In vitro ANTIOXIDANT ACTIVITY OF *Phyllanthus niruri* LEAF EXTRACT

SARANYA.J

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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

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Parvathi

Signature of the
Head of the Department

P. Rathi

Signature of the
Supervisor

Acknowledgement

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Introduction

1. INTRODUCTION

Oxygen is essential for survival. It is very stable in the ground state. Molecular oxygen react rapidly with free radicals to become reactive oxygen species (ROS) inevitable to living cells and highly associated with wide range of pathogenesis such as diabetes, cancer, liver damage, inflammation, ageing and neurological disorder (Saha and Tamrakar, 2011).

ROS cause damage to cell structures, DNA, lipids and protein. The ROS are generated continuously by both endogenous and exogenous sources. Almost all the organism possess defence mechanism against free radical induced oxidative stress, which involve preventive mechanisms, repair mechanisms, physical defences and antioxidant defences (Khatoon *et al.,* 2013).

ROS including free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and non-free radical species such as hydrogen peroxide (H_2O_2), various form of activated oxygen are often generated by oxidation product of biological reaction or exogenous factor. There is a balance between generation of ROS and antioxidant system in organism. In pathological condition, ROS are overproduced and result in lipid peroxidation and oxidative stress. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in cellular membrane or intracellular molecules (Awah *et al*., 2010).

The oxygen free radicals initiate lipid peroxidation and inflict damage to macromolecular components of cell (Ragavendra *et al.,* 2007). During oxidative stress and exposure to radiation, excessive free radicals are produced which are known to cause damage to biomolecules (Naik *et al*., 2008). Due to detection of many bioactive compounds in food with possible antioxidant activity, there has

been increased interest in relationship between antioxidants and disease risks (Tarhan *et al.*, 2007).

The antioxidant compounds may function as free radical scavengers, potential complexing of pro-oxidant metals and quenching of singlet oxygen. Antioxidants may offer resistance against the toxic oxidative reaction inhibiting the lipid peroxidation and by other mechanism and thus prevent diseases (Umamaheswari *et al*., 2008).

The plant kingdom is an abundant source of phytochemicals having important properties. Plants have developed a complex antioxidative defence and many antioxidant compounds, naturally occurring from plant sources have been identified as free radical or active oxygen scavenger (Youweie *et al.*, 2008). As plant produce significant amount of antioxidants to prevent the oxidative stress they represents a potential source of new compounds with antioxidant activity.

Consumption of dietary antioxidants from plant materials has been associated with a lower incidence of diseases due to oxidative stress from free radicals accordingly, dietary antioxidant have recently garnered increased research interest (Wojchikowski, 2008).

Traditional herbal medicines form an important part of the health care system of India. Ayurveda, supposed to be the oldest medical system in the world provides potential leads to find active and therapeutically useful compounds from plants (Hazra *et al*., 2009). One of the most challenging pursuits in the realm of pharmaceutical and medical success is the search for newer and more potent drugs with little toxic effects, self-administrable, less expensive and completely reversible. Much of these properties are observed in the drugs of plant origin (Raiz *et al*., 2011).

One such popularly used plant that is reported to have antitumor, anti-carcinogenic, hypolipidaemic, hepatoprotective, antiviral used for the treatment of urolitic disease, as a diuretic is *Phyllanthus niruri*, which is commonly known as stone breaker. Several bioactive molecules such as lignin, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins are present in *Phyllanthus niruri* (Sabir and Rocha, 2008). In all the medical preparations, it is the whole plant, fruits and leaves that are used.

In order to better understand the antioxidant activity of *Phyllanthus niruri* leaves, three different extracts (aqueous, methanol and chloroform) were prepared and assessed for their radical scavenging effects.

Thus the objective of the present study was set as to assess the free radical scavenging effects of aqueous, methanol and chloroform extracts of *Phyllanthus niruri* leaves using various free radicals and oxidants.

The literature relevant to the present study was studied and a brief review of the same is presented in the next chapter.

Review of literature

2. REVIEW OF LITERATURE

Oxygen is an element obligatory for life. Living system has evolved to survive in the presence of molecular oxygen and for most biological systems. Oxidative properties of oxygen play a vital role in diverse biological phenomena. Oxygen has double edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events (Mahantesh *et al.*, 2012).

FREE RADICALS

Biological combustion produces harmful intermediates called free radicals. Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. The family of free radical generated from the oxygen is called reactive oxygen species (ROS), which cause damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen. There is an extensive evidence to involve ROS in the development of degenerative diseases (Chanda and Dave, 2009).Compounds especially from natural sources are capable of providing protection against free radicals (Rosalind *et al*., 2013).

Free radicals can cause a wide range of toxic oxidative reaction like initiation of the peroxidation of the membrane lipids, inhibition of mitochondrial respiratory chain enzymes, fragmentation or random cross linking of molecules like DNA, enzymes and proteins which ultimately leads to cell death (Sharma *et al.*, 2012).

There is an increased evidence for the participation of these free radicals in the aetiology of diseases like cancer, diabetes, cardiovascular disease, autoimmune disorders, neurodegenerative disorders, ageing etc (Poongodi *et al.*, 2012).

SOURCES OF REACTIVE OXYGEN SPECIES

A diverse group of physical and chemical agents can lead to the production of ROS. The ROS can be formed in living things both from exogenous and endogenous sources. Endogenous sources of free radicals include normal aerobic respiration, peroxisomes and stimulation of polymorph nuclear leucocytes and macrophages. The exogenous source includes ionizing radiation, tobacco smoke, pollutants, pesticides and organic solvents (Oboh *et al.*, 2011).

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 (Huy *et al*., 2008). ROS including free radicals such as superoxide anion radicals($O_2^{\cdot -}$), hydroxyl radicals (OH^-), singlet oxygen ($^1O_2$) and non-free radical species such as hydrogen peroxide (H_2O_2) are various forms of activated oxygen (Shilva *et al*., 2010).

TYPES OF FREE RADICALS

SUPEROXIDE ANION:

It is the first product of oxygen. It regulates metabolites capable of signalling and communicating important information to the cellular genetic machinery. O_2^- is non-reactive and dismutase to H_2O_2. Superoxide anion can be produced by either univalent reduction of oxygen or by univalent oxidation of H_2O_2. As it is unreactive, it can diffuse through a long way and at low pH it becomes protonated (HO_2), which is reactive. They increase under stress conditions and various disease states (Gutowski and Kowalczyk, 2013).

HYDROGEN PEROXIDE

It is the most stable reactive oxygen molecule, least reactive and can be easily detected. H_2O_2 is the primary product formed by the reduction of oxygen by numerous oxidases, such as xanthine oxidase and D-amino acid oxidase. H_2O_2 can easily cross cell membrane and attack different sites by converting into OH. H_2O_2can cause DNA damage in the form of both single strand and double strand break, which is believed to be the initial step in the induction of cancer (Kumar and Kumar, 2009).

HYDROXYL RADICAL

It is highly reactive and short lived. It reacts with any molecule present in cells and due to this it does not diffuse through the cells. The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine base and also deoxyribose backbone (Gul *et al.,* 2013).

REACTIVE OXYGEN SPECIES AND FREE RADICALS MEDIATED DAMAGE

ROS are continuously produced during normal physiologic events and removed by antioxidant defence mechanism. There is a balance between generation of ROS and antioxidant system in organisms. In pathological condition ROS are overproduced and result in lipid peroxidation and oxidative stress (Saritha *et al.,* 2010).

Oxidative stress depicts the existence of products called free radicals and ROS which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous system. In fact, oxidative stress results from an imbalance between the generation of reaction oxygen species and endogenous antioxidant system (Constantini *et al.,* 2009).

ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* and create oxidative stress, which results in numerous diseases and disorders. Excessive amount of ROS is harmful because they initiate bimolecular oxidation which leads to cell death and creates oxidative damage to cellular system (Chanda and Dave, 2009).

ROS formed *in vivo* such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. They are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signalling and immune function. ROS are regulated by endogenous superoxide dismutase, glutathione peroxidase and catalase but due to over production of reactive species, induced by exposure to external oxidant substances or a factor in the defence mechanisms, damage to cell structure DNA, lipids and proteins, occur which increases rest of more than thirty different disease processes (Ali *et al*.,2008).

INVOLVEMENT OF FREE RADICALS IN DISEASES

Free radicals may lead to cell injury and death ,which may contribute to many disorders like cancer, hepatic ailments, cardiovascular disease, cataracts, immune system decline, diabetes mellitus, inflammation, renal failure, brain dysfunction and the process of aging (Tippani *et al*., 2010).

The diseases and disorders in which free radicals production appears to be involved are:

CARDIOVASCULAR DISEASE

Cardiovascular disease can be defined as a class of diseases affecting the heart and/or blood vessels. Oxidative stress is involved in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis,

hypertension, diabetes and heart failure.Free radical damage has been implicated in the development of cardiovascular disease and in order to reduce, a number of antioxidant nutrients are considered to be important for the body's defence systems. Diets rich in fruit and vegetables, which contain antioxidants such as vitamin C, vitamin E and beta-carotene, are said to be associated with reduced risk of developing cardiovascular disease (Oguntibeju *et al.*, 2009).

NEUROLOGICAL DISORDER:

Reactive species play an important part in neuronal death in major neurodegenerative diseases. Neurodegenerative diseases comprise a condition in which nerve cells from brain and spinal cord are lost leading to either fundamental loss (ataxia) or sensory dysfunction (dementia). The major neurodegenerative diseases are Alzheimer's disease, Huntington disease, Parkinson's disease, etc (Uttara *et al*., 2009).

Antioxidants may have neuroprotective (preventing apoptosis) and neurogenerative roles by reducing or reversing cellular damage and slowing progression of neuronal loss. Antioxidants such as carotenoids and in particular lycopene, a potent antioxidant in tomatoes and tomato products, are potentially useful agents in the management of human neurodegenerative disease (Hamid *et al.,* 2010).

DIABETES

Diabetes mellitus is a state of sustained hyperglycaemia due to absolute or relative insulin deficiency or inactivity. Although diabetes mellitus is defined in terms of high blood glucose level, it is associated with multiple metabolic, endocrine and hematological derangements which are important in the pathogenesis of the disease and its complications (Wali *et al.,* 2011). Deficiencies

of micronutrients may increase susceptibility of diabetic mellitus and the associated complications. Complex antioxidant mechanism including antioxidant vitamins and trace elements exists to limit the effects of these reactions (Wali *et al.*, 2013).

CANCER

Cancer is a multistep disease incorporating environmental, chemical, physical, metabolic and genetic factors which play a direct and / or indirect role in the induction and deterioration of cancers. It is increasingly proposed that ROS and reactive nitrogen species (RNS) play a key role in human cancer development. Antioxidants may prevent or delay the onset of some types of cancers (Baghel *et al*., 2012).

ROS and RNS have been shown to possess many characteristics of carcinogens. Mutagenesis by ROS and RNS could contribute to the initiation of cancer, cause structural alteration in DNA, and affect cytoplasmic and nuclear signal transduction pathways (Klaunig *et al*., 2010). Intake of fresh fruit and vegetables which are the main sources of these antioxidants is correlated with cancer of the stomach, pancreas, oral cavity and oesophagus and to lesser extent of the breast, cervix, rectum and lung.

CATARACT

Cataract remains the leading cause of visual disability. Lipid peroxidation (LPO) within the lens may contribute to the development of cataract. LPO in eye is due to impaired enzyme defences, leading to the accumulation of ROS; induce LPO in lentiallar membrane (**Oduntan *et al*., 2011**).

AGING

Aging is associated with a general decline in physiological functions including cognitive functions. Oxidative stress contributes to age related impairment in learning and memory (Herrera *et al*., 2009). This is supported by a correlation between age, memory impairment and the accumulation of oxidative damage to cellular molecules. ROS are necessary components of signal transduction cascades during normal physiological process (Serrano *et al.*, 2009). According to the free radical theory, aging can be considered as a progressive, inevitable process partially related to the accumulation of oxidative damage into biomolecules - nucleic acids, lipids, proteins or carbohydrates due to an imbalance between prooxidants and antioxidants in favour of the former (Narang *et al.*, 2011).

ANTIOXIDANTS

Antioxidants may be defined as radical scavengers, which protect the human body against free radicals that may cause pathological conditions. Antioxidant delay or inhibit the oxidation of lipids or other biomolecules by inhibiting the initiation or propagation of oxidative chain reaction. Antioxidants prevent free radicals from doing harm to our DNA, proteins, and cells by donating electrons to stabilize and neutralize the harmful effects of the free radicals. This action helps in protecting the body from degenerative diseases (Wali *et al*., 2011).

Antioxidant based drugs/formulation are used in the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer (Ara and Nur, 2009).

MEDICINAL PLANTS -SOURCE OF ANTIOXIDANTS

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine (Ravi *et al.*, 2009). India has such tradition in the case of medicinal plant for the development of the therapeutic materials. Plants are potent biochemical factories and have been components of phytomedicines in recent times immemorial. Plant based natural constituents can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds etc. Any part of the plant may contain active components. The beneficiate medicinal effect of plant material typically results in the secondary products present in the plant.

Many plant products are used as important therapeutic agent (Kaushik and Jalapure, 2011). Medicinal plants play a vital role in pharmaceutical field to produce new drugs to cure disease. Flavonoids present in plant exhibit several biological effects such as anti-inflammatory, hepatoprotective, antidermatitic (Samantha *et al*., 2011).

Plant constitutes an important source of active natural products which differ widely in terms of structure and biological properties (Uhegbu *et al*., 2011). Even in modern times, plant based systems continue to play an essential role in health care (Ashafa *et al*., 2010).

The protective effects of plant products are due to the presence of several components which have distinct mechanisms of action, some are enzymes and proteins and other low molecular weight compounds such as vitamin, carotenoids, flavonoids, anthocyanin and other phenolic compounds. They are important in the

plant for normal growth and development and defence against infection and injury (Arab and Salem, 2010). Plants used for traditional medicine contains a wide range of substance that can be used to treat chronic as well as infectious diseases (Sasidharan *et al*., 2011).

Plants are an untapped reservoir of various awaiting intensive exploitation of their biological properties (Ramana *et al*., 2007). Many plants are distributed in our country of rich biodiversity, the potential of which are not fully utilized for want of knowledge. In this connection we have chosen to investigate the free radical scavenging activity of extracts of *Phyllanthus niruri* of the Indian Pharmacopeia, which find immense importance in Indian Ayurveda.

Phyllanthus niruri is a perennial herb distributed throughout India. Whole plant, fresh leaves and fruits are used to treat various ailments particularly hepatitis (Nair *et al.*, 2010).

With this background of literature, the methodology adopted for the present study is detailed in the next chapter.

PLATE 1

Phyllanthus niruri

Methodology

3. METHODOLOGY

Phyllanthus niruri (Family: *Euphorbiaceae*) is a perennial herb distributed throughout India. Whole plant, fresh leaves and fruits are used to treat various ailments particularly hepatitis. It is found to possess antitumor, anticarcinogenic, hypolipidaemic, antiviral and hepatoprotective activity. *Phyllanthus niruri* traditionally is claimed to be useful in the treatment of liver ailments.

The aim of the present study is to assess the free radical scavenging activity of three different extracts of leaves of *Phyllanthus niruri.*

COLLECTION OF PLANT SAMPLE

The fresh leaves of *Phyllanthus niruri were* collected within our university campus. The leaves were collected, washed in running tap water to remove the surface contaminants and blotted dry between folds of filter paper.

PREPARATION OF METHANOL AND CHLOROFORM EXTRACTS

1g of the fresh leaves was homogenized in 10 ml of methanol and chloroform using mortar and pestle. The homogenate was centrifuged at lower rpm to clarify the extract. The supernatant corresponding to the concentration of 1mg/µl was used for assay. The supernatant was transferred to a preweighed beaker and evaporated at 60ºC protected from light. The residue was weighed and dissolved in dimethyl sulphoxide (DMSO) at a concentration of 5mg/µl. The extracts were tested for their ability to scavenge the free radicals.

PREPARATION OF AQUEOUS EXTRACT

Aqueous extract was prepared fresh when experiments were performed.

FREE RADICAL SCAVENGING EFFECTS OF *Phyllanthus nirruri* LEAF EXTRACTS

The free radical scavenging effects of *Phyllanthus niruri* leaf extracts was assessed by analyzing its ability to scavenge DPPH, ABTS, H_2O_2, hydroxyl radicals and by determining its reducing property.

DPPH RADICAL SCAVENGING ACTIVITY - DOT PLOT ASSAY

The ability of the leaf extracts to scavenge the DPPH radical was tested in a rapid dot-plot screening and quantified using a spectrophotometric assay. The rapid screening assay was performed by the method outlined in Appendix I proposed by Soler-Rivas *et al.* (2000).

DPPH RADICAL SCAVENGING ACTIVITY - SPECTROPHOTOMETRIC METHOD

The ability of the different extracts to scavenge stable free radical DPPH (1,1 diphenyl – 2 – picrylhydrazyl) was assessed. In this method a commercially available and stable free radical DPPH, which is soluble in methanol is used. In its radical form DPPH has absorption at 517nm and can be estimated according to the procedure outlined in Appendix II by Mensor *et al.* (2001).

ABTS RADICAL SCAVENGING ACTIVITY

The antioxidant activity of the leaf extract was studied by the ability to scavenge the free radicals ABTS (2,2' azino-bis 3 ethyl benzthiazoline 6 sulphonic acid) and can be estimated as per the procedure in Appendix III (Shirwaikar *et al*., 2006).

HYDROXYL RADICAL SCAVENGING ACTIVITY

The damage to deoxyribose, which is the backbone of DNA, induced *in vitro* by H_2O_2 in the presence and absence of plant extracts was quantified as TBARS and was estimated according to the procedure in Appendix IV (Elizabeth and Rao, 1990).

H_2O_2 SCAVENGING ACTIVITY

The H_2O_2 scavenging ability of different extracts of leaves of *Phyllanthus niruri* was determined according to the procedure in Appendix V (Ruch *et al*., 1989).

ASSAY OF REDUCING POWER

The reducing property was determined according to the modified method outlined in Appendix VI of (Oyaizu, 1986).

DETERMINATION OF IC_{50} VALUE

The extract concentration providing 50% inhibition (IC50) was calculated from the plot of inhibition (%) against extract concentration. Ascorbic acid standard was used for comparison.

The results thus obtained are presented and discussed in the next chapter.

Results and discussion

4. RESULTS AND DISCUSSION

Oxygen is highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “Free Radicals”. A free radical may be defined as a molecule or molecular fragments containing one or more unpaired electron in its outermost atomic or molecular orbital and are capable of independent existence (Sen *et al*., 2010). They are highly reactive species capable of wide spread, indiscriminate oxidation and peroxidation of protein, lipids and DNA, which can lead to significant cellular damage and even tissue and/or organ failure (Saha and Tamrakar, 2011).

Antioxidants are compounds which, interfere with the production of free radicals and also play a key role to inactivate them (Prakash *et al*., 2007). The human body is equipped with antioxidant defence system that deactivates these highly reactive free radicals. Antioxidants and antioxidant nutrients takes up all excess energy that these free radicals have, turning them into harmless particles that can be metabolized. So these antioxidant nutrients are functional components of food that have extra health benefits in the body (Dadheech *et al.*, 2006).

Four thousand years ago, the medicinal knowledge of the Indian subcontinent was termed as Ayurveda. It remains an important system of medicine and drug therapy in India. Today the pharmacologically active ingredients of many Ayurvedic medicines are being identified and their usefulness in drug therapy is being determined (Samy *et al.*, 2008).

The plant used in the present study *Phyllanthus niruri* is one such medicinal plant belonging to the family *Euphorbiaceae*. The present study “*In vitro* antioxidant activity of *Phyllanthus niruri* leaf extract” was formulated to assess the

free radical scavenging activity of the plant extract against a battery of radicals such as DPPH, ABTS, H_2O_2, OH and to determine its reducing power.

The results thus obtained is presented and discussed below.

DPPH RADICAL SCAVENGING ACTIVITY OF *Phyllanthus niruri* LEAF EXTRACTS

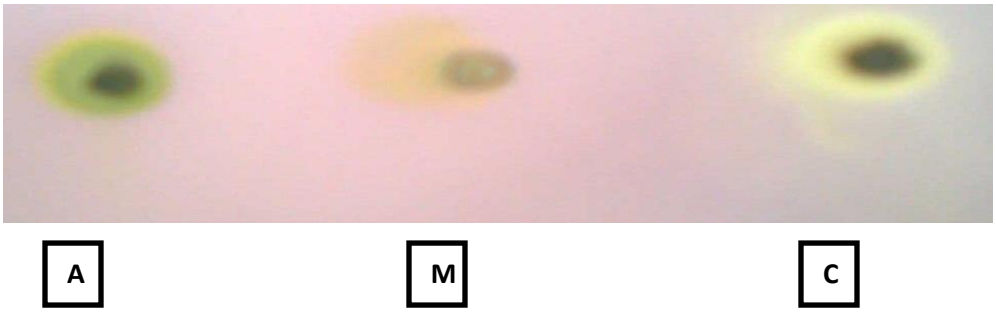
DPPH DOT PLOT ASSAY

The DPPH radical reacts with suitable reducing agents and electrons become paired off and such reactivity has been widely used to test the ability of compounds/plant extracts to act as free radical scavengers. The ability of the leaf extracts to scavenge the DPPH radical was tested in a rapid dot-plot screening. Plate 2 shows the results obtained in the dot plot assay.

Among the three different extracts, aqueous extract of *P. niruri* leaves showed maximum radical scavenging activity. The appearance of the yellow color in the extract is an indication of its scavenging activity.

PLATE 2

DPPH – DOT PLOT ASSAY



A – Aqueous extract

M – Methanol extract

C – Chloroform extract

DPPH SPECTROPHOTOMETRIC ASSAY

The stable DPPH radical is widely used to evaluate antioxidant activities. The addition of extracts to the DPPH solution causes a rapid decrease in the optical density at 515nm. The degree of discoloration is indicative of the scavenging capacity of the extracts. The result of the present study is shown in Figure 1.

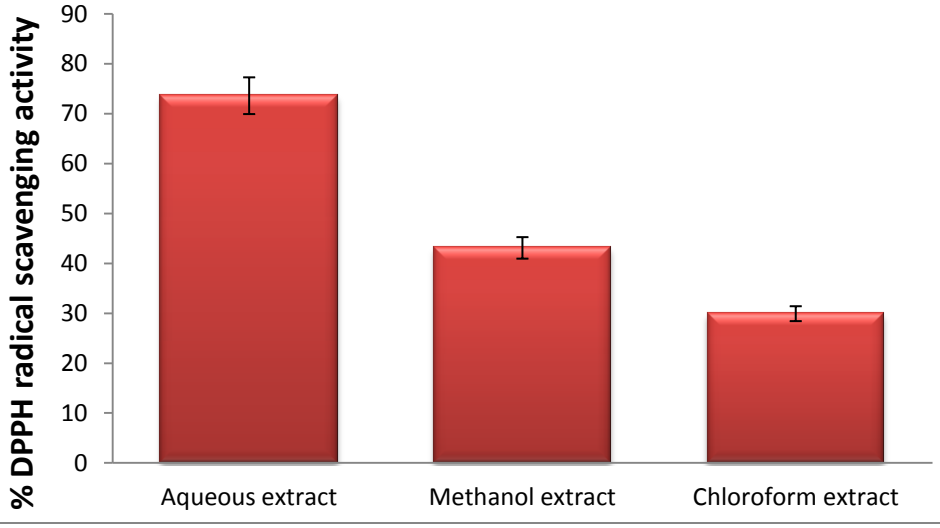
Among the three extracts, the aqueous extract of *P. niruri* leaves exhibited strong DPPH radical scavenging activity compared to methanol and chloroform extract. The IC_{50} value represents the concentration of the extract that caused 50% inhibition in the initial DPPH concentration. The IC_{50} values obtained for DPPH scavenging was found to be 0.4mg/20µl which were comparable for the reference standard, ascorbic acid and the results are discussed in Figure 2.

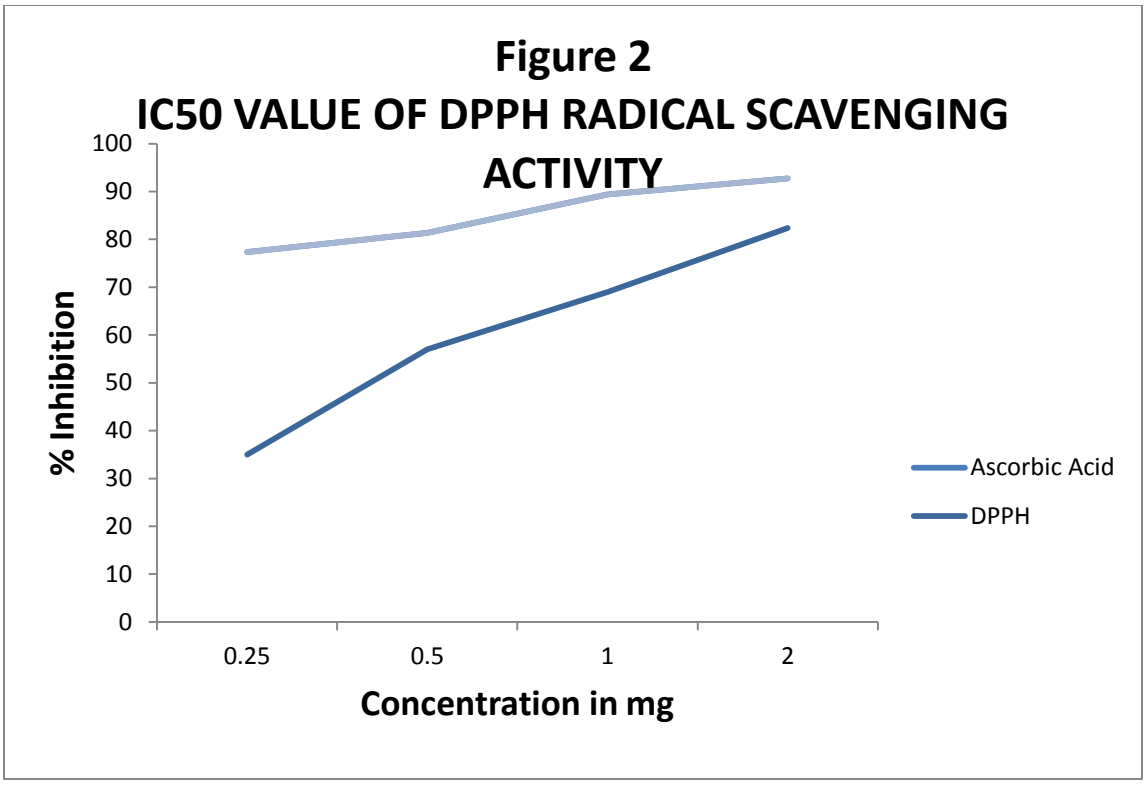
Clitoria ternatea and *Alternanthra sessilis* leaves were tested for their free radical scavenging activity against DPPH. The maximum extents of DPPH scavenging activity was elicited by the methanol extract followed by the ethanol extract and then by the aqueous extract (Balakrishnan *et al*., 2011). Different extracts of *Polygalia myrtifolia* leaves were tested for their DPPH radical scavenging activity and aqueous extract exhibited maximum radical scavenging activity (Gupta *et al*., 2011).

Free radical scavenging activity of three coumarin compound I, II and III were evaluated. The coumarin compound I, II and III were able to reduce the stable free radical DPPH to the yellow coloured diphenylpicrylhydrazine exhibiting better free radical scavenging activity (Rajesh and Natvar, 2011).

Figure 1

DPPH RADICAL SCAVENGING ACTIVITY OF *Phyllanthus niruri* LEAF EXTRACTS





Leaves of *Aleo vera, Bacopa monniera, Moringa Oleifera and Rhizome* of *Zingiber officinale* were evaluated for its DPPH radical scavenging activity. Among the four medicinal plants *Zingber Officinale* has maximum scavenging activity (Padmanaban and Jangle, 2012). The methanolic extracts of the roots of *Clerodendrum viscosum showed* maximum activity when compared to leaves and stem (Dey *et al*., 2012).

In our present study too, among the three different leaf extracts of *Phyllanthus niruri,* aqueous extract exhibited maximum DPPH scavenging activity followed by methanol and chloroform extract which shows that the leaves of *Phyllanthus niruri* possess strong radical scavenging activity.

ABTS RADICAL SCAVENGING ACTIVITY OF *Phyllanthus niruri* LEAF EXTRACTS

The radical scavenging ability of the extracts was tested with yet another radical ABTS. This assay is applicable for both lipophilic and hydrophilic antioxidants. The ABTS radical scavenging activity results are depicted in Figure 3. The IC_{50} was determined for ABTS radical scavenging activity and the results are discussed in Figure 3.

The ABTS scavenging ability of the different extracts of *Phyllanthus niruri* leaves were analysed using a spectrophotometric assay. Among the three different extracts, the aqueous extract was found to be a better scavenger of free radical followed by methanol and chloroform. The IC_{50} value was found to be 0.33mg/20µl for ABTS radical scavenging activity. It was compared with the standard ascorbic acid. The result of the present study is shown in Figure 4

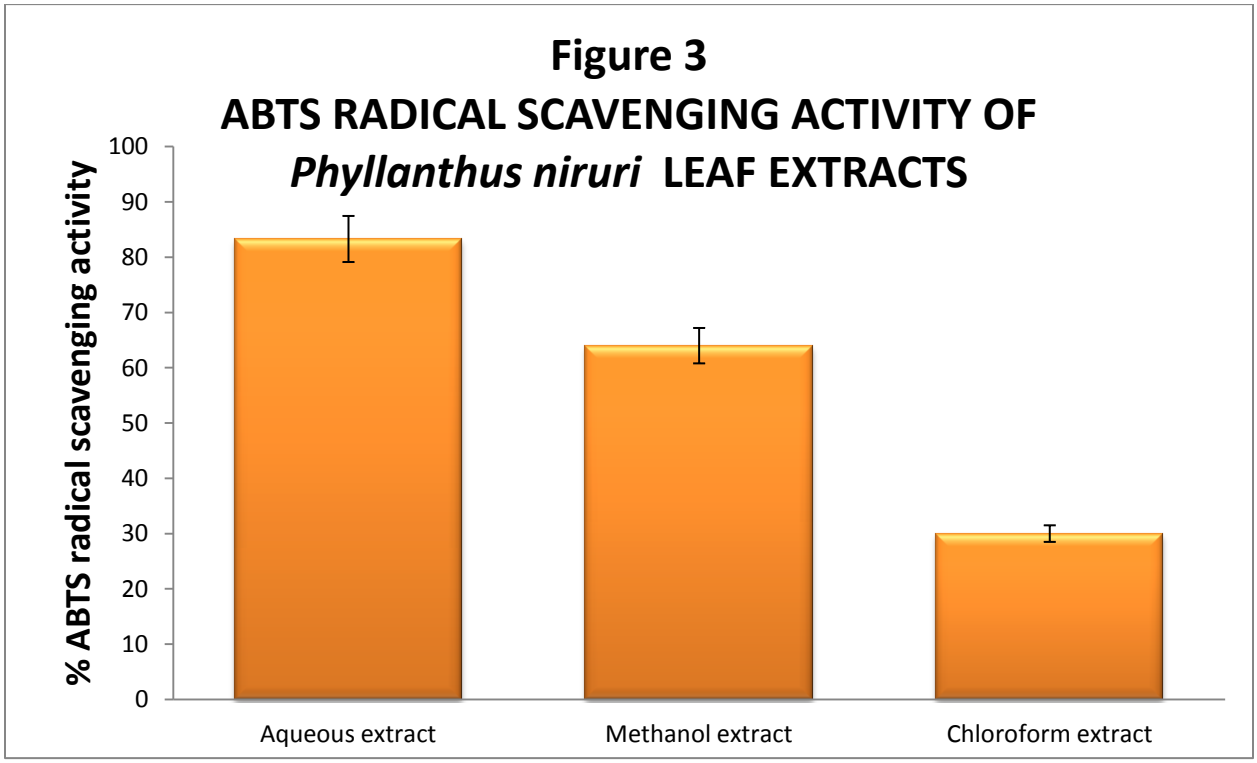
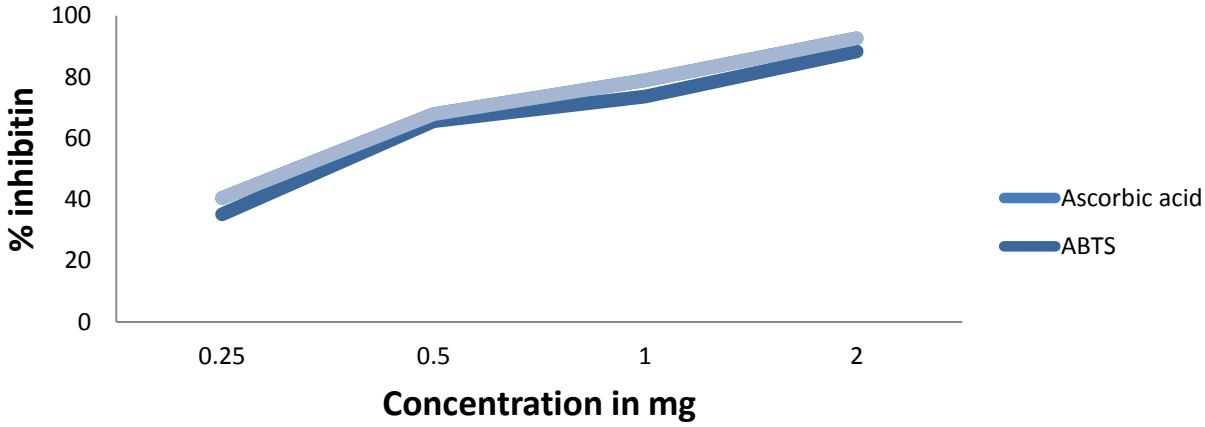


FIGURE 4
IC50 VALUE OF ABTS RADICAL SCAVENGING ACTIVITY



Aqueous extracts of three moss species *Brachythecium rutabulam*, *Calliergonella cuspidata*, *Hypnum mammillatum* have been studied in regard to antioxidant activity. *B. rutabulam* had the highest radical scavenging activity (Pejin and Pristov, 2012). Aqueous extract of *Andrographis paniculata* exhibited strong antioxidant potential *in vitro* against the stable free radical ABTS (Tripathi *et al.*, 2007).

ABTS assay has also been valuable in reporting the free radical scavenging activities of polyherbal formulation for triphala (Naik *et al.*, 2008). The ABTS radical scavenging activity in several population of *Teucrium polium* (TBP, TPS,

TPP, and TPM) was studied. TPB show the highest ABTS radical scavenging activity (Aghdam *et al.*, 2011).

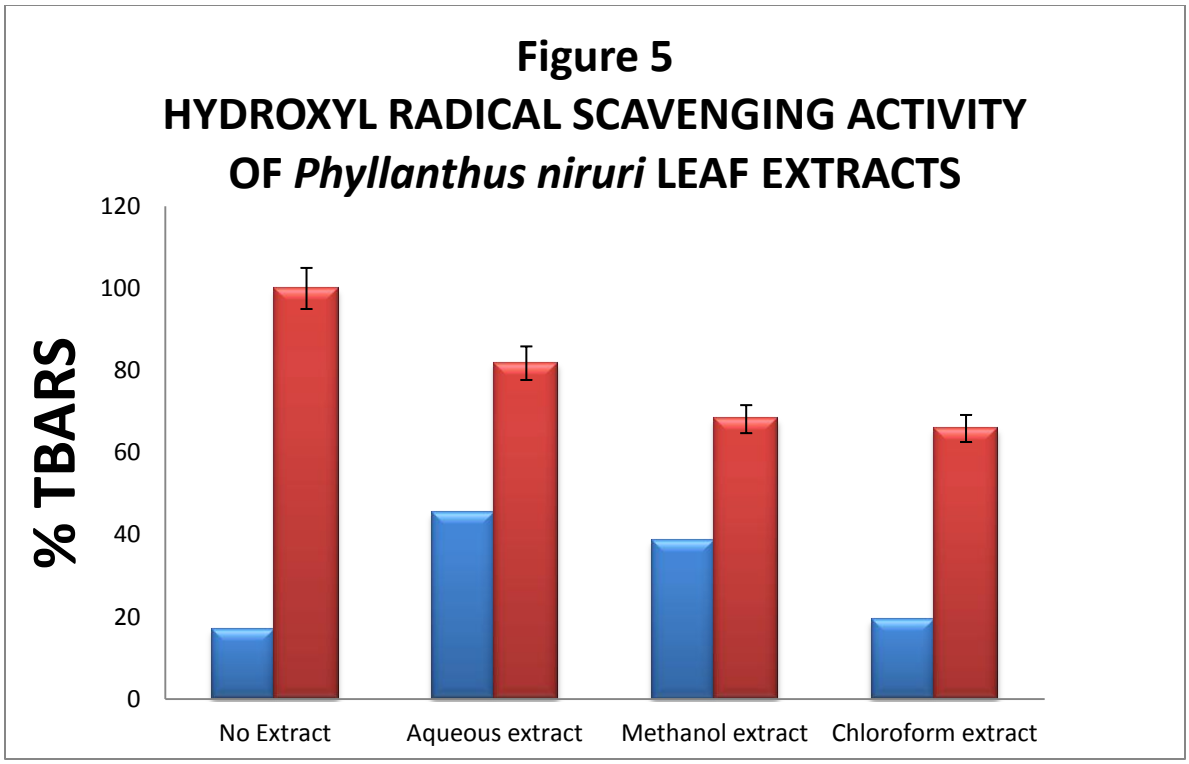
These facts support our finding, where the different extracts showed varying amounts of radical scavenging activity.

HYDROXYL RADICAL SCAVENGING ACTIVITY OF *Phyllanthus niruri* LEAF EXTRACTS

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage. The effect of aqueous, methanol and chloroform extracts in scavenging hydroxyl radical is depicted in Figure 5.

The exposure to H_2O_2 caused the maximum damage to deoxyribose and the damage was very effectively rectified by the treatment of the leaf extracts of *Phyllanthus niruri.* The aqueous extract of leaves of *Phyllanthus niruri* was more effective than the other two extracts .The crude protein extracts of *Leucas linifolia* showed maximum inhibition of hydroxyl radical induced degradation (Ramakrishna *et al*., 2012). The methanolic extract of *Borreria hispida* was found to be more effective in scavenging hydroxyl radical than petroleum ether and ethyl acetate extracts (Shajiselvin and Muthu, 2010).

The aqueous extract of *Chenopodium album* leaves prevented H_2O_2 induced deoxyribose degradation. The percentage of hydroxyl radical scavenging was significantly increased with the increasing concentrations of extracts (Kumar and Kumar, 2009).



In the extent of all these reports and their inferences, the ability of *Phyllanthus niruri* leaves to scavenge hydroxyl radical gains a lot of significance in establishing the strong antioxidant activity of the leaves.

HYDROGEN PEROXIDE SCAVENGING ACTIVITY OF *Phyllanthus niruri* LEAF EXTRACT

The measurement of hydrogen peroxide scavenging activity is one of the useful methods for determining the ability of antioxidants to decrease the level of prooxidants such as hydrogen peroxide.

The three different extracts of *Phyllanthus niruri* showed considerable H_2O_2 scavenging activity. The aqueous extract of *Phyllanthus niruri* showed better scavenging of H_2O_2 than the other two extracts. The values obtained are represented in Figure 6.

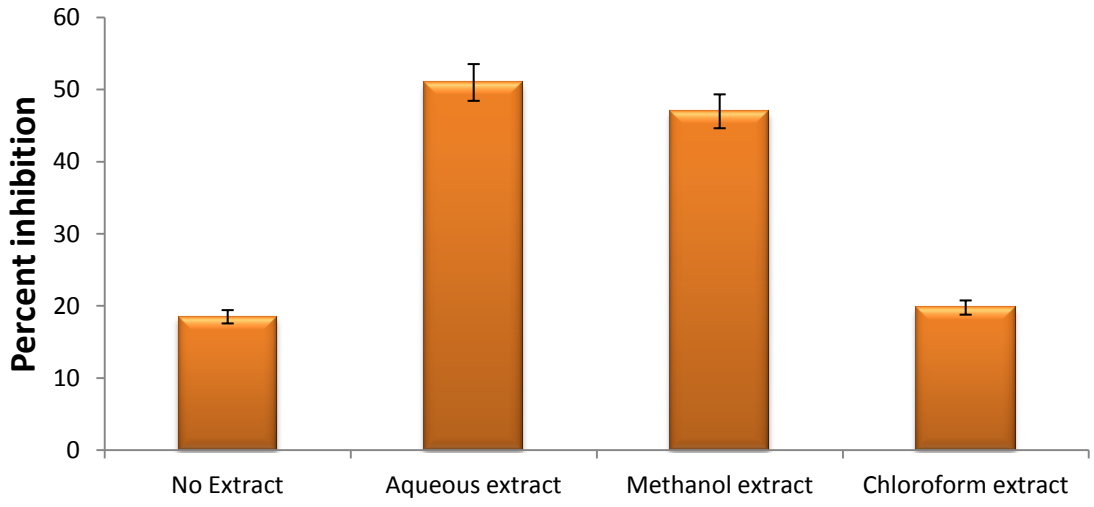
Several studies have reported the H_2O_2 scavenging action of plant extracts. The H_2O_2 scavenging activity of ethanolic extract of *Kalanchoe pinnata* was found to be significant compared to that of standard ascorbic acid (Mohan *et al.*, 2012). Water and ethanolic extracts of *Crataegus monogyna* showed strong scavenging activity of hydrogen peroxide (Kesar *et al*., 2012). *Acalypa torta* fractions have high H_2O_2 radical scavenging activities when compared to standard ascorbic acid.

The observations made show that the leaves of *Phyllanthus niruri* can effectively scavenge H_2O_2.

Thus from the results of the present study, it is found that the leaves of *Phyllanthus niruri* possess very effective antioxidant and radical scavenging activity.

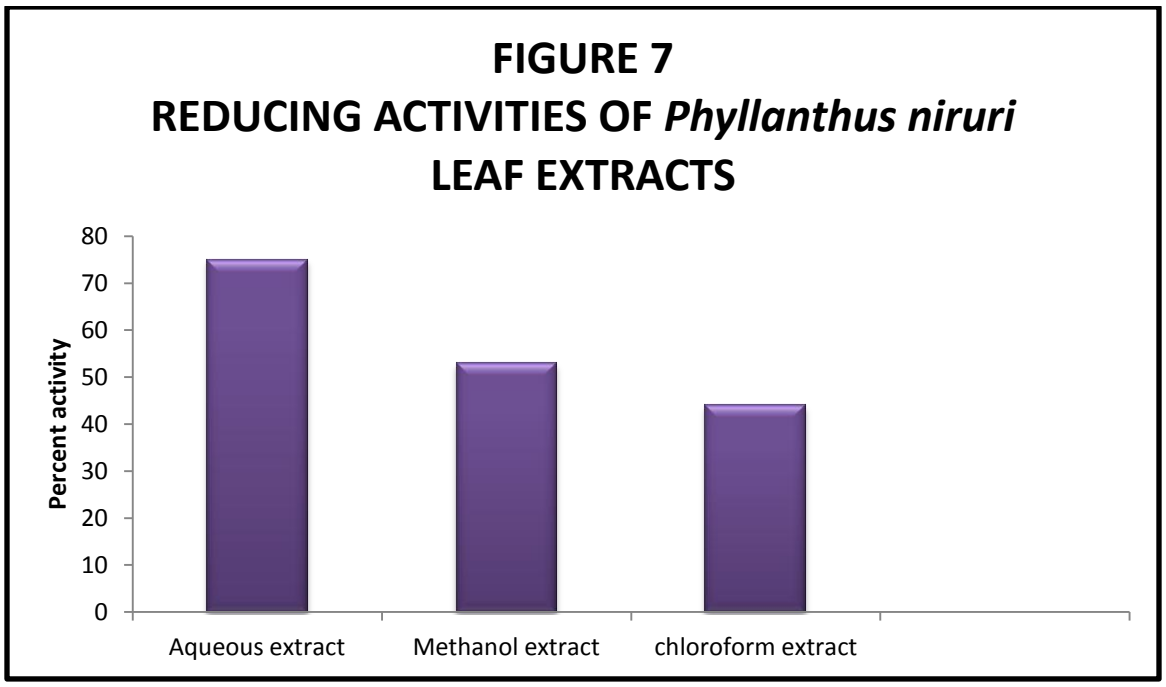
Figure - 5

HYDROGEN PEROXIDE RADICAL SCAVENGING ACTIVITY OF THE DIFFERENT EXTRACTS OF *Phyllanthus niruri*



ASSAY OF REDUCING PROPERTY:

The FRAP assay is a direct test of "total antioxidant power". Reducing power reflects the electron donating capacity of bioactive compounds, is associated with antioxidant activity. Reducing power was measured by the direct reduction of ferric cyanide to ferrous cyanide, and was determined by measuring absorbance resulting from the formation of the Perl's Prussian Blue complex followed by the addition of excess ferric ions (Fe^{3+}). In this method, higher absorbance values indicate greater reducing capacity of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. The FRAP of the leave extracts of *Phyllanthus nirruri* was determined and the results are presented in Figure 7.



The result indicated that the aqueous extract was very effective in reducing metal ion followed by methanol and chloroform extract.

Lyophilized extract of medlar (LEM) fruits (*Mespilus germanica L*.) showed better reducing activity (Gulcin *et al*., 2011). Reducing activity of ten different

Chinese herbs was determined. All of the aqueous extracts and ethanol extracts from these Chinese herbs exhibit reducing activity but to different degrees (Wang and Dai, 2012).

Toona sinensis extracts and gallic acid possessed antioxidant properties, which was expressed by their reducing power (Hseu *et al*., 2008). The result reported by Kirkova *et al*. (2007) revealed that *Celecoxile* and *Amtolmetic guacyl* possess antioxidant activity, which might contribute to their beneficial anti-inflammatory activity effects. Mulberry extracts possessed higher reducing power than BHT (Bae and suh, 2007).

The conclusions drawn from the present study are summarized in the next chapter.

Summary and Conclusion

5. SUMMARY AND CONCLUSION

The relationship between man and nature is long standing and man has always had the wisdom to turn to Mother Nature for many of his needs – right from the food that he consumes to healing of ailments that he develops. From this exquisite relationship was born the practice of ancient times – the Ayurveda.

Ayurveda is considered to be the science of life, of longevity and holistic well-being. The importance of Ayurveda and the medicinal properties of natural sources like plants are being rediscovered today. Moreover, much evidence is mounting for the fact that herbs have the ability to manage or prevent the onset of chronic diseases. Herbs are also perceived as being natural, healthful, non-toxic and safe.

The use of plant compounds for pharmaceutical purposes has gradually increased in India. About 80% of the developed countries use traditional medicine, which involves compounds derived from medicinal plants. Therefore, such type of plants should be investigated to understand better about their properties.

Of all the medicinal plant used in India, one of the important herb in Ayurveda, *Phyllanthus niruri*, is attributed to number of medicinal properties also known as stone breaker. All the parts of the plants are used for the treatment of different ailments. It is used as one of the components of a multiherbal preparation for treating liver ailments. In the present study, an effort was made to test the efficacy of the leaves of the plant to screen the free radical scavenging activity of *Phyllanthus niruri* leaf extract under *in vitro* condition.

The scavenging activity was tested against a battery of free radicals such as DPPH, ABTS, H_2O_2, hydroxyl radicals and by determining its reducing property

using three different extracts of *Phyllanthus niruri* leaves namely aqueous, methanol and chloroform.

The free radical scavenging activity of the leaf extracts were evaluated using DPPH activity. The stable DPPH radical is widely used method to evaluate antioxidant activities. The results revealed that among all the extracts, the aqueous extract exhibited strong DPPH scavenging activity while methanol and chloroform extract showed moderate scavenging activity. The IC_{50} value obtained for DPPH scavenging was 0.4mg/20µl which were comparable for the reference standard, ascorbic acid.

ABTS scavenging ability of P*hyllanthus niruri* was analyzed. The result of this assay followed the same trend as observed in the case of DPPH. The extent of scavenging of ABTS radical was more effective in the aqueous extract of the leaves, followed by methanol and chloroform. This observation strengthened the free radical scavenging activity of the leaves.

The hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+}/Ascorbate / EDTA / H_2O_2. The extent of damage to deoxyribose both in the presence and absence of the leaf extracts was studied. The extent of TBARS formation with deoxyribose was effectively reduced in the presence of aqueous extract of the leaves while the other two extracts showed moderate activity.

The ability to tackle the non – radical oxidant H_2O_2 was analysed. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to the cells which may give rise to hydroxyl radical in the cell. Thus, removal of H_2O_2 and O_2 is very important for normal cellular function. The ability of the extracts to effectively

scavenge H_2O_2 was determined. The aqueous extracts of the leaves showed better scavenging of H_2O_2 than the other two extracts.

The metal reducing activity of the leaf extracts of *Phyllanthus niruri* was determined. The aqueous extracts exhibited the maximum activity. The reducing activities of aqueous extract were higher followed by methanol and chloroform extracts.

All the three extracts of *Phyllanthus niruri* leaves effectively scavenged or inhibited all the radicals tested. Among the three extracts the aqueous extract was most effective scavenger, followed by methanol and chloroform. Thus, the results showed that *Phyllanthus niruri* leaf extracts exhibited antioxidants and radical scavenging activity *in vitro.*

SUGGESTION FOR FUTURE STUDY:

- The active component in the aqueous extract may be isolated and checked for its radical scavenging effects.
- The efficacy of extracts can be tested in various *in vitro* models that are alternative to laboratory animals.
- Cytotoxicity of the extracts can be tested so that they can be used as anticancer agents.

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Appendices

APPENDIX - I

DPPH SCAVENGING ACTIVITY

DPPH DOT PLOT ASSAY

(Soler-Rivas *et al*., 2000)

The rapid screening assay was performed by the method proposed by Soler-Rivas *et al*, (2000).

REAGENTS

1. TLC plates (silica gel 60 F_{254}-Merck)
2. DPPH (0.4mM) in methanol

PROCEDURE

Aliquots of plant extracts (3µl) were spotted carefully on TLC plates and dried for 3 minutes. The sheets bearing the dry spots were placed upside down for 10 seconds in a 0.4mM DPPH solution and the layer was dried. The stained silica layer revealed a purple background with yellow spots, which showed radical scavenging capacity.

APPENDIX-II

DPPH SCAVENGING ACTIVITY

DPPH PHOTOMETRIC ASSAY

(Mensor *et al*., 2001)

Mensor *et al*. (2001) have proposed an assay for the determination of antioxidant activity of compounds by their ability to scavenge the stable free radical DPPH.

REAGENTS

1. 0.3mM DPPH in methanol
2. Methanol

PROCEDURE

3ml of 0.3mM DPPH in methanol solution was added to 2.5µl and 5.0µl of the plant sample. DPPH solution with methanol was used as a positive control and methanol alone acts as blank. When DPPH reacts with antioxidant, it is reduced and the color changes from deep violet to light yellow measured at 517mm. The percent inhibition was calculated by the following formula

$$\text{Scavenging capacity \%} = A_0\text{-}A_1 / A_0 \times 100$$

A_0 = Absorbance of control

A_1 = Absorbance in the presence of sample of plant extract and standard.

APPENDIX-III

ABTS SCAVENGING ACTIVITY

(Shirwaikar *et al*., 2006)

Shirwaikar *et al*., 2006 have proposed an assay for the determination of antioxidant activity of compounds by their ability to scavenge ABTS cation.

REAGENTS

ABTS solution; ABTS solution (7mM) with 2.45mM ammonium persulphate.

PROCEDURE

ABTS radical cation ($ABTS^{+}$) was produced by treating ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Different extracts of aqueous, methanol and chloroform extracts (0.5ml) were added to 0.3ml of ABTS solution and the final volume was made upto 1ml with ethanol. The absorbance was read at 745nm and the percentage inhibition was calculated using the formula.

Inhibition (%) = control-test/control × 100

APPENDIX-IV

HYDROXYL RADICAL SCAVENGING

Elizabeth and Rao, 1990

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extracts for hydroxyl radical generated with Fe^{3+}/ ascorbate EDTA/H_2O_2 system. The hydroxyl radicals attack deoxyribose which eventually results in TBARS formation.

REAGENTS

1. Deoxyribose (2.8mM)

2. $FeCl_3$ (0.1mM)

3. EDTA (0.1mM)

4. H_2O_2 (1mM)

5. Ascorbate (o.1mM)

6. KH_2PO_4– KOH buffer (20mM, pH-7.4)

7. TBA (1%)

8. HCl (25%)

PROCEDURE

The reaction mixture contained deoxyribose (2.8mM), $FeCl_3$ (0.1mM), EDTA (0.1mM), H_2O_2 (1mM), Ascorbate (0.1mM) and KH_2PO_4-KOH buffer (20mM, pH -7.4). 20µl of plant extract was added such that the final volume was 1ml. The reaction mixture was incubated for 1hr at 37ºC. Deoxyribose degradation

was measured as TBARS by addition of 0.5ml of TBA and 0.5ml of HCl and kept in boiling water bath for 20 minutes. After cooling the absorbance was measured at 532nm.

APPEXDIX-V

H_2O_2 SCAVENGING ACTIVITY

(Ruch *et al*., 1989)

Ruch *et al*., (1989) have proposed an assay for the determination of antioxidant activity of compounds by their ability to scavenge the oxidant H_2O_2.

REAGENTS

1. Phosphate buffer (0.1mM, pH - 7.4)

2. H_2O_2 in phosphate buffer (40mM)

PROCEDURE

A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH - 7.4). Different extracts at the concentration of 10mg /10µl were added to a H_2O_2 solution (0.6ml, 40mM). The total volume was made up to 3ml .The absorbance of the reaction mixture was recorded at 230nm. A blank solution that contained phosphate buffer without H_2O_2 served as the blank. The percentage H_2O_2 scavenging activity of plant extracts was calculated using

$$\% \text{ Scavenging } (H_2O_2) = A_o\text{-}A_1/A_o \times 100$$

APPEXDIX-VI

ASSAY OF REDUCING PROPERTY

(Oyaizu, 1986)

Potassium ferricyanide and ferric chloride, when subjected to reduction by an antioxidant, give a coloured complex, which can be read spectrometrically at 700nm.

REAGENTS

1. Phosphate buffer (20mM, pH 6.6)
2. Potassium ferricyanide (1%)
3. Trichloroacetic acid (10%)
4. Ferric chloride (0.1%)

PROCEDURE

20mg leaf extracts were taken with 500µl of phosphate buffer (20mM, pH 6.6) and 500µl of 1% potassium ferricyanide was added and incubated at 50°C for 20 minutes. TCA was added to the mixture to a final concentration of 0.5% and centrifuged at 2000rpm for 15 minutes. The supernatant were mixed with 500µl of distilled water and ferric chloride (0.1%).The color obtained was measured at 700nm.