseases (Hajjar and Gotto 2013b). Monocytes and vascular endothelial cell interactions play a pivotal role in atherosclerosis progression (Mestas and Ley 2008). Monocytes are key immune cells which gets activated due to various adverse external factors and sets the stage of inflammation by secreting various proinflammatory cytokines. These secreted cytokines causes endothelial dysfunction by activating the adhesion markers expression on the surface of endothelium and migrates into the endothelium with the help of adhesion molecules, differentiates into macrophages and later converts to lipid loaded foam cells. Foam cells activate smooth muscle cells (SMCs) by secreting various proinflammatory cytokines and ROS which leads atherosclerosis complications *via* thrombus formation (Libby 2002). Inflammation

in atherosclerosis also invoke apoptotic response in early and latter stages of atherosclerosis. During initial stages of atherosclerosis, SMCs and macrophages undergo apoptosis and delays atherosclerosis but in advanced stages, apoptosis leads to thrombus formation through plaque rupture. *Tinospora cordifolia* is used in Ayurveda for the treatment of various inflammatory diseases which was attributed due to richness in phytochemicals belonging to various classes. Therefore in our study, we tested the effect of TC extracts on both oxidative and inflammatory stress markers in THP-1 and made attempt to unravel molecular mechanism for its activities.

MTT assay has shown that cell viability and growth of THP-1 cells were unaffected when treated with tested concentration's TC extracts (**Fig. 4.5)**. Our results also reveals the scavenging effect of TC extracts against ROS induced by arachidonic acid in THP-1 cells. TCAE showed concentration dependant scavenging of ROS whereas TCWE showed drastic effect in presence of arachidonic acid (**Fig. 4.6)**. Gene expression of antioxidant enzymes like catalase, glutathione peroxidase and sodium oxide dismutase also got upregulated by TC extracts in presence of arachidonic acid. Both TCAE/TCWE activated transcripts of CAT and GPx whereas SOD was upregulated by TCAE only (**Fig. 4.7)**. In addition, catalase protein activity was increased by both extracts in THP-1 cells which correlates with CAT transcript analysis **(Fig. 4.8)**. Again CAT enzyme was highly promoted by TCWE even at lower dose (0.4 mg dw/mL) suggesting components of TCWE are more active in dissipating oxidative stress in THP-1 cells.

Inflammation is a biological response of host to harmful stimuli such as pathogens, damaged cells or irritants. Lipopolysaccharide is a prototypic endotoxin of gram negative bacteria which induces inflammation by binding to Toll like receptor-4 (TLR-4) *via*. lipopolysaccharide binding protein (LBP) and upregulates the NF-κB signalling pathway by ubiquitin-26S proteosomal degradation of inhibitory subunit (I-κB) of NF-κB protein and latter translocates the NF-κB from to the nucleus where it binds to promoter region of various pro-

inflammatory mediators such as TNF-α, MCP-1, IL-6 and IL-8 etc and their expression leads to various chronic disorders (Chow et al., 1999).

TNF-α is the most important cytokine secreted by the activated monocytes/ macrophages which up regulates the expression of sticky adhesive proteins like VCAM, ICAM and E-Selectin present on the surface layer of endothelium (Kim et al., 2008). TNF-α being pleiotropic in nature, it initiate's inflammation through the release of other mediators such as IL-6, IL-8 and MCP-1. Among these secretory products, IL-6 and IL-8 also have significant roles in inflammation, rapidly causes rolling of monocytes to firmly adhere to monolayer's expressing E-selectin. IL-8 is also a mitogenic and chemo tactic for vascular smooth muscle cells (SMCs), as it stimulates the concentration-dependent cell proliferation and DNA synthesis in both human and rat aortic SMCs. MCP-1, a chemokine released by smooth muscle and endothelial cell promotes the transmigration and recruitment of monocytes and macrophages to the site of inflammation. Sticky molecules like VCAM-1, ICAM-1 and E-selectin present on the surface of endothelium helps in the recruitment and migration of activated monocytes to endothelial intima (Chen et al. 2001). Proinflammatory cytokines like MCP-1, IL-8 and IL-6 were found abundantly in atherogenic lesions (Tedgui and Mallat 2006). MCP-1, IL-8 and IL-6 aids in the chemotaxis, recruitment, rolling and adhesion of monocytes during the pathogenesis (Gerszten et al. 1999). Our results with extracts on LPS induced proinflammatory markers at protein and transcript levels in THP-1 cells showed significant attenuation of TNF-α both at protein and transcript levels whereas MCP-1. IL-6 and IL-8 were unaffected (**Fig. 4.9 and 4.10)**.

In order to unveil the molecular mechanism of TC extracts in attenuating TNF-α, we tested their effects on LPS induced NF-κB translocation into nucleus and hypothesized that TCAE significantly inhibited the LPS induced TNF-α both at protein and transcript levels using ELISA and RT-qPCR methods through the sequestration of inactive p-65 subunit in the cytosol

by Western blot and Immunofluorescence studies (**Fig. 4.11 and 4.12)**. Our significant findings revealed antioxidant and antiinflammatory activities of TC dry leaf extracts *via* inhibiting NF-κB pathway can be explored in the area of drug discovery for the treatment of inflammatory diseases.

Chapter – 5

Results and Discussion

Metabolite profiling of *T. cordifolia dry* leaf extracts and identification of active metabolites responsible for their effects in activated human monocytes (THP-1)

Chapter-5

Results and Discussion

Results

Metabolites identification in *T. cordifolia* dry leaf extracts and quantification of 20-Hydroxyecdysone

Metabolite profiling of dry leaf extracts of *T. cordifolia* done by employing LC-QTOFMS revealed for the presence of several metabolites and also authenticated the plant material used. Positive ion and Negative ion modes of LC-QTOF/MS detected 11 abundant metabolites including both TCAE and TCWE respectively. MS-MS fragmentation spectra of the metabolites and other details including m/z values, retention time, MS/ MS fragment's and etc. are given in the **Tables. 5.1 and 5.2**. Metabolites identified in TC extracts include alkaloids, vitamins, phenolics, flavonoids and phytosteroids. Tembetarine, 20-hydroxyecdysone, magnoflorine and syringin were commonly detected in both TCAE and TCWE. Pantothenic acid, Quercitin, and N-trans-ferulotyramine were exclusively detected in TCAE while columbin, palmatine and tetrahydropalmatine were detected in TCWE **(Fig. 5.1)**. Some of these metabolites were reported to be present in aerial parts of *T. cordifolia,* e.g. 20-hydroxyecdysone, Tembetarine, magnoflorine, palmatine and tetrahydropalmatine (Saha and Ghosh 2012; Kapil and Sharma 1997). Specific metabolites like amritosids, tinocordioside, cordioside, tinosponone and palmatosides (Saha and Ghosh 2012; Kapil and Sharma 1997; Jahfar and Azadi 2004) were detected in the *TC* extracts but their MS/MS fragmentations patterns are not matching with mass banks and databases.

Metabolites identified in TCAE and TCWE by LC-MS/MS

Pantothenic acid, $C_9H_{17}NO_5$ (Figure. 5.1 A). Chemically it is vitamin in nature and yielded peaks at RT 10.8 min from TCAE. Pantothenic acid was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 220.11.The compound was identified and confirmed by

matching the MS/MS spectral data with the mass bank ID KO009182. Matched mass fragmentation pattern of Pantothenic acid were m/z 116, 128, 162 and 164.

Tembetarine, $C_{20}H_{27}NO_4$ (Figure. 5.1 B). Chemically it is alkaloid in nature and yielded peaks at RT 13.7 min from TCAE and 11.2 from TCWE. Tembetarine was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 345.19.The compound was identified and confirmed by matching the MS/MS spectral data with Zhang et al. 2006 and Yan et al. 2013 references. Matched mass fragmentation pattern of tembetarine were m/z 137, 143, 151, 175 and 207.07.

Palmatine, $C_{21}H_{21}NO_4$ (Figure. 5.1 C). Chemically it is alkaloid in nature and yielded peak at RT 15.8 min from TCWE. Palmatine was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 353.18. The compound was confirmed and identified by mass bank IDKO009210. Matched mass fragmentation pattern of palmatine were m/z 158, 174, 264, 279 and 281.

Tetrahydropalmatine, $C_{21}H_{25}NO_4$ (Figure. 5.1 D). Chemically it is alkaloid in nature and yielded peak at RT 15.6 min from TCWE. Tetrahydropalmatine was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 355.18. The compound was confirmed and identified by Metlin ID 84992 and mass bank IDKO009283. Matched mass fragmentation pattern of Tetrahydropalmatine were m/z 165,177, 191.08, 248, 264, 265 and 281.

20-hydroxyecdysone, $C_{27}H_{44}O_7$ (Figure. 5.1 E). Chemically it is phytosteroid in nature and yielded peak at RT 15.63 min from TCAE and 14.4 from TCWE. 20-hydroxyecdysone was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 481.31. The compound was identified and confirmed by matching the spectral data with references (Destrez et al., 2009; Bajpai et al. 2016; Zhang et al., 2006). Matched mass fragmentation pattern of 20-hydroxyecdysone were m/z 165.12, 179.14, 261.02, 283.2, 301 and 379.

N-Trans-Ferulotyramine, $C_{18}H_{19}NO_4$ (Figure. 5.1 F). Chemically it is alkaloid in nature and yielded peak at RT 20.5 min from TCAE. The compound was not detected in TCAE and was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 314.13. The compound was identified by matching the MS/MS spectral data with references (Park 2009; Bajpai et al.,2016). Matched mass fragmentation pattern of N-Trans-Ferulotyramine were m/z 122 and 178.

Columbin, $C_{20}H_{22}O_6$ (Figure. 5.1 G). Chemically it is Furano diterpene lactone in nature and yielded peak at RT 21.9 min from TCWE. Columbin was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 357.12. The compound was identified by matching the MS/MS spectral data with references (Shi et al., 2007). Matched mass fragmentation pattern of columbin were m/z 121, 131 and 201 respectively.

Quercitin, $C_{15}H_{10}O_7$ (Figure. 5.1 H). Chemically it is a flavonoid and yielded peak at RT 24.1 min from TCAE. It is not detected in TCWE and was identified and confirmed by mass bank ID PB000202. The compound was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 325.14. Matched mass fragmentation pattern of quercetin were m/z 105.07, 128.06, 129.07, 131.08, 142.07, 155.06, 157.10, 166.07 and 169.10.

Magnoflorine, $C_{20}H_{25}NO_4$ (Figure. 5.1 I). Chemically it is alkaloid in nature and yielded peak at RT 26.7 min from TCAE. It is not detected in TCWE and was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 343.17. The compound was identified by matching the MS/MS spectral data with reference. Matched mass fragmentation pattern of magnoflorine were m/z 115, 153, 165, 179 and 191 respectively (Govindarajan et al., 2003).

Phloretin, $C_{15}H_{14}O_5$ (Figure. 5.1 J). Chemically it is phenol in nature and yielded peak at RT 28.1 min from TCWE. It is not detected in TCAE and was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 275.09. The compound was confirmed and identified by

matching the spectral data with mass bank ID TY0000158. Matched mass fragmentation pattern of phloretin were m/z 105, 128 and 169.

Syringin, $C_{17}H_{24}O_9$ (Figure. 5.1 K). Chemically it is a glucoside of synapyl alcohol in nature and yielded peak at RT 29.7 min from TCAE and 29.4 from TCWE. Syringin was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 373.16. The compound was confirmed and identified by matching the spectral data with mass bank ID TY000098 and with references (Ma et al., 2013; Eyles et al., 2007). Matched mass fragmentation pattern of syringin were m/z 209, 194, 179 and 151.

Amritosid-A, $C_{34}H_{26}O_{10}$. Chemically it is diterpene glucoside in nature and yielded peaks at RT 10.86 and 5.56 min from TCAE and TCWE. It was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 556.19.

Tinocordioside, $C_{21}H_{32}O_9$. Chemically it is diterpenoid and yielded peak at RT 26.14 min from TCAE. It is not detected in TCWE and was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 427.24.

Tinosponone, $C_{21}H_{20}O_5$. Chemically it is sesquiterpene and yielded peaks at RT 30.77 from TCAE and 30.1 min from TCWE. It was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 330.14.

Palmatoside-C, $C_{32}H_{22}O_4$. Chemically it is diterpene glycoside and yielded peak at RT 25.7 min from TCAE. It is not detected in TCWE and was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 448.17.

Cordioside, $C_{26}H_{34}O_{12}$. Chemically it is diterpene glycoside and yielded peak at RT 20.1 min from TCWE. It is not detected in TCAE and was confirmed by the mass spectrum exhibiting ions [M-H] - with m/z 538.27.

Table. 5.1. Q - TOF profile of metabolites identified in positive mode of TCAE and TCWE by LC-MS/MS

Metabolite	Nature	Molecular formula	Molecular weight	RT AE	RT WE	Abundance AE	Abundance WE
Pantothenic acid	vitamin	$C_9H_{17}NO_5$	219.23	10.8		166909	
Tembetarine	alkaloid	$C_{20}H_{27}NO_4$	343.17	13.7	11.2	56180	45960
Palmatine	alkaloid	$C_{21}H_{23}NO_4$	353.13	ND	15.8	ND	275000
Tetrahydropalmatine	alkaloid	$C_{21}H_{25}NO_4$	355.17	ND	15.9	ND	78510
20-Hydroxyecdysone	Phyto-ecdysteroid	$C_{27}H_{44}O_7$	480.3	15.6	14.4	443763	66439
N-Trans-Ferulotyramine	Phenyl propanoids	$C_{18}H_{19}NO_4$	313.13	20.8	ND	482058	ND
Quercitin	Flavonoid	$C_{15}H_{10}O_7$	302.15	24.1	ND	46477	ND
Magnoflorine	alkaloid	$C_{20}H_{25}NO_4$	343.17	26.7	27.06	147078	121493
Syringin	Glucoside of Synapyl alcohol	$C_{17}H_{24}O_9$	373.15	29.7	29.4	97549	95134
Phloretin	Phenol	$C_{15}H_{14}O_5$	274.26	ND	28.1	ND	23772
Amritosid-A	diterpene glucoside	$C_{34}H_{26}O_{10}$	556.19	10.8	5.56	39622	65377
Tinocordioside	diterpenoid	$C_{21}H_{32}O_9$	427.24	26.1	ND	29509	ND
Tinosponone	sesquiterpene	$C_{21}H_{20}O_5$	330.14	30.7	30.1	880166	84461
Palmatoside-C	diterpene glycoside	$C_{32}H_{22}O_4$	448.17	25.7	ND	55853	ND
Cordioside	diterpene glycoside	$C_{26}H_{34}O_{12}$	538.27	ND	20.17	ND	1201

Table. 5.2. Q - TOF profile of metabolites identified in negative mode of TCAE and TCWE by LC-MS/MS

Metabolite	**Nature**	**Molecular formula**	**Molecular weight**	**RT**		**Abundance**	
				AE	**WE**	**AE**	**WE**
20-Hydroxyecdysone	Phyto-ecdysteroid	$C_{27}H_{44}O_7$	480.27	18.4	ND	9596	ND
N-Trans-Ferulotyramine		$C_{18}H_{19}NO_4$	313.13	20.8	ND	60661	ND
Columbin	Furano diterpene lactone	$C_{20}H_{22}O_6$	357.0	ND	21.9	ND	4307
Tembetarine	alkaloid	$C_{20}H_{26}NO_4$	344.21	ND	19.03	ND	1448
Tinosponone	sesquiterpene	$C_{21}H_{20}O_5$	330.23	23.7	ND	9233	ND

* ND: Not detected

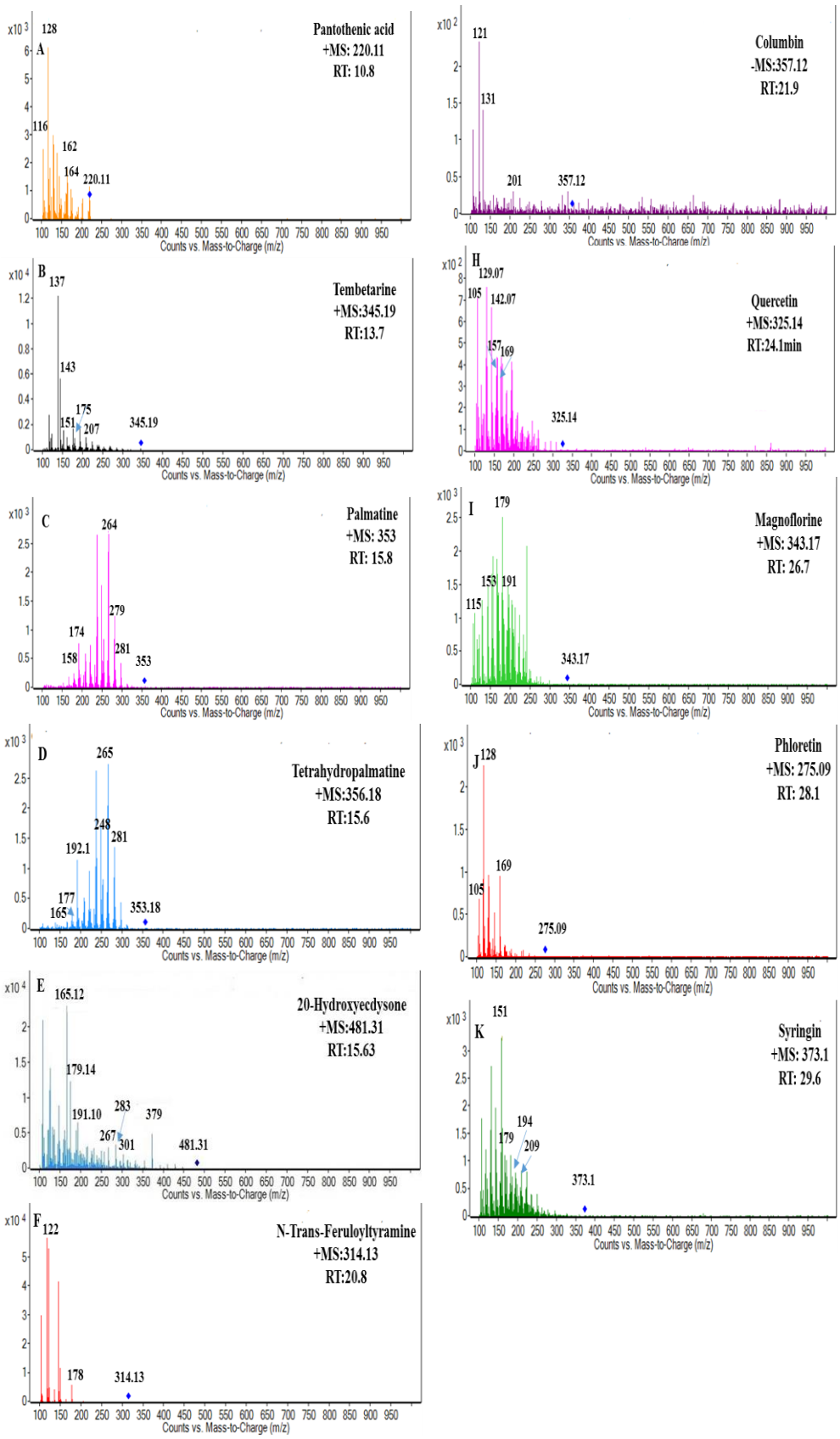
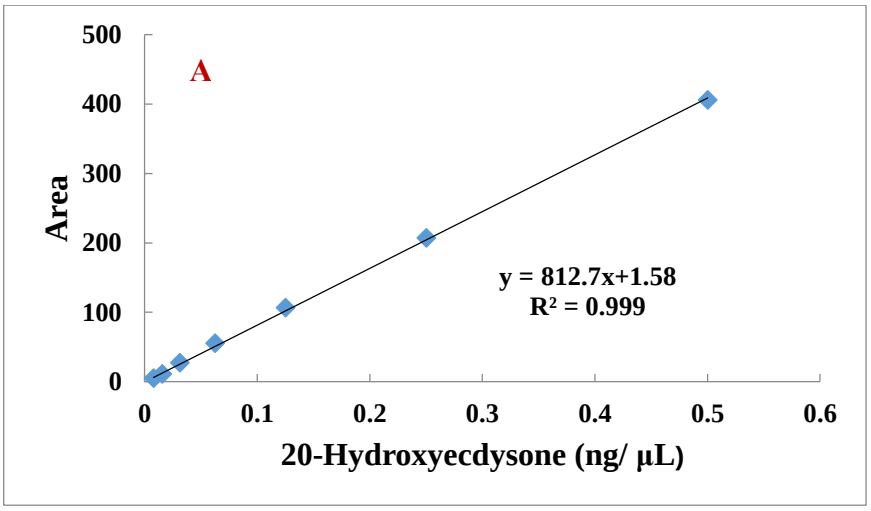


Fig. 5.1. Positive ion (+) and Negative ion (-) ESI/MS/MS of alcoholic extract (AE) and water extract (WE) of TC dry leaf extracts.

Quantification of 20-Hydroxyecdysone in TCAE by LC-MS

A typical LC-MS method with gradient elution was employed to quantify the marker metabolite 20-Hydroxyecdysone (20-HOD) in TCAE. Under experimental conditions standard compound 20-HOD was eluted at retention time 17 min. Standard calibration curve of 20-HOD ranging from 0.1 -0.5 ng/µL was constructed by measuring peak areas **(Fig. 5.2 A)**. LC- MS analysis of TCAE (0.4 and 0.8 mg dwt/mL) showed peaks corresponding to standard 20- HOD with retention times of 17.12 and17.19 min respectively. Using standard regression plot, concentrations of 20-HOD in TCAE (0.4 and 0.8 mg dwt/mL) was found to be 8 and 16 µM respectively **(Fig. 5.2 B)**.



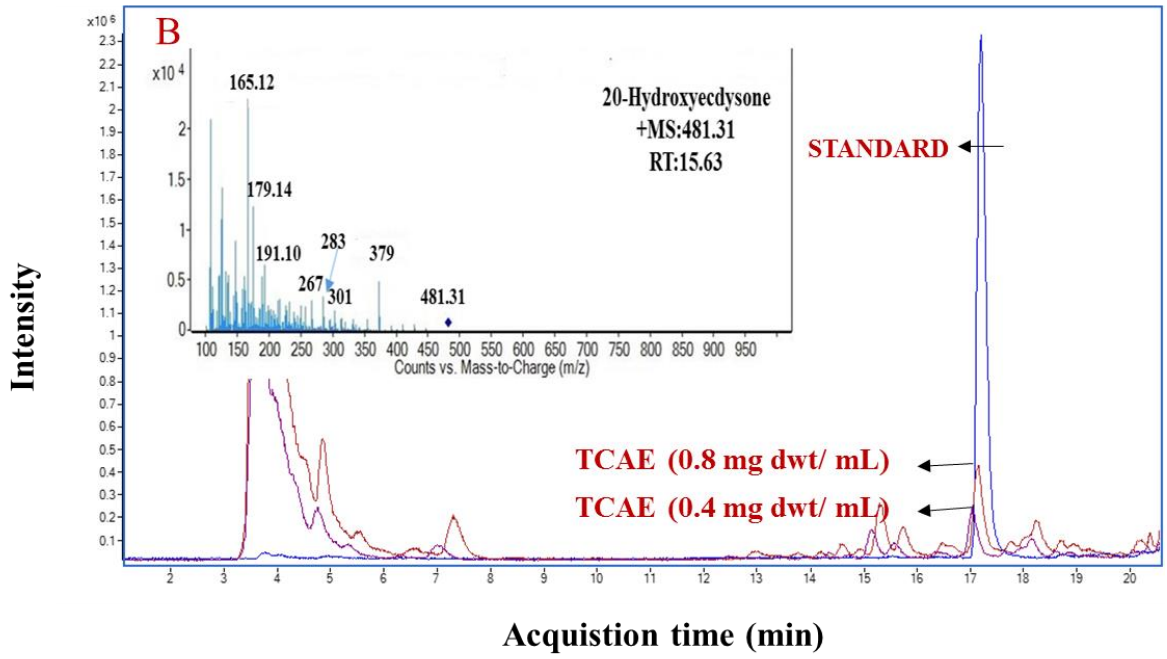


Fig. 5.2. LC-MS analysis for quantification of 20-Hydroxyecdysone in TCAE extract. (A) Standard regression plot of standard 20-HOD ranging from 0.1 - 0.5 ng/µL. (B) An example chromatogram of standard compound 20-HOD and TCAE showing peaks with retention time period of 17 min. 20-HOD content in plant extracts was determined according to the peak areas.

Effect of 20-HOD and SGY (Syringin) on cell viability - MTT assay

Cell viability of THP-1 pretreated with quantified various concentrations of 20-HOD 8 and 16 µM (10 and 20 ng/mL) and SGY (50 µM) was tested by trypan blue and then confirmed the results with MTT assay. Results of MTT assay showed the non-toxic effect of both metabolites on cell lines with cell viability more or less equal to control/ untreated cells (>95 %) **(Fig 5.3)**. The concentrations of HOD and syringin did not exceed the mentioned concentrations for their antioxidant and antiinflammatory activities.

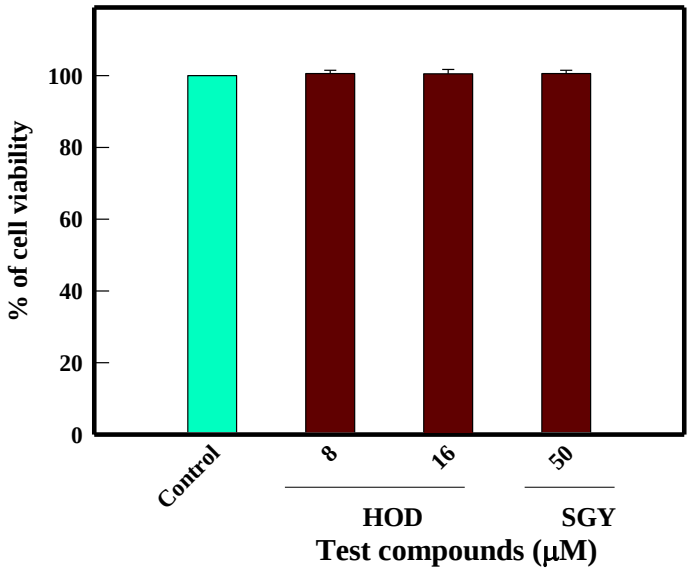


Fig. 5.3. MTT assay 20-HOD and SGY in THP-1. Percentage of cell viability after treating THP-1 cells with tested concentrations of 20-HOD/SGY for 16 h at 37 °C and 5 % CO_2 was quantified by MTT assay. Media alone treated with compounds were used as respective blanks. Data represent mean ± SD of 3 independent experiments.

Effect of 20-HOD / SGY on AA induced intracellular ROS and antioxidant enzymes in THP-1 cells

THP-1 cells on induction with arachidonic acid (100 µM) for 10 min increased H_2DCF-DA fluorescence intensity when compared to untreated control cells **(Fig. 5.4 B)**. Increase in fluorescence of H_2DCF-DA is directly proportional to generation of increased intracellular ROS. 20-HOD / SGY pretreated cells in presence of arachidonic acid did not show increase in H_2DCF-DA fluorescence **(Fig. 5.4. C-H)**. Spectrofluorimetric studies **(Fig. 5.4 I)** further revealed the ROS scavenging activity of 20-HOD and SGY at tested concentrations. Cells on stimulation with AA had increased fluorescence intensity by 3.92 folds to that of control cells (un-induced) whereas THP-1 cells pretretaed with 20-HOD (16 µM)/ SGY (50 µM) scavenged ROS significantly to 1.84 and 1.18 folds respectively. Cells treated with 20-HOD / SGY were also tested.

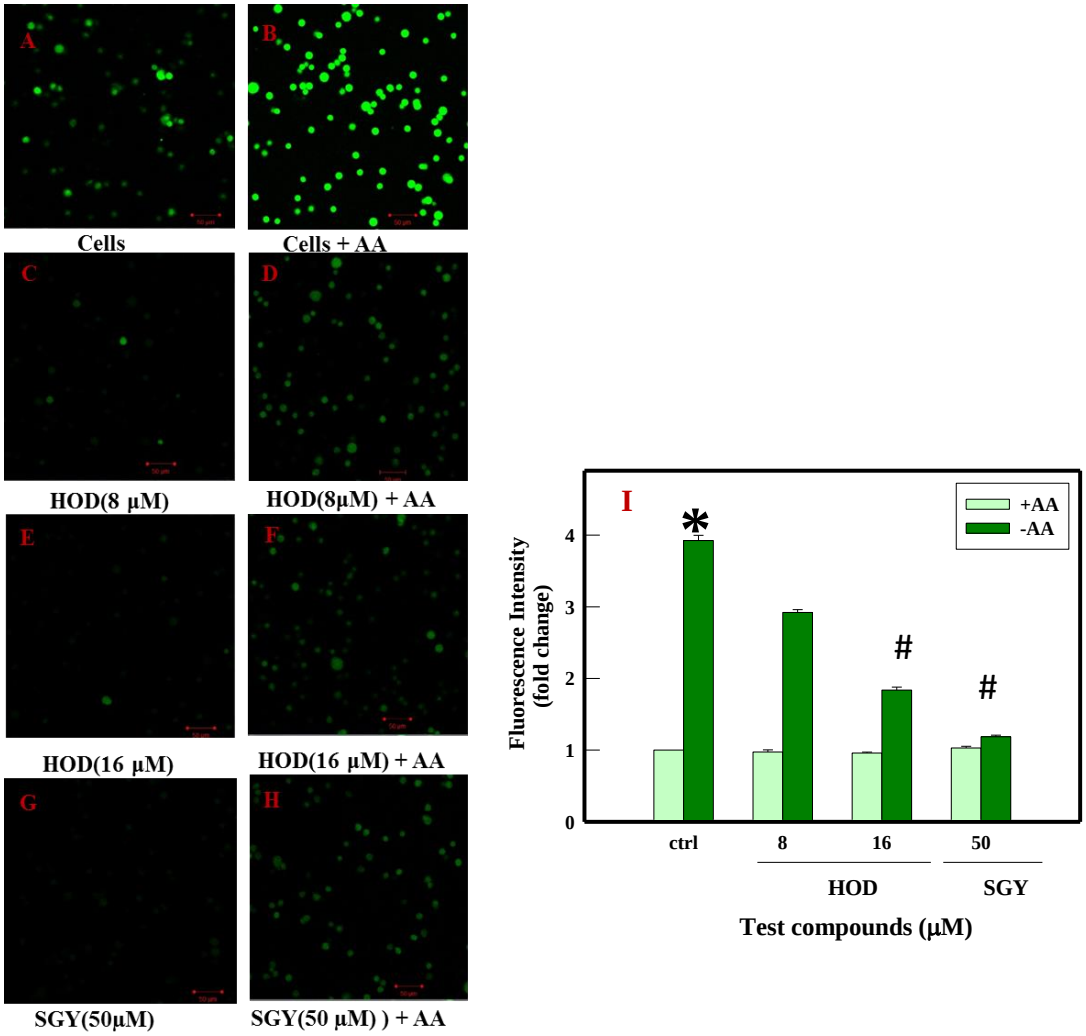


Fig. 5. 4. Effect of HOD and SGY on arachidonic acid induced ROS in THP-1 cells. THP-1 cells were pretreated for 16 h with 20-HOD/ SGY prior to induction with arachidonic acid (100 µM) for 10 min followed by incubation with 5 µM H_2DCF dye for 15 min. After incubation, cells were washed twice with growth medium. Fluorescence images were captured by confocal microscope and further fluorescence intensity was measured by spectrofluorimetry. Data represent mean ± SD of 3 independent experiments.

20-HOD increased the gene expression of antioxidant enzymes such as CAT (2.79 folds) and Gpx (4.20 folds) at 16 µM in presence of arachidonic acid which correlates with its ROS scavenging activity of 20-HOD. Cells induced with arachidonic acid alone has little or no effect on the above transcripts when compared to uninduced control cells **(Fig. 5.5)**. Catalase

activity in THP-1 cell lysates was promoted by 20-HOD significantly at both 8 and 16 µM. It also increased catalase activity s to 1.5 and 1.8 folds in the presence of arachidonic acid whereas cells induced with arachidonic had equivalent to uninduced cells **(Fig. 5.6)**.

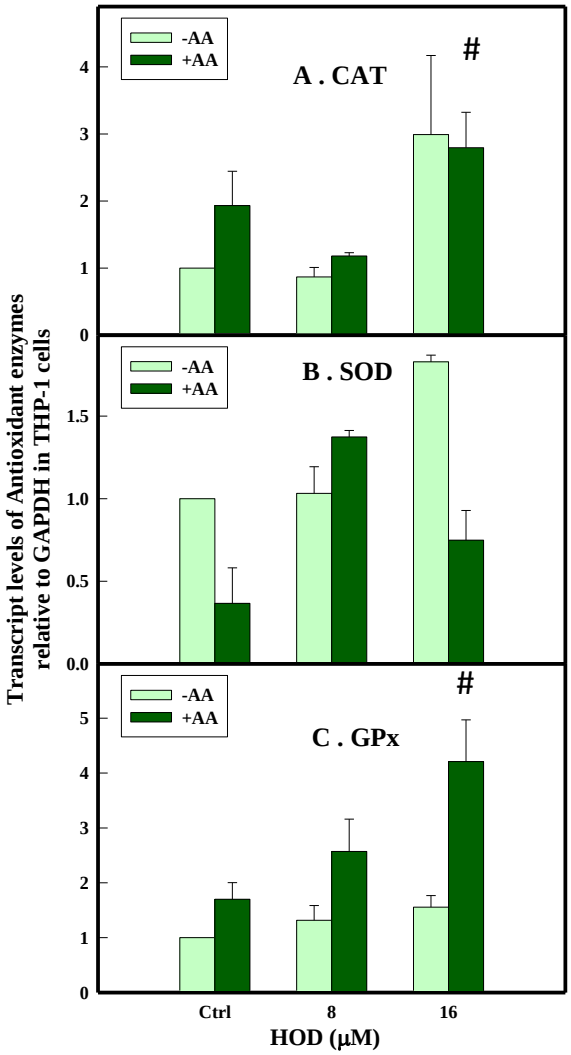


Fig. 5. 5. Real time measurement of gene transcripts of antioxidant enzymes (CAT, SOD and GPx). THP-1 cells were treated with 20-HOD (8 and 16 µM) and induced with arachidonic acid (100 µM) for ten minutes. At the end of the treatment, RNA was isolated using TRIzol reagent. Gene expressions of CAT (A), SOD (B) and GPx (C) were analysed by quantitative real time PCR. Data represent mean of ± SD of 3 independent experiments. # $p<0.001$ compared between treated and untreated with controls, *$p<0.001$ between the induced and untreated control cells.

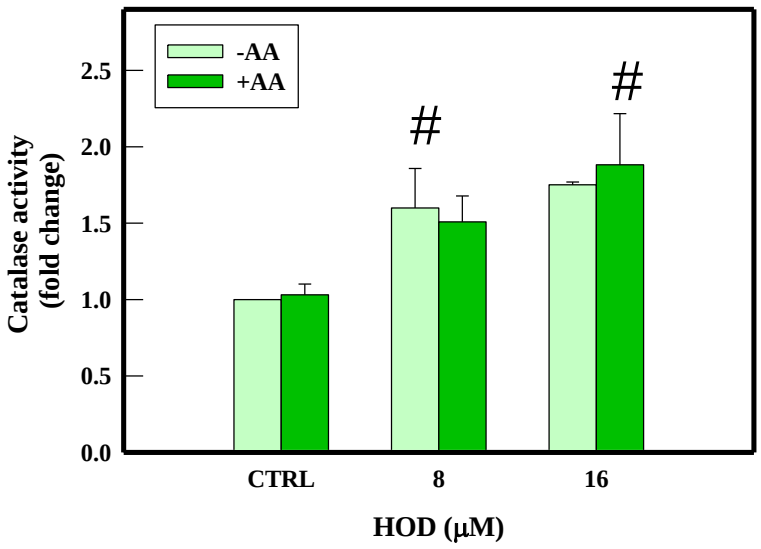


Fig. 5. 6. Effect of 20-HOD on catalase enzymatic activity in THP-1 cells exposed to arachidonic acid. THP-1 cells were treated with 20-HOD (8 and 16 μM) and induced with arachidonic acid (100 μM) for ten minutes. At the end of the treatment, cell lysates were tested for catalase enzyme activity on exposure to 7.5 mM H_2O_2 using multimode microplate reader. Rate of consumption or decrease in absorbance was measured at λ 240 nm. Data represent mean of ± SD of 3 independent experiments. # $p<0.001$ represents significant comparison of test compound treated/ untreated with controls, *$p<0.001$ between the control groups.

Effect of 20-HOD and SGY on LPS induced proinflammatory protein markers

We tested the inhibitory effect of 20-HOD (8, 16 μM) and SGY (50 μM) on LPS induced inflammatory cytokines using ELISA. Our results demonstrated the attenuatory effect of HOD on TNF-α and IL-6 release whereas no effect on and IL-8 secretions. As compared to the control cells, LPS induction increased the release of TNF-α (283.24 ± 31 fold increase) and IL-6 (5.25± 0.9 fold increase) whereas treatment of 20-HOD in presence of LPS significantly attenuated the release of TNF-α and IL-6 by 50 % i.e. induction of LPS by HOD treated cells resulted only 145 ± 8.2 folds of TNF-α and 2.97 ± 1.18 folds of IL-6 to that of uninduced cells

and SGY was not equivalently effective (205 ± 1.7; 4.03 ± 1.7 fold changes) **(Fig. 5.7A)**. Therefore, further experiments were continued with 20-HOD.

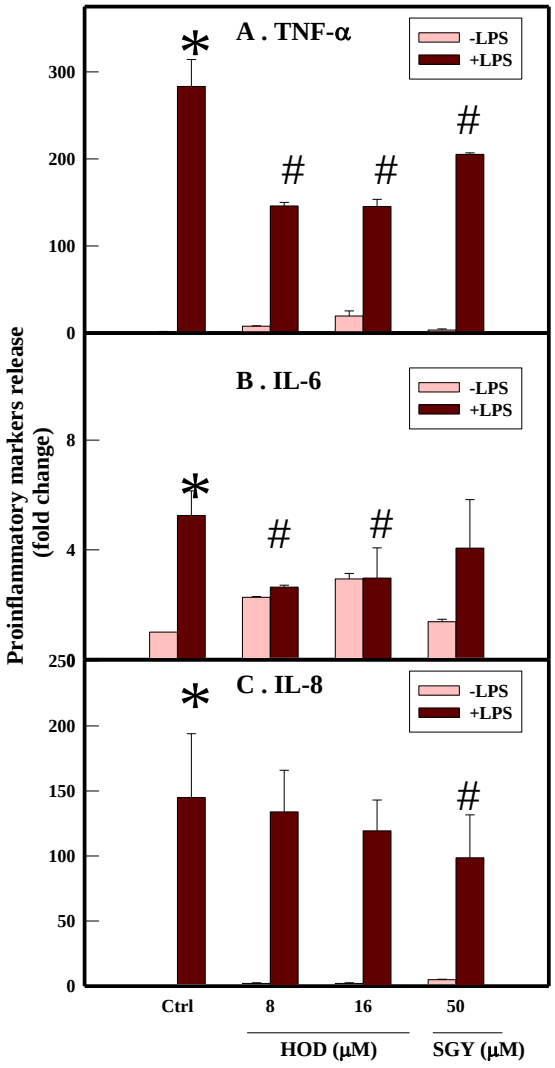


Fig. 5.7. Effect of 20-HOD and SGY on LPS induced proinflammatory protein markers. THP-1cells pre-treated with or without 20-HOD and SGY were induced with LPS for 3 h and 12 h. At the end of the treatment, cells were spun and collected the culture supernatant and subjected to ELISA. Protein levels of the secreted markers into the supernatant of TNF-α (A), IL-6 (B) and IL-8 (C) were quantified by ELISA. Data represent mean ± SD of 3 independent experiments and # $p<0.001$ signifies the # $p<0.001$ signifies the comparision of test compound treated and untreated with controls whereas *$p<0.001$ denotes the significance between the control groups.

Effect of 20-HOD on LPS induced proinflammatory gene transcripts

Transcriptional regulation is the control point for the secretion of these inflammatory cytokines at the protein levels. Results in **Fig. 5.8** demonstrates TNF-α (24.2 folds), IL-6 (1.98 folds) and IL-8 (8.87 folds) gene transcripts were significantly upregulated in LPS treated cells when compared to uninduced control cells whereas pretreatment with 20-HOD significantly inhibited TNF-α expression to 14.62 folds at 16 µM concentration, however did not show the effect on expression of IL-6 and IL-8 transcripts induced by LPS in THP-1 cells at both 8 and 16 µM concentrations.

Effect of 20-HOD on LPS induced NF-κB translocation

Transcripts of various proinflammatory cytokines were regulated by redox-sensitive transcription factor NF-κB. In order to determine whether 20-HOD would attenuate pro-inflammatory markers expression through the suppression of NF-κB activation, we tested the effect of 20-HOD on NF-κB targeting into the nucleus by immuno-fluorescence method shown in **Fig. 5.9**. Immunofluorescence studies have shown that 20-HOD at 8 and 16 µM sequestered the inactive p65 in the cytosol **(Fig. 5.9 G to L)** which is unlike in LPS induced cells in which most of p65 protein is translocated into nucleus **(Fig. 5.9 D to F)**. Uninduced cells retained p65 protein in an inactive state in cytosol shown in **Fig. 5.9 A to C**. Based on these results it is demonstrated that 20-HOD possess anti-inflammatory activities via attenuating the translocation of p65 into nucleus in THP-1 cells.

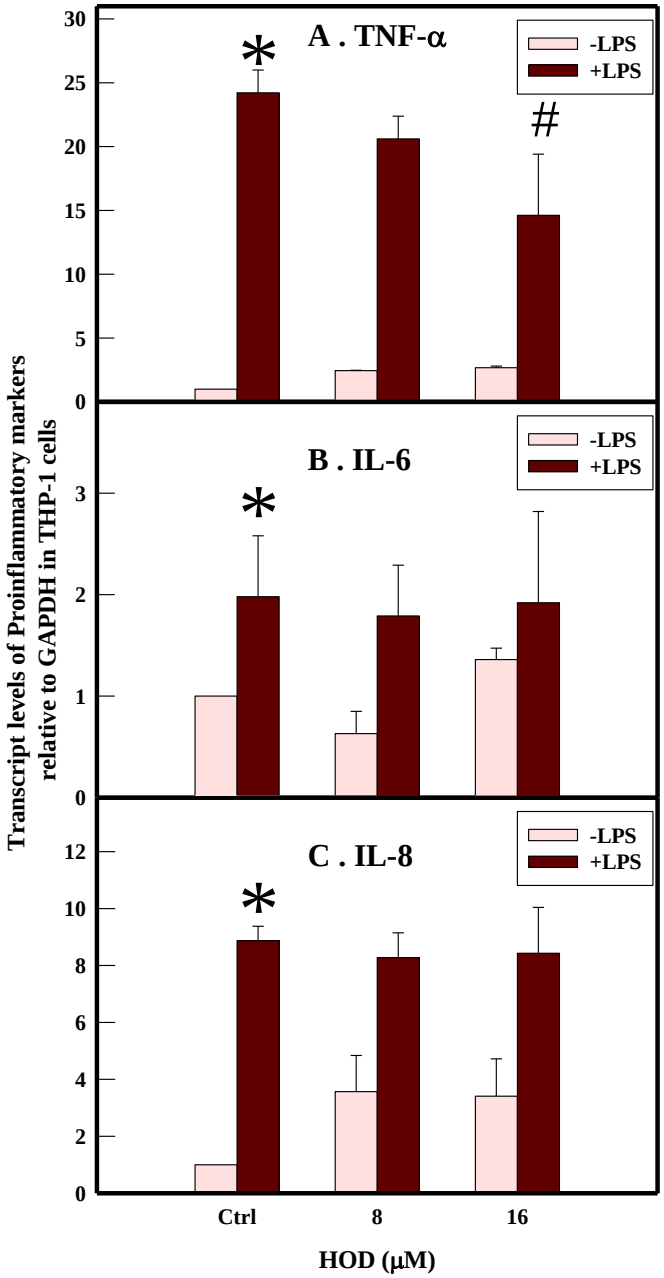


Fig. 5.8. Effect of 20-HOD on LPS induced proinflammatory gene transcripts. THP-1 cells pre-treated with 20-HOD and induced with LPS for 3 h. At the end of the treatment, RNA was isolated using TRIzol reagent. Gene transcripts TNF-α (A), IL-6 (B) and IL-8 (C) quantified by real time PCR (qRT-PCR) and were normalized with GAPDH. Data obtained represents mean ± SD of 3 independent experiments and # $p<0.001$ signifies the comparison of test compound treated and untreated with controls whereas *$p<0.001$ denotes the significance between the control groups.

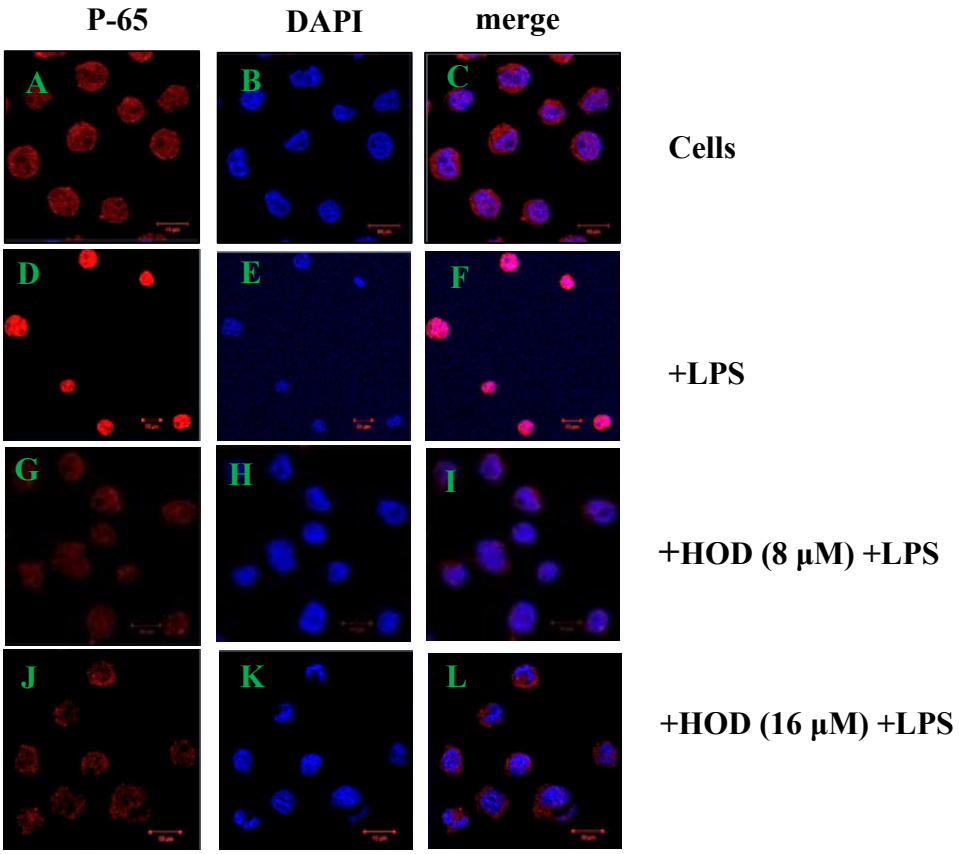


Fig 5.9. Effect of HOD on LPS induced nuclear translocation of NF-κB P-65 subunit in THP-1 cells. THP-1 cells induced with LPS (0.5µg/mL) in presence of HOD (8, 16 µM). NF-κB p-65 subunit was stained with alexa-fluor 594 conjugated secondary antibody and nuclei were counterstained with DAPI. Images were captured under confocal microscope (Ziess). A, D, G, J are Alexa fluor labelled cells; B, E, H, K are DAPI stained cells and C, F, I, L are merged images of respective Alexa fluor p-65 and DAPI stained cells.

Effect of 20-HOD on ox-LDL induced foam cell formation

Foam cell formation was considered to be the characteristic feature of atherosclerosis, therefore in our study we tested the effect of 20-HOD (8 and 16 µM) on foam cell formation in THP-1 cells using oil red staining method. At first THP-1 monocytic cells were induced to differentiated macrophages by treating with PMA **(Fig. 5.10 A).** Subsequent treatment with Ox-LDL (100 µg/mL) for 48 h lead to foam cell formation and visualised under microscope at

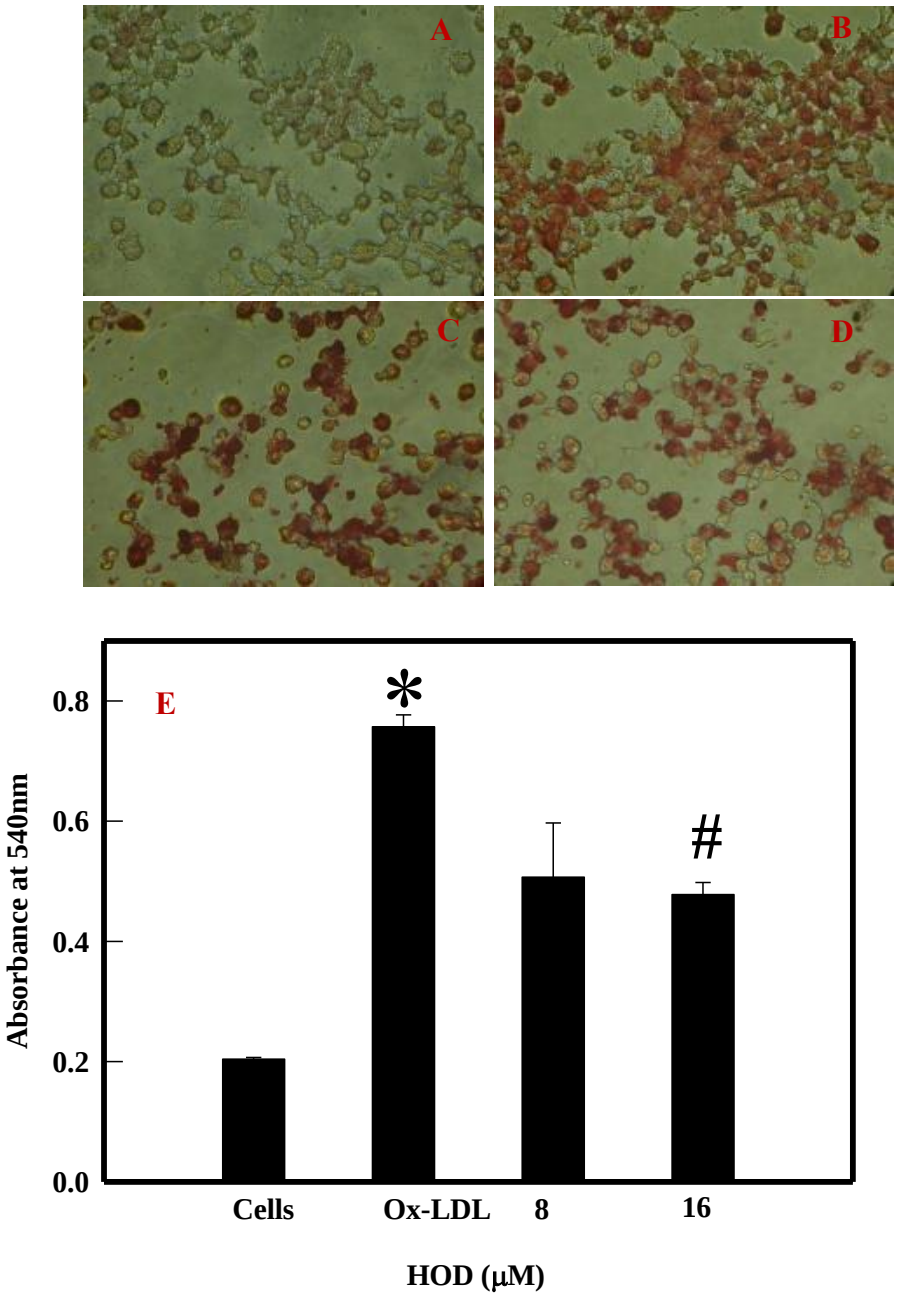


Fig 5.10. Effect of 20-HOD on ox-LDL induced foam cell formation from differentiated (THP-1) macrophages. PMA differentiated THP-1 cells were stimulated with ox-LDL (50 µg/mL) for 48h in presence of HOD (8, 16 µM) and stained with oil red stain for foam cells. Images for foam cells were visualised under inverted microscope. A. Cells, B. Ox-LDL, C. HOD (8 µM), D. HOD (16 µM). Further Spectrophotometric quantification of oil red stain at 540 nm was measured (F).

20x after staining with oil red stain **(Fig. 5.10 B)**. 20-HOD at 16 µM effectively inhibited ox-LDL induced foam cell formation **(Fig. 5.10 D)**. Oil red stain quantification further quantitated inhibitory effect of 20-HOD on foam cell formation at 16 µM concentration by measuring the absorbance of the dye spectrophotometrically at 540 nm **(Fig. 5.10 E)**.

Effect of mixture of metabolites (20-HOD+SGY+QUE and 20-HOD + SGY +PAL) on LPS induced proinflammatory markers

TCAE and TCWE mimicking mixtures H+S+Q and H+S+P attenuated TNF-α expression significantly in presence of LPS. THP-1 cells on stimulation with LPS enhanced the gene expression (24.22 fold change) of TNF-α whereas pretreatment of THP-1 cells with H+S+Q (TCAE mimicking mixture) and H+S+P (TCWE mimicking mixture) attenuated TNF-α to 14.8 and 13.6 fold changes **(Fig. 5.11)**.

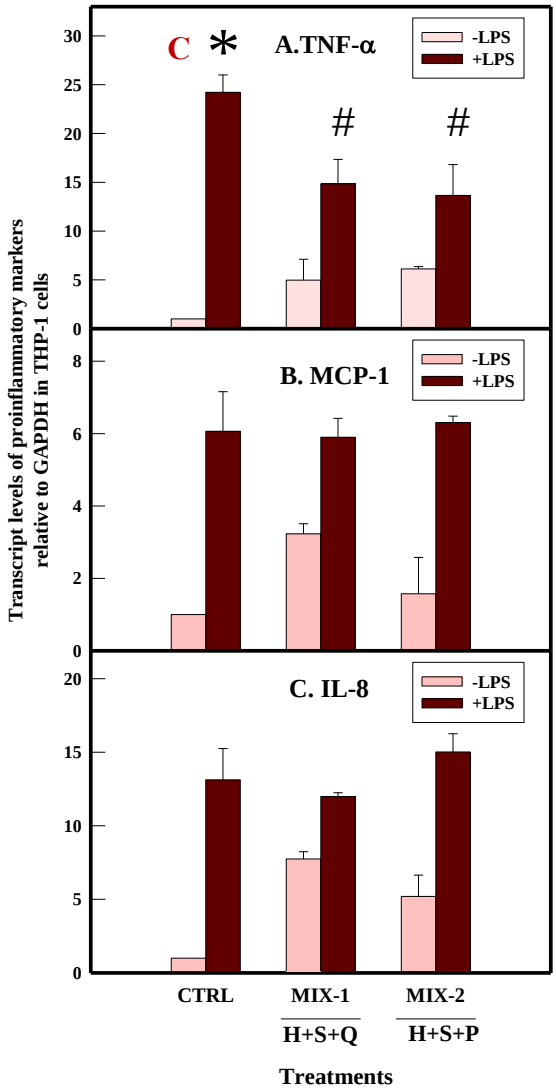


Fig. 5.11. Effect of cumulative mixtures on LPS induced proinflammatory markers in THP-1. THP-1 cells pre-treated with mixture of metabolites (H+S+Q, H+S+P) and induced with LPS for 3 h. At the end of the treatment, cells were spun, isolated RNA using Trizol reagent. Transcript and protein level of proinflammatory genes were quantified by RT-PCR. Data represent mean ± SD of 3 independent experiments and # $p<0.001$ compared between treated mixture and untreated with controls, *$p<0.001$ between the control groups.

Effect of cumulative mixture 20-HOD+SGY+QUE on LPS induced NF-κB translocation

Transcripts of various proinflammatory cytokines were regulated by redox-sensitive transcription factor NF-κB. Western blotting studies in **Fig. 5.12** shows the stimulation of cells with LPS translocates NF-κB into the nucleus as opposed to control cells, retained the protein in an inactive state in the cytosol. However, pretreatment of THP-1 cells with 20-HOD (8 μM) alone showed marginal attenuation whereas 8 μM HOD in combination with syringin (1.7 μM) and quercetin **(**0.8 μM) significantly sequestered NF-κB in the cytosol in inactive state similar to un-induced control cells. Similar results were yielded with 0.4 mg dwt /mL of TCAE as shown in **Fig. 4.11**

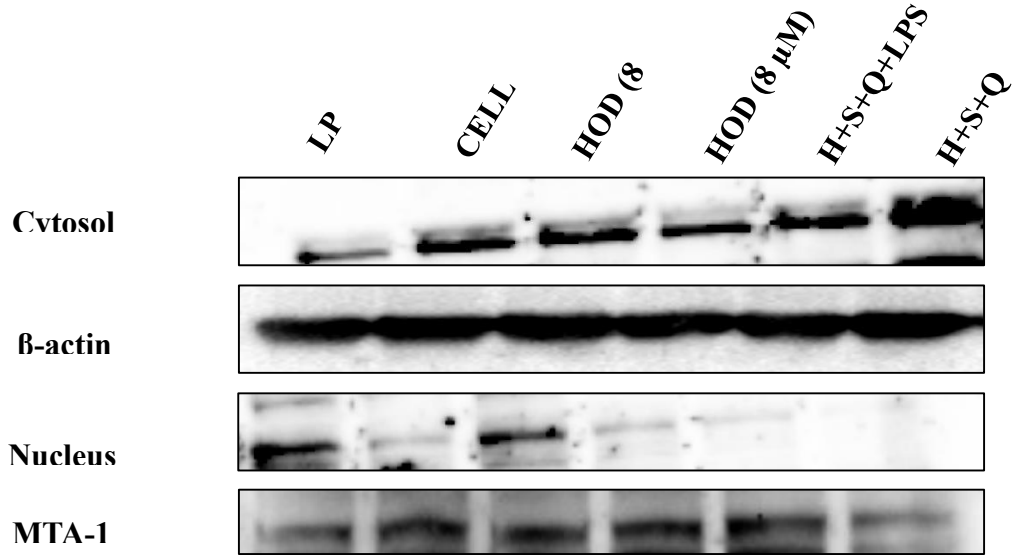


Fig. 5.12. Effect of HOD and H+S+Q on LPS induced NF-κB P-65 subunit translocation. THP-1 cells stimulated with LPS (0.5μg/mL) in presence of HOD (8 μM)/ 8 μM HOD in combination with syringin (1.7 μM) and quercetin **(**0.8 μM) and sub cellular localisation of p-65 subunit were detected by western blotting analysis. Proteins of equal amount were loaded to detect the expression of β-actin and MTA-1 an internal references for cytosol and nuclear extracts.

Discussion

Out study carried in the first objective with hydroalcoholic and water extracts of *T. cordifolia* revealed that both the extracts had significantly inhibited TNF-α secretion by LPS induced THP-1 cells, however, TCAE was more effective in attenuating NF-κB translocation. In order to know these differential effects of TCAE and TCWE, the extracts were subjected to metabolite profiling by LC-MS. Phytochemical analysis by LC-MS revealed the presence of metabolites belonging to the classes of alkaloids, phenols and phytosteroids in both the extracts. **(Table. 5.1, 5.2 and Fig. 5.1)**. However, abundance of the metabolites greatly varied among the two types of extracts. We selected 2 metabolites for further analysis, (1) 20-Hydroxyecdysone (HOD) which is a phytosteroid, highly abundant in TCAE compared to TCWE and (2) syringin (SGY), which is equally got extracted into TCAE and TCWE.

Phytosteroids are known for their antioxidant and antinflammatory activities. Recent reports has shown that ethanopharmacological properties of plants *Vitex doniana* (Ochieng et al., 2013) and *Leuzea charthamoides* (Peschel et al., 2011) extracts in animal models are attributed due to the richness of phytoecdysteroids. HOD is also abundant in several other medicinal plants like *Achyranthes bidentata* Blume and *Cyanotis arachnoidea* which were reported to possess strong antioxidant property in scavenging free radicals both *in vitro* and *in vivo* (Hu et al., 2012n). 20-HOD was shown to exhibit significant antilipidperoxidation activity in rat microsomal and liver mitochondria similar to vitamin D (Kholodova et al., 1997). In another study, HOD was shown to possess antilipidperoxidation similar to thylenediamine tetraacetate and diethyl paraphenylenediamine inhibitors (Cai et al., 2002). It also shown to exhibit neuroprotection by scavenging oxidative stress through NF- κB pathway (Hu et al., 2012).

Syringin or eleutheroside-B is a glycoside of synapyl alcohol found in the stem bark of *Eleutherococcus senticosus* is reported to be responsible for radical scavenging potential of Eleutherococcus extracts. Syringin in *Eleutherococcus* extracts reduced H_2O_2 oxidative damage in rat cardiomyocytes by antilipidperoxidation activity and upregulating antioxidant enzymes (Liang et al., 2009). Glycosides of *Eleutherococcus* plant extracts are shown to be responsible for providing protection against oxidative stress induced by CCL_4, acetaminophen (Lin and Huang, 2000) and ter-butyl hydroperoxide (Wang et al., 2010) in mice. Syringin also attenuated *in vitro* cytochrome P450 activity in microsomes of rat liver (Guo et al., 2014) and reported to possess neuroprotection potential against oxidative stress induced by Abeta (Yang et al., 2010). Having known antioxidant potential of these compounds but no reports with THP-1 cells in the context of oxidative stress and atherosclerosis, prompted us to investigate their activity in arachidonic acid induced THP-1 cells. Our Results demonstrated ROS scavenging potential of 20-HOD and SGY in THP-1 cells induced by arachidonic acid **(Fig. 5.4)**. Further 20-HOD upregulated CAT and GPx gene expression and also significantly, promoted enzyme activity of catalase in AA induced THP-1 cells **(Fig. 5.5 and 5.6)**.

Antiinflammatory effect of 20-HOD is reported in the literature using cellular and animal models however there are no reports in THP-1 cells.. A scientific study has shown that 20-HOD is an abundant marker metabolite in fermented *Achyranthes japonica* while reporting its antiinflammatory activities in LPS activated RAW cells (Lee et al., 2012). Similarly, plant *Quinona* enriched with 20-HOD reduced obesity through modulation of adipokines expression (Foucault et al., 2012). Hypoglycemic nature of 20-HOD has been demonstrated in glucagon, alloxan and high fat diet treated mice (Chen et al., 2006; Kizelsztein et al., 2009; Yoshida et al., 1971). Syringin was also reported to reduce inflammatory markers like INOS, COX-2, prostaglandins-E2 and matrix metalloproteinases (MMP) (Jung et al., 2007; Yamazaki et al., 2007) . Syringin enriched stem extracts of *Eleutherococcus* showed antinflammatory activity

by supressing INOS in LPS + γ-interferon activated RAW cells *via* NF-κB pathway (Lin et al., 2008). There are strong evidences proving that diabetes and obesity promote cardiovascular inflammation due to the secretion of proinflammatory cytokines which in turn causes thrombosis. Hence inhibitors to proinflammatory cytokines are therapeutics in treating various metabolic diseases. In the present study, 20-HOD and SGY were validated for their anti-inflammatory effects with respect to the markers TNF-α, MCP-1, IL-8 and IL-6 of THP-1 cells induced by LPS. 20-HOD effectively attenuated TNF- α and IL-6 whereas SGY showed marginal inhibitory activity towards TNF- α. MCP-1 and IL-8 were unaffected by both the metabolites at protein and transcript levels in presence of LPS **(Fig. 5.7 and 5.8)**. Similarly metabolite mixtures were tested for their inhibitory activity towards TNF-α and observed both the mixtures mimicking TCAE and TCWE attenuated TNF-α significantly **(Fig. 5.11)**.

Our study using western blot and immunofluorescent studies unveiled the molecular mechanism of 20-HOD and cumulative mixture (H+S+Q) in attenuating LPS induced TNF-α through the attenuation of p-65 NF-κB subunit translocation into the nucleus (**Fig. 5.9 and 5.12)**.

Foam cells play a critical role in the pathogenesis of vascular inflammatory diseases like atherosclerosis. LDL passes through the endothelium and prone to oxidation in lumen of artery. Ox-LDL induces activated macrophages to intake lipid molecules by means of scavenging receptors and forms lipid loaded macrophages known as foam cells. Foam cells initiates the formation of atherosclerotic plaque which latter ruptures and leads to thrombosis. In this context of 20-HOD w highly effective in inhibiting Ox-LDL induced foam cell formation from THP-1 cells **(Fig. 5.10)**. In summary, our study concludes that 20-HOD is a potent inhibitor of activation of human monocytic cells in response to oxidative stress and inflammatory stimuli.

Chapter – 6

Results and Discussion

Molecular basis for antioxidant and antiinflammatory activities of Berberine (BBR), an alkaloid of *T. cordifolia* in activated human monocytes (THP-1)

Chapter-6

Results and Discussion

Results

Cell viability of Berberine – MTT assay

Cell viability of the THP-1 cells treated with various concentrations of berberine were tested by MTT assay. Cell viability of the tested concentrations (up to 50 µM) were >95 %. Based on the cytotoxicity results, berberine concentration restricted to 50 µM in THP-1 cells (**Fig. 6.1)**.

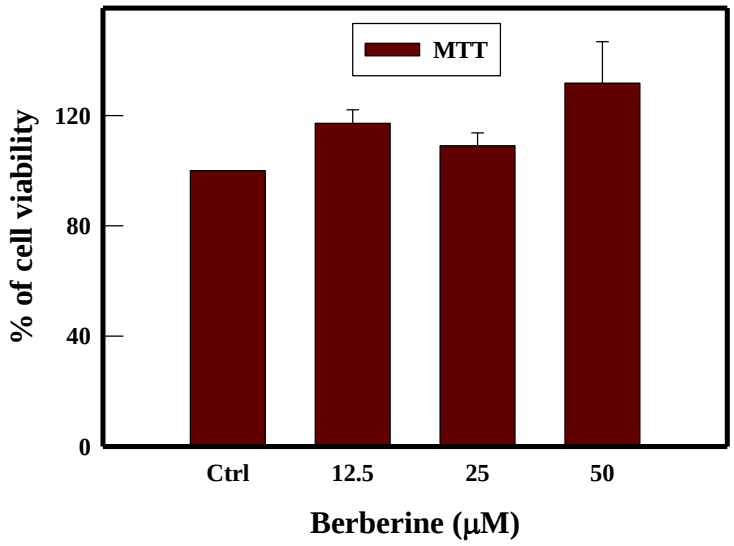


Fig. 6.1. Effect of berberine on cell viability of THP-1 cells by MTT assay. Cell viability after treating with different concentrations of berberine ranging from 12.5-50 µM for 16 h at 37 °C and 5 % CO_2 was checked by MTT assay. Media alone treated with berberine were used as respective blanks. Data represent mean ± SD of 3 independent experiments.

Effect of berberine on AA induced ROS and antioxidant enzymes in THP-1cells

Cells on stimulation with arachidonic acid induced ROS production in THP-1 cells **(Fig. 6.2 B)** whereas pretreatment with BBR (25, 50 µM)) attenuated arachidonic acid induced ROS generation. Increase in H_2DCF-DA fluorescence is directly proportional to increase in

intracellular ROS. Confocal studies have shown that BBR pretreated cells did not show increase in H_2DCF-DA fluorescence in the presence of arachidonic acid **(Fig. 6.2 C-F)**. Spectrofluorimetric studies further revealed the radical scavenging activity of berberine in a dose dependent manner **(Fig. 6.2 G)**. Cells induced with AA had increased fluorescence by five folds to that of control cells (un-induced). BBR treated cells showed only 4 and 2 fold increase at 25 and 50 µM concentrations respectively. Cells alone pretreated with berberine and dye were also tested.

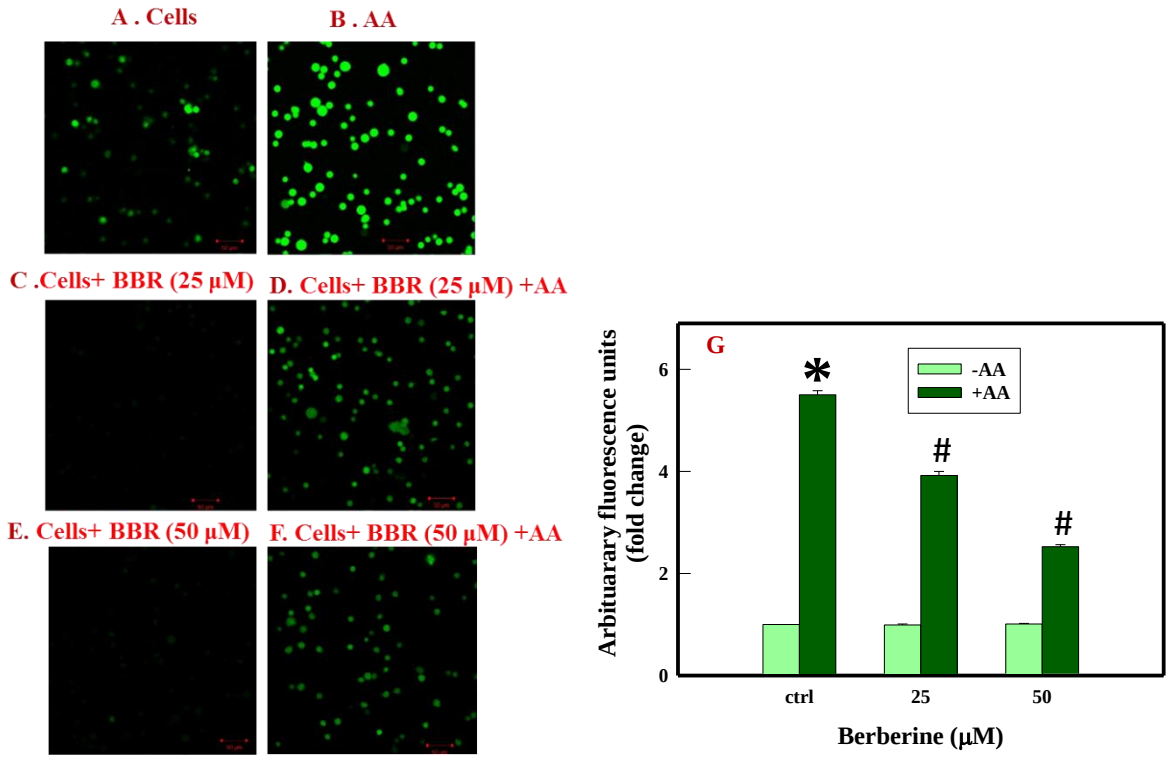


Fig. 6.2. Measurement of arachidonic acid induced ROS. THP-1 cells were pretreated with berberine prior to induction with arachidonic acid (100 µM) for 10 min followed by incubation with 5µM H_2DCF dye for 15 min. After incubation cells were washed twice with growth medium. Fluorescence images were captured by confocal microscope (5.2 A-F) and further fluorescence intensity was measured by spectrofluorimetry (5.2 G). Data represent mean ± SD of 3 independent experiments and # $p<0.001$ compared between treated test compound and untreated with the controls, *$p<0.001$ between the control groups.

Berberine has effectively upregulated antioxidant enzymes such as CAT (2.41 folds), GPx (3.14 folds) and SOD (1.95 folds) at 50 µM concentration in presence of arachidonic acid which correlates with ROS scavenging activity of BBR. At 25 µM BBR, only CAT transcripts were significantly increased and the increase in the other transcripts were not significant. Cells induced with arachidonic acid alone has little or no effect on the above transcripts when compared to normal control cells **(Fig. 6.3)**. Since CAT transcript was upregulated, catalase enzyme activity in THP-1 cell lysates was also increased by BBR at 50 µM concentration **(Fig. 6.4)**. Catalase protein activity was increased by BBR at 50µM concentration to 1.80 folds in presence of arachidonic acid whereas untreated control cells (1 fold) and arachidonic acid induced cells (1.03 folds) did not show any significant effect on catalase activity.

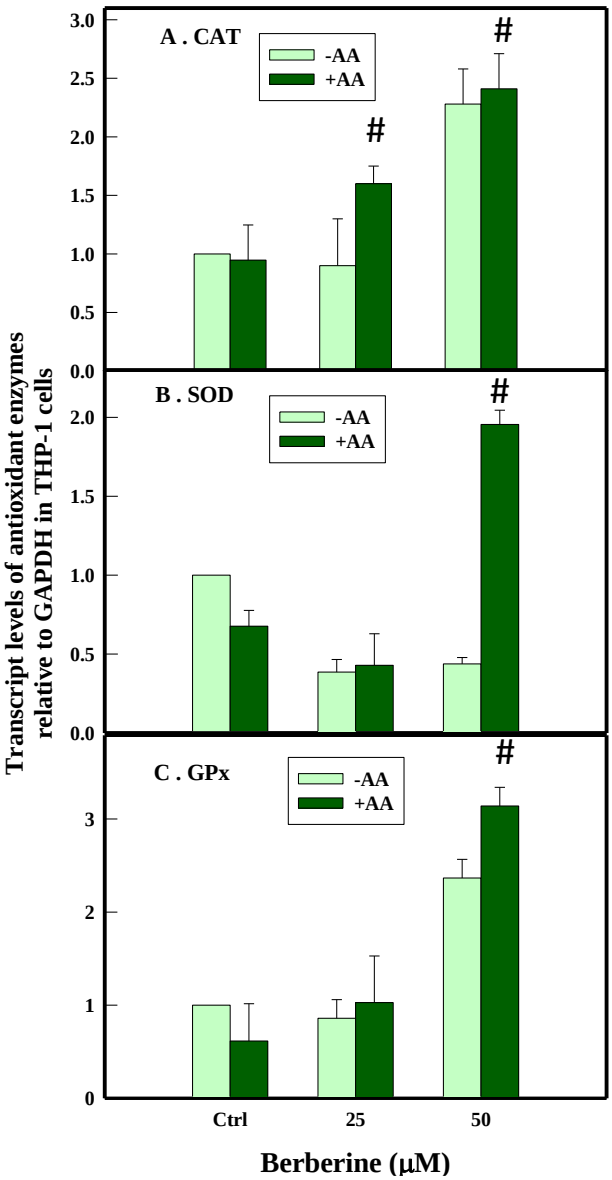


Fig. 6.3. Real time measurement of gene transcripts of antioxidant enzymes (CAT, SOD and GPx). THP-1 cells were pretreated with berberine for about 16 h and induced with arachidonic acid (100 μM) for ten min. At the end of the treatment, RNA was isolated using TRIzol reagent. Gene expressions of CAT (A), SOD (B) and GPx (C) were quantified by quantitative real time PCR. Data represent mean of ± SD of 3 independent experiments. # $p<0.001$ compared between untreated and treated test compound with the controls, *$p<0.001$ between the control groups.

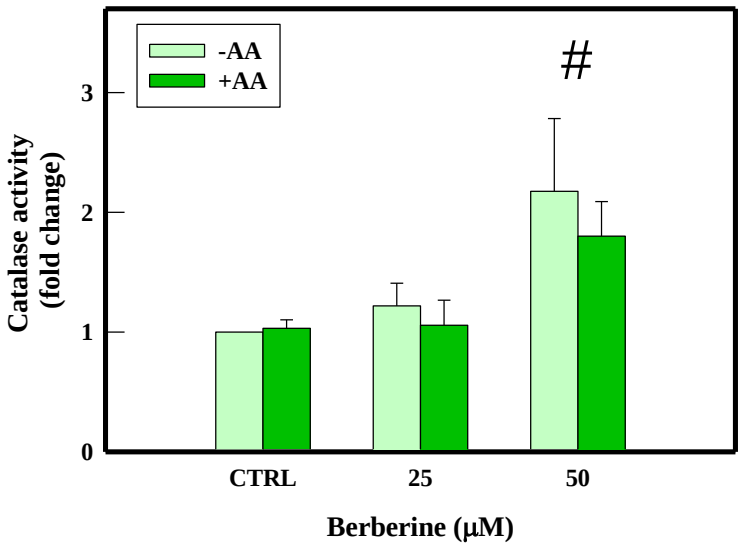


Fig. 6.4. Effect of BBR on catalase activity in THP-1 cells exposed to arachidonic acid. THP-1 cells were treated with BBR and induced with arachidonic acid (100 µM) for ten min. At the end of the treatment, cell lysates were tested for catalase activity on exposure to 7.5 mM H_2O_2 using multimode microplate reader. Rate of consumption of H_2O_2 i.e. decrease in absorbance at λ 240 nm was measured. Data represent mean of ± SD of 3 independent experiments. # $p<0.001$ compared between treated test compound and untreated with the controls whereas *$p<0.001$ refers to the significance between induced and non-induced controls.

Effect of berberine on AA induced proinflammatory markers

Earlier scientific reports have shown that AA induces the secretion of proinflammatory markers in various cell lines. We tested the inhibitory effect of berberine (25, 50 µM) on AA induced inflammatory cytokines and COX-2 using ELISA and real time PCR. Results clearly shows the above tested markers were significantly attenuated by berberine at 25 and 50 µM. Cells on induction with AA increased the release of MCP-1 (73.94 pg/mL) and IL-8 (29.14 pg/mL) and

pretreatment of cells with berberine significantly inhibited AA induced release of MCP-1 (39 and 16.36 pg/mL) and IL-8 (20.14 and 14.60 pg/mL) shown in **Fig. 6.5 A and B**. Control cells released pg/mL of MCP-1 and pg/mL of IL-8. Control/ uninduced cells released 6.99 pg/mL of MCP-1 and 8.49 pg/mL of IL-8.

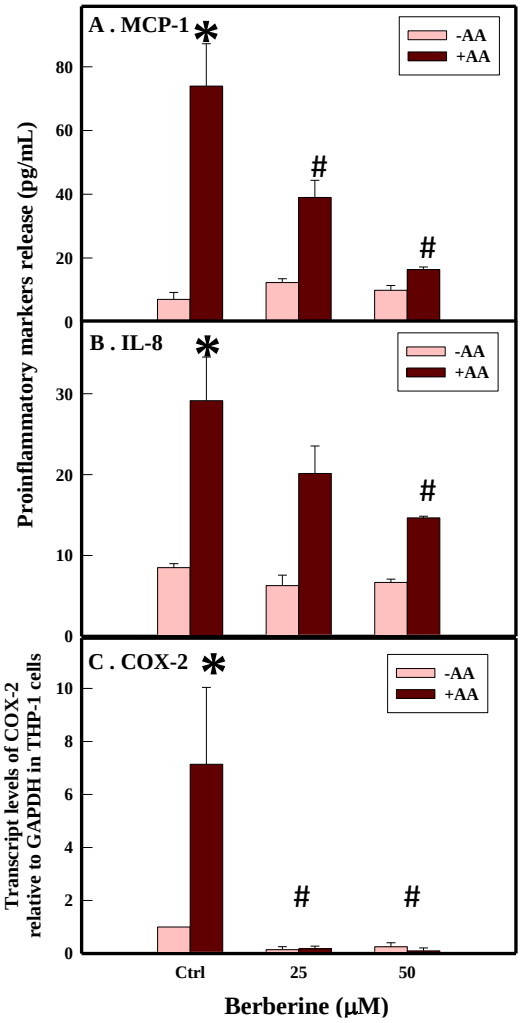


Fig. 6.5. Effect of berberine on AA induced proinflammatory markers. THP-1 cells pre-treated with berberine and followed by AA induction for 24 h. Pelleted cells were subjected to RNA extraction for quantitative real-time PCR and cell supernatants subjected to quantification of protein level. Protein levels of MCP-1 (A) and IL-8 (B) were quantified by ELISA and gene transcripts of COX-2 (C) assayed by RT-PCR. Data obtained represents mean ± SD of 3 independent experiments and # $p<0.001$ signifies the comparison of treated test compound and untreated with the controls whereas *$p<0.001$ between the control groups.

COX-2 transcript was significantly upregulated to 7.14 folds by arachidonic acid when compared to control cells whereas pretreatment with berberine completely attenuated such induction of COX-2 at both 25 µM and 50 µM **(Fig. 6.5 C)**.

Effect of berberine on LPS induced proinflammatory markers

The activated monocytes secrete various proinflammatory cytokines and chemokines (e.g. TNF-α, IL-6, IL-8 and MCP-1) most importantly in atherogenic lesions. LPS induced the secretion of TNF-α (283.24 ± 31 fold change), MCP-1(141 fold change), IL-6 (5.25 ± 0.9) and IL-8 (56.9 fold change) by several folds in THP-1 cells. Pretreatment with berberine significantly attenuated LPS induced secretion of TNF-α (105 ± 10.4 and 86.2 ± 8.9 fold change), MCP-1 (99.5 ± 22.8 and 84.7 ± 16) and IL-6 (1.55 ± 0.3 and 1.54 ± 0.3 fold change) at 25 and 50µM but did not show any protection towards IL-8 as shown in **Fig. 6.6A-D**.

Transcriptional regulation is the control point for the secretion of these inflammatory cytokines at the protein levels. We demonstrated the effect of berberine on m-RNA levels of these genes in THP-1 cells attenuated to a great extent in the presence or absence of LPS (0.5µg /mL). Results in **Fig. 6.7** demonstrates upregulation of TNF-α (7.27 folds), IL-6 (2.27 folds), and MCP-1(5.59 folds) gene transcripts in LPS treated cells when compared to normal control cells whereas pretreatment of berberine at 25 and 50 µM concentrations significantly down regulated MCP-1 (1.46, 1.43 folds) and IL-6 (0.57, 1.70 folds) genes, however, unaffected the induction of TNF-α (8.15, 8.0 folds) transcript in THP-1 cells.

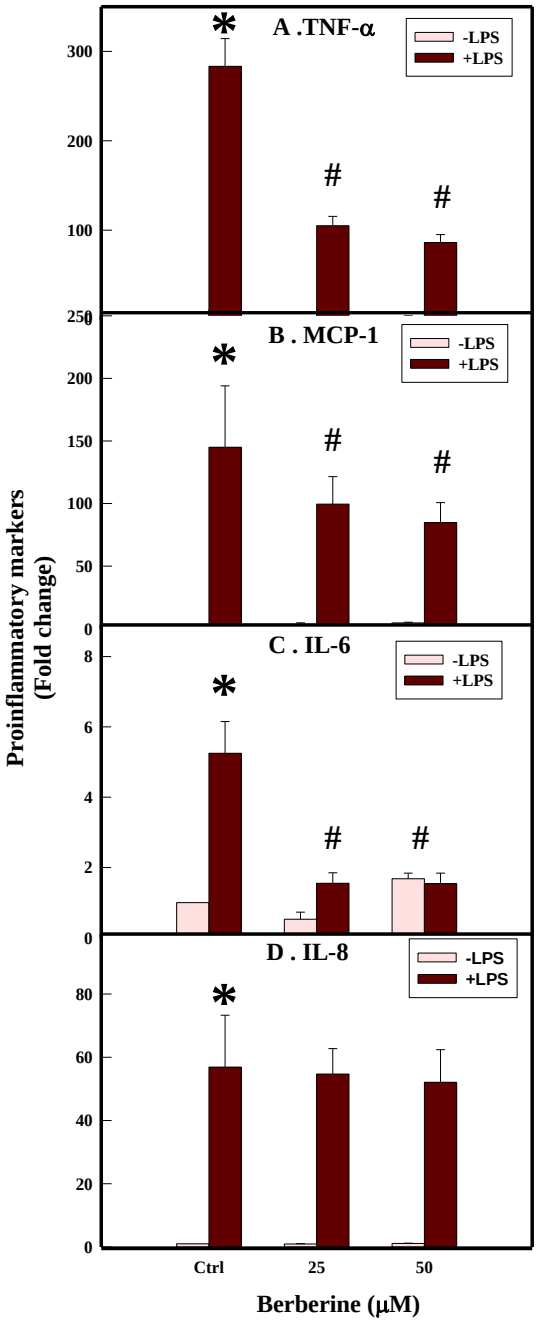


Fig. 6.6. Effect of berberine on LPS induced proinflammatory markers by ELISA.

THP-1 cells pre-treated with berberine and induced with LPS for 3 h. At the end of the treatment, cells were spun and collected the culture supernatant. Protein levels of the markers TNF-α (A), MCP-1 (B), IL-6 (C) and IL-8 (D) were quantified by ELISA. Data represent mean ± SD of 3 independent experiments and # $p<0.001$ compared between treated test compound and untreated with the controls, *$p<0.001$ between the control groups.

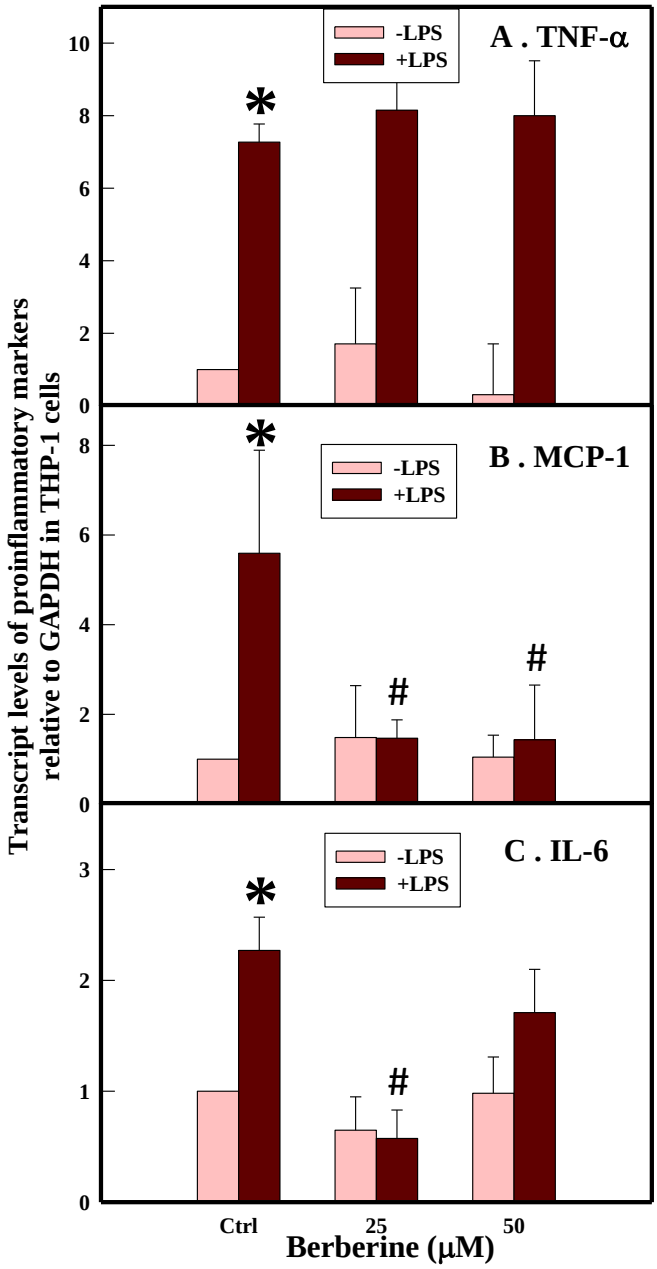


Fig. 6.7. Effect of berberine on LPS induced proinflammatory gene transcripts. THP-1 cells pre-treated with berberine and induced with LPS (0.5 μg/mL) for 3 h. At the end of the treatment, RNA was isolated using TRIzol reagent. Gene transcripts normalized with GAPDH of TNF-α (A), MCP-1 (B) and IL-6 (C) were quantified by real time PCR. Data represent mean ± SD of 3 independent experiments and # $p<0.001$ compared between treated test compound and untreated with the controls, *$p<0.001$ between the control groups.

Effect of berberine on LPS induced NF-κB p-65 translocation in THP-1 cells.

Transcripts of various proinflammatory cytokines were regulated by redox-sensitive transcription factor NF-κB. In order to determine whether BBR would attenuate pro-inflammatory markers expression through the suppression of nuclear translocation of NF-κB, we tested the effect of BBR on NF-κB targeting into the nucleus by Western blot and immuno-fluorescence methods shown in **Fig. 6.8 and 6.9**. As expected, stimulation of THP-1 cells with LPS translocated the p65 subunit of heteromeric NF-κB protein into the nucleus whereas uninduced cells retained the p65 protein in the cytosol. However pretreatment of cells with BBR (25 and 50 μM) attenuated the p65 translocation which is confirmed by the presence of clear bands in the cytosol and marginal reduced band in the nucleus. Cytosolic and nuclear fraction protein samples were confirmed by the presence of marker proteins, GAPDH and histone respectively.

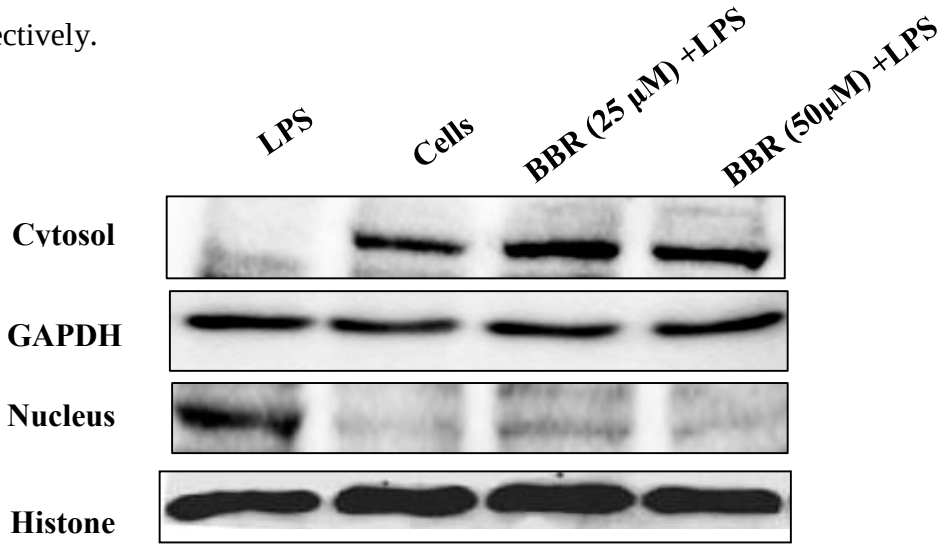


Fig. 6.8. Effect of BBR on LPS induced NF-κB P-65 subunit translocation. THP-1 pretreated with BBR were induced with LPS (0.5 μg/mL) and extracted protein lysate using NE-PER buffer. Lysates were loaded onto 10 % SDS PAGE and transferred to blotting membrane, probed with primary antibody of NF-κB P-65 and anti-rabbit and mouse HRP secondary antibodies. Finally NF-κB levels were detected by ECL reagents using versadoc. For loading controls antibodies against GAPDH and histone were used for cytosolic and nuclear fractions respectively.

In addition Immunofluorescence studies has shown that BBR at 25 and 50 µM clearly sequestered the inactive p65 in the cytosol **(Fig. 6.9 G to L)** which is unlike in LPS induced cells in which most of p65 protein is translocated into nucleus **(Fig. 6.9 D to F)**. Uninduced cells retained p65 protein in an inactive state in cytosol shown in **Fig. 6.9 A to C**. Based on these results it is demonstrated that BBR possess anti-inflammatory activities via attenuating the translocation of p65 into nucleus in THP-1 cells. Nuclei were stained with DAPI and merged fluorescence image distinguished nuclear portions from cytosolic portions of the cells.

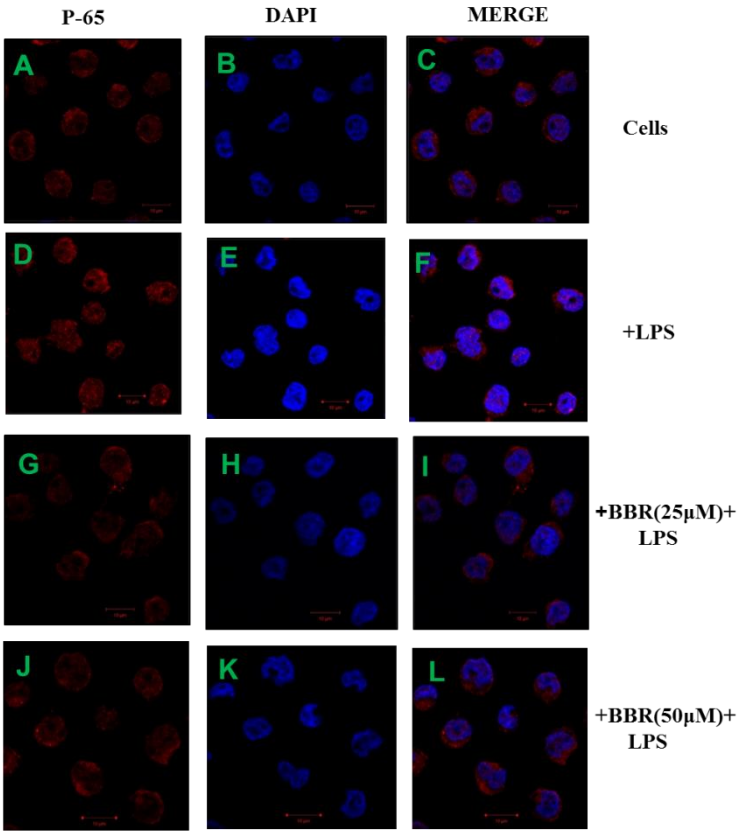


Fig. 6.9. Effect of berberine on LPS induced NF-κB P-65 subunit translocation. THP-1 cells stimulated with LPS (0.5 µg/mL) in presence of berberine (25, 50 µM) and identified P-65 subunit cellular localisation by immunofluorescence assay. p-65 subunit was stained with alexa-fluor 594 conjugated secondary antibody and nucleus was counterstained with DAPI. Images were captured under confocal microscope.

Discussion

Berberine belonging to the class of proto-berberine isoquinoline alkaloids found in many medicinal plants like *Berberis vulgaris* (Imanshahidi and Hosseinzadeh, 2008), *Coptidis rhizoma* (Tang et al., 2009), *Coscinium fenestratum* (Rojsanga et al., 2010), *Hydrastis Canadensis* (Ettefagh et al., 2011) *and Tinospora cordifolia* (Khan et al., 2011). Berberine enriched plants have been used in both Chinese and Indian traditional medicinal for the treatment of cardiovascular, diabetes, neurodegenerative disorders and etc. There is an accumulated evidence in the literature about the pharmacological activities of berberine such as anti-cancer (Hou et al., 2011), cardio protective (Li et al., 2014), anti-atherosclerotic (Chen et al., 2014; Wang et al., 2011), anti-diabetic (Lee et al., 2006), hypolipidemic (Jia et al., 2014), neuroprotective (Cui et al., 2009) and hepatoprotective (Domitrović et al., 2011) properties.

Berberine has been clinically tested to treat chronic inflammatory diseases such as diabetes (Xie and Du, 2011; Yin et al., 2008), hypertension (Affuso et al., 2010) and hyperlipidemia (Kong et al., 2008; Zhang et al., 2008). Clinical studies or applications of isoquinoline alkaloid berberine has been focussed more in recent years. Clinical studies has shown potential therapeutic applications of berberine. Antidiabetic studies in animals and human trials has been successful using berberine which showed effect similar to metformin (a standard pharmaceutical drug in treating type-2 Diabetes). Berberine activated adenosine monophosphate kinase (AMPK) similar to metformin in treating diabetes (Lee et al., 2006). In the year 2008, two clinical trials on 36 diabetic patients in 1^{ST} phase and 48 patients in 2^{nd} phase were done in which subjects were given 500 mg of berberine and metformin separately 3 times /day and observed hypoglycaemic effects similar to metformin (Yin et al., 2008). Similarly 14 human trials of berberine on 1068 patients was successful in showing its hypoglycaemic effect similar to standard drug metformin (Dong et al., 2012). Also a clinical study in china has shown the synergetic action of berberine and metformin in reducing diabetes. Lipid lowering

application of berberine similar to statins was also reported in 32 hypercholesteraemic patients and these studies showed the safety and efficacy of the drug in performing other clinical trials related to cancer, CVD and etc (Kong et al., 2004). Most of the studies projected berberine as an antidiabetic drug but its lipid lowering properties may improve cardiovascular risk by improving endothelial function and suppressing proinflammatory cytokines. Hence we mainly studied antioxidant and antinflammatory properties of berberine in activated THP-1 cells in relevance to vascular disease, atherosclerosis.

Oxidative stress plays a key role in pathogenesis of several human diseases by damaging macromolecules leading to cell death (Uy et al., 2011; Vladykovskaya et al., 2012). Berberine was reported in treating oxidative stress mediated diseases like liver cancer due to its strong antioxidant and hepatoprotective properties (Li et al., 2015). Literature has shown that berberine reduced ethanol and CCL_4 induced liver damage *via* oxidative stress in mouse model through decreased phosphorylation of ERK (Feng et al., 2011; Zhang et al., 2015). Berberine was shown to upregulate antioxidant enzymes (CAT, GPx and SOD) in liver and serum of diabetic rats through the expression of elongation factor which indicates the protective nature of beberine against oxidative stress mediated diabetes (Zhou and Zhou, 2011). With this background we checked the effect of berberine against arachidonic acid induced oxidative stress and inflammation and our results demonstrated ROS scavenging effect of berberine by activating antioxidant enzymes **(Fig. 6.2, 6.3 and 6.4)**. Further, berberine attenuated AA induced proinflammatory markers like MCP-1, IL-8 secretion and COX-2 gene expression in THP-1 cells **(Fig. 6.5)**.

Previous studies of berberine have shown to reduce LPS induced lung injury by attenuating VCAM-1 in both *in vitro* (HUVECs) and *in vivo* (Male Spray Daley rats) models, Literature strongly supports berberine antinflammatory activity (Wang et al., 2012; Spatuzza et al., 2014). Berberine was also reported to possess anticoagulant activity through attenuation

of tissue factor release *via* NF- κb pathway in THP-1 cells (Gao et al., 2014). Recent study has showed that berberine carried out anti-inflammatory effects post transcriptionally against TNF-α and IL-6 in Human blood mononuclear cells (Spatuzza et al., 2014). In extension to these studies we tested the effect of berberine on LPS activated proinflammatory cytokines in THP-1 (monocytes) and elucidated the molecular mechanism, which is the first study to the best of our knowledge. In our study, berberine significantly attenuated LPS induced proinflammatory cytokines in THP-1 cells proving its antiinflammatory potential in monocytic cells **(Fig. 6.6 and 6.7).** Our study also unveiled the molecular mechanism of berberine in attenuating the above LPS induced inflammatory genes both at protein and transcript levels which are NF-κb target genes through the sequestration of inactive p-65 subunit in the cytosol by immunofluorescent and western blotting studies **(Fig. 6.8 and 6.9)**.

Our findings support that therapeutic properties of berberine can be exploited for the treatment of atherosclerosis. However, the concentration of BBR required for antinflammatory activity in THP-1 cells is significantly higher than hydroxyecdysone, which is a phytosteroid abundantly present in aerial parts of *T. cordifolia*.

Chapter -7

Summary and Conclusions

Chapter-7

Summary and Conclusions

Present work was mainly emphasized on (1) evaluation of antioxidant and ant- inflammatory activities of *Tinospora cordifolia* dry leaf extracts in activated human monocytic (THP-1) cells; (2) to identify the active principles responsible for their activities; (3) evaluation of antioxidant and anti-inflammatory activities of berberine, root alkaloid of *T. cordifolia* in activated human monocytic (THP-1) cells. Atherosclerosis is defined as chronic inflammatory vascular disease associated with the hardening and thickening of blood vessel by which blood flow is affected. Pathophysiology of atherosclerosis is complexed and it is widely proved that atherosclerosis is an inflammatory disease, which is strongly affected by oxidative stress and inflammation and primarily due to the interaction between activated monocytic cells in the blood and vascular endothelium. *Tinospora cordifolia* is a shrub that is native to India is known for its various medicinal properties in folk medicine. Its root, stem and leaves are used in Indian traditional Ayurvedic medicine for the treatment of cardiovascular diseases (CVD), diabetes and many inflammatory diseases etc. The following objectives were framed to prove therapeutic properties of TC dry leaf extracts and berberine in relevance to oxidative stress and inflammation at molecular level using human monocytic (THP-1) cells.

Objectives of the present study:

Following objectives were designed for the present study with the selection of *Tinospora. cordifolia* leaves based on its traditional applications in Indian medicinal system and its scientific validations :

1. Evaluation of antioxidant and antiinflammatory properties of *Tinospora cordifolia* dry leaf extracts in activated human monocytic (THP-1) cells.
2. Metabolite profiling of *T. cordifolia* leaf extracts and identification of active metabolites responsible for their effects in activated human monocytes (THP-1).

3. Molecular basis for antioxidant and anti-inflammatory activities of berberine (BBR), an alkaloid of *T. cordifolia*, in human monocytic (THP-1) cells.

Results

The results of the above mentioned experiments are organised into three sections, which correspond to the three objectives of the study.

Objective 1: Evaluation of antioxidant and antinflammatory activities of *Tinospora cordifolia* dry leaves in activated human monocytic (THP-1) cells.

Estimation of total phenolic and flavonoid content in TC extracts

Water and hydroalcoholic extracts of dry leaves of *Tinospora cordifolia* (TCWE and TCAE) showed positive for phenolics and flavonoids upon estimation using FC reagent and aluminium chloride ($AlCl_3$) methods. Total phenolic and flavonoid content of both the extracts were obtained by extrapolating standard curves of gallic acid and quercetin respectively. TCWE showed total phenolic and flavonoid content of 3.1 ± 0.3 mg GAE/g dw and 0.178 ± 0.05 mg QE/g dw whereas TCAE exhibited 3.56 ± 0.5 mg GAE/g dw and 0.307 ± 0.1 mg QE/g dw. In brief, both modes of extracts had similar amounts of total phenolic content but TCAE had higher flavonoid content when compared to TCWE.

Total antioxidant activity

Total antioxidant activity was evaluated by ammonium molybdate reagent and revealed that both TCWE and TCAE had significant antioxidant activity. TCWE showed an antioxidant activity of 3.55 ± 0.2 mg ASE/g dw whereas TCAE showed 4.0 ± 0.3 mg ASE/ g dw.

Free radical scavenging studies of TC extracts

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

Both TCAE and TCWE effectively scavenged DPPH in a concentration dependant manner. TCWE and TCAE scavenged 90 % of DPPH radical with an IC_{50} values of 3.8 ± 0.4 µg dw/ mL and 11.9 ± 2.2 µg dw/mL. When compared to IC_{50} values, TCAE is more effective in

scavenging DPPH than TCWE. Gallic acid is used as a positive reference which scavenged DPPH radical effectively in a concentration dependent manner with IC_{50} being 1.32µg/mL.

Hydrogen peroxide (H_2O_2) scavenging assay

Dry leaf extracts of *T. cordifolia* tested for its *in vitro* radical scavenging potential towards hydrogen peroxide. Both TCWE and TCAE showed efficient scavenging towards H_2O_2 with the increase in concentrations. TCWE and TCAE scavenged 90 % of H_2O_2 with an IC_{50} Values of 18 ± 2.7 µg dw/mL and 5.4 ± 0.9 µg dw/mL. Standard metabolite gallic acid scavenged H_2O_2 in a concentrated dependent manner with 50% scavenging at 4.4 µg/mL.

Anti-lipidperoxidation activity of TC extracts in rat tissue homogenates

TC extracts exhibited potent anti-liver and heart lipid peroxidation against $Fecl_3$ stimulation in a concentrated dependent manner. Both TCWE and TCAE exhibited 90 % inhibition of lipid peroxidation in liver homogenate with an IC_{50} values of 37 µg dwt/mL and 56 µg dwt/mL respectively. TCWE and TCAE also scavenged lipid peroxide formation in heart homogenate effectively with 50 % inhibition at 1.1 and 0.64 mg dwt/mL. These results showed the efficacy of TC extracts in suppressing the levels of lipid peroxides in rat liver and heart homogenates. Reference compounds like gallic acid and ascorbic acid are used as positive controls for liver and heart lipid peroxidation. Gallic acid exhibited 90 % anti-lipidperoxidation activity in liver tissue with an IC_{50} value of 6 µg/mL whereas ascorbic acid exhibited 80 % antiheart lipidperoxidation with an IC_{50} value of 6.97µg/mL.

Effect of T. cordifolia on cell viability - MTT assay

Cytotoxicity of TC extracts on THP-1 cells was determined by MTT assay. In this study, dosage of the extracts for cell treatments were determined, that up to 0.8 mg dwt/mL of both TCWE and TCAE showed no toxic effect on THP-1 cells, cell viability was more or less equal to the control cells. Hence subsequent experiments, dosage of plant extracts for cellular treatments did not exceed 0.8 mg dwt/mL.

Effect of T. cordifolia on arachidonic acid induced ROS and antioxidant enzymes in THP-1 cells

We evaluated the efficacy of plant extracts in attenuating arachidonic acid (AA) induced ROS generation in monocytic (THP-1) cell line. Exposure to AA of 100 µM concentration significantly generated intracellular ROS to 1.6 folds compared to the unexposed cells. Pretreatment of cells with TCAE or TCWE markedly attenuated the AA induced ROS generation, in a dose-dependent manner. Elevated transcripts of antioxidant enzymes such as CAT, GPx and SOD correlated with lower levels of intracellular ROS in TC extracts pretreated cells. Enzymatic activity of catalase was also increased by plant extracts in THP-1 cell lysates in presence of arachidonic acid. Cells induced with arachidonic acid alone has little or no effect on the above transcripts and catalase activity when compared to normal untreated control cells.

Effect of dry leaf extracts of T. cordifolia on LPS induced proinflammatory markers in THP cells

Proinflammatory markers like TNF-α, MCP-1, IL-6 and IL-8 were secreted by activated monocytes which plays a key role in the formation of atherogenic lesions in vascular wall. Proinflammatory cytokines / chemokines in pretreated cell culture supernatants were measured by ELISA. Induction of THP-1 cells with LPS for respective time points significantly enhanced the release of the proinflammatory mediators like TNF-α (2111.46 pg/mL: 283.24 ± 31 fold change), MCP-1 (1031 pg/mL: 26.5 ± 0.1 fold change), IL-6 (30.42 pg/mL: 5.25 ± 0.9 fold change) and IL-8 (1693.18 pg/mL: 145 ± 49 fold change). Such LPS dependent induction in TNF-α secretion was significantly inhibited by TCWE and TCAE pretreatments, however, did not show the inhibitory effect on MCP-1, IL-8 and IL-6 release. LPS induced TCWE treated cells released 1075 pg/mL (156.5 ± 5.41fold change), 1199 pg/mL (174.58 ± 19.441fold change) of TNF-α at concentrations of 0.4 and 0.8 mg dwt /mL whereas TCAE secreted 1424 pg/mL (177 ± 6.8 fold change) and 1332 pg/mL (152 ± 0.08 fold change).

Effect of T. cordifolia dry leaf extracts on LPS induced transcripts of proinflammatory markers in THP-1 cells

Cells induced with LPS for 3 h enhanced the transcripts of TNF-α (4.8 folds), MCP-1 (8.4 folds, IL-6 (2.43 folds) and IL-8 (148.47 folds) genes significantly when compared to normal control cells. Both TCWE and TCAE pretreatments decreased the expression of TNF-α gene transcript (1.99, 1.33 folds) significantly whereas MCP-1, IL-6 and IL-8 transcripts were not inhibited by plant extracts in the presence of LPS.

Effect of T. cordifolia dry leaf extracts on LPS induced NF-κB Translocation/ I-κB degradation in THP-1 cells

Expression of many proinflammatory genes were controlled by NF-κB signalling pathway. We tested the effect of plant extracts on NF-κB targeting into the nucleus by western blotting and immuno-fluorescence methods. As expected, stimulation of THP-1 cells with LPS translocated the p65 subunit of heteromeric NF-κB protein into the nucleus whereas uninduced cells retained the p65 protein in the cytosol. Pretreatment of cells with TCAE (0.4 mg dwt /mL) completely suppressed the p65 translocation, which is confirmed by the presence of clear intensified band in the cytosol and its absence in the nucleus but TCWE (0.4 mg dwt /mL) did not show such response. Further tested the effect of TC extracts on LPS induced phosphorylation and degradation of I-κB and found that TCAE significantly inhibited the degradation of I- κB but not TCWE.

In addition immunofluorescence studies has shown that TCAE pretreatment helped sequestering the inactive p65 in the cytosol of LPS treated cells similar to uninduced cells. Based on these results it is demonstrated that TCAE possess anti-inflammatory activities against LPS *via* inhibiting p65 translocation in THP-1 cells.

Effect of T. cordifolia extracts on PMA induced monocyte to macrophage differentiation

Human monocytic THP-1 cells are spherical and suspension in nature and do not adhere to the plastic surfaces of the culture plates. On stimulation with PMA (25 ng/ml) for 48 h, monocytic cells were differentiated into macrophage like cells which adhered to substratum of the culture dish. Such PMA induced differentiation process was not inhibited by the plant extracts, of TCAE or TCWE (0.4 and 0.8 mg dwt/mL). Cell differentiation markers, toll- like receptors-4 (TLR 4) was upregulated when treated with TC extracts in the presence of PMA. Therefore, further studies were not focused on this phenomenon.

Objective 2: Metabolite profiling of *T. cordifolia dry* leaf extracts and identification of active metabolites responsible for their effects in activated human monocytes (THP-1).

Metabolite profiling of T. cordifolia dry leaf extracts and quantification of 20-hydroxyecdysone

LC-QTOF/MS was employed in identifying the phytochemicals or active metabolites responsible for biological activities of TC extracts. Positive ion and negative ion modes of LC-QTOF/MS detected 11 abundant metabolites including both TC extracts. Compounds were confirmed based on the MS-MS fragmentation spectra of the metabolites. Of these, tembetarine, 20-hydroxyecdysone, magnoflorine and syringin were commonly detected in both TCAE and TCWE. Within the detection limits, pantothenic acid, quercitin, and N-trans-ferulotyramine were exclusively detected in TCAE while columbine, palmatine and tetrahydropalmatine were detected in TCWE.

20-Hydroxyecdysone (20-HOD) in TCAE was most abundant in TCAE, therefore, LC-DAD method with gradient elution was employed to quantify the marker metabolite 20-hydroxyecdysone (20-HOD) in TCAE. Under experimental conditions standard compound 20-

HOD was eluted at RT (retention time) 17 min. Standard calibration curve of 20-HOD ranging from 0.1 -0.5 ng/µL was constructed by measuring peak areas. LC- MS analysis of TCAE (0.4 and 0.8mg dwt/mL) showed peaks corresponding to standard 20-HOD with retention times of 17.12 and17.19 min respectively. Using standard regression plot, concentrations of 20-HOD in TCAE of 0.4 and 0.8 mg dwt/mL, was found to be 8 and 16 µM respectively. Besides 20-HOD, other metabolites like syringin (SGY) also tested for their antioxidant and anti-inflammatory activity in THP-1 cells.

Dosage of 20-HOD and SGY for THP-1 cell treatments-MTT assay

Cytotoxicity of 20-HOD (8, 16 µM) and SGY (50 µM) on THP-1 cells treatments were determined based on MTT assay and trypan blue exclusion methods. Results of MTT assay showed that the above mentioned concentration of 20-HOD amd SGY did not show cytotoxicity, cell viability was more or less equal to control or untreated cells (>95 %).

Effect of 20-HOD/ SGY on AA induced intracellular ROS and antioxidant enzymes (CAT, GPx and SOD) in THP-1 cells

Cells on stimulation with arachidonic acid induced ROS production in THP-1 cells whereas pretreatment with 20-HOD (8, 16 µM) and SGY (50 µM) attenuated arachidonic acid stimulated ROS production. Cells on stimulation with AA had increased fluorescence intensity by 3.92 folds to that of control cells (un-induced). THP-1 cells, on pretreatment with 20-HOD (1.84 fold) or SGY (1.18 fold) inhibited ROS generation significantly.

20-HOD increased the gene expression of antioxidant enzymes such as CAT (2.79 folds) and Gpx (4.20 folds) at 16 µM in presence of arachidonic acid which correlated with ROS scavenging activity of 20-HOD. Catalase in THP-1 cell lysates was significantly increased by 20-HOD at 16 µM concentration.

Effect of 20-HOD and SGY on LPS induced proinflammatory markers in THP-1 cells

It is well established that LPS induces the secretion of proinflammatory markers by various cell lines including THP-1 cells. We tested the inhibitory effect of 20-HOD (8, 16 µM) and SGY (50 µM) on LPS induced inflammatory cytokines using ELISA. Our results clearly shows the attenuation of the TNF-α was significant by 20-HOD whereas IL-6 and IL-8 secretions were unaffected. As compared to cells on induction with LPS release of TNF-α (283.24 ± 31 fold increase) whereas treatment of 20-HOD in presence of LPS significantly attenuated the release of TNF-α to 146 ± 4.1 and 145 ± 8.2 fold change, whereas SGY was not equivalently effective (205 ± 1.7 Fold change). Therefore, further experiments were continued with 20-HOD.

Effect of 20-HOD on of LPS induced proinflammatory transcripts in THP-1 cells

Transcriptional regulation is the control point for the secretion of these inflammatory cytokines at the protein levels. Our results demonstrated that TNF-α (24.2 folds), IL-6 (1.98 folds) and IL-8 (8.87 folds) gene transcripts were significantly upregulated in LPS treated cells when compared to normal control cells whereas pretreatment of 20-HOD significantly inhibited TNF-α expression (14.62 folds) at 16 µM concentration, however unaffected the expression of IL-6 and IL-8 transcripts induced by LPS in THP-1 cells at both 8 and 16 µM concentrations, which is in agreement with the results obtained from TCAE treated THP-1 cells.

Effect of 20-HOD on LPS induced NF-κB translocation

Transcripts of various proinflammatory cytokines were regulated by redox-sensitive transcriptional factor NF-κB. Pretreatment of the cells with HOD at 16 µM concentration strongly inhibited LPS dependent translocation of NF-κB into the nuclei whereas 8 µM showed marginal inhibition.

Effect of 20-HOD on Ox-LDL induced Foam cell formation

Foam cell formation was considered to be crucial in the pathogenesis of atherosclerosis, therefore in our study we tested the effect of 20-HOD (8 and 16 µM) on foam cell formation in THP-1 cells using oil red staining method. Up on stimulation of macrophages (PMA differentiated THP-1 cells) with Ox-LDL (100µg/mL) for 48 h induced the formation of foam cells when visualised under microscope at 20x after staining with oil red stain. 20-HOD at 16 µM effectively inhibited Ox-LDL (100µg/mL) induced foam cell formation. Oil red stain quantification measured spectrophotometrically at 540 nm for calculating statistical significance.

Effect of metabolite mixture (20-HOD+SGY+QUE) on LPS induced proinflammatory markers

TCAE mimicking metabolite mixture *20-HOD+SGY+QUE* attenuated TNF-α expression significantly as effective as TCAE in the presence of LPS by THP-1 cells. Similar to the effect of TCAE, metabolite mixture markedly attenuated LPS dependent induction of TNF-α at gene transcript level and did not inhibit gene expression of MCP-1 and IL-8 markers.

Effect of cumulative mixture 20-HOD and 20-HOD+SGY+QUE on LPS induced NF-κB translocation

Immunoblotting results shows the stimulation of THP-1 with LPS translocate NF-κB into the nucleus unlike control cells, which retained the protein in an inactive state in the cytosol. However, pretreatment of THP-1 cells with HOD (8 µM) alone showed marginal attenuation whereas cumulative mixture HOD+SGY+QUE **(**H+S+Q) significantly sequestered NF-κB in the cytosol in inactive state similar to un-induced control cells. These results were similar to the results obtained with 20-HOD of 16 µM.

Objective 3. Molecular basis for antioxidant and anti-inflammatory activities of berberine (BBR), an alkaloid of *T. cordifolia* in activated human monocytes (THP-1)

Cell viability of Berberine – MTT assay

Cell viability of the THP-1 cells treated with various concentrations of berberine were also tested by MTT assay. Cell viability at these concentrations were >95 % was ensured by MTT assay. Based on the cytotoxicity results, berberine concentration restricted to 50 µM in THP-1 cells.

Effect of berberine on AA induced ROS and antioxidant enzymes in THP-1 cells

Cells on stimulation with arachidonic acid induced ROS production in THP-1 cells whereas pretreatment with BBR (25, 50 µM)) attenuated arachidonic acid induced ROS generation. Confocal studies has shown BBR pretreated cells did not show ROS dependent increase in fluorescence when stimulated with arachidonic acid. Spectrofluorimetric studies further revealed the radical scavenging activity of berberine in a dose dependent manner. Cells induced with AA had increased fluorescence by five folds to that of control cells (un-induced). BBR treated cells showed only 4 and 2 fold increase at 25 and 50 µM concentrations respectively. Cells alone pretreated with berberine and dye were also tested.

Berberine has effectively upregulated antioxidant enzymes such as CAT (2.41 folds), GPx (3.14 folds) and SOD (1.95 folds) at 50 µM concentration in presence of arachidonic acid which correlates with ROS scavenging activity of BBR. Cells induced with arachidonic acid alone has little or no effect on the above transcripts when compared to normal control cells. Catalase enzyme activity in THP-1 cell lysates was also increased by BBR at 50 µM concentration.

Effect of berberine on AA induced proinflammatory markers

We tested the inhibitory effect of berberine (25, 50 µM) on AA induced inflammatory cytokines and COX-2 using ELISA and real time PCR. Results clearly shows the attenuation of the above markers was significant by berberine at 25 and 50 µM. Cells on induction with AA increased the release of MCP-1 (73.94 pg/mL) and IL-8 (29.14 pg/mL) and pretreatment of cells with berberine significantly inhibited AA induced release of MCP-1 (39 and 16.36 pg/mL) and IL-8 (20.14 and 14.60 pg/mL).

COX-2 transcript was significantly upregulated to 7.14 folds by arachidonic acid when compared to control cells whereas pretreatment with berberine (25µM and 50 µM) significantly attenuated such induction of COX-2.

Effect of berberine on LPS induced proinflammatory markers

In comparison to the LPS induced the secretion of TNF-α (283.24 ± 31 fold change), MCP-1 (145 ± 49 fold change), IL-6 (5.25 ± 0.9 fold change) and IL-8 (56.9 ± 16.4 fold change) by THP-1 cells, pre-treatment with berberine significantly attenuated such LPS induced secretion of TNF-α (105 ± 10.4 and 86.2 ± 8.9 fold change), MCP-1 (99.5 ± 22, 84.7 ± 16 pg/mL) and IL-6 (1.55 ± 0.3 and 1.54 ± 0.3 fold change) at 25 and 50 µM but did not show any protection towards IL-8.

Since transcriptional regulation is the control point for the secretion of these inflammatory cytokines, we tested the effect of berberine on m-RNA levels of these genes in THP-1 cells stimulated in presence or absence of LPS (0.5 µg /mL). Results demonstrated upregulation of TNF-α (7.27 folds), IL-6 (2.27 folds), and MCP-1(5.59 folds) gene transcripts in LPS treated cells when compared to normal control cells whereas pretreatment of berberine at 25 and 50 µM concentrations significantly down regulated MCP-1 (1.46, 1.43 folds) and IL-6 (0.57, 1.70 folds) genes, however, unaffected the induction of TNF-α (8.15, 8.0 folds) transcript in THP-1 cells.

Effect of berberine on LPS induced NF-κB p-65 translocation in THP-1 cells.

Since transcripts of various proinflammatory cytokines are regulated by redox-sensitive transcription factor NF-κB, berberine effect was tested on its translocation. Immunoblotting and immunofluorescence studies has shown that THP-1 cells upon stimulation with LPS, NF-κB translocates into the nucleus, such LPS induced NF-κB translocation was inhibited in BBR (25, 50 μM) pretreated cells, NF-κB was sequestered in the cytosol in inactive state similar to un-induced control cells.

Highlights of the work

- Dry leaf extracts of T. *cordifolia* practically revealed remarked antioxidant and anti-lipid peroxidation activity in liver and heart tissues of male Wistar rats (*in vitro* study).
- Both TC extracts inhibited arachidonic acid induced ROS in THP-1 cells *via* upregulation of antioxidant enzymes CAT, GPx and SOD.
- Both TCAE and TCWE attenuated LPS induced proinflammatory TNF-α secretion and gene expression in THP-1 cells.
- TCAE inhibited LPS induced NF- κB translocation into nucleus, however, TCWE was not effective.
- Metabolite profiling by LC-Q-TOF-MS/MS revealed that TC dry leaf extracts are enriched with the presence of several bioactive compounds like alkaloids, diterpene glucosides, phenolics and phytosteroids.
- 20-Hydroxyecdysone (HOD) was identified as one of the abundant metabolite detected in TCAE. The amount of dry weight extracts of TCAE (0.4 and 0.8 mg dwt/mL) used for THP-1 cell pretreatments contained 8 and 16 μM concentrations of HOD.
- Pure compound, 20-hydroxyecdysone (HOD) detected in TC exhibited attenuation towards AA induced oxidative stress *via* upregulation of CAT and GPx.

- 20-HOD showed anti-inflammatory activity by attenuating LPS induced TNF-α secretion and gene expression in THP-1 cells.
- Ox-LDL induced foam cell formation was inhibited by 20-HOD.
- Metabolite mixture (20-HOD+SGY+QUE) mimicking TCAE suppressed LPS induced TNF- α both at protein and transcript levels in THP-1 cells and also suppressed LPS induced NF-κB translocation into the nucleus at molecular level which indicated the beneficial role of other metabolites in TCAE.
- Berberine, a TC-root alkaloid showed ROS scavenging effect by upregulating antioxidant enzymes. Further berberine attenuated AA induced proinflammatory markers like MCP-1, IL-8 secretion and COX-2 gene expression in THP-1 cells.
- Berberine significantly attenuated LPS proinflammatory markers in THP-1 by inhibiting NF-κB translocation into nucleus. However, BBR at 8 μM was not as effective as HOD.

Concluding remarks

Our findings demonstrated antioxidant and anti-inflammatory activities of *T. cordifolia* dry leaf extracts in activated human monocytic (THP-1) cells. Metabolite analyses have shown that the leaf extracts are enriched with several bioactive compounds. Investigation with one of the abundant bioactive metabolites, 20-Hydroxyecdysone, exhibited significant antioxidant and antiinflammatory activities in human monocytic (THP-1) cells. It is effective at much lower concentration compared to berberine, an alkaloid, which is abundant in the roots of *T. cordifolia* and is been widely investigated. BBR exhibited antioxidant and anti-inflammatory activities in activated human monocytic cells but required higher concentration than 20-HOD. In conclusion, the present study supports traditional use of *T. cordifolia* for therapeutic purpose, also highlighted for the first time, the pharmacological activity of 20-hydroxyecdysone in THP-1 cells. In the perspective of medicinal plant conservation, the use of aerial parts are preferred

over underground parts. Hence the present study suggests that 20-HOD can preferably be explored as a potential drug candidate for human use since it is an abundant metabolite present in the aerial parts of the plant.

Schematic representation of the summary of the present study

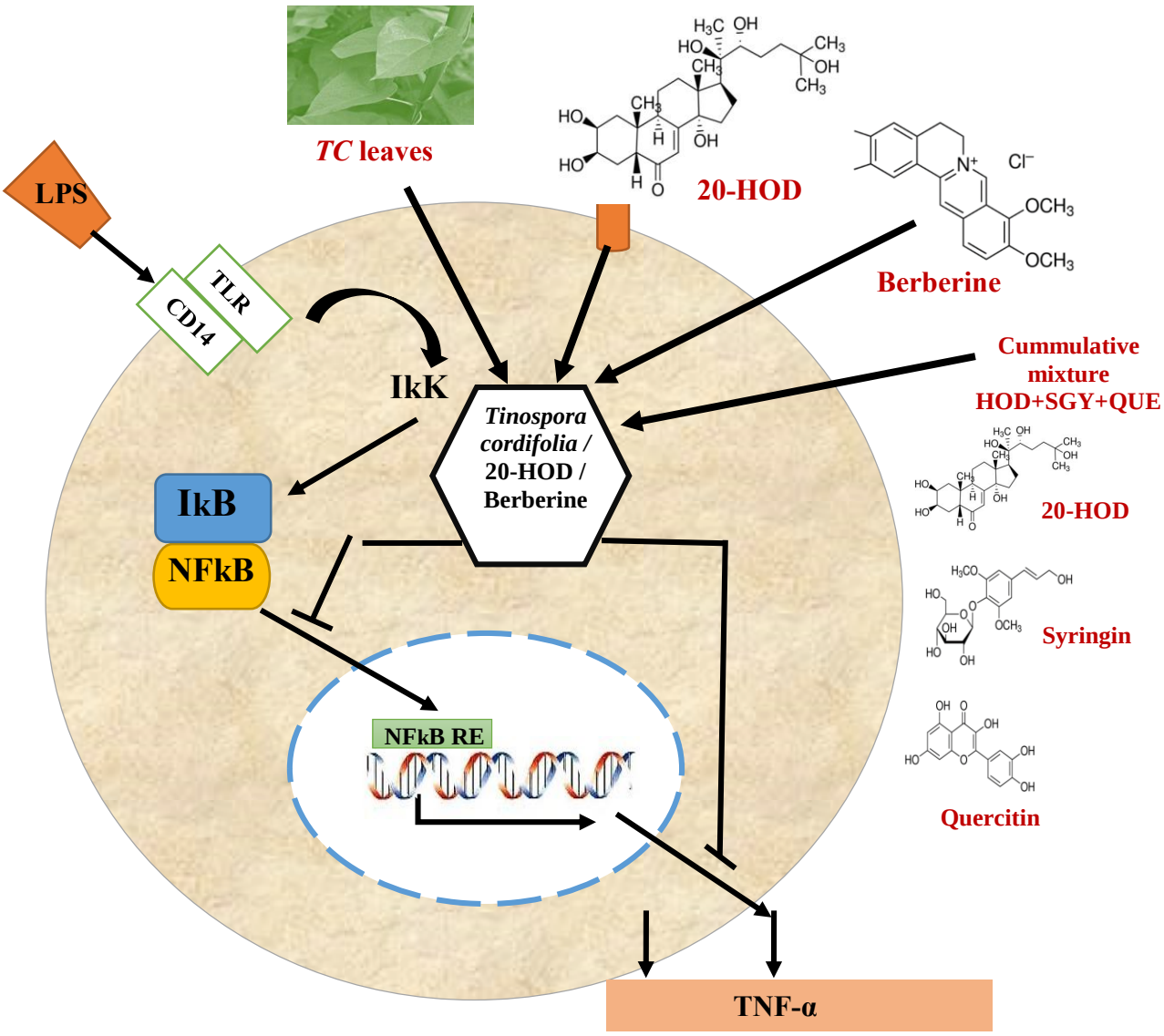


Fig. 7.1. Inhibitory effect of *T. cordifolia* and its metabolites on LPS induced TNF-α secretion *via* NF-κB signalling pathway.

Chapter-8

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Annexure I

Research papers published

Kumar, R.K., Basu, S., Lemke, H.-D., Jankowski, J., Kratz, K., Lendlein, A., Tetali, S.D., 2015. Effect of extracts of poly(ether imide) microparticles on cytotoxicity, ROS generation and proinflammatory effects on human monocytic (THP-1) cells. Clin. Hemorheol. Microcirc.

Posters presented

Reddi K. Kumar[1], Sayantani Basu[2,3], Karl Kratz[2], Andreas Lendlein[2,3], Sarada D. Tetali[1]. Examining the influence of poly(ether imide) microparticles on cytotoxicity and proinflammatory effects on human monocytic cells as well as apoptosis of human aortic endothelial cells.International congress of clinical hemorheology and micricirculation 27th Nov 2015, Uniklinikum Regensberg, Germany.

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Effect of extracts of poly(ether imide) microparticles on cytotoxicity, ROS generation and proinflammatory effects on human monocytic (THP-1) cells

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Abstract. Current haemodialysis techniques are not capable to remove efficiently low molecular weight hydrophobic uremic toxins from the blood of patients suffering from chronic renal failure. With respect to the hydrophobic characteristics and the high level of protein binding of these uremic toxins, hydrophobic adsorber materials might be an alternative to remove these substances from the plasma of the chronic kidney disease (CKD) patients. Here nanoporous microparticles prepared from poly(ether imide) (PEI) with an average diameter of 90 ± 30 μm and a porosity around $88 \pm 2\%$ prepared by a spraying/coagulation process are considered as candidate adsorber materials. A prerequisite for the clinical application of such particles is their biocompatibility, which can be examined i.e. indirectly in cell culture experiments with the particles' extracts. In this work we studied the effects of aqueous extracts of PEI microparticles on the viability of THP-1 cells, a human leukemia monocytic cell line, as well as their macrophage differentiation, reactive oxygen species (ROS) generation and inflammation.

A high cell viability of around $99 \pm 18\%$ and $99 \pm 5\%$ was observed when THP-1 cells were cultured in the presence of aqueous extracts of the PEI microparticles in medium A and medium B respectively. The obtained microscopic data suggested that PEI particle extracts have no significant effect on cell death, oxidative stress or differentiation to macrophages. It was further found that the investigated proinflammatory markers in THP-1 cells were not up-regulated. These results are promising with regard to the biocompatibility of PEI microparticles and in a next step the hemocompatibility of the microparticles will be examined.

Keywords: Chronic kidney disease (CKD), cytotoxicity, human monocytic (THP-1) cells, poly(ether imide) microparticles, reactive oxygen species (ROS)

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1. Introduction

Chronic renal failure is considered to be a condition, in which kidneys malfunction to remove uremic toxins and fluids from the body. Chronic kidney disease (CKD) is associated with inflammation and as a consequence with oxidative stress. Both inflammation and oxidative stress cause adverse health effects in CKD patients especially cardiovascular diseases (CVD). It is anticipated that retained uremic metabolites are one potential cause of oxidative stress and inflammation [4]. Hemodialysis (HD), hemofiltration (HF), hemodiafilteration (HDF) and kidney transplantations are commonly used procedures for treatment of CKD patients, but current conventional, high flux haemodialysis techniques, which are operated under aqueous conditions, are less efficient in removing low molecular weight hydrophobic uremic solutes from the blood of CKD patients. In uremic erythrocytes certain protein kinases or endothelial NO synthase are activated, which play a significant role in maintaining the mechanical and morphological properties of the erythrocyte membrane [14].

With respect to the hydrophobic characteristics and the high level of protein binding of such uremic solutes, some scientific studies investigated therapeutic approaches like combined usage of hydrophobic adsorber materials, such as apheresis particles, along with dialysis as an artificial kidney [28]. Recently, nanofibrous polymeric membranes prepared from poly(ethylene-co-vinyl alcohol) zeolite composites by electrospinning have been reported to adsorb uremic solutes like creatinine in a blood purification system [15]. Another approach for the removal of hydrophobic substances from the blood of patients is the utilization of hydrophobic polymers such as poly(ether imide) (PEI), which are applied as highly porous microparticulate adsorber materials. These porous, spherically shaped PEI microparticles can be prepared by a spraying/coagulation process having a mean diameter in the range of 70–100 μm and a total porosity based on weight in the range of 75–80% depending on the polymer solution composition [1]. PEI microparticles are of high mechanical robustness and steam-sterilisable, while the pore sizes of these microparticles are well defined so that the binding of uremic toxins of various sizes should be very effective. For qualifying such microparticles in view of potential future clinical applications their biocompatibility needs to be demonstrated *in vitro* and *in vivo*. In a first step typically aqueous extracts of the adsorber materials are investigated regarding *in vitro* effects on human cell lines, which are caused by leachable toxic substances originating from the particle processing, before studying the application of the adsorber *in vivo*. It is further essential to ensure that adsorber materials are free of bacterial contaminations and do not release any toxic chemical substances, which otherwise evoke inflammation and lead to failure of the device in patients. Human monocytes are sensitive and get activated on exposure to various factors like toxins released by infectious agents and cause inflammation by secreting various proinflammatory markers including cytokines and chemokines. These secreted proinflammatory products lead to onset as well as progression of chronic inflammatory disorders. In this study, we investigated potential toxic effect of PEI microparticle extracts, on human monocytic (THP-1) cells by assaying cytotoxicity, intracellular ROS levels, gene expression of proinflammatory markers at transcripts and protein levels and differentiation of monocytes to macrophages.

2. Materials and methods

2.1. Particle preparation

Poly(ether imide) (trade name Ultem® 1000) was purchased from SABIC Deutschland GmbH (Düsseldorf, Germany) and used without any further purification. PEI microparticles were prepared

by a spraying/coagulation process with a 130 μm spinneret utilizing a water coagulation bath according to the method previously reported [1]. Afterwards, the obtained PEI particles were sterilized via steam sterilization (121°C, 2.0 bar, 20 minutes) using a Systec Autoclave D-65 (Systec GmbH, Wettenberg, Germany).

2.2. Particle characterization

Shape and size of the prepared microparticles were characterized by scanning electron microscopy (SEM) with a Phenom G2 pro (L.O.T. - Oriel, Darmstadt, Germany) after coating with a conductive layer. For analysis of the average particle diameters the image processing software, Image J (Version 1.48v, Wayne Rasband, USA) [23] was applied and the standard deviation (error) was determined by measuring 350 particles. In addition, the particles‘ surface and cross section was visualized by scanning electron microscope (SEM) at higher magnification with a Gemini Supra 40 VP (Zeiss AG, Oberkochem, Germany). The cross sectional samples were prepared via cryo-ultramicrotomy. The frozen samples were cut into sections of 500 nm thickness and coated with a 5 nm thin conductive layer before SEM investigation.

Hg-porosimetry was applied to determine the overall porosity of the microparticles as well as the cumulative pore volume, the average pore diameter and the pore size distribution utilizing a PASCAL 140–440 porosimeter (POROTEC, Germany). A powder dilatometer type CD3P was filled with ca. 0.1 g of the dried microparticles and examined in the low pressure range of 0.013 to 0.4 MPa with the 140 PASCAL system and in the pressure range from 0.1 to 400 MPa with the PASCAL 440 system. Data analysis was conducted with the SOL.I.D Software Version 1.4.1., while applying the “sphere” surface area model.

2.3. Cell culture experiments

THP-1 cells procured from NCCS (Pune, India) were cultured in RPMI-1640 (Invitrogen, New York, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biowest, Kansas, USA) and 1% (v/v) Penstrep (Invitrogen, New York, USA). Cells were maintained in an incubator at 37 ° C and 5% CO_2. THP-1 cells were incubated with the prepared PEI particle extracts (as described below) for 5 h and subjected to the assays described in the following.

2.4. Preparation of PEI microparticle extracts

200 mg of steam sterilized PEI microparticles were suspended in 12 mL of THP-1 cell growth medium without (medium-A) and with FBS of 10% (v/v) (medium-B) and agitated for 5 h on a rotatory shaker for proper mixing. Then the media were centrifuged at 3000 rpm for 5 min at room temperature and the collected supernatants. FBS (10% v/v) was added to medium-A. Medium A and B were prepared to distinguish the toxic or inflammatory effects, if any, of PEI particles on THP-1 cells, due to the depletion of essential components of the medium by binding to serum albumin or release of toxic substances, which may cause toxicity to the cells.

2.5. Cell viability- trypan blue method and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

THP-1 cells at a density of 5×10^5/ mL were incubated with PEI extracts for 5 h at 37°C and 5% CO_2. The treated cells were tested for their viability by trypan vital staining exclusion method and MTT assay. For the trypan vital assay 20 μL of the PEI treated cell suspension were mixed with 10 μL of trypan blue

solution and the morphology of cells observed under an inverted microscope using a hemocytometer [10]. For the MTT assay 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) were added to the PEI treated cell suspension and incubated for 4 h. The cells were washed twice with growth medium after the incubation period to remove excess dye. Purple coloured insoluble formazans were dissolved in 100 μL of dimethyl sulfoxide (DMSO) and the absorbance was quantified at 570 nm with the reference wavelength at 630 nm.

2.6. Macrophage differentiation

The effect of PEI microparticle extracts on THP-1 cells differentiation into macrophages was investigated by microscopic analysis. Differentiation of cultured THP-1 cells into macrophages can be achieved by stimulating THP-1 cells with phorbol-myristate acetate (PMA) for 48 h [18]. Phenotype of PEI treated THP-1 cells after 5 h was observed microscopically using an inverted microscope (Olympus) at a magnification of 20X. THP-1 cells induced with PMA for 48 h were used as positive control [18, 26].

2.7. Intracellular reactive oxygen species (ROS) production

PEI extracts pretreated cells were loaded with 5 μM 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) fluorescent probe for 15 min and the fluorescence intensity observed under a confocal microscope (Zeiss NLM 710, Carl Zeiss MicroImaging GmbH, Germany) [11]. Cells stimulated with 100 μM arachidonic acid (AA) for 10 min were used as positive control [13].

2.8. Quantification of cytokines by ELISA

Cells pretreated with PEI extracts for 5 h were centrifuged at 3000 rpm for 3 min and the supernatants collected. Secretory porinflammatory cytokines like tumor necrosis factor (TNF-α), monocyte chemotactic protein (MCP-1) and interleukins (IL-6, IL-8) in supernatants were quantified by using BD-Bioscience (San Diego, USA) ELISA kits according to the manufacturer's protocol. THP-1 cells induced with lipopolysaccharide (LPS) were applied as positive control for comparison [31].

2.9. Gene expression studies of proinflammatory and cell differentiated markers by quantitative real-time PCR

RNA was isolated from the cells using Trizol reagent and c-DNA was synthesized by Iscript c-DNA synthesis kit (Bio-Rad laboratories, Hercules, USA). The gene expression levels of TNF-α, MCP-1, IL-6, IL-8, and toll like receptor (TLR-4) were analysed by using qRT-PCR, which was performed by using SYBER Green PCR kit. The primer sequences for the tested genes were purchased from Eurofins genomics (Bangalore, India) and are given in the Table 1 [6]. The 10 μl reaction mixture consisted of 2 μl of cDNA template and 8 μl of cocktail mix (5 μl of 1x SYBR Green, 0.5 μl of 1x forward and reverse primers, 2 μl of water). The mRNA levels of all genes were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Negative controls contained 8 μl of cocktail mix without cDNA and 2 μl of water.

Table 1
Primer sequences for studying gene expression of TNF-α, MCP-1, IL-6, IL-8, and TLR-4

S.no	Genes		Sequence
1.	TNF-α	Forward	5′-CCCAGGGACCTCTCTCTAATC-3′
		Reverse	5′-ATGGGCTACAGGCTTGTCACT-3′
2.	MCP-1	Forward	5′-GCCAAGGAGATCTGTGCTGAC-3′
		Reverse	5′-CATGGAATCCTGAACCCACTTC-3′
3.	IL-6	Forward	5′-TGGATTCAATGAGGAGACTTGC-3′
		Reverse	5′-CAGGAACTGGATCAGGACTT-3′
4.	IL-8	Forward	5′-GTGTAAACATGACTTCCAAGCTGG-3′
		Reverse	5′-GCACCTTCACACAGAGCTGC-3′
5.	TLR-4	Forward	5′-TGGATACGTTTCCTTATAAG-3′
		Reverse	5′-GAAATGGAGGCACCCCTTC-3′
6.	GAPDH	Forward	5′-CACCAACTGCTTAGCACCCC-3′
		Reverse	5′-TGGTCATGAGTCCTTCCACG-3′

2.10. Statistics

All data were expressed as mean value of minimum of 3 independent experiments ± standard deviation.

3. Results

In this study we explored the viability, the monocytic differentiation, the proinflammatory behaviour and the intracellular ROS levels of human monocytic cells, which were treated with extracts prepared from porous PEI microparticles.

3.1. Microparticle characterization

Morphological characterization by SEM and Hg-porosimetrie revealed that spherical, highly porous particles with a bulk porosity of $88 \pm 2\%$ and an average diameter of $90 \pm 30\,\mu m$ were obtained by the spraying/coagulation process. In Figs. 1A, C and D representative SEM images of PEI microparticles are shown, visualizing their spherical shape as well as the porous morphology of the particles. Here a nanoporous structure becomes obvious from SEM images taken at high magnification at the particles' cross section. The particle diameter distribution obtained from the analysis of 350 particles is shown in Fig. 1B.

Hg-porosimetry was employed for quantification of the particles' bulk density, cumulative pore volume, average pore size and pore size distribution as well as the pore accessibility. The microparticles exhibited a low bulk density of $0.12 \pm 0.02\ g \times cm^3$, a mean pore diameter of $320 \pm 20\,nm$ and a cumulative volume of $1.33 \pm 0.07\ g \times cm^3$, while a relative low pore accessibility of $43 \pm 2\%$ was found. In Fig. 1E the pore size distribution and the cumulative pore volume are displayed.

3.2. Effect of microparticle extracts on cell viability

Trypan blue exclusion method and MTT assays were performed to assess cell viability of PEI extract treated THP-1 cells. Integrity of the cell membrane can be detected by the Trypan blue assay. Dead cells

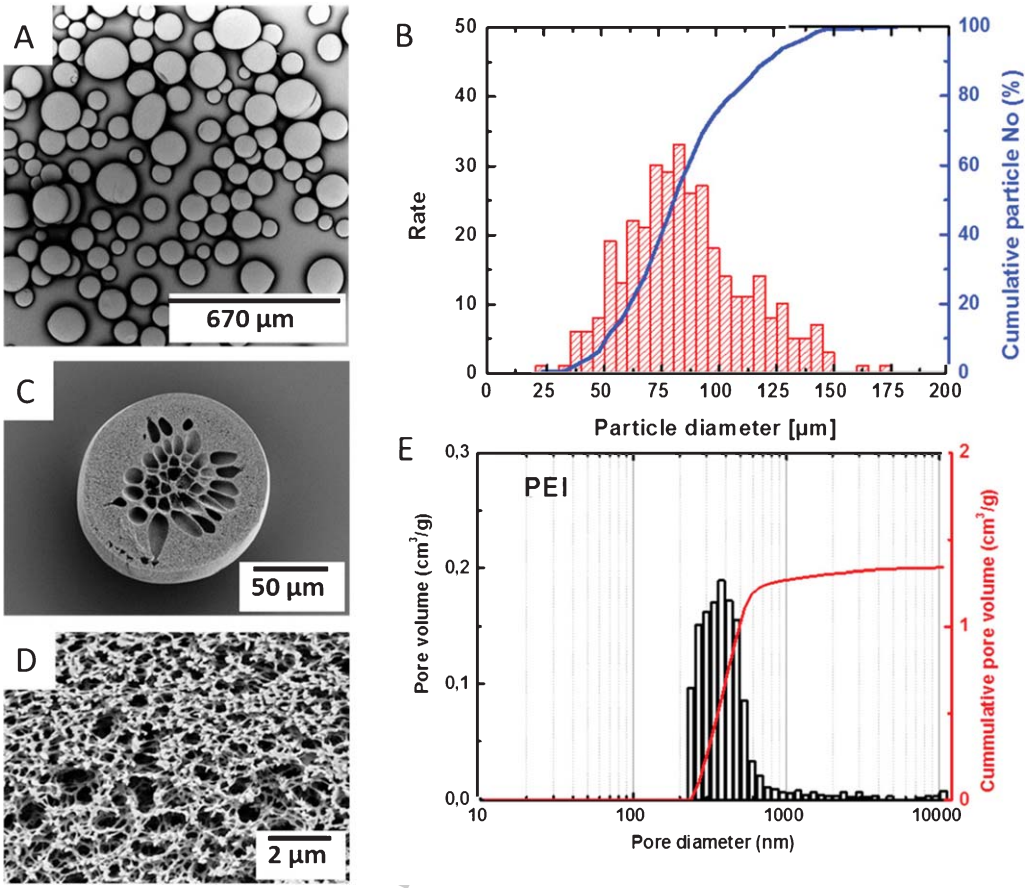

Fig. 1. Scanning electron microscopy images of PEI microparticles (A), cross section of a single microparticle (C) and the outer part of the cross section at higher magnification (D). Particle diameter distribution obtained from SEM image analysis (B) and pore-size distribution as well as cumulative pore volume determined by Hg-porosimetry.

take up the dye and as a result the cytosol appears blue in colour, while the cytosol of viable cells remains unstained. A high viability around 99% was observed when THP-1 cells were cultured in both medium extracts A ($99 \pm 18\%$) and B ($99 \pm 5\%$) of the PEI microparticles. This suggests that extracts of PEI particles did not cause a significant cell death rate (data not shown). The MTT assay further confirmed no toxic effects of PEI particle extract treated cells since $100 \pm 5\%$ cell viability was seen similar to untreated cells (Fig. 2).

3.3. Influence of microparticle extracts on ROS production in THP-1 cells

Pretreatment of THP-1with PEI microparticle extracts (medium A and medium B) for 5 h did not increase intracellular ROS production as indicated by confocal microscopy images (Fig. 3C, D) similar to control cells (Fig. 3A) whereas AA treated cells showed high amount of ROS production (Fig. 3B) within 10 min of stimulation.

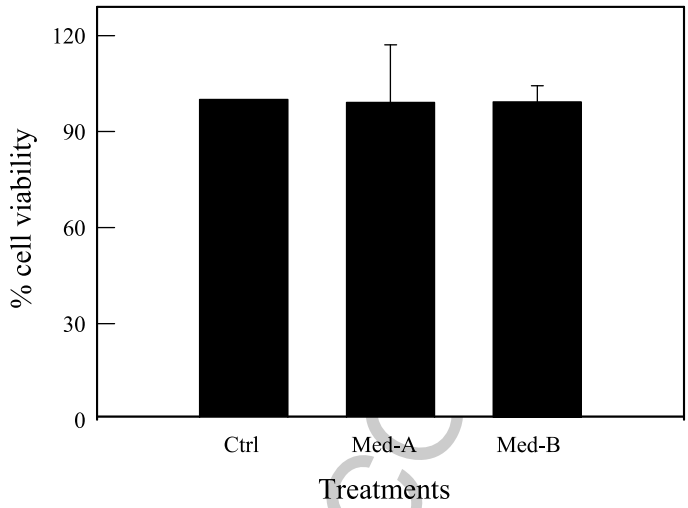

Fig. 2. Cell viability of THP-1 cells pre-treated with poly(ether imide) microparticle extracts of medium-A and medium-B for 5 h as determined by MTT assay. Data represent mean values ± standard deviation of 3 independent experiments.

3.4. Effect of microparticle extracts on proinflammatory markers and differentiation to macrophages

Transcripts of proinflammatory genes like TNF-α, MCP-1, IL-6 and IL-8 were quantified using real time PCR. Bacterial endotoxin or LPS upregulated the gene transcripts of proinflammatory markers while PEI mircoparticle extracts treated cells did not show such upregulation (Figs. 4A–D). Secretion of the protein markers TNF-α, MCP-1, IL-6 and IL-8 were quantified using ELISA and observed at basal level in control cells as well as in microparticle extracts treated cells, whereas LPS treatment enhanced the secretion of above proinflammatory markers by several folds (Figs. 5A–D).

Differentiation of monocytes to macrophages is a well-established biomarker indicating inflammatory stress. Macrophages are key immune cells generating ROS and secreting various proinflammatory cytokines. Phenotype of THP-1 suspension cells (Fig. 6A) treated with PMA for 48 h changed to adherent macrophage like structures (Fig. 6B). In contrast, no macrophage differentiation was observed for THP-1 cells treated with PEI extracts for 5 h (Figs. 6C and D). At the molecular level PMA enhanced the gene expression of TLR-4, which is a marker for differentiated macrophages, whereas PEI microparticle extract treated cells had a low level of TLR4 similar to the undifferentiated THP-1 cells (Fig. 6E).

4. Discussion

Chronic kidney disease (CKD) is described as a serious condition associated with ageing, diseased or failure of kidneys in removing the waste from the body. CKD is preferentially recognized in aged persons, who were affected with diabetes and hypertension [16]. Patients suffering from CKD have a high risk of experiencing cardiovascular diseases like stroke, heart diseases or peripheral arterial diseases, which account for approximately 30% mortality worldwide [21]. Dialysis is a common procedure employed in reducing the progression of the disease, but existing dialysis procedures can only help the patients partially. A major limitation of conventional hemodialysis therapies is that such treatments can only remove

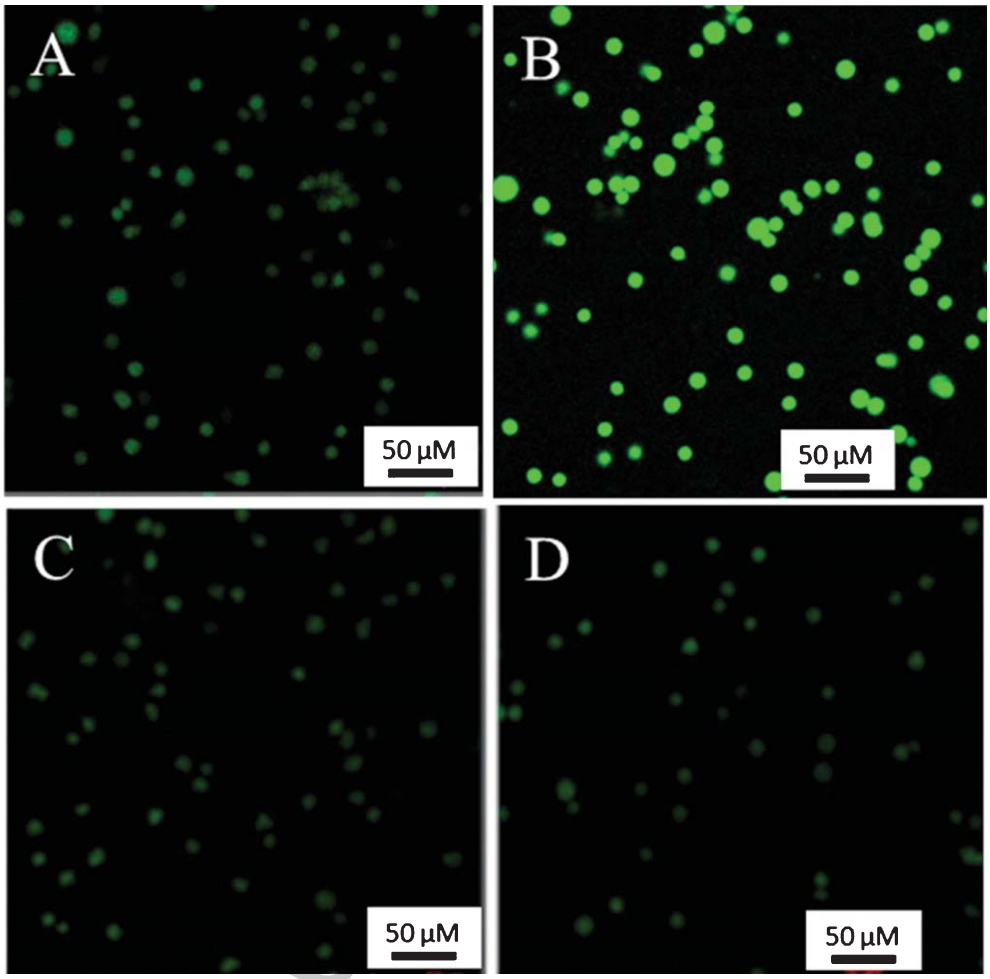

Fig. 3. Representative fluorescence microscope images visualizing the amount of reactive oxygen species (ROS) generated in THP-1 cells without pre-treatment (A) and after exposure to arachidonic acid for 10 min (B) and medium-A (C) as well as medium-B (D) for 5 h.

hydrophilic substances efficiently, while hydrophobic or protein bound substances such as uremic toxins remain in the patients' blood. Recently, a good *in vitro* absorption capacity for protein bound middle and high molecular weight uremic toxins could be demonstrated for a nanoporous activated carbon monolith [20]. Another approach for the removal of hydrophobic substances from the blood of patients is the utilization of highly porous, microparticulate adsorber materials prepared from hydrophobic polymers such as poly(ether imide) (PEI). Such steam-sterilisable and mechanical robust PEI microparticles possess well defined pore sizes so that the binding of uremic toxins with various molecular weights should be possible. For qualifying such microparticles in view of potential future clinical applications, the absorber particles have to be tested extensively for their side effects on toxicity or activation of human monocytic cells, since human plasma subjected to hemodialysis will get in contact with blood cells. It is established that activation of monocytic cells participates in onset and progression of inflammation processes and in turn is responsible for atherosclerosis [24].

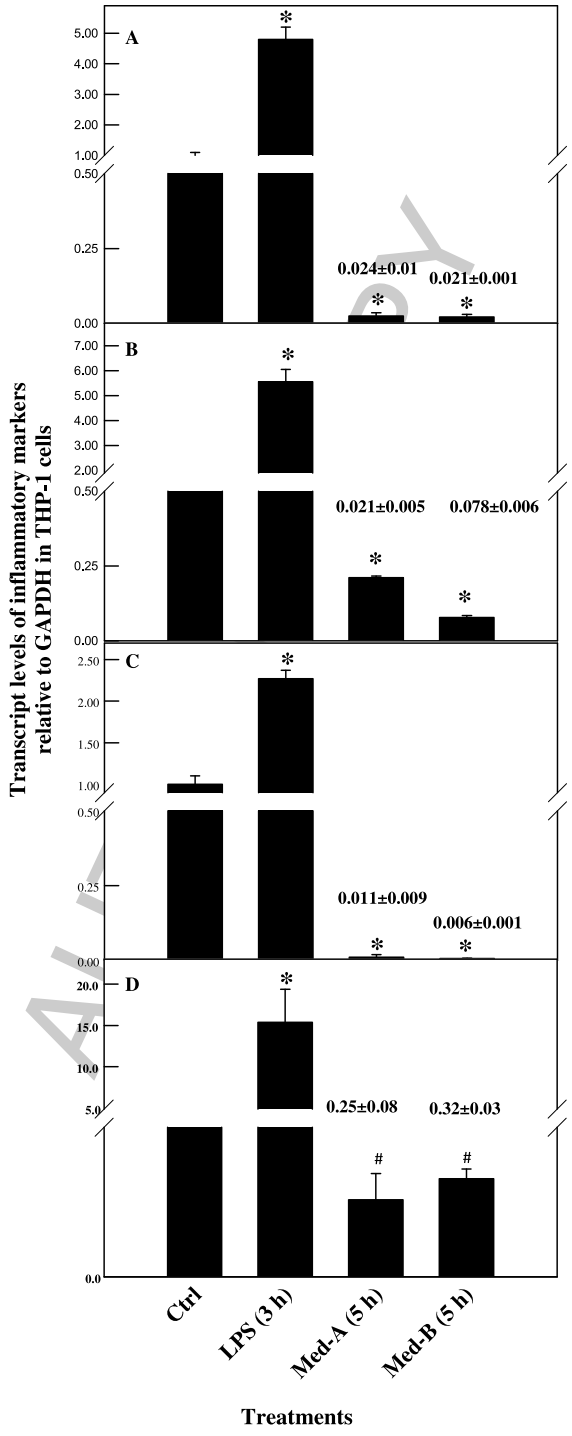

Fig. 4. Effect of microparticles extracts on inflammatory gene transcripts of THP-1 cells. Gene expressions of TNF-α (A), MCP-1 (B), IL-6 (C) and IL-8 (D), were quantified by real time PCR. Data represent mean ± SD of 3 independent experiments and $^{*}p < 0.001$ compared between treated and untreated cells.

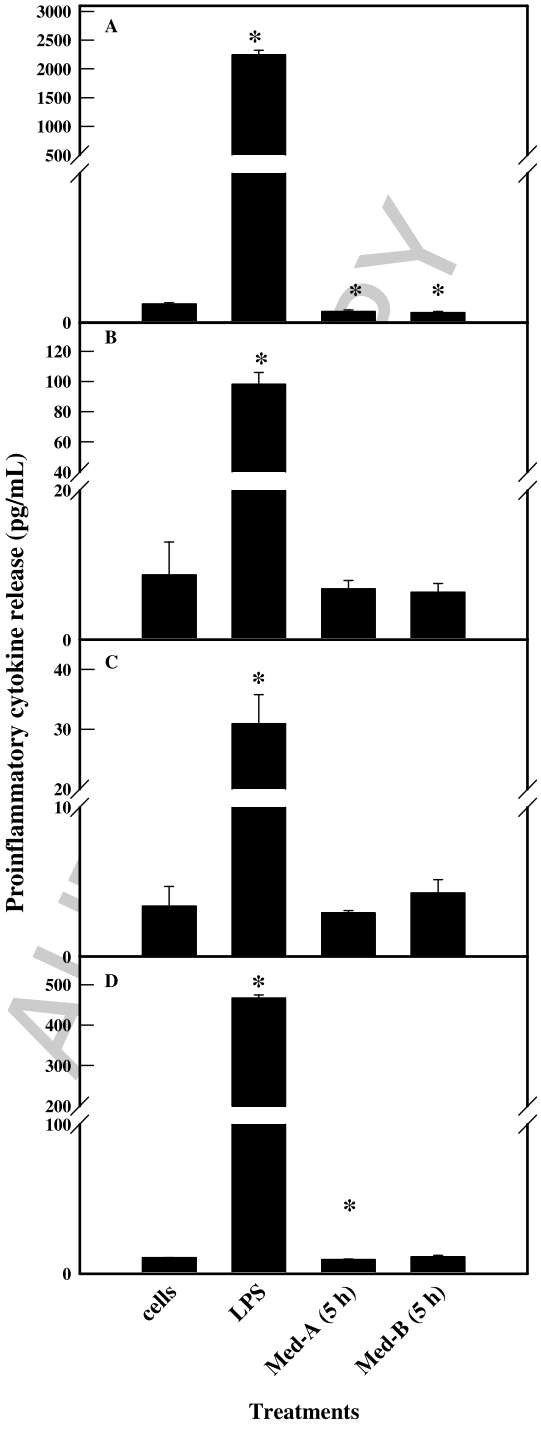

Fig. 5. Influence of microparticles extracts on the release of proinflammatory markers like TNF-α (A), MCP-1 (B), IL-6 (C) and IL-8 (D) in supernatants measured by ELISA. Data represent mean ± SD of 3 independent experiments and $^{*}p < 0.001$ between treated and untreated cells.

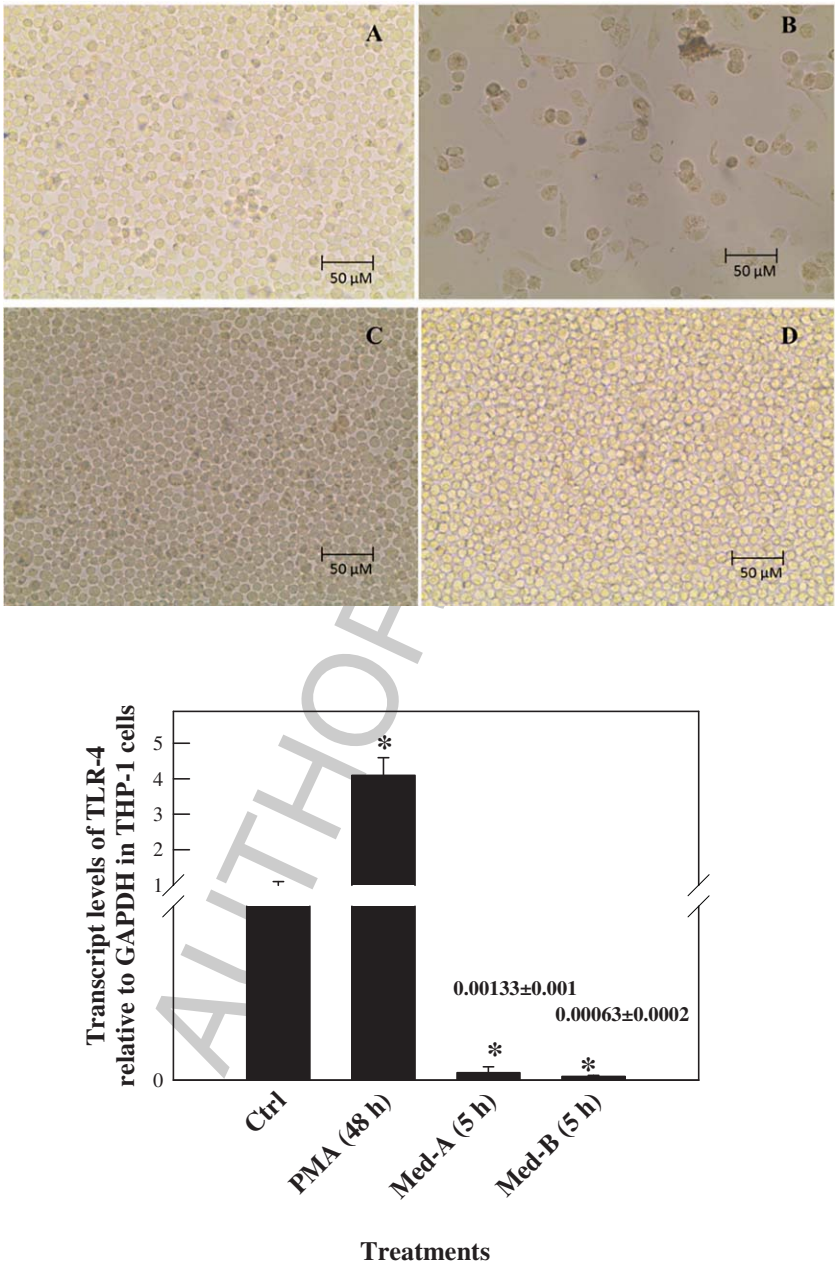

Fig. 6. Representative microscopic images visualizing the macrophage differentiation of THP-1 cells without pre-treatment (A) and after exposure to PMA for 48 h (B) and medium-A (C) or medium-B (D) for 5 h. Gene expression of cell differentiation marker TLR-4 (E) quantified using real time PCR. Data represent mean ± SD of 3 independent experiments and $^{*}p < 0.001$ compared between treated and untreated cells.

Trypan blue staining and MTT assay have shown that the cell viability of THP-1 were not affected when treated with PEI microparticle extratcs for 5 h. Differentiation of monocytes to macrophages initiates the progression and pathogenesis of various chronic diseases [29] as macrophages are major sources of ROS generation [3] and cytokine secretions upon activation [25]. Macrophages are capable to form foam cells, which are considered to be a crucial step in leading to atherosclerosis [17]. However PEI microparticles extracts did not affect the phenotype of THP-1 cells on incubation for 5 h which is equivalent to the time of a single hemodialysis treatment.

Activation of key immune cells like monocytes can be triggered by various factors when they come in contact with endotoxins, cytokines or dietary assimilates of glucose and polysaccharides. These factors can induce an oxidative stress by generating intracellular ROS, which attack macromolecules of the cells like nucleic acids, proteins and lipids. Lipids present in the membrane form peroxide adducts upon oxidation [12], which activate various stress signaling pathways by modifying enzymes like caspases leading to apoptosis [27]. Oxidative stress can lead to many metabolic disorders including atherosclerosis [19]. Oxidative stress generated by ROS has been implicated in the pathological systems of both CKD and CVD, most importantly chronic inflammation through activation of proinflammatory cytokines *via* NF-κb signaling pathway. In this study THP-1 cells pretreated with PEI microparticle extracts for 5 h did not show enhanced ROS, the cells were physiologically similar to untreated control cells, whereas THP-1 cells treated with arachidonic acid had higher levels of intracellular ROS.

Inflammatory response is characterized by the secretion of various proinflammatory cytokines like TNF-α, MCP-1, IL-1 IL-6, IL-8, and IL-18 etc. through the activation of TLR-2 and TLR-4 [22]. TLRs act as sensors for bacterial contaminants and activate the cells through NF-κb signaling pathway [9]. These secreted products lead to various chronic inflammatory diseases like CKD and CVD. It is essential to ensure that PEI microparticle extracts are free of bacterial contamination and do not release any toxic chemical substances, which otherwise evoke inflammation and lead to failure of the device applied in patients.

Endotoxins of gram negative bacteria or LPS bind to TLRs and evoke inflammatory response by activating translocation of NF-κb into the nucleus [2, 8]. NF-κb binds to the promoters of various proinflammatory genes and initiates inflammation. Proinflammatory cytokines upregulate adhesion molecules like vascular cell adhesion molecules (VCAM-1), intracellular adhesion molecules (ICAM-1) and endothelial selectin (E-selectin) and can lead to atherosclerosis [5, 7, 30]. Therefore, we analysed the inflammatory response of PEI microparticle extracts in THP-1 cells and we observed that the particle extracts did not activate any of the inflammatory markers both at protein and transcript levels.

5. Conclusion

The obtained results clearly demonstrate that the extracts of PEI microparticles did not exhibit negative side effects on the viability, the differentiation and the proinflammatory behaviour of human monocytic cells. In a next step the hemocompatibility of the presented PEI microparticle will be examined. These results support that PEI microparticles are interesting absorber material candidates in the context of CKD.

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