

**PROTECTIVE EFFECT OF *Phyllanthus niruri* LEAF
EXTRACTS ON OXIDANT INDUCED DAMAGE TO
BIOMOLECULES *in vitro***

MEERA, R.

11PBT05

A thesis submitted to Avinashilingam Institute for Home Science and Higher
Education for Women, Coimbatore.

In partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

May, 2013

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**Signature of the
Head of the Department**


**Signature of the
Supervisor**

Acknowledgement

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Introduction

1. INTRODUCTION

Molecular oxygen is an essential component for all living organisms, where it helps in the process of oxidation, which is a basic component of aerobic life and of our metabolism (Aiyegoro and Okoh, 2010). A part of the oxygen taken into living cells is converted to several harmful reactive oxygen species and free radicals. Once formed, free radicals can start a chain reaction, leading to the formation of more free radicals (Wu *et al.*, 2011).

Free radical is defined as any atom or molecule which possesses unpaired electrons (Sen *et al.*, 2010). Free radicals tend to be reactive and participate in chain reactions, in which a single free radical initiation event can be propagated to damage multiple molecules. Free radicals from both endogenous and exogenous sources are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer (Alberto *et al.*, 2013).

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^\bullet) plus the related peroxynitrite ($ONOO^-$) molecule are generally thought to cause cell dysfunction and ultimately death due to alteration of i) metabolic pathway activity (Newsholme *et al.*, 2009) and ii) the structure of cellular membrane, DNA or Proteins (Limon - Pacheco and Gonsebatt, 2009).

Oxidative stress is a harmful condition that occurs when there is an excess of ROS and a decrease in antioxidant levels (Mahantesh *et al.*, 2012). It can cause damage to all molecular targets: DNA, lipids and protein (Halliwell, 2011). Oxidative stress initiated by free radicals, such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite play a vital role in damaging various cellular macromolecules. This damage may result in many

diseases, including diabetes mellitus, atherosclerosis, myocardial infarction, arthritis and neurodegenerative diseases (Oyedemi *et al.*, 2010).

Biochemical oxidation of lipid is referred to as LPO. Lipid peroxidations mediated by free radicals are being considered a primary mechanism of cell membrane destruction and cell damage. The oxidation of unsaturated fatty acids in the biological membrane leads to a reduction of membrane structure and functions (Sivakumar *et al.*, 2008). Much attention has been focused on the use of antioxidants especially natural antioxidants to inhibit lipid peroxidation or to protect the human body from the oxidative damage by free radicals (Gupta *et al.*, 2007).

Oxidative damage to DNA is widely thought to be a significant contributor to the age related development of major cancer (Polo and Jackson, 2011). Although background levels of oxidative by damaged DNA always exist, oxidative stress can lead to an increase in the damage, and it is this increase which has been linked to various pathological conditions such as ageing, carcinogenesis, neurodegenerative and cardiovascular diseases. Antioxidant supplements may be used to help the human body to reduce oxidative damage (Jena, 2012).

An antioxidant is any substance that at relatively low concentrations, in relation to the oxidizable substrate, significantly delays or prevents oxidation of the substrate. Antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxy and thus reduce the level of oxidative stress and prevent the development of complications associated with oxidative stress related disease (Brand, 2010). The uniqueness of the antioxidant defence system lies in capability to maintain the reactive species at certain steady state levels and thereby create and maintain the balance between beneficial and injurious effects of reactive species (Lushchak, 2010).

Medicinal plants are natural sources, yielding valuable herbal products, which are often used in the treatment of various ailments (Sudhahar *et al.*, 2007). Medicinal plants have been traditionally used in the treatment of several human diseases and their pharmacological and therapeutic properties have been attributed to different chemical constituents isolated from their crude extracts. Of particular importance, chemical constituents with antioxidant activity can be found at high concentrations in plants and can be responsible for their preventive effects in various degenerative diseases, including cancer, neurological and cardiovascular diseases (Krishna *et al.*, 2010). Thus, the antioxidant properties of plants have a full range of perspective applications in human healthcare (Pereira *et al.*, 2010).

Drugs available for treatment are mainly herbal combinations. Ancient systems of medicine like Ayurveda, Unani etc., have used plants of the genus *Phyllanthus* in treating various liver disorders. These plants are also known to have diverse biological activities like hypoglycaemic, hypotensive, diuretic, antioxidative and anti-inflammatory (Nekweti *et al.*, 2013).

Phyllanthus niruri is a common Kharif (rainy season) weed found in both cultivated fields and wastelands. It is a small herb distributed throughout the tropical and subtropical regions of both hemisphere. This plant is popular in folk medicine. Whole plant, fresh leaves and fruits are used in the treatment of various diseases, particularly hepatitis and numerous ailments like dysentery, influenza, vaginitis, tumors, diabetes, diuretic, jaundice, kidney stone, dyspepsia, antihepatotoxic, antihepatitis B, antihypoglycemic and also as antiviral and antibacterial (Paithankar *et al.*, 2011).

The present study is an attempt to study the effect of aqueous, methanol and chloroform extracts of leaves of *Phyllanthus niruri* on the oxidant induced damage to lipids and DNA.

The objective of the present study is thus to determine the effect of *Phyllanthus niruri* leaf extracts on the

(i) inhibition of lipid peroxidation in

- Goat liver homogenate
- RBC ghosts
- Goat liver slices

(ii) extent of DNA damage in

- Lambda DNA
- pUC18 DNA
- Herring sperm DNA

A brief review of literature pertaining to the present study is given in the next chapter.

Review of literature

2. REVIEW OF LITERATURE

Reactive oxygen species (ROS) is a collective term used for a group of oxidation, which are either free radicals or molecular species capable of generating free radicals (Kunwar and Priyadarsini, 2011). ROS are highly reactive molecules that originate mainly from the mitochondrial electron transport chain. ROS can damage or cause complete degradation of essential complex molecules in the cells, including fat molecules, proteins, and DNA (Alfadda and Sallam, 2012).

Free Radicals

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. Various metabolic processes, UV radiations, smoke, etc. trigger the production of free radicals (Halliwell, 2011). Free radical is an atom, molecule or compound that is highly unstable because of its atomic or molecular structure. As a result, free radicals are very reactive as they attempt to pair up with other molecular atoms or even individual electrons to create a stable compound (Mahantesh *et al.*, 2012).

Free radicals reactions are ubiquitous in food and biological systems. They play major roles in biochemical pathways and food degradation. There is escalating evidence for the fundamental role of free radicals in diseases. Free radicals can be divided into reactive oxygen species such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical (OH^{\cdot}), hydrogen peroxide (H_2O_2) and Reactive Nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($ONOO^-$) (Fukai and Ushio-Fukai, 2011).

Excessive production of free radicals leads to oxidative stress. The diseases associated with the ROS mainly depend on the balance of the pro-oxidant and the antioxidant concentration in the body. Free radicals are important factors for several pathological conditions such as cardiovascular

disease, inflammation, atherosclerosis and degenerative diseases (Wagesdrama, 2012).

Types of free radicals

Oxidative stress is initiated by ROS such as superoxide anion ($O_2^{\cdot -}$), perhydroxy radical (HOO^{\cdot}) and hydroxyl radical (HO^{\cdot}). These radicals are formed by one electron reduction process of molecular oxygen (Kreslavski *et al.*, 2012).

Superoxide anion ($O_2^{\cdot -}$)

Superoxide anion radical ($O_2^{\cdot -}$) is one of the strongest reactive oxygen species among the free radicals that are generated first after oxygen is taken into living cells. O_2 changes to other harmful reactive oxygen species and free radicals such as hydrogen peroxide and hydroxyl radical (Guo and Chen, 2012). Major features of $O_2^{\cdot -}$ includes, it is relatively unreactive intermediate, being able to act at best as reactant in physiological condition and indeed, only the interaction with nitric oxide (NO) to give peroxynitrite is able to transform superoxide into H_2O_2 (Navo and Parola, 2008).

Hydroxyl radical (OH^{\cdot})

OH^{\cdot} has a short half life and is the most reactive and damaging ROS. It is known to be capable of abstracting hydrogen atoms from membranes (Cardenas-Redriguez *et al.*, 2013). They are the major active species causing lipid oxidation and enormous biological damage (Gupta *et al.*, 2007).

Hydrogen peroxide (H_2O_2)

H_2O_2 has important roles in oxidative damage and carcinogenesis, since it is stable and diffuses easily through biological membranes, thus reaching other cellular compartments, further producing cellular injury, especially if it

becomes converted to the highly reactive OH^\bullet (Aiyegoro and Okoh, 2010). It is a weak oxidizing agent and can inactivate a few enzymes by oxidizing their thiol groups. It can probably react with Fe^+ and Cu^+ ions in the cell to form hydroxyl radical, ultimately resulting in its damaging effects (Holley *et al.*, 2011).

Oxidative stress

Oxidative stress is well known to be involved in the pathogenesis of lifestyle – related diseases, including atherosclerosis, hypertension, diabetes mellitus, ischemic diseases and malignancies. Oxidative stress has been defined as harmful because oxygen free radicals attack biological molecules such as lipids, proteins and DNA (Burhans and Heintz, 2011).

Oxidative stress occurs in a cellular system when production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system. Oxidative stress is known to play an important role in the pathogenesis. (Samundeeswari and Rajadurai, 2012). Under normal conditions, the magnitude of oxidant production is balanced by the rate of oxidant elimination. Overproduction of oxidants that overwhelm the cellular antioxidant capacity results in pathogenic oxidative stress (Grossman and Ehad, 2012).

Oxidative stress plays an important contributory role in the process of aging and pathogenesis of numerous diseases like diabetes, cancer, neurodegenerative diseases, and respiratory tract disorders. Improved antioxidant status helps to minimize the oxidative damage, and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases (Karuna *et al.*, 2009).

Oxidative stress can cause damage to all molecular targets; DNA, proteins and lipids. Often, it is not clear which is the first point of attack, since injury mechanisms overlap widely. When H_2O_2 is added to many mammalian cells, increased DNA strand breakage occurs before detectable lipid

peroxidation or oxidative protein damage. This DNA damage appears to involve conversion of H_2O_2 into OH in the cell nucleus, by reaction with transition metal ions (Halliwell, 2011).

Oxidative Damage to Biomolecules

ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2 . ROS are well recognized for playing a dual role as both deleterious and beneficial species depending on their concentration in plants. At high concentration ROS cause damage to biomolecules, whereas at low/moderate concentration it acts as second messenger in intracellular signaling cascades that mediate several responses in plant cells (Leonard *et al.*, 2012).

Lipid peroxidation

Lipid peroxidation involves series of free radical mediated chain reaction processes, which also is associated with several biological damages. Lipid peroxidation mainly affects biological membranes of especially liver, brain, spinal cord, containing highly oxidizable PUFA (Polyunsaturated fatty acids). Malondialdehyde (MDA) is a volatile B – scission product formed as major product of lipid peroxidation along with other aldehydes and ketones (Ujwala *et al.*, 2012). Lipid peroxidation can result in the formation of reactive products that themselves can react with and damage proteins and DNA (Defeng and Cederbaum, 2003). The consequences of lipid peroxidation are cross linking of membrane proteins, change in membrane fluidity and formation of lipid – protein, lipid – DNA adduct which may be detrimental to the functioning of the cell (Repetto *et al.*, 2012).

When a hydrogen atom is removed from a fatty acid molecule for some reason, the free radical chain reaction proceeds. Thus, radicals that can be involved in the extraction of hydrogen atoms from lipids include the hydroxyl

radical (HO^\bullet), the hydroperoxyl radical (HOO^\bullet), the lipid peroxy radical (LOO^\bullet), and the alkoxy radical (LO^\bullet). The process by which lipid radicals (L^\bullet) thus generated react immediately with oxygen, resulting in the formation of LOO^\bullet , which attacks another lipid and remove a hydrogen atom from it, resulting in the formation of lipid hydroperoxide (Lipid peroxide LOOH) and another L^\bullet . This new L^\bullet also reacts with oxygen and forms LOO^\bullet , which attacks another lipid to generate lipid peroxide, so lipid peroxide accumulates as the chain reaction proceeds (Farooqui and Farooqui, 2011).

DNA Damage

DNA is repaired with great efficiency in living cells. But this repair ceases upon death of organisms or preservation of sample (Zimmermann *et al.*, 2011). DNA damage occurs both as a result of normal metabolism as well as by exogenous inputs such as UV and IR at a rapid rate in every cell (Raman *et al.*, 2012). Reactive oxygen species (ROS) are capable of damaging biological macromolecules such as DNA, carbohydrates and proteins (Subhadradevi *et al.*, 2011).

The oxidative induced DNA damage associated with ROS typically are apurinic/apyrimidinic DNA sites, oxidized purines and pyrimidines, single strand (SSBs) and double strand (DSB) DNA breaks. Two of the most common endogenous DNA base modification are 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamine-4-hydroxy 5-formamidopyrimidine. Both original from the addition of the hydroxyl radical to the C8 position of the Guanine ring producing a 8-hydroxy-7,8-dihydroguanyl radical which can be either oxidized to 8-oxoGua or reduced to give the ring- opened FapyGua (Altieri *et al.*, 2008).

Diseases associated with ROS

Reduction or oxidation caused by addition or loss of any electron is responsible for alterations in functional and structural profile of molecule,

hence, changing signalling mechanism (Riccioni *et al.*, 2012). ROS are involved in signal transduction which is related to relaxation and contraction of blood vessels, migration, growth and death of vascular cells, and also extracellular matrix (ECM) alteration. ROS are not only strongly associated with lipid peroxidation, leading to food deterioration, but are involved in cellular aging, mutagenesis, carcinogenesis, coronary heart disease, diabetes and neurodegeneration (Moskovitz *et al.*, 2008).

Cancer

ROS are considered oncogenic usually. They are increased in cancer cells due to oncogene activation, relative lack of blood supply or other variances and ROS do involve in initiation, progression and metastasis of cancers (Wang and Yi, 2008). This can be manifested by the significant over expression of several antioxidant/repair enzymes in some malignant tissues compared to controls in response to the high- oxidant status (Kobayashi and Suda, 2011).

Neurodegenerative diseases

High levels of oxidative stress can cause necrosis, ATP depletion and prevention of controlled apoptic death. Oxidative stress is an important factor in neurodegenerative diseases, as the damage of the neurons could be due to either an increase in oxidative process or a decrease in antioxidant defences or both. It is extremely difficult to distinguish whether mitochondrial derived oxidative stress is the primary cause of toxicity or just refelt the consequence of neurodegeneration (Shukla *et al.*, 2011).

Diabetes mellities

Diabetes mellitus (DM) is a metabolic disorder characterized by impaired action, secretion of insulin or both, resulting in hyperglycemia. The disease is progressive and is responsible for micro and macro vascular

complication of diabetes mellitus (Pavana *et al.*, 2012). Increased levels of the products of oxidative damage to lipids, proteins and DNA have been detected in the serum of patients with diabetes, and their presence is correlated with the development of diabetes – associated complications (Pan *et al.*, 2011).

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of oxidized lipoproteins, increased cell death and hypertrophic degeneration of the arterial lipoprotein (Nakashima *et al.*, 2007). DNA alteration are present in the atherosclerotic tissues and can play a fundamental role in the pathogenesis of this disease (Olinski *et al.*, 2012).

ANTIOXIDANT DEFENSE

Antioxidant acts as a major defence against radical – mediated toxicity by protecting the damages caused by free radicals (Erejuwa *et al.*, 2010). They are present a low concentrations and significantly delays or prevents oxidation of the oxidizable substrate. Antioxidants are effective because they can donate their own electrons to ROS and there by neutralizing the adverse effects of the latter. An antioxidant in the body may work at three different levels a) prevention – keeping formation of reactive species to a minimum e.g. desferrioxamine b) interception – scavenging reactive species either by using catalytic and non – catalytic molecules e.g. ascorbic acid, alpha tocopherol and c) repair – repairing damaged target molecules e.g. glutathione (Willcox *et al.*, 2010).

The antioxidant systems are classified into two major groups, enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants present in the body include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), that act as body's first line of defence against ROS by catalyzing their conversion to less reactive or inert species (Rahimi *et al.*, 2012).

Non-enzymatic antioxidants include Vitamin E and C, thiol antioxidant (glutathione, thioredoxin and lipoic acid, melatonin, carotenoids, natural flavonoids) and other compounds (Miller *et al.*, 2009).

Antioxidants such as α -tocopherol, ascorbic acid, carotenoids, amino acid, peptides, proteins, flavonoids and other phenolic compounds play a significant role as physiological and dietary antioxidants, thereby augmenting the body's natural resistance to oxidative damage (Arulpriya *et al.*, 2010).

Enzymic Antioxidants

Superoxide dismutase (SOD)

Superoxide dismutase is one of the most important antioxidant enzymes present in all oxygen metabolizing cells (Vendelle and Delledomae, 2011). SOD is an enzyme that catalyzes the conversion of superoxide free radicals to oxygen and hydrogen peroxide. In biological systems, the sources of electrons are generally enzymes and reducing substances. Reducing substances act as antioxidants by reducing less reactive species via reactions which are typically mediated by the cyclical reduction/oxidation of transition-metal ions.

SOD and catalase catalyze the dismutation of superoxide and hydrogen peroxide, respectively. Peroxide and superoxide can also react in the presence of a metal ion to produce hydroxyl radical and molecular oxygen (Li and Zhou, 2011).

Catalase (CAT)

Catalase is an iron-containing enzyme found primarily in the small membrane-enclosed cell components called peroxisomes. It serves to detoxify hydrogen peroxide and various other molecules. One way that catalase eliminates hydrogen peroxide is by catalyzing a reaction between two hydrogen peroxide molecules, resulting in the formation of water and O₂. Catalase can

promote the interaction of hydrogen peroxide with compounds that can serve as hydrogen donors so that the hydrogen peroxide can be converted to one molecule of water and the reduced donor becomes oxidised (Bailey *et al.*, 2012).

Glutathione Peroxidase (GPx)

The antioxidative enzyme glutathione peroxidase (GSH-Px), catalyze the breakdown of inorganic and organic peroxides (Liang *et al.*, 2011). GSH-Pxs prevent lipid peroxidation and protect the cell membranes from oxidative damage (Rosa *et al.*, 2009).

The glutathione peroxidase system consist of several components, including the enzymes glutathione peroxidase and glutathione reductase and the cofactors glutathione (GSH) and reduced nicotinamide adenosine dinucleotide phosphate (NADPH). Together, these molecules effectively remove hydrogen peroxide (Rolo *et al.*, 2012).

Non-enzymic Antioxidants

Vitamin E

Vitamin E is used as a generic designation for a group of eight lipid-soluble compound synthesised by plants. These compounds fall into two classes, tocopherols and tocatrienols, which exhibit the biological antioxidant acitivity of vitamin E. The effects of vitamin E are consistent with an antioxidant role. Vitamin E is thought to have basic functional importance in the maintenance of membrane integrity in virtually all cells of the body. Non-antioxidant functions have also been proposed for α - tocopherol including modification of gene transcription and expression (Yusuf *et al.*, 2012).

Vitamin C

Vitamin C in humans must be ingested for survival. Vitamin C is an electron donor, and this property accounts for all its known functions. As an electron donor, vitamin C is a potent water-soluble antioxidant in humans. Antioxidant effects of vitamin C have been demonstrated in many experiments *in vitro*. Vitamin C is called an antioxidant because, by donating its electrons, it prevents other compounds from being oxidized (Padayatty *et al.*, 2012).

Vitamin A

Carotenoids have received considerable attention for their possible clinical use in diseases associated with reactive oxygen species such as cancer. Lycopene and β -carotene possess antioxidant and antiproliferative properties and anticancer activity in humans (Dahan *et al.*, 2008). Lycopene is considered the carotenoid with the greatest capacity to eliminate the singlet oxygen. Lycopene protects lipid molecules, low-density lipoproteins, protein and DNA against free radical attack, playing an essential role in the protection against disease (Oliveira *et al.*, 2010).

Glutathione

Cellular redox state is regulated by numerous components. The thiol-disulfide compound, glutathione, is considered to be one of the significant, owing to its antioxidant power and potential influence over protein structure and function (Mhamdi and Noctor, 2013). Glutathione in mammalian cells maintains the intracellular thiol redox state and detoxifies exogenous and endogenous reactive molecules. Depletion of intracellular GSH predisposes cells to proapoptotic stimuli and can also activate apoptosis in the absence of such stimuli (Valko *et al.*, 2007).

Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in plants. They have been shown to possess a variety of biological activities at nontoxic concentration in organisms. Dietary flavonoids has a role in cancer prevention. Flavonoids have important effects on cancer chemoprevention and chemotherapy. Many mechanisms of action have been identified, including carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and several of multidrug resistance or a combination of these mechanisms (Duthie and Morrice, 2012).

Medicinal plants

Man ever since his first appearance on Earth, has used plant throughout his historical development as a source of medicine. Medicinal plants have formed the basis of the folkloric medicine which is the main source for new medicines discoveries (Newman *et al.*, 2011). By the middle of the nineteenth century at least 80% of all medicines were derived from plants (Kumarasamyraja *et al.*, 2012).

Herbal drugs are prescribed widely because of their effectiveness, fewer side effects and are relatively low in cost (Odhav *et al.*, 2010). Plants have traditionally served as man's most important weapon against pathogens. India is respository of herbal medicines and there are evidences that herbs are predominant in the treatment of various diseases for revitalizing body system from ancient civilization. Recently, considerable attention has been paid to utilize eco friendly and bio friendly plant based products for the prevention and cure of different human diseases. Interest in medicinal plants is increasing as an alternative to modern medicine (Annalakshmi *et al.*, 2012).

Phyllanthus niruri

Phyllanthus niruri is an annual erect glabrous herb that is found in tropical and subtropical regions of the world. It has been in herbal medicine worldwide for centuries where it grows for the treatment of various ailments including diabetes, malaria, colic, fever, jaundice, kidney and gall bladder stones, urinary tract infections and viral infections (Suleiman *et al.*, 2013).

Traditional uses of *Phyllanthus* have been confirmed by a number of biochemical, pharmacological and clinical studies. These activities have been attributed to the flavonoids terpenes, benzenoids, lignans, lipids, vitamin C, steroids etc that have been purified from the plant (Soh, 2009).

The species *Phyllanthus niruri* is being used for the treatment of jaundice extensively in folklore medicine. Experiments in animal models and humans have shown the plant extract to possess inhibitory activity against Hepatitis B viruses (Tirkey *et al.*, 2005). In majority of cases, extract from the whole plant were used for curing jaundice, followed by root and fruit (Annalakshmi *et al.*, 2012).

The present study is an attempt to study the protective effect of *Phyllanthus niruri* leaves against oxidant induced damage to biomolecules.

The methodology adopted in the present study is presented in the following chapter.

PLATE I

Phyllanthus niruri



Methodology

3. METHODOLOGY

Phyllanthus niruri (plate I) is a widespread tropical plant commonly found in coastal areas, best known by the common names stone breaker or seed-under-leaf. It is an important plant of Indian Ayurvedic system of medicine which is used for problems of the stomach, genitourinary system, liver, kidney and spleen. The plant has also been used to reduce the level of urinary calcium (Patel *et al.*, 2011).

The present study focuses on the effect of aqueous, methanol and chloroform leaf extracts of *Phyllanthus niruri* on the inhibition of *in vitro* lipid peroxidation and on DNA damages mediated by oxidants.

COLLECTION OF PLANT SAMPLE

Fresh leaves of *Phyllanthus niruri* were collected within our University campus. The collected leaves were cleaned under running tap water and dried between the folds of filter paper.

PREPARATION OF METHANOL AND CHLOROFORM EXTRACT

1 g of *Phyllanthus niruri* leaves was homogenized with a total volume of 10 ml of methanol / chloroform. Centrifuged and the supernatant was transferred to a preweighed beaker. The extract was dried at 60°C protected from light. The residue obtained was weighed and dissolved in dimethyl sulphoxide (DMSO) to obtain a final concentration of 5 mg/µl.

PREPARATION OF AQUEOUS EXTRACT

Aqueous extract was prepared fresh on the day of experiment.

TREATMENT GROUPS

The treatment groups set up to study the effect of *Phyllanthus niruri* leaf extracts on oxidant induced damage to lipids and DNA are

Group I - Untreated (negative) control

Group II - H₂O₂ treated (positive) control

Group III - Aqueous extract

Group IV - H₂O₂ + Aqueous extract

Group V - Methanol extract

Group VI - H₂O₂ + Methanol extract

Group VII - Chloroform extract

Group VIII - H₂O₂ + Chloroform extract

The above treated groups were analysed for the following parameters.

PARAMETERS ANALYSED

***In vitro* LIPID PEROXIDATION**

The extent of lipid peroxidation was assessed in three different membrane preparations namely goat liver homogenate, RBC ghosts and liver slices.

LPO IN GOAT LIVER HOMOGENATE

In vitro lipid peroxidation in goat liver homogenate was assessed by the method of Okhawa *et al.* (1979) as described in Appendix I.

LPO IN RBC GHOST

The extent of lipid peroxidation in RBC ghost was estimated by the method of Dodge *et al.* (1963). The procedure is explained in Appendix II.

LPO IN GOAT LIVER SLICES

The estimation of lipid peroxidation in goat liver slices was assessed by the method of Okhawa *et al.* (1979) as detailed in Appendix III.

DNA STRAND BREAKS IN HERRING SPERM DNA

The extent of damage in Herring sperm DNA was measured by an increase in absorbance at 532nm as per the procedure explained in Appendix IV (Aeschlach *et al.*, 1994).

DNA STRAND BREAKS IN λ DNA AND pUC18 DNA

The damage to deoxyribose sugar unit that make up the backbone of DNA was quantified according to Chang *et al.* (2002) in λ DNA and pUC 18 DNA. The experimental details of the same are presented in Appendix V.

The results obtained are presented and discussed in the following chapter.

Results and discussion

4. RESULTS AND DISCUSSION

Oxygen is essential for the aerobic life process. Oxygen has double edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events. Free radicals are for dangerous substances produced in the body along with toxins and waste which are formed during the metabolic process of the body. Over production of free radicals are responsible for tissue injury (Mahantesh *et al.*, 2012).

Oxidative stress results from imbalance between formation and neutralization of prooxidants. It is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA, which leads to protein and DNA damage along with lipid peroxidation in healthy human cells (Dnyaneshwar *et al.*, 2012). Eventually these changes lead to many chronic diseases such as cancer, diabetes, aging, atherosclerosis, cardiovascular diseases, inflammatory diseases and other degenerative diseases in human (Olayinka and Anthony, 2010).

A variety of herbs and herbal extracts contain different phytochemical with biological activity that can be of valuable therapeutic index. Phytochemicals are non – nutritive plant chemicals that have protective properties. Plant produces these chemicals to protect itself against diseases (Kumar *et al.*, 2009).

Phyllanthus niruri, a common Kharif weed, has many effective traditional uses for a wide variety of diseases. Some of the medicinal uses have been supported in experimental models, suggesting that the plant extract possess various pharmacological properties. Due to its impressive preclinical therapeutic potential, extracts of species of the genus *Phyllanthus* have been evaluated to treat hypertension, jaundice, diabetes, hypercalciuria, and urolithiasis (Calixto *et al.*, 2012).

Therefore the present study is a scientific loom to allow the traditional use of *Phyllanthus niruri* and employ its antioxidant activity.

The *in vitro* models that are used in the present study being cost effective and ethics. The *in vitro* models used were goat liver homogenate, RBC ghosts, and goat liver slices and include purified biological such as λ DNA, pUC18 and Herring sperm DNA. They were all purchased from commercial suppliers, apart from the goat liver homogenate, RBC ghosts and goat liver slices, which represent various membrane preparations.

The effect of *Phyllanthus niruri* leaf extract on the extent of lipid peroxidation was tracked in the goat liver homogenate, RBC ghosts and goat liver slices. The goat liver homogenate contains combination of lipids comprising plasma membrane and other intracellular membranes. The RBC ghost was prepared by hypotonic lyses of RBC which constitutes plasma membrane lipids. Goat liver slices contain predominant intact cells and it mimics direct *in vivo* model. Three different membrane preparations were used in order to test whether the nature of the membrane lipid influences the effect of plant extract. The presence of antioxidant in plant suppresses the formation of free lipid radical and thus prevents the formation of endoperoxidation (Sharma *et al.*, 2007).

EFFECT OF *Phyllanthus niruri* LEAF EXTRACTS ON OXIDATIVE DAMAGE TO MEMBRANE LIPIDS

The level of LPO is a measure of membrane damage. Lipid peroxidation has been implicated in the pathogenesis of hepatic injury (Chodon *et al.*, 2008).

The theme of the study was to compare the extent of inhibition of LPO in the presence of *Phyllanthus niruri* leaf extracts. The extent of lipid peroxidation and the effect of *P. niruri* leaf extracts were studied in three different membrane models *in vitro* comprising goat liver homogenate, RBC ghost and goat liver

slices. The percentage inhibition of LPO *in vitro* by the plant extracts is presented in the Table I and depicted in Figure 1.

TABLE I

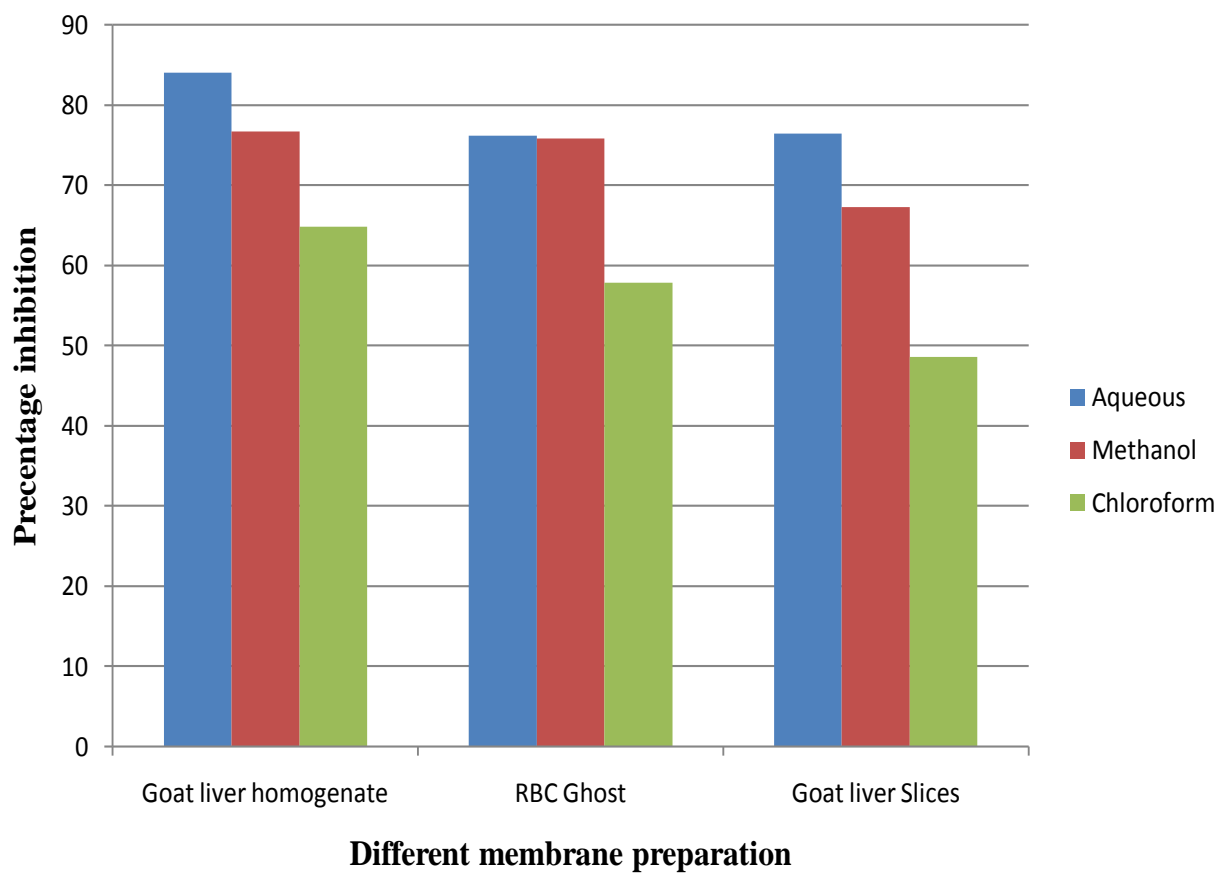
EFFECT OF *Phyllanthus niruri* LEAF EXTRACTS ON LIPID PEROXIDATION IN DIFFERENT MEMBRANE PREPARATIONS

EXTRACT	% INHIBITION OF LPO		
	GOAT LIVER HOMOGENATE	RBC GHOST	GOAT LIVER SLICES
Aqueous	84.1	76.22	76.43
Methanol	76.77	75.87	67.3
Chloroform	64.77	57.9	48.6

From the values tabulated it is clear that the aqueous extract of *P. niruri* leaves exhibited maximum inhibition of lipid peroxidation. This was followed by methanol and chloroform extract in all the three membrane preparations. The inhibition was also found to be higher in goat liver homogenate when compared to the other two membrane preparation.

Chloroform extract of *Costus pictus* leaves possess inhibition of lipid peroxidation and potent antioxidant activity *in vitro* (Kavitha, 2013). The ethanolic extract of the *Piper trioicum Roxb.* and *Physalis minima L.* suppressed the lipid peroxidation in goat liver homogenate and it was also found that *Piper trioicum Roxb.* extract had more activity than *Physalis minima L.* extract (Dinakaran *et al.*, 2011).

Figure 1
Effect of *Phyllanthus niruri* leaf extracts on lipid peroxidation in different membrane preparation



The ethanol extract of *Rumex vasicarius L.* exhibited significant *in vitro* lipid peroxide inhibition activity on compared with standard antioxidant Quercitin (Prasad and Ramakrishnan, 2012). The methanolic extract of *Borreria hispida* had significant *in vivo* antioxidant and lipid peroxidation activity when compared with petroleum ether and ethyl acetate extracts (Shajiselvin *et al.*, 2010). The methanolic extract of *Hygrophila auriculata* inhibited LPO thereby suppressing the vigour of development of hepatocellular carcinoma in rat (Premkumari *et al.*, 2012).

Thus, form our results it is clear that the leaf extracts of *P. niruri* were very effective in inhibiting lipid peroxidation in different membrane preparations.

EFFECT OF *P. niruri* LEAF EXTRACTS ON OXIDANT INDUCED DAMAGE TO DNA MOLECULES

The extent of oxidation damage caused by H₂O₂ to various DNA molecules and the possible protection rendered by the leaf extracts of *P. niruri* were followed in pure DNA preparations and were compared.

EFFECT OF *P. niruri* LEAF EXTRACTS ON DAMAGE INDUCED BY H₂O₂ TO λ DNA AND pUC18 DNA

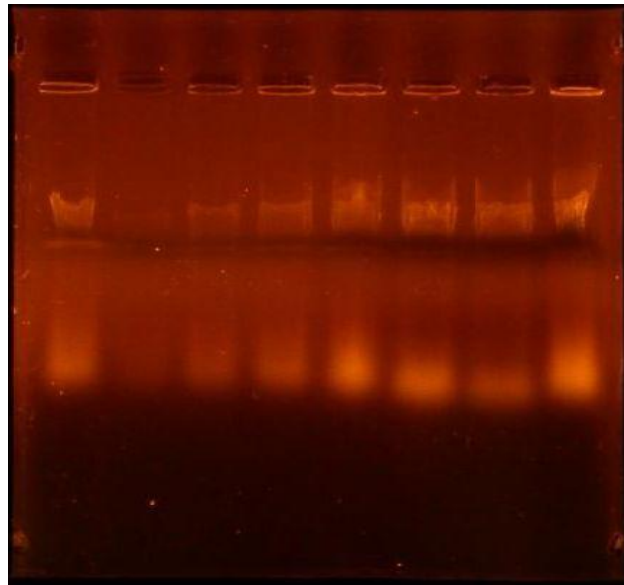
The extent of damage caused to λ DNA and pUC18 DNA after the exposure of H₂O₂ *in vitro* in the presence and absence of *Phyllanthus niruri* leaf extracts is presented in Plate II and Plate III.

H₂O₂ damaged both λ DNA and pUC18 DNA. The absence of significant bands in lane 2 (DNA treated with H₂O₂) suggests that DNA was severely damaged, resulting in very small fragments that cannot be visualized in the gel.

PLATE II

EFFECT OF *P. niruri* LEAF EXTRACTS ON DNA DAMAGE INDUCED BY H₂O₂ TO λ DNA

1 2 3 4 5 6 7 8



LANE 1- CONTROL

LANE 2- H₂O₂

LANE 3- AQUEOUS EXTRACT

LANE 4- AQUEOUS EXTRACT + H₂O₂

LANE 5- METHANOL EXTRACT

LANE 6- METHANOL EXTRACT+ H₂O₂

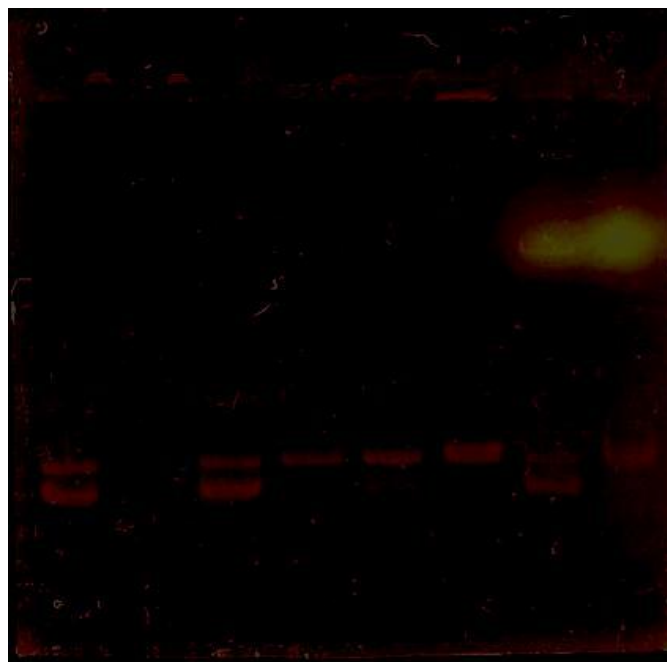
LANE 7- CHLOROFORM EXTRACT

LANE 8- CHLOROFORM EXTRACT+ H₂O₂

PLATE III

EFFECT OF *P. niruri* LEAF EXTRACTS ON DNA DAMAGE INDUCED BY H₂O₂ TO pUC18 DNA

1 2 3 4 5 6 7 8



LANE 1- CONTROL

LANE 2- H₂O₂

LANE 3- AQUEOUS EXTRACT

LANE 4- AQUEOUS EXTRACT + H₂O₂

LANE 5- METHANOL EXTRACT

LANE 6- METHANOL EXTRACT+ H₂O₂

LANE 7- CHLOROFORM EXTRACT

LANE 8- CHLOROFORM EXTRACT+ H₂O₂

The presence of specific bands in lanes (lane 3- Aqueous extract, lane 5- Methanolic extract and lane 7-Chloroform extract) suggests that plant extracts did not cause any damage in both the samples. The leaf extracts of *Phyllanthus niruri* reduced the damage caused by H₂O₂ (lane 4- aqueous extracts + H₂O₂, lane 6- Methanolic extract + H₂O₂ and lane 8- Chloroform extract + H₂O₂), as evidenced by the presence of sharp DNA bands. Aqueous extract exhibited maximum protection of λ DNA and pUC18 DNA.

Aqueous extracts of leaf, fruit and seed of *Moringa oleifera* significantly inhibited the OH-dependent damage of pUC18 plasmid DNA (Singh *et al.*, 2009). The aqueous extract of the edible sea weed *Gracilaria tenuistipitata* reduced H₂O₂- induced plasmid DNA breaks (Yang *et al.*, 2012).

DNA damage induced by H₂O₂ in HeLa cells was inhibited by the methanolic extract of *Viscum album* L. (Onay-Ucar *et al.*, 2012). Increased lipid peroxidation and DNA damage in CCl₄ exposed rat was significantly improved with the co-administration of methanolic extract of *Sonchus asper* (L). Hill, close to those seen in control rats (Khan, 2012).

Chemopodium album's ethanolic leaf extract protect the DNA of both yeast and mononuclear leukocytes against the damaging effect of hydrogen peroxide (Korcan *et al.*, 2013). Sage tea protected colon cells against oxidative and alkylating DNA damage (Ramos *et al.*, 2012).

Methanolic extract of Cyclotrichium niveum exhibited strong concentration dependent inhibition of oxidative DNA damage (Emen *et al.*, 2009). Methanolic extract of *Nelumbo nucifera* inhibited H₂O₂- induced damage on fatty acid peroxidation and plasmid DNA damage in Swiss albino mice (Wang *et al.*, 2010).

EFFECT OF *Phyllanthus niruri* LEAF EXTRACTS ON DAMAGE INDUCED BY H₂O₂ TO HERRING SPERM DNA

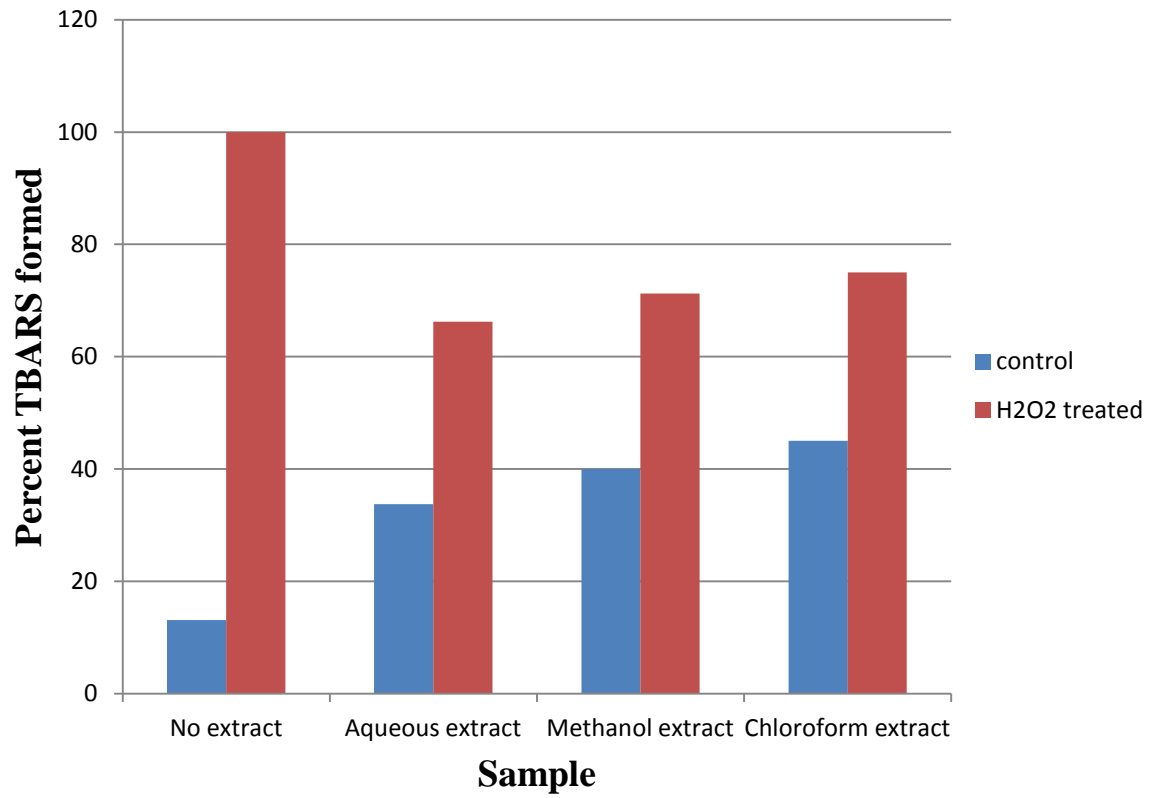
The next level of hierarchy of DNA molecules used in the study included Herring sperm DNA. The extent of DNA damage induced by H₂O₂ to Herring sperm DNA was quantified using spectrophotometer and the values are presented in Table II and Figure 2.

TABLE II

EFFECT OF *Phyllanthus niruri* LEAF EXTRACTS ON H₂O₂ INDUCED DAMAGE TO HERRING SPERM DNA

SAMPLE	PERCENT TBARS PRODUCED	
	CONTROL	H₂O₂ TREATED
No extract	13.12	100
Aqueous extract	33.75	66.25
Methanol extract	40	71.25
Chloroform extract	45	75

Figure 2
Effect of *Phyllanthus niruri* leaf extracts on H₂O₂ Induced damage to Herring Sperm DNA



The amount of TBARS formed in the group treated with H₂O₂ alone was fixed as 100% and the extent of damage caused to other groups were calculated relative to this value. The leaf extracts of *P. niruri* decreased the damage inflicted by H₂O₂.

The aqueous extract of *P. niruri* leaves showed maximum protection against H₂O₂ induced damage in Herring sperm DNA compared to the other two extracts.

Vishnupriya *et al.* (2012) reported the strong antioxidant and DNA damage protecting potential of the aqueous extract of *Curcuma amada*. The aqueous extract of *Lantana camara* possess high antioxidant and DNA damage inhibition potential (Kalita *et al.*, 2012). Oxidative DNA damage has been shown to be reverted upon treatment with many antioxidants (Chen *et al.*, 2010).

The results of the present study exhibit the extent of protection rendered by the *Phyllanthus niruri* leaf extracts to lipids and DNA. Thus our study shows that *Phyllanthus niruri* leaves are an excellent source of natural antioxidants with the potential of lipid and DNA damage inhibition.

The results presented in this chapter are summarized and conclusions that can be drawn from them are presented in the next chapter.

Summary and Conclusion

5. SUMMARY AND CONCLUSION

Traditional system of medicine depends primarily on the use of plant products, either directly or indirectly. Medicinal plants have played an essential role in the development of human health care. Plants are directly used as resources of new drugs, but also in understanding plant toxicity and protecting humans and animals from natural poisons. Herbal medicines are very different from well-defined synthetic drugs. In modern medicine, herbal medicines are more often used to treat chronic diseases, and avoid the occurrence of undesirable side effects.

Free radicals such as reactive oxygen species are formed during a variety of biochemical reactions and cellular functions, which may involve mitochondrial metabolism. The steady state formation of pro-oxidants (free radicals) is normally balanced by a similar rate of consumption by antioxidants. Reaction between cellular components and free radicals lead to DNA damage, mitochondrial malfunction, cell membrane damage and eventual cell death.

The generation of free radicals and the degree to which they cause oxidative injury plays an important role in the process of various free radicals associated diseases. The detoxification of free radicals and hydrogen peroxide consequently is of prime importance in many defense mechanisms. Antioxidants are molecules that act as free radical scavengers. Antioxidants bind with free radicals and protect against oxidative stress and prevent damage to cells.

Most of the medicinal plants possess antioxidant properties. *P. niruri* is one of the most important medicinal plants used in different regions in the world for the treatment of various diseases. In order to view the medicinal property of the active compounds, leaf extracts of the candidate plant of

different polarity (water, methanol and chloroform) were used. The leaf extracts were analysed for their effect against LPO and DNA protecting effects.

The effects of the plant extracts on lipids were studied, as damage to lipid is an important end point of biological oxidative damage. The extent of lipid peroxidation (LPO) was analysed in three different membrane systems, representing different groups of lipid.

The first was the goat liver homogenate, which was a mixture of plasma membrane and internal membrane constituents. The second system was the goat RBC ghost, which constituted the plasma membrane components. The third system was the goat liver slices, which were intact liver cells assaulted by the oxidant H_2O_2 .

The results observed several that the plant extracts are efficient in protecting all the three membrane preparations. The effect of aqueous extract was maximal when compared with methanol and chloroform extracts.

Following there finding, in the second part of the study, the effects of the leaf extracts of *Phyllanthus niruri* was determined for their DNA- protective effects using λ DNA (phage DNA), pUC18 DNA (plasmid, circular, bacterial DNA) and Herring sperm DNA (haploid, high molecular weight, eukaryotic DNA).

The extent of damage to λ DNA and pUC18 DNA in presence and absence of plant extracts of *Phyllanthus niruri* were analysed by following the migration pattern of the treated DNA on the agarose gels.

The results showed that exposure to H_2O_2 caused damage to λ DNA and pUC18 DNA. H_2O_2 damage both types of DNA significantly. The leaf extract by themselves, did not caused any damage to the DNA molecules. Additionally, the presences of extracts caused a significant protection both the DNA molecules. The leaf extracts of *Phyllanthus niruri* in presence of H_2O_2 caused a

significant protection to both DNA, signified by the presence of bands compared to the untreated control.

H₂O₂ caused maximum damage to Herring sperm DNA. On treatment with the plant extracts, the results showed that the maximum protection was offered by the aqueous extract of *Phyllanthus niruri* than the other two extracts. Thus, from the observation all the three extracts were effective in protective of biomolecules among which extract of *Phyllanthus niruri* leaves was very effective in protecting lipids and DNA under oxidatively stressed conditions.

Our results indicate that the nature of oxidative damage and its protection vary depending on the sequence and nature of the DNA molecule. Thus it becomes imperative to study several sources of DNA, before concrete conclusions can be drawn regarding the oxidative DNA damage and its protection by plant extracts.

Plant medicine appears to be safer because of its proximity to nature. Now there is growing trend of acceptance of plant medicine worldwide because many synthetic and or chemical drugs proving to be toxic in both short term and long term use.

The outcome of the present study reveals that the leaf extracts of *Phyllanthus niruri* possess inhibitory effect on LPO and protecting moieties. This study strongly warrants closer attention to this traditional plant for the development of drugs to treat various complications initiating by free radicals.

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Appendices

APPENDIX I

ESTIMATION OF LIPID PEROXIDATION IN GOAT LIVER

HOMOGENATE

(Okhawa *et al.*, 1979)

Goat liver was procured fresh from a slaughter house and washed free of blood using Tris-HCl buffer (40 mM, pH-7). A 20% homogenate of the liver was prepared in the same buffer using a motorized Teflon homogenizer. The homogenate was clarified to remove debris and used as the membrane source for assessing lipid peroxidation.

REAGENTS:

1. FeSO₄ (10μM)
2. TBA (1%)
3. Tris- buffered saline (TBS) - 10mM Tris, 0.15M NaCl, pH 7.4
4. Ethanol - 70%
5. Acetone

PROCEDURE

The reaction mixture (0.5 ml) contained 0.05 ml each of liver homogenate, leaf extract (50μl) and TBS. A blank containing no lipid source but only FeSO₄, no plant extract and TBS to a final volume of 0.5ml was prepared.

An assay medium corresponding to 100% oxidation was prepared by adding all the other constituents except the plant extract. The experimental medium corresponding to auto oxidation contained only the liver homogenate. All the tubes were incubated at 37°C for 1 hr. After incubation, 0.5ml of 70% ethanol was added to all tubes to arrest the reaction. 1 ml of 1% TBA was added

to all tubes. The contents were incubated in a boiling water bath for 20 minutes. After cooling to room temperature, added 0.5ml of acetone. The intensity of pink color produced was measured at 535nm in a spectrophotometer.

APPENDIX II

ESTIMATION OF LIPID PEROXIDATION IN RBC GHOSTS

(Dodge *et al.*, 1963)

REAGENTS

1. Isotonic KCl - 1.15%
2. Hypotonic KCl - 0.5%
3. Phosphate buffered saline (PBS)

PREPARATION OF GOAT RBC GHOSTS

About 50 ml of fresh venous whole blood was collected into a clean sterile bottle. The blood was defibrinated immediately using acid washed stones. The defibrinated blood was then transferred to sterile centrifuge tube and diluted 1:1 with sterile isotonic KCl. It was centrifuged at 3000 rpm for 10 minutes at 4°C to pellet the cells and the supernatant was discarded. The pellet of RBC was washed with isotonic KCl thrice. The washed pellet was then treated with hypotonic KCl (0.5%) and incubated at 37°C for one hour for lysis to occur. The lysate was centrifuged at 5000 rpm for 15-20 min at 4°C. The pellet obtained was washed repeatedly with hypotonic KCl until most of hemoglobin was washed off. The pale pink pellet containing the erythrocyte ghosts was then suspended in 1.5 ml PBS and used as a source of membrane lipid.

The determination of LPO in erythrocytes was carried out as for the liver homogenate.

APPENDIX III

ESTIMATION OF LIPID PEROXIDATION IN GOAT LIVER SLICES

(Okhawa *et al.*, 1979)

REAGENTS:

1. Phosphate buffered saline (PBS)
2. HBSS – Hank's Balanced salt solution
3. H₂O₂ - 30%

PREPARATION OF GOAT LIVER SLICES

The goat liver was collected fresh from a slaughter house, plunged into cold sterile PBS and maintained at 4°C till use. Then slices of 1mm thickness were cut using a sterile scalpel.

PROCEDURE

250mg portion of the slices were used for the assay. The slices were taken in 1ml HBSS and treated with H₂O₂ (5µl of 30% solution), with or without 20µl of the leaf extract. The slices were incubated at 37°C in a water bath for one hour. At the end of the incubation period, the slices were taken into a homogenizer tube along with the incubated HBSS and homogenized using a Teflon homogenizer. The homogenate was clarified using low speed centrifugation and an aliquot taken for the assay of TBARS formed. The LPO reaction in all the tubes was arrested by the addition of 500µl of 70% ethanol. 1 ml of 1% TBS was added to all tubes and heated in a boiling water bath for 20

minutes. After cooling to room temperature, added 500 μ l of acetone and measured the TBARS at 535nm in a spectrophotometer.

APPENDIX IV

EVALUATION OF DNA STRAND BREAKS IN HERRING SPERM DNA (Aeschlach *et al.*, 1994)

The damage caused by H₂O₂ in Herring sperm DNA was studied in the presence and absence of plant extracts.

REAGENTS

- 1) Herring sperm DNA (0.5mg/ml in 10mM Tris buffer, pH 7.4)
- 2) H₂O₂ (30%)
- 3) MgCl₂
- 4) FeCl₃ (50 μ M)
- 5) EDTA (0.1M)
- 6) TBA (1%)
- 7) HCl (25% v/v)

PROCEDURE

The reaction mixture (0.5ml) contained, Herring sperm DNA (0.05ml), H₂O₂ (0.167ml), MgCl₂ (0.05ml), FeCl₃ (0.05ml) and leaf extract (5 μ l). The mixture was incubated at 37°C for 1 hr. The reaction was terminated by the addition of 0.05ml of 0.1M EDTA. The color was developed by adding 0.5ml of TBA (1%) and 0.5ml HCl (25%) followed by incubation at 37°C for 15 minutes. After centrifugation the extent of DNA damage was measured by the increase in absorbance at 532nm.

APPENDIX V

ESTIMATION OF DNA DAMAGE USING λ DNA AND pUC18 DNA

(Chang *et al.*, 2002)

The DNA damage to deoxyribose sugar unit that make up the backbone of DNA, induced by H₂O₂ in the presence and absence of the plant extracts was quantified in λ DNA and pUC 18 DNA.

REAGENTS

1. λ DNA
2. pUC18 DNA
3. Tris buffer (30mM, pH 7.4)
4. H₂O₂ (30%)
5. FeCl₃ (500 μ M)
6. Agarose (1% in 1X TAE buffer)
7. EtBr (10mg/ml)
8. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol)
9. 50X TAE buffer (Tris base 24.2 g, EDTA 18.612 g, glacial acetic acid 5.7 ml, in a total volume of 100 ml, pH 8.0)

PROCEDURE

The reaction was conducted in a total volume of 30 μ l containing 5 μ l of 50mM Tris buffer (pH 7.4), 5 μ l of λ DNA or pUC18 plasmid DNA (2 μ g concentration), and 5 μ l of Tris buffer or plant extract. Then 10 μ l of 30% H₂O₂ and 5 μ l of 500 μ M FeCl₃ were added and incubated at 37°C for 15 min for pUC18 DNA and 30 min for λ DNA. The reaction mixture was then placed in 1% agarose gel and run at 100V for 15 min in a submarine gel electrophoresis

apparatus. The DNA was visualized and photographed using an alpha digidoc digital gel documentation system.