Antioxidants and free radical scavenging activity of *Tabebuia rosea*

J.S. EVANGELIN ESTHER

(16PBT003)

A Thesis submitted to Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore- 641043

In partial fulfilment of the Requirement for the Degree of

Master of Science in Biotechnology

April 2018

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1.0 INTRODUCTION

Medicinal plants have been used in virtually all cultures as a source of medicine. Assurance of the safety, quality, and efficacy of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. The widespread use of herbal remedies and healthcare preparations is described in the Vedas and the Bible.Medicinal Plants have been used for thousands of years to flavor and conserve food, to treat health disorders and to prevent diseases including epidemics. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Active compounds produced during secondary metabolism are usually responsible for the biological properties of plant species used throughout the globe for various purposes, including treatment of infectious diseases. Currently, data on the phytochemical activity of numerous plants, so far considered empirical, have been scientifically confirmed. Products derived from plants may potentially have phytochemical activity in diverse situations and in the specific case of disease treatment, numerous studies have aimed to describe the chemical composition of these plantsphotochemical and the mechanisms involved in conventional methods(Singh 2015).

The use of plants for medicinal purposes is as old as the emergence of human species on earth. Historically, first civilizations realized that some plants contained active ingredients in their essences, which empirically revealed their healing power when they were tested in disease (Vasconcelos*et al.,* 2014).Because of the importance of oxidative stress in the pathophysiology of most of the hard curable diseases, the use of medicinal plants with antioxidant properties is important and should be considered. Drug therapy and even drug discovery should also be focused more than before on this source. Multidisciplinary team work including ethnobotanists, pharmacologists, physicians and phytochemists are essential

for the fruitful outcome of medicinal plants research. More importantly, regulations are also needed to ensure efficacy, quality and safety of herbal medicines(Kopaei, 2012).

Free Radicals are molecules with an unpaired electron and are important intermediates in natural processes involving cytotoxicity, control of vascular tone and neurotransmission. Free radicals are very unstable and react quickly with other compounds, and try to capture the needed electron to gain stability. A chain reaction thus gets started. Once the process is started, it can cascade, and finally results in the disruption of a living cell. Generally, harmful effects of reactive oxygen species on the cell are most often like damage of DNA, oxidations of polydesaturated fatty acids in lipids, oxidations of amino acids in proteins, oxidatively inactivate specific enzymes by oxidation of co-factors. Free radicals cause many human diseases like cancer, Alzheimer's disease, cardiac reperfusion abnormalities, kidney disease and fibrosis. The free radicals formed in our body are combated by antioxidants that safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Excessive exercise has been found to increase the free radical level in the body and causes intense damage. Regular physical exercise enhances the antioxidant defense system and protects against exercise induced free radicaldamage(Sarma *et al*.,2010).

A radical is any molecule that contains one or more unpaired electrons. Radicals are normal products of many metabolic pathways. Some exist in a controlled form as they perform essential functions. Others exist in a free form and interact with various tissue components. Such interactions can cause both acute and chronic dysfunction, but can also provide essential control of redox regulated signaling pathways. The potential roles of endogenous or xenobiotic-derived free radicals in several human pathologies have stimulated extensive research linking the toxicity of numerous xenobiotics and disease processes to a free radical mechanism. In recent years, improvements in analytical methodologies, as well as the realization that subtle effects induced by free radicals and oxidants are important in modulating cellular signaling, have greatly improved our understanding of the roles of these reactive species in toxic mechanisms and disease processes. However, because free radical-mediated changes are pervasive, and a consequence as well as a cause of injury, whether such species are a major cause of tissue injury and human disease remains unclear. This concern is supported by the fact that the bulk of antioxidant defenses are enzymatic and the findings of numerous studies showing that exogenously administered small molecule antioxidants are

unable to affect the course of most toxicity and diseases purported to have a free radical mechanism (Kehrer*et al* ., 2015).

Oxidative stress is a high-profile element among the risk factors for aging. Although several stress markers have been proposed for the evaluation of oxidative stress, there remains much room for improvement in testing and evaluation methods. Several markers are available for evaluation of the oxidative stress status. Methods of detecting oxidatively modified substances as new markers by mass spectrography have been developed. Oxidative modifications of highly reactive cysteine residues in several target proteins such as tyrosine phosphatase and thioredoxin-related proteins control the functions of relevant molecules, and thereby play an important role in signal transmission. Advanced glycation endproducts (AGEs) gradually accumulate with aging and are involved in the development of diabetic complications, Alzheimer's disease, and arteriosclerosis. Basic studies of the indicators of glycation are also important(Naito *et al.*,2010).

Reactive oxygen species (ROS), highly reactive molecules, are produced by living organisms, a result of normal cellular metabolism and environmental factors, and can damage nucleic acids and proteins, thereby altering their functions. The human body has several mechanisms to counteract oxidative stress by producing antioxidants. A shift in the balance between oxidants and antioxidants in favor of oxidants is termed as "oxidative stress". Paradoxically, there is a large body of research demonstrating the general effect of oxidative stress on signaling pathways, less is known about the initial and direct regulation of signaling molecules by ROS, or what we term the "oxidative interface." The molecular mechanisms through which ROS directly interact with critical signaling molecules to initiate signaling in a broad variety of cellular processes, such as proliferation and survival , ROS homeostasis, and antioxidant gene regulation (Gupta *et al*.,2014).

Antioxidants are molecules that inhibit or quench free radical reactions and delay or inhibit cellular damage.Though the antioxidant defenses are different from species to species, the presence of the antioxidant defense is universal. Antioxidants exists both in enzymatic and non-enzymatic forms in the intracellular and extracellular environment.Normal biochemical reactions, increased exposure to the environment, and higher levels of dietary xenobiotics result in the generation of ROS and reactive nitrogen species (RNS).ROS and RNS are responsible for the oxidative stress in different pathophysiological conditions. Cellular constituents of our body are altered in oxidative stress conditions, resulting in various diseased states. The oxidative stress can

be effectively neutralized by enhancing cellular defenses in the form of antioxidants.Certain compounds act as *in vivo* antioxidants by raising the levels of endogenous antioxidant defenses. Expression of genes encoding the enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) increases the level of endogenous antioxidants.It can be categorized in multiple ways. Based on their activity, they can be categorized as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in presence of cofactors such as copper, zinc, manganese, and iron. Non-enzymatic antioxidants work by interrupting free radical chain reactions. Few examples of the non-enzymatic antioxidants are vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione(Nimse *et al.,* 2015).

Antioxidants act as an inhibitor of the oxidation process even at relatively small concentration and thus have diverse physiological role in the body. They are important in prevention of cellular damage, disease prevention in both plants and animals and play an important role in the body's defense system. These compounds quench dreadful free radicals and stop oxidation chains *invivo* as well, so they have become viewed by many as nature's answer to environmental and physiological stress, aging, atherosclerosis, and cancer. Consumers believe that foods rich in antioxidants may afford a degree of protection against free radical damage not only in foods, but also in the human body, protecting against cardiovascular diseases, damage of nucleic acids, and other deteriorative processes(Akbarirad*et al* .,2016).

The Bignoniaceae is a wide spread family and commonly called the trumpet vine or trumpet creeper family. It gets its name from genus Bignonia and some of the well known members of the family are Tecoma, Catalpa, Tabebuiya and Jacaranda. Many species are important ornamentals and have large spectacular flowers, leaves are typically opposite or whorled(Choudhury*et al*.,2011).

Bignoniaceae comprises 82 genera and 827 species distributed mostly in tropical and subtropical regions, with a few species in temperate climates, and is most diverse in South America. According to Gentry (1990), it is the most ecologically important liana family in the American tropics, and so constitutes a good model for studying the great diversity of tropical plant communities(Silva *et al.,* 2016). Plant species belonging to this family are distributed worldwide, but most of them occur in the tropical and sub-tropical countries. Although the family is small, the Bignoniaceae plants are important for their reported bio-active constituents and diverse pharmacological activities. Bignoniaceae family plants are also

widely used in traditional medicinal systems of number of countries, including Bangladesh, where folk and tribal medicinal practitioners use a number of species for treatment of diverse ailments. Since folk medicinal practitioners form the first tier of primary health care in Bangladesh. Accordingly, a survey was conducted among traditional medicinal practitioners of Bangladesh, which included folk medicinal practitioners (catering to the mainstream population and otherwise known as Kavirajes) as well as tribal medicinal practitioners of various tribes residing within the country. It was observed that the traditional medicinal practitioners use a total of seven Bignoniaceae family species for treatment of ailments like cancer, snake bite, skin disorders, gastrointestinal disorders, respiratory tract disorders, gynecological disorders, hepatic disorders, epilepsy, cholera, pain, urinary problems, malaria, heart problems, and sexually transmitted diseases. The seven species of Bignoniaceae family plants in use were *Crescentiacujete, Heterophragmaadenophyllum, Oroxylumindicum, Stereospermumsuaveolens, Tabebuiaargentea, Tecomagaudichaudi, and Tecomastans.* Since the available scientific literature validates the use of these plants for the ailments they are prescribed for by the Kavirajes and tribal medicinal practitioners, the plants present excellent potential for further scientific studies, which may result in discovery of novel compounds of therapeutic interest(Rahmatullah *et al.*,2010). The objectives set for the present study are as follows

Objectives

The main aim of the present study is as follows

- To assess the antioxidant and free radical scavenging efficacy of different extracts of *Tabebuiarosea* leaves
- To identify the phytochemical constituents present in the different solvent extractsof the leaves of *Tabebuia rosea*

The brief review of literature pertaining to the current topic is presented in next chapter.

2.0 Review of literature

Numerous studies support the fact that many diseases are caused by ‘oxidative stress’ resulting from a discrepancy in the neutralization and configuration of pro-oxidants. Human bodies naturally produce free radicals. Chemicals that obstruct the action of free radicals are termed as antioxidants. These overloaded free radicals contradict with biological macromolecules, such as lipids, proteins, and DNA, in healthy human cells. This results in the stimulation of carcinogenesis, cardiovascula disease, atherosclerosis, aging and inflammatory diseases (Halliwell 1996). Currently, an increasing number of studies are being conducted to explore natural compounds rich in antioxidants and antimicrobial properties because of their significance in treating various chronic disorders, such as cancer and cardiovascular disease. Approximately two-thirds of drugs approved worldwide are predicted to be plant derivatives (Patridge *et al.* 2016).

Natural antioxidants are often added in foods to prevent the radical chain reactions involving oxidation by inhibiting the initiation and propagation step leading to the termination of the reaction and a delay in the oxidation process .Reactive oxygen species, such as the superoxide anion (O_2^-), hydroxyl radicals (OH^-), and nitric oxide (NO), inactivate enzymes and damage vital cellular components, causing injury. Antioxidants may provide resistance against oxidative stress by scavenging free radicals. Therefore, compounds with antioxidative properties may be useful in the treatment of various disorders. (Parim *et al.,* 2015).

Natural products have played an important role in the development of drugs and drug leads for various diseases including cancer. The secondary metabolites from natural sources

are good component for drug development because it is elaborated with the living systems, which exhibit more similarities to drugs and show more biological friendliness than totally synthetic drugs (Ramalakshmi *et al*., 2011).The review of literature related to the study is discussed under the following headings.

2.1 Free radicals

Oxygen is one of the most important molecules on Earth mainly because of the biochemical symmetry of oxygenic photosynthesis and aerobic respiration that can maintain homeostasis within our planet's biosphere. Oxygen can also produce toxic molecules and Reactive Oxygen Species. ROS play a dual role in biological systems, since they can be either harmful or beneficial to living systems. They can be considered a double-edged sword because at moderate concentrations, nitric oxide, superoxide anion, and related reactive oxygen species play an important role as regulatory mediators in signalling processes. Many of the ROS-mediated responses actually protect the cells against oxidative stress and re-establish "redox homeostasis". On the other hand, overproduction of ROS has the potential to cause damage. In the recent decades, ROS has become a focus of interest in most biomedical disciplines and many types of clinical research. Increasing evidence from research on several diseases shows that oxidative stress is associated with the pathogenesis of diabetes mellitus, obesity, cancer, cardiovascular diseases, inflammation, ischaemia/reperfusion injury,

obstructive sleep, neurodegenerative disorders, hypertension and ageing (Gutowski *et al* ., 2013).

A free radical may be defined as a molecule or molecular fragment containing one or more unpaired electrons in its outermost atomic or molecular orbital. These when formed can be highly reactive and can start a chain reaction. The sources of free radicals can be endogenous and exogenous in nature. Endogenous sources of free radicals are intracellularly generated from auto-oxidation or inactivation of small molecules. Exogenous sources of free radicals are tobacco smoke, certain pollutants, organic solvents, anesthetics and pesticides. The sites of free radical generation encompass all cellular constituents including mitochondria, lysosomes, peroxisomes, endoplasmic reticulum, plasma membrane and sites within the cytosol. Apart from this, certain medications metabolized to free radical intermediate products also cause oxidative damage within the target tissues (Rao *et al*., 2011).

ROS including free radicals such as superoxide anion radicals, hydroxyl radical species, singlet oxygen, and hydrogen peroxide, are active oxygen species that are often generated by biological oxidation reactions of exogenous factors. These ROS are known to cause severe damage to biological molecules. Several phenolic compounds from plants can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (Motlhanka *et al*., 2008).

2.2 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. However, oxidative stress also has a useful role in physiological adaptations and in the regulation of intracellular signal transduction. Therefore, a more useful definition of oxidative stress may be “a state where oxidative forces exceed the antioxidant systems due to loss of the balance between them.” The biomarkers that can be used to assess oxidative stress *in vivo* have been attracting interest because the accurate measurement of such stress is necessary for investigation of their role in lifestyle diseases as well as to evaluate the efficacy of treatment. Many markers of oxidative stress have been proposed, including lipid hydroperoxides, 4-hydroxynonenal, isoprostan, 8-hydroxyguanine, and ubiquinol-10. To prevent the development of lifestyle diseases, advice on how to lead a healthy life should be given to individuals based on the levels of

oxidant and antioxidant activity assessed by pertinent biomarkers. Individual genetic information should also be taken into consideration in such cases (Yoshikawa *et al*., 2002).

Oxidative stress is defined as a misbalance in cell redox reactions resulting in the increase of ROS and/or decreased antioxidant defence. Crucial part of oxidative stress, which avoids protective effects of antioxidants, is lipid peroxidation (LPO). Since LPO comprises several chain reactions allowing the spread of ROS-caused damage to the macromolecules (proteins, nucleic acids and lipids) and it is important in pathogenesis of various diseases such as cardiovascular diseases, neurodegenerative diseases, diabetes mellitus and cancer. On the other hand, research on physiology and pathology of lipid peroxidation revealed that not only ROS but also the LPO products such as 4-hydroxynonenal (HNE) are involved in physiological homeostasis of various tissues. This leads to the conclusion that LPO and oxidative stress are not only pathological but also physiological processes. Accordingly, HNE was revealed as biomarker, growth regulating factor and signalling molecule. In this light, both natural as well as synthetic antioxidants could be considered as biological response modifiers maintaining oxidative homeostasis. Accordingly, some antioxidants might eventually become important components of advanced individual and integrative biomedicine (Gasparovic *et al*., 2010)

2.3 Disease caused by free radical damage

Free radicals, which are generated in several biochemical reactions in the body, have been implicated as mediators of many disease including cardiovascular diseases, carcinoma, Alzheimer's disease, Stroke and neurodegenerative disease (Peiyuvan *et al*., 2010).

2.3.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death in the United States. Approximately 1.5 million cases of myocardial infarction are diagnosed and approximately 800,000 deaths related to the coronary artery disease and other CVDs occur each year. In most cases, myocardial infarction occurs because of rupture or fissuring of a coronary artery, atherosclerotic plaque containing a lipid-rich necrotic core with superimposed thrombosis causing reduced blood flow and consequent ischemic myocardial cell death (Santo *et al* ., 2016).

2.3.2 Alzheimer's disease

Alzheimer Disease (AD) is characterized by a progressive decline in cognitive function. AD is substantially increased among people aged 65 years or more, with a progressive decline in memory, thinking, language and learning capacity. AD should be differentiated from normal age-related decline in cognitive function, which is more gradual and associated with less disability. Disease often starts with mild symptoms and ends with severe brain damage. People with dementia lose their abilities at different rates (Duthey, 2013).

2.3.3 Stroke

Stroke, also called brain attack, is a brain injury caused by a sudden interruption in the blood supply of the brain. It occurs when part of the brain does not receives the needed blood flow for one of two reasons either the blood supply to part of the brain is suddenly interrupted, or because a blood vessel in the brain ruptures and blood invades the surrounding areas. The brain is the central information-processing organ of the body responsible with the control of multiple complex functions. Without the blood supply, the brain cells start to die (cerebral infarction) leading to brain damage. Approximately two million brain cells die every minute during a stroke which increases the risk of brain damage, disability, and death. The level of disability varies from patient to patient according to the type of stroke suffered, the part of the brain affected, and the size of the damaged area. Stroke is the third leading causes of death in United States and UK after heart disease and cancer, and the number one cause of adult disability. In the United States, over 160,000 American adults die of stroke each year. In Europe, approximately 650,000 people die of stroke. In the United States, the majority of people that have a stroke are over the age of 65. However, 28 percent of all stroke cases occur in people younger than 65. The risk of having a stroke more than doubles each decade after the age of 55. A stroke is a medical emergency that can affect individuals of all age categories including children and babies. Among adults age 45 to 65, 8 to 12 percent of the ischemic stroke cases and 37 to 38 percent of hemorrhagic stroke cases result in death within 30 days. Within a year, 25 percent of those that had a transient ischemic attack will die and this percentage increases among seniors 65 and older. On average in the United States, someone suffers a stroke every 45 seconds and at every 3 to 4 minutes someone dies of a stroke. Every seven minutes a person dies of heart disease or stroke in Canada, while every five minutes somebody suffers a stroke in the UK. According to the World Health Organization, 15 million people worldwide have a stroke every year of which, 5 million die

and 5 million are permanently disabled. However, medical research shows that every year 80 per cent of the all cases of stroke can be prevented (Gund *et al*., 2013).

2.3.4 Neurodegenerative Diseases

Neurodegenerative Diseases (NDDs) are traditionally defined as disorders with selective loss of neurons and distinct involvement of functional systems defining clinical presentation. Comprehensive biochemical, genetic and molecular pathological examinations have expanded this definition. During the last century, the application of different silver staining techniques has demonstrated that intra- and extracellular structures that are associated with many forms of neurodegenerative diseases. This suggested that proteins with altered physicochemical properties are deposited in the human brain in neurodegenerative diseases. In addition, glial cells as well as neurons also accumulate these proteins. The involvement of proteins has led to the definition of the concept of conformational diseases. According to this, the structural conformation of a physiological protein changes, which results in an altered function or potentially toxic intra- or extracellular accumulation. Mutations in the encoding genes are linked to hereditary forms of disease. The pathological protein conformers are also called misfolded proteins. Disruption of the homeostasis of the endoplasmic reticulum leads to the misfolding of proteins. The two major elimination pathways, which control the quality of cellular components and maintain cell homeostasis, are the Ubiquitin–Proteasome System (UPS) and the autophagy–lysosome pathway. Chaperones and stress-response proteins are in close relation to protein-processing systems, which have a central role in the pathogenesis of neurodegenerative diseases. Widespread molecular pathological and biochemical studies have revealed that there are modifications of proteins intrinsic to disease (species that are, for example, phosphorylated or nitrated, oligomers, proteinase resistant, with or without amyloid characteristics, or cleavage products) (Kovacs *et al*., 2015)

2.3.5 Cancer

Free radicals can damage DNA and cause mutagenicity and cytotoxicity and thus play a key role in carcinogenesis. It is believed that ROS can induce mutations and inhibits DNA repair process that results in inactivation of certain tumor repressor genes, leading to cancer. Lipid Peroxidation plays an important role in control of cell division. The end product of lipid peroxidation, Malondialdehyde (MDA) due to its high cytotoxic and inhibitory action on

protective enzymes is suggested to act as a tumor promoter and a co-carcinogenic agent. (Shinde *et al*., 2015)

2.4 Antioxidants

Antioxidants slow down the process of degradation so that the energetic action of the environment can lead to higher sustainability. They interact with free radicals, making possible their reaction with oxygen. Antioxidants can be grouped into two classes–synthetic antioxidants and natural antioxidants. The difference between the two categories is that most synthetic antioxidants generate substances that develop cancer or other diseases. This can also be catogerized into Enzymic and non-enzymic antioxidants (Butnarlu *et al*., 2012).

2.4.1 Enzymatic antioxidants

The enzymatic antioxidants Superoxide dismutase, Catalase, Glutathione-S-transferase, Glutathione peroxidise and Peroxidase activities (Starlin *et al*., 2013).

Catalase

Catalase is an antioxidant enzyme ubiquitously present in aerobic cells. It catalyses the decomposition of hydrogen peroxide to water and oxygen. Catalase has one of the highest turn over rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (Rahman, 2007). High concentrations of hydrogen peroxide is deleterious to cells, and its accumulation causes oxidation of cellular targets such as DNA, proteins and lipids, leading to mutagenesis and cell death (Valko *et al.*, 2007).

Superoxide dismutase (SOD)

Superoxide dismutase is produced at a relatively high rate by cell during normal metabolism, its low intercellular level is maintained by either spontaneous dismutation and or catalytic break down by the enzyme SOD. It is one of the most effective intracellular enzymatic antioxidants and it catalyses the conversion of superoxide anion to oxygen and hydrogen peroxide. Assessment of SOD activity and identification of the various forms of the enzyme and their organismal origin were conducted by gel electrophoresis and staining for SOD

activity. To ensure that the activities assessed from gels correlated with the actual enzymatic rates (Wessiman *et al.*, 1975).

Peroxidase

Peroxidases are widely distributed in the plant kingdom, and have been associated with many metabolic and physiological changes in tissues. The enzyme has also been involved in deteriorative changes in flavor, texture and color in raw and processed fruits and vegetables (Neves *et al*., 2001).

Glutathione-S-transferase

Glutathione S-transferases promote the inactivation and degradation of a wide range of compounds by the formation of glutathione conjugates. Enzymes of this family are generally characterized by broad substrate specificity and low affinity (high Km values). A low catalytic 6 efficiency (Vmax/Km) is a hallmark of the functional flexibility of the family and has probably been integral to the evolution of GSTs as detoxifiers of a broad spectrum of endogenous and environmental chemicals (Todorova, 2007).

Glutathionine peroxidase

Glutathione peroxidase (GPx) is a selenium-containing antioxidant enzyme that effectively reduces H_2O_2 and lipid peroxides to water and lipid alcohols, respectively, and in turn oxidizes glutathione to glutathione disulfide. Reduced glutathione plays a major role in the regulation of the intracellular redox state of vascular cells by providing reducing equivalents for many biochemical pathways. In the absence of adequate GPx activity or glutathione levels, hydrogen peroxide and lipid peroxides are not detoxified and may be converted to OH-radicals and lipid peroxyl radicals, respectively, by transition metals (Fe^{2+}). The GPx/glutathione system is thought to be a major defence in low-level oxidative stress. Four isoforms of GPx have been identified and characterized: GPx-1 (cellular GPx) is ubiquitous and reduces H_2O_2 and fatty acid peroxides and has been inversely associated with increased cardiovascular risk. Esterified lipids are reduced by membrane-bound GPx-4 (phospholipids hydroperoxide GPx), which can use several different low-molecular-weight thiols as reducing equivalents. GPx2 (gastrointestinal GPx) is localized to gastrointestinal epithelial cells. GPx-3 (extracellular GPx) is the only member of the GPx family that exists in the extracellular compartment, and is believed to be the most important extracellular antioxidant enzyme in mammals (Tabet *et al*., 2007).

2.4.2 Non -enzymatic antioxidants

The non-enzymatic antioxidant like ascorbic acid, α-tocopherol and reduced glutathione were determined in the present study.

Ascorbic acid

It is a non-enzymatic endogenous antioxidant and water-soluble vitamin. It works synergistical with vitamin E to quench free radicals and generates the reduced form of vitamin E. It also has anti-atherogenic, anti-carcinogenic, immunomodulator properties (Rao *et al.,* 2011).

Reduced glutathioine

Glutathione, the dominant intracellular thiol, plays an important protective role against oxidative stress. The accidental findings of increased reduced glutathione level post prandially as compared to post absorptive level prompted the design of certain research. Reduced Glutathione levels were estimated in 50 healthy individuals in post absorptive and postprandial phase by taking whole blood. It also shows positive correlation between these two GSH levels. The post absorptive specimen collection is preferable over random or postprandial as the former reflects the true basal level of reduced glutathione (Chavan *et al*., 2005).

α-Tocopherol

α-Tocopherol are chemical compounds which are components of coenzymes and non-protein ingredients of enzymes. They are essential for life and normal functioning of the body. Vitamins are supplied with food as they cannot be synthesised by the body. Bad diet, impaired vitamin absorption or the use of substances such as caffeine, nicotine and medicines may lead to hypovitaminosis or vitamin deficiency. This causes weakness, lowered immunity, muscular and joint pains, fragility of blood vessels, apathy and skin lesions. It also speeds up the ageing process. One of the most effective antioxidants which slow down the ageing of cells is vitamin E. It can penetrate the skin and is absorbed by the intercellular cement, thus protecting against epidermal damage and decreasing the sensitivity of epidermis to UV radiation. The term vitamin E describes a group of α-tocopherol derivatives exhibiting similar physiological activity (Zielinska *et al*., 2014).

2.5.2 Medicinal uses

More than 3000 plants officially used for medicinal application and nearly 6000 plants used n folk, herbal and traditional medicinal system in India . Although for the study of antioxidant, there are many reports showed good antioxidant activity especially in the northern district of the Tamilnadu district, India and Around 104 plant species belonging to 45 families were studied for phytochemical analysis and antioxidant activity through DPPH scavenging activity. Hence, there is an increasing interest in finding out the phytochemical analysis and antioxidant activity and carried out phytochemical analysis, antioxidant activity (Madhumitha *et al* ., 2015).

2.5 *Tabebuia rosea*

Tabebuia rosea commonly known as 'pink trumpet tree' can grow up to 15 m and well known for its beautiful flowers. The timber is widely used for general construction and carpentry in many European countries. The fruits are green, long and bean pod-like with a length of 20-40 cm (8-16 inch). The fruits turn dark brown when ripe and contain flat, heart-shaped seeds with tiny wings. The graceful beauty is a treat for the eyes, but the tree has medical uses as well. Tea made from the leaves and bark is known to have a fever reducing effect. *Tabebuia rosea* plant belongs to Bignoniaceae and commonly known as Pink trumpet tree grown as an ornamental tree for its pink or purple flowers with different shades of colours. In the traditional days, aerial parts of the tree were used for the treatment of malaria and uterine cancer. A decoction of the cortex of the tree utilized for anaemia and constipation. The flowers, leaves and roots also were used to reduce fever, pain, cause sweating, tonsil inflammation and many other disorders. A lapachol is a botanical product that has been isolated from *T.rosea* considered to be an anticancer drug and also recommended for anti-malarial and anti-panasomal effects. More recently this plant gained interest to discover new anticancer drugs and increasing the understanding of their biological importance. It has been suggested that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antiviral, antitumor and antimicrobial agents. The selection of crude plant extracts for screening programs has the potential of being more successful in initial steps than the screening of pure compounds isolated from natural products (Saravanam *et al* ., 2011).

A large number of medicinal plants are claimed to be useful in treating skin diseases in all traditional system of medicine. Studies were carried out to investigate the antimicrobial effect of the sample isolated from the ethylacetate fraction of flowers of *Tabebuia rosea*. The

phytochemical activity of the compound isolated from ethyl acetate fraction is almost comparable with standard solvent control Chloromphenicol (Solomon *et al*., 2016).

Tabebuia rosea, chloroform fractions have significant herbicidal potency, which might be due to the presence of allelochemicals. Thus isolation of individual compounds from extracts can be used in the anticancer studies (Ramalakshmi*et al*., 2015).

Systematic position

Kingdom: Plantae

Division: angiosperms

Class: dicotyledonae

Order: lamiales

Family: Biogoniaceae

Genus: *Tabebuia*

Species: *rosea*

Plate 1 *Tabebuia rosea*

2.6 Phytochemicals

Phytochemicals are bioactive compounds found in vegetables, fruits, cereal grains, and plant based beverages such as tea and wine. Phytochemical consumption is associated with decrease in risk of several types of chronic diseases due to their antioxidant and free radical scavenging effects. Recent research has also highlighted their potential role in improved endothelial and increased vascular blood flow (Ashley *et al*., 2016).

Phenols

Phenolic acids contain carboxylic acid in the chemical composition. Hydroxycinnamic and hydroxybenzoic acids are both main pillars of phenolic acids. Moreover, scientists have noted that caffeic, ferulic and sinapic acids are main components of the hydroxycinnamic acids (Altemim, 2017).

Alkaloids

Alkaloids are classified in several families that present totally different biosynthetic pathways. Four major families, for which the biosynthesis and the regulation are more particularly studied, these being Monoterpene Indole Alkaloids (MIA), BenzylisoQuinoline Alkaloids (BQA), Tropane and Nicotine Alkaloids (TNA) and purine alkaloids. Despite their chemical diversity, alkaloids share the fact that they originate commonly from primary metabolites such as amino acids or bases. Except for *Nicotiana tabacum*, no genome sequencing project exists for the major alkaloid-producing plants. Therefore, most of the enzymatic steps have been identified using classical biochemical and molecular biology studies (Guirimand *et al*., 2010).

Flavonoids

Flavonoids are characterized by a phenylbenzopyran chemical structure. The general structure includes a C15 skeleton joined to a chroman ring (benzopyran moiety). The heterocyclic benzopyran ring is known as the C ring, the fused aromatic ring as the A ring, and the phenyl constituent as the B ring. The A ring can be of two types: phloroglucinol type that is meta trihydroxylatedor a resorcinol type that is meta-dihydroxylated. The B ring can be mono-hydroxylated, ortho di-hydroxylated or vicinaltri-hydroxylated. The center heterocycle most commonly exists in one of three forms: pyran, pyrilium (Pereira *et al.,* 2011).

Saponins

Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains. Consumer demand for natural products coupled with their physicochemical (surfactant) properties and mounting evidence on their biological activity (such as anticancer and anticholesterol activity) has led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceutical sectors. The realization of their full commercial potential requires development of new processes/processing strategies to address the processing challenges posed by their complex nature (Ustandag *et al*., 2007).

Medicinal plants as a source of pharmaceuticals

Medicinal plants have many characteristics when used for treatment are as follows:

• Synergic medicine- The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects

. • Support of official medicine- In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective.

• Preventive medicine- It has been proven that the component of the plants also characterize by their ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment (Hassan 2012).

Plants are the backbone of life on Earth and an essential resource for humans. Everything humans consume is obtained directly or indirectly from plants. Plants are the fortitude of all habitats; wildlife is dependent on plants, except for humans. Plants regulate the water cycle, distributing and purifying water through transpiration. Plants also recycle the air people breathe. They store carbon and regulate the amounts of carbon dioxide and oxygen in the air. Plants provide significant contribution in the field of medicine. Ever since times immemorial plants are accustomed to cure illness or strengthen physical Health in humans (Thalluri 2016).

Natural products, especially those derived from higher plants, in terms of drug development. It describes the main strategies for obtaining drugs from natural sources, fields of knowledge involved, difficulties and perspectives (Rates, 2001). Therefore the present study focuses on the antioxidant assessment and free radical scavenging ability of the plant

extracts for the treatment of free radical mediated diseases. The methodology adopted to carry out the study is described in the next chapter.

3.0 Methodology

Medicinal plants represent a constant interest as sources of new antioxidant substances.The large majority of substances isolated from plants with antioxidant activities include alkaloids, flavonoids, phenols, terpenoids and steroids. These compounds are able to scavenge free radicals and thus inhibit oxidative mechanisms that lead to oxidative stress. Oxidative stress can lead to inactivation of cellular components and can have serious effects on the cells, probably leading to ageing as well as several diseases (Mothanka *et al.*, 2008). The large majority of The methodology adopted for the present study is discussed under the following headings.

3.1 Collection of the plant materials

3.2 Preparation of the extracts

3.3 Phytochemical analysis

3.3.1 Qualitative Analysis

3.4 Antioxidant activity

3.4.1 Assay of enzymatic antioxidant activity

3.4.2 Assay of non – enzymatic antioxidant content

3.5 Free radical scavenging activity

3.5.1 DPPH scavenging activity

3.5.2 ABTS scavenging activity

3.5.3 Hydroxyl radical scavenging activity

3.5.4 H_2O_2 Scavenging activity

3.6 Inhibition of free radical generation

3.6.1 Inhibition of Nitric oxide generation

3.6.2 Inhibition of Superoxide generation

3.1 Collection of Plant materials

The *Tabebuia rosea* leaf sample was collected in the regions in and around Coimbatore. The collected fresh samples were dried, ground, powdered and then stored in plastic bottles and used for the experiments of the present study.

3.2 Preparation of the leaf extract

About 5g dried powdered plant material of *Tabebuia rosea* was extracted with solvents in Soxhlet extractor.The solvents used were petroleum ether, chloroform, hydroethanol and aqueous. Each extract was evaporated to dryness. The extracts obtained were subjected to qualitative test for the identification of various phytoconstituents, enzymatic, non enzymatic antioxidant potential and free radical scavenging activity of the extracts were also evaluated.

3.3 QUALITATIVE ANALYSIS

The qualitative analysis is depicted in Appendix I

TABLE 1

METHODS ADOPTED FOR THE ASSESSMENT OF PRELIMINARY PHYTOCHEMICAL ANALYSIS

S.NO	PHYTOCONSTITUENTS	METHODS	APPENDICES
1.	Alkaloid	Raaman, 2006	I
2.	Carbohydrate	Iyengar, 1995	I
3.	Saponin	Siddiqui and Ali, 1997	I
4.	Phytosterols	Siddiqui and Ali, 1997	I
5.	Phenols	Raaman, 2006	I
6.	Flavanoids	Raaman, 2006	I
7.	Proteins	Raaman, 2006	I
8.	Terpenoids	Siddiqui and Ali, 1997	I
9.	Glycosides	Raaman, 2006	I
10.	Tannin	Raaman, 2006	I

3.4 Antioxidant Activity

A substance that inhibits oxidation, especially one used to counteract the deterioration of stored food products.The antioxidant activity of the leaves of *Tabebuia rosea*was arrested by determining enzymatic and non – enzymatic antioxidant status.

3.4.1 Enzymatic antioxidant activity

The enzymatic antioxidant activity is determined for the following enzymes superoxide dismutase, catalase and peroxidase.

SUPEROXIDE DISMUTASE

The activity of superoxide dismutase of the leaf extarcts of *Tabebuia rosea* were estimated according to Misra *et al*. (1972). The detailed procedure is briefly described in Appendix II

CATALASE

The activity of catalase in the leaf samples was estimated according to the method of Luck. (1974). the detailed procedure is explained in Appendix III.

PEROXIDASE

The peroxidase activity was estimated according to the procedure of the Reddy *et al.* (1995). The detailed procedure is explained in Appendix IV.

3.4.2 Non – enzymatic antioxidant activity

Non enzymatic antioxidant activity of *Tabebuia rosea* leaf samples were determined for α – tocoperol, ascorbic acid and reduced glutathione.

α – TOCOPHEROL

Non – enzymatic activity of α – tocoperol of leaf sample is proposed by the Engel *et al.*(1938). The protocol isdetailed in Appendix V

ASCORBIC ACID

Ascorbic acid activity of leaf sample was determined by the Keuther *et al*.(1943). This procedure is described in Appendix VI

REDUCED GLUTATHIONE

Reduced glutathione activity of *Tabebuia rosea* leaf sample was done by Moron *et al.*(1979) method. The method is given in Appendix VII

3.5 FREE RADICAL SCAVENGING ACTIVITY

Free radicals scavenging activity of*Tabebuia rosea* leaf sample was determined using four different methods for the assessment of radical scavenging assays such as DPPH, ABTS, hydroxyl radical and hydrogen peroxide scavenging activity.

3.5.1 DPPH SCAVENGING ACTIVITY

The effect of samples on 2,2–diphenyl-2-picryhydrazyl (DPPH) radical was estimated according to Mensor *et al.*(2001). The procedure is fully explained in Appendix VIII

3.5.2 ABTS SCAVENGING ACTIVITY

The 2,2 – azine – bis (3-ethabenzothiazloine-6- sulpfonic acid radical ection ($ABTS^{+}$) decoloration test is a spectrophotometric method widely used assessment of the antioxidant activity of various substance. The $ABTS^{+}$ scavenging activity was measured using the Shirwaikar *et al.,* 2006. The detailed procedure is given by Appendix IX

3.5.3 HYDROXYL RADICAL SCAVENGING ACTIVITY

The hydroxyl radical scavenging activity was currently done by Elizabath *et al.,* 1990. The procedure is elaborated in Appendix X

3.5.4. HYDROGEN PEROXIDE SCAVENGING ACTIVITY

The hydrogen peroxide scavenging activity is done by using the procedure given in Ruch *et al*., 1989. The full procedure is given in Appendix XI

3.6 Inhibition of free radical generation

Inhibition of free radical generation of *Tabebuia rosea* leaf sample was determined by two methods such as nitric oxide generation and superoxide generation.

3.6.1 NITRIC OXIDE GENERATION

The nitric oxide scavenging activity was done by using the procedure given by Grenn and Hill (1984). The full procedure is presented in Appendix XII

3.6.2 SUPEROXIDE GENERATION

The superoxide generation activity on the leaf samples of *Tabebuia rosea* was measured using the Winterbourn *et al.,* 1975. The detailed procedure is given in Appendix XIII

The result and discussion of the present study is present in the next chapter.

4.0 Results and Discussion

Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject for very intense pharmacological studies and this has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutic value and as sources of lead compounds in the drug development (Ramanath *et al.,* 2006).

Free radicals induced by peroxidation have gained much importance because of their involvement in several pathological conditions such as atherosclerosis, ischemia, liver disorder, neural disorder, metal toxicity and pesticide toxicity (Baskar *et al.,* 2007). Free radicals cause damage to physiological structures such as lipids, DNA and proteins. Living organisms have developed a number of mechanisms to repair some of this damage. (Upagandawar *et al.,* 2007).

Oxidative stress, increased lipid levels and disturbances in glucose metabolism are important risk factors for diabetes, cardiovascular, oncologic and many other diseases (Rostislav *etal.,*2007). Reactive oxygen species which include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radical ($\cdot OH$) and non-freeradicals species such as H_2O_2 and singlet oxygen ($\cdot O_2$) are various forms of activated oxygen. The importance of free radicals and ROS has attracted increasing attention over the past decades. The active molecules are exacerbating factors in cellular injury and aging process (Kumar *et al.,* 2006).

Plants contain a wide variety of antioxidant phytochemicals or bioactive molecules, which can neutralize the free radicals and thus retard the progress of many chronic diseases associated with oxidative stress and ROS. The intake of natural antioxidants has been associated with reduced risk of cancer, cardiovascular disease, diabetes and diseases associated with aging. Studies on dietary free radical scavenging molecules have attracted the attention to characterize phenolic compounds and other naturally occurring phytochemicals as antioxidants (Ani *et al.,* 2006).

Thus the present study involves the analysis of phytochemical constituents of the leaves of *Tabebuia rosea.* After determining the phytocompounds, the leaves were extracted using different solvents (petroleum ether, chloroform, hydro ethanol and water) which were then analysed for their antioxidant status and radical scavenging potential against a team of radicals such as DPPH, ABTS, Hydroxyl radical and H_2O_2 and also tested for their ability to prevent radical generation such as Nitric oxide and Super oxide.

The results of these parameters analysed are presented and discussed below.

Phytochemical Analysis of *Tabebuia rosea*

Preliminary phytochemical analysis of *Tabebuia rosea* leaf extracts were done using appropriate test procedures to confirm the availability of active phytochemicals in the various solvent extracts. The healthful properties of edible plants and perhaps due to the presence of a variety of phytoconstituents such as alkaloids, flavonoids, phenols, terpenoids, tannins, glycosides, volatile oils, saponins and carbohydrates. The preliminary screening tests are useful in the detection of these bioactive constituents. The result of phytochemical analysis is depicted in Table I. In this phytochemical screening, secondary metabolites such as alkaloids, phenolics, flavonoids, tannins, glycosides, terpenoids, steroids and saponins have screened in petroleum ether, chloroform, hydroethanol, and aqueous leaf extracts of *Tabebuia rosea.*

Table 1

Phytochemical Analysis of the leaf extracts of *Tabebuia rosea*

Solvents	**Petroleum ether**	**Chloroform**	**Hydro ethanol**	**Aqueous**
Alkaloids	+	++	+	+
Flavonoids	++	++	+	+
Phenols	+	+	+	+
Terpenoids	+	+	+	++
Tannins	+	+	+	+
Glycosides	++	+	+	+
Volatile oils	-	-	-	-
Saponins	+	+	+	+
Carbohydrates	+	+	++	++

‘+’ – Presence; ‘–’ - Absence

The results reveal that all the different solvent extracts possess all the phytochemicals tested except volatile oils. Thus the results of phytochemical analysis provide justification for the use of the plants in folk medicine to treat various diseases.

Table 1 indicates that all the solvent extracts of *Tabebuia rosea* analysed indicated the presence of secondary metabolites. Hemamalini *et al*. (2013) reported that their studies on

preliminary phytochemical investigations of *Tabebuia rosea* leaves revealed the presence of chemical constituents such as saponins, tannins, phenolic acids, flavonoids and alkaloids.

The extract of root bark of *Delonix regia* revealed the presence of tannins, terpenoids, alkaloids, glycosides, carbohydrates and sterols suggesting that the root bark can be used for curing various ailments such as cancer, diabetic and cardiovascular diseases (Sama and Raja, 2011).

All the selected medicinal plants were found to contain tannins and flavonoids. Moreover, terpenoids were also present in all the selected plants except *P.dactylifera*. On the other hand, saponins and steroids were absent in all plants except *S.chirata* and phlobatannins were absent in all plants except *R.sativus*. In addition, carbohydrates, glycosides and coumarins were present in all the selected plants except *P.dactylifera* and *R.sativus*. Alkaloids were present in all the selected plants except *F.religiosa, P.dactylifera* and *R.sativus*. Proteins were present only in *F.religiosa* and *S.chirata*, whereas emodins, anthraquinones, anthocyanins and leuco-anthocyanins were absent in all the selected six plant (Yadav *et al*., 2014).

The literature cited above added credit to the current findings on *Tabebuia rosea* leaf extract which carries a good source of pharmaceutically valuable components like alkaloids, flavonoids, sterols, terpenoids, tannins, saponins, phenols and carbohydrates in the qualitative screening performed. Hence *Tabebuia rosea* leaf extract can very well be considered for the formulation of pharmaceutical products to be used for the treatment of various diseases and disorders.

Antioxidant Activity of *Tabebuia rosea*

One of the biological mechanisms to combat oxidative stress is by producing antioxidants. Thus the harmful effect of oxidative stress is counteracted by the action of both antioxidant enzymes and non-enzymatic antioxidants.

Enzymatic Antioxidant Activity

Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to Hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in presence of cofactors such as copper, zinc, manganese, and iron. Non-enzymatic antioxidants work by interrupting free radical chain reactions. Few examples of the non-enzymatic antioxidants are vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione (Nimse *et al.,* 2015).

Table 2

Enzymatic Antioxidant Activity of the Leaves of *Tabebuia rosea*

S.No.	Enzymes	Activity
1.	Catalase (U/g)[1]	0.42±0.03
2.	Peroxidase (U/g)[2]	0.63±0.03
3.	Superoxide dismutase (U/g)[3]	0.31±0.02

The values are mean ± S.D. of triplicate

[1]1Unit- amount of enzyme required to decrease the absorbance at 240nm by 0.05 units/min

[2]1 Unit- change in absorbance at 430nm/min

[3]1 Unit- Amount of enzymes that causes 50% reduction in NBT oxidation

Enzymatic antioxidant activities determined in the leaves of *Tabebuia rosea* revealed that the leaf extracts exhibited considerable enzymatic activity including the activities of catalase, peroxidase and superoxide dismutase and are presented in Table 2.

Some plant extracts and essential oils were examined in relation to their value, while others were tested for their antioxidant capacity. IC50 values of *Nepetamelissi folia*, *Mentha pulegium* and *Phlomis lanata* were found to be similar to BHT and ascorbic acid (Proestos *et al*., 2013).

Enzymes are the best known substances of the antioxidant protection system and are important components of the protection or defence mechanisms achieved by eliminating toxic metabolites from the system. The considerable enzymatic antioxidant potential of *Tabebuia rosea* shown in the present study could alleviate the toxicity rendered by reactive species.

Non- Enzymatic Antioxidants

Non-enzymatic antioxidants are of two types which include nutrient antioxidants and metabolic antioxidants. In the present study, ascorbic acid and tocopherol levels were assessed under nutrient antioxidants and the estimation of glutathione for metabolic antioxidant status.

Table 3

Non-enzymatic Antioxidant Levels of the leaves of *Tabebuia rosea*

S.No.	Non-Enzymatic Antioxidants	Levels
1.	Ascorbic acid (mg/g)	0.81±0.03
2.	α-tocopherol (μg/g)	0.31±0.02
3.	Reduced glutathione (nmoles/g)	0.15±0.02

The values are mean ± S.D. of triplicate

Non-enzymatic antioxidant such as Ascorbic acid, α-Tocopherol and Reduced glutathione were assessed in the leaves of *Tabebuia rosea* and the levels of Non Enzymatic contents of *Tabebuia rosea* is depicted in Table 3.

The results indicate that the leaves of *Tabebuia rosea* is found to be a good source of non-enzymatic antioxidants as they exhibit a moderate levels of non-enzymatic antioxidants such as ascorbic acid, tocopherol and reduced glutathione

Kumari and Achal (2008), reported that the non-enzymatic antioxidants vitamin C, E and A were shown to be present in Oyster mushroom. Tiwari (2012) has brought to light that *Punica granatum* could be considered as good source of antioxidants due to the abundance of Vitamin C, B5 and polyphenols.

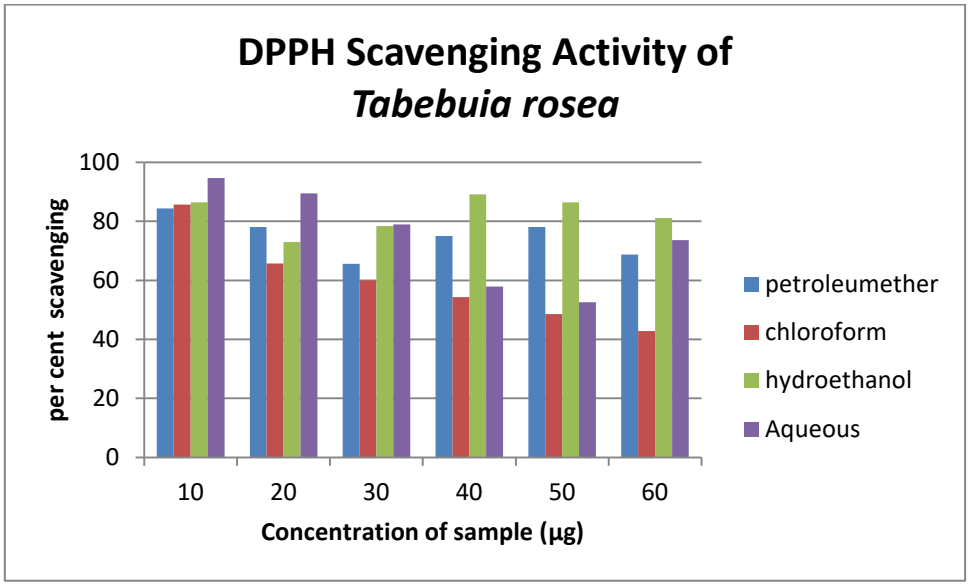
A study reported by Starlin and Gopalakrishnan, (2013) revealed that *Tylophora pauciflora* has an excellent source of enzymatic and non-enzymatic antioxidants and has the capability to scavenge the free radicals and protect against oxidative stress causing diseases

Free radical Scavenging Activity

Free radicals that are generated in several biochemical reactions in the body have been implicated as mediators of many diseases, including cancer, and several diseases and disorders. Although these free radicals can be scavenged by the *in vivo* produced antioxidant compounds, the endogenous antioxidants are insufficient to completely remove them and maintain a balance and hence, the analysis and measurement of radical quenching ability by these dietary or medicinal plants becomes necessary.

DPPH Radical Scavenging Activity of the leaves of *Tabebuia rosea*

Figure 1

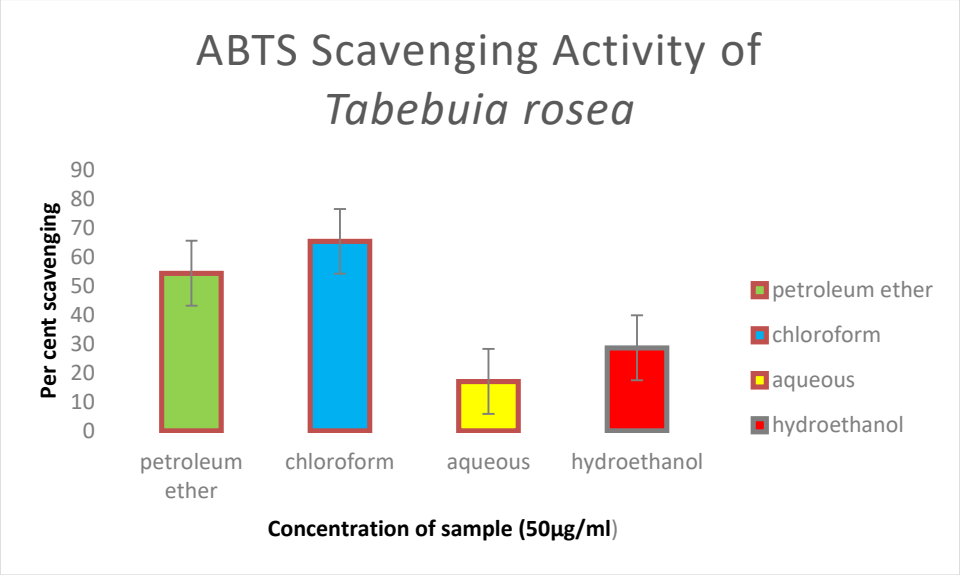


From the above Figure 1, it is clear that antioxidant activity of various extract shows moderate levels of radical scavenging at all concentrations tested. Among the various extracts tested, Aqueous shows maximum scavenging potency followed by hydroethanol, chloroform and water. The order of DPPH Activity of the leaves of *T.rosea*as represented in the figure 2 is Aqueous>Hydroethanol>Chloroform>Petroleum ether respectively.

DPPH has been widely used for free radical-scavenging assessments due to its ease and convenience. In the present study, all the extracts tested were found to be the effective scavengers against DPPH radical. Li *et al*. (2010) reported that the ethyl acetate extracts showed the highest DPPH radical scavenging activity, while the weakest scavengers were the petroleum ether extracts. On the other hand, the extracts obtained by cold-extraction exhibited stronger DPPH radical scavenging ability than the corresponding extracts obtained by Soxhlet extraction.

ABTS Scavenging Activity of the Leaves of *Tabebuia rosea*

Figure 2

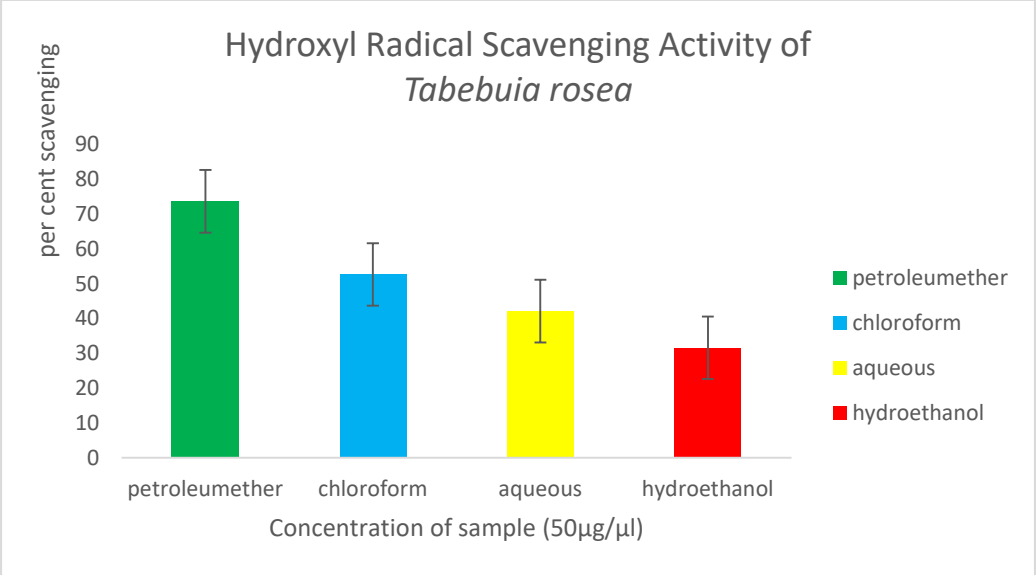


It is clearly evident from the Figure 2, that among the various extracts tested, chloroform shows maximum activity followed by petroleum ether, hydroethanol, and aqueous. The order of ABTS Activity of the leaves of *T.rosea* as represented in the figure is Chloroform>Petroleum ether>Hydro ethanol>Aqueous respectively.

The technique for the generation of ABTS described involves the direct production of the blue/green ABTS chromophore through the reaction between ABTS and potassium persulfate. This has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm, as reported previously as well as the more commonly used maximum at 415 nm. Addition of antioxidants to the pre-formed radical cation reduces it ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus the extent of decolorization as percentage inhibition of the ABTS radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox as a standard, under the same conditions. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts (Roberta *et al., 1998).*

Hydroxyl Radical Scavenging Activity of the Leaves of *Tabebuia rosea*

Figure 3



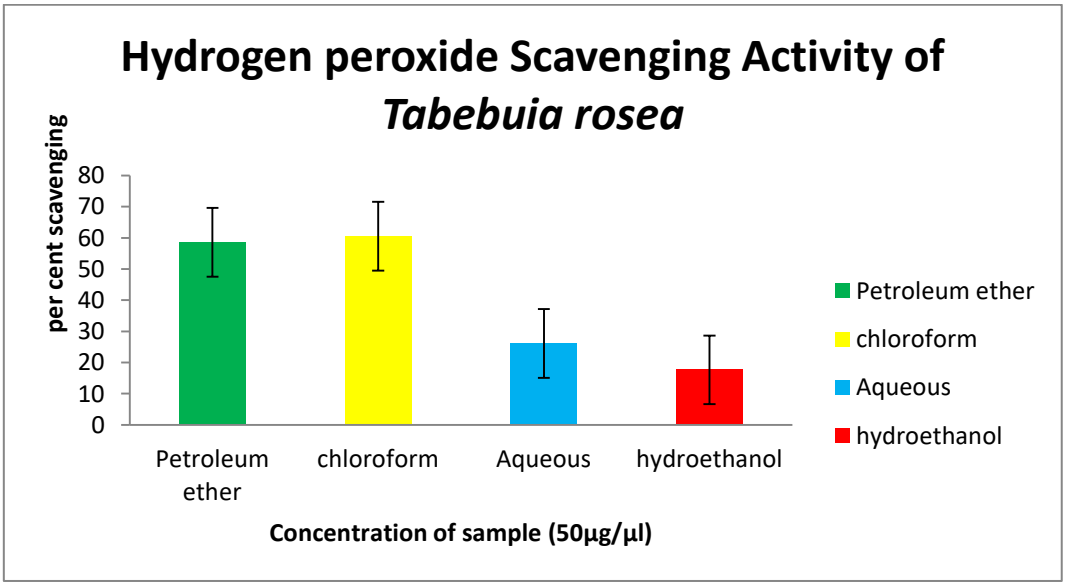
The percentage scavenging of Hydroxyl radicals of various extracts of the leaves of *T.rosea* is presented in Figure 3. The extracts used were petroleum ether, chloroform, aqueous and hydroethanol. The scavenging activity is higher in petroleum ether. The order of Hydroxyl radical scavenging activity of the leaves of *T.rosea is found to be* Petroleum ether> Chloroform>Aqueous> Hydroethanol.

Hydroxyl radicals scavenging activity were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH Ferric – EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4 while the addition of methanol extract to the reaction mixture found that they removed hydroxyl radical from the sugar and prevented their degradation. The methanol extract of *P.florida* mushroom showed potent hydroxyl radical scavenging activity.

In a study carried out by Menaga *et al*. (2013), the hydroxyl radical-scavenging effect of the ethanol extract in a concentration of 500 µg/ml which was found to be 59.2% and ascorbic acid was used as a standard since it is reported to be significantly effective in inhibition of hydroxyl radicals, which shows 63% scavenging effect at the same concentration. The value of *P. florida*and ascorbic acid was found at the concentration of 410µg/ml and 360µg/ml respectively, further the shown hydroxyl radical scavenging activity as dose dependent.

Hydrogen Peroxide Scavenging Activity of the Leaves of *Tabebuia rosea*

Figure 4



The percentage scavenging of hydrogen peroxide by the various leaf extracts of *T.rosea* is shown in the Figure 4. The extracts used are petroleum ether, chloroform hydroethanol and water. The hydrogen peroxide scavenging activity is higher in chloroform extract. The order of Hydrogen peroxide scavenged by the leaves of *T.rosea i*s chloroform> petroleum ether> aqueous > hydroethanol.

The scavenging ability of water and ethanol extracts of *C.monogyna* on hydrogen peroxide is compared with BHA and α-tocopherol as standards. The *C.monogyna* extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner (Kesar *et al*., 2012).

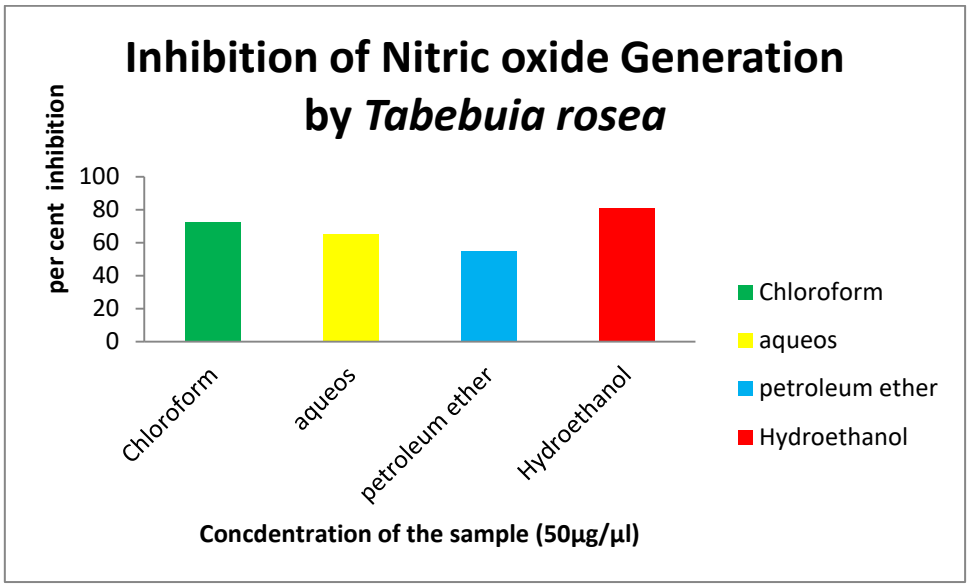
Kumaran and Kumarakarnan, (2006) suggested that significant antioxidant activity and Free radical scavenging property of *Coleus aromaticus* may be one of the mechanisms by which this drug is useful as a food stuff as a traditional medicine. In agreement with the above literatures cited the leaves of *Tabebuia rosea* exhibits radical quenching ability and hence can be used in pharmaceutical preparations for the treatment of diseases associated with free radicals.

The results of radical scavenging assays reveal that the leaf extracts of *Tabebuia rosea* could scavenge the battery of radicals such as DPPH, ABTS, Hydroxyl radical and

hydrogen peroxide tested in the present study to a better extent indicating that the considerable antioxidant potential of the plant extract might be responsible for their scavenging properties which further enable them to be used in pharmaceutical preparations to alleviate free radical and oxidative stress mediated diseases.

Inhibition of Nitric oxide Radical generation by the Leaves of *Tabebuia rosea*

Figure 5



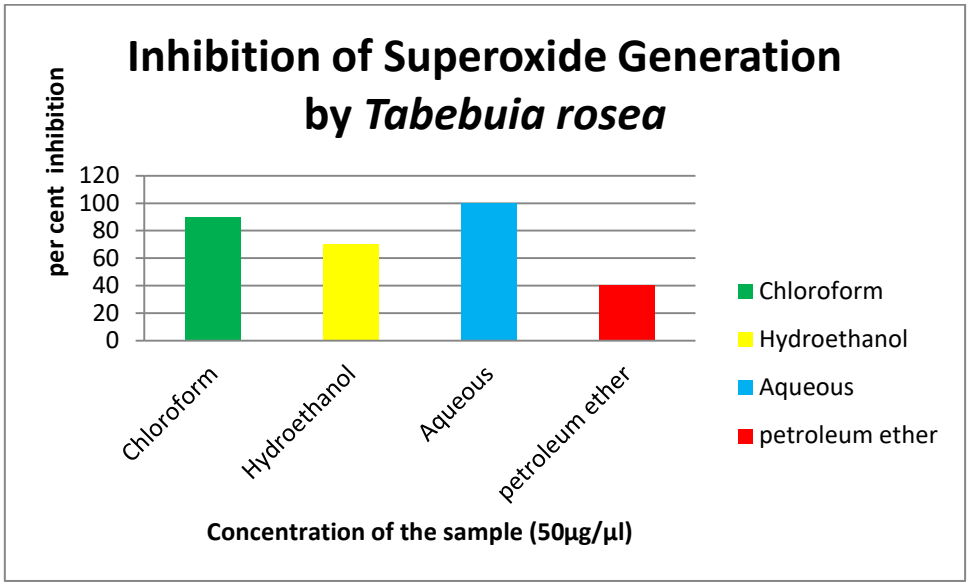
The percentage inhibition of nitric oxide generation activity of various extracts of leaf of *T.rosea* is represented in Figure 5. The extracts used are petroleum ether, chloroform, hydroethanol and water. The Nitric oxide radical generation is inhibited to a greater extent in hydroethanol extract followed by chloroform extract.

Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3, and NO_2 are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 250ºC for 2 h resulted in linear time-dependent nitrite production, which is reduced by the tested methanolic extracts of *Phyllanthus freternus* leaves, barks and roots of *Triumfetta rhomboidae* and barks of *Casuarina littorea*. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. It is to be noted that *Phyllanthus freternus*, a greater

inhibition comparative to other plant extracts but less than ascorbic acid which has shown 96.27% inhibition of nitric oxide (Parul *et al*., 2013).

Inhibition of Superoxide Radical generation by the Leaves of *Tabebuia rosea*

Figure 6



The percentage inhibitory activity of superoxide generation of various leaf extracts of *T.rosea* is presented in Figure 6. The extracts used were petroleum ether, chloroform, hydroethanol and water. The Superoxide generation is highly inhibited by the aqueous extract. The order of Superoxide generation of activity of *T.rosea is* found to be aqueous> chloroform> hydroethanol> petroleum ether.

The extracts of *Thermopsis turcica* were determined to have a certain level of radical scavenging effect, proportional to their level of phenolic content. Thus indicating that the plant can be used in pharmaceuticals products as a source of natural antioxidants (Aksoy *et al.*, 2013).

The study indicates that the ethanol extract of *Carthamus tinctorius*has significant superoxide anion radical scavenging activity. It can be used as easily accessible source of natural antioxidants and as a possible food supplement industry and pharmaceutical industry. However, the compounds responsible for superoxide anion radical scavenging activity of ethanol extract of the marshmallow are currently unclear. Therefore, it is suggested that further study could be performed on the isolation and characterisation of the antioxidant content of the *Carthamus tinctorius* (Shareef *et al*., 2007).

The inhibitory efficiency of various solvent extracts of the leaves of *T.rosea* revealed that both Nitric oxide and superoxide radical's generation were inhibited to a greater extent proving their therapeutic efficacy.

The content of total phenolic compounds, flavonoids and flavanols were measured in some medicinal and aromatic plant extracts in a study conducted by Miliauskas *et al*. (2004) indicated a correlation between scavenging capacities of extracts with total phenolic compound content was observed.

Hameed, 2009 suggested that his study provide evidence that all the six *Ficus* species have radical scavenging activity and antioxidant capacity. The results reveal that most phenolic compounds are the major components of *Ficus* species and the antioxidant properties were attributed to them and these plant species may have great relevance in the prevention and therapies of diseases in which oxidants or free radicals are implicated after more *in vitro* studies for understanding their mechanism of action as antioxidants.

Thus it can be concluded that the leaves of *Tabebuia rosea* exhibited a good antioxidant potential and could scavenge the radicals readily which might be due to the presence of various phytoconstituents in the leaves of the candidate plant. More *in vivo* and *in vitro* studies along with detailed spectrophotometric and chromatographic investigations are needed in the hope to make use of these plant extracts in the prevention and therapies of diseases in which oxidants or free radicals are involved.

5.0 Summary and Conclusion

The use of plants for medicinal purposes is as old as the emergence of human species on earth. Historically, first civilizations realized that some plants contained active ingredients in their essences, which empirically revealed their healing power when they were tested in disease. Because of the importance of oxidative stress in the pathophysiology of most of the hard curable diseases, the use of medicinal plants with antioxidant properties is important and should be considered. Drug therapy and even drug discovery should also be focused more than before on this source. Multidisciplinary team work including ethnobotanists, pharmacologists, physicians and phytochemists are essential for the fruitful outcome of medicinal plants research. More importantly, regulations are also needed to ensure efficacy, quality and safety of herbal medicines.

A radical is any molecule that contains one or more unpaired electrons. Radicals are normal products of many metabolic pathways. Some exist in a controlled form as they perform essential functions. Others exist in a free form and interact with various tissue components. Such interactions can cause both acute and chronic dysfunction, but can also provide essential control of redox regulated signaling pathways. The potential roles of endogenous or xenobiotic-derived free radicals in several human pathologies have stimulated extensive research linking the toxicity of numerous xenobiotics and disease processes to a free radical mechanism.

The human body has several mechanisms to counteract oxidative stress by producing antioxidants. A shift in the balance between oxidants and antioxidants in favor of oxidants is termed as “oxidative stress”. Paradoxically, there is a large body of research demonstrating the general effect of oxidative stress on signaling pathways, less is known about the initial and direct regulation of signaling molecules by ROS, or what we term the “oxidative interface.” The molecular mechanisms through which ROS directly interact with critical signaling molecules to initiate signaling in a broad variety of cellular processes, such as proliferation and survival , ROS homeostasis, and antioxidant gene regulation. Cellular constituents of our body are altered in oxidative stress conditions, resulting in various diseased states. The oxidative stress can be effectively neutralized by enhancing cellular defenses in the form of antioxidants. Certain compounds act as *in vivo* antioxidants by raising the levels of endogenous antioxidant defenses.

The present study focuses on the determination of antioxidant status and radical scavenging ability of the various solvent extracts of *Tabebuia rosea*. Preliminary phytochemical analysis of *Tabebuia rosea* leaf extracts were also done using appropriate test procedures to confirm the availability of active phytochemicals in the various solvent extracts. The healthful properties of edible plants and perhaps due to the presence of a variety of phytoconstituents such as alkaloids, flavonoids, phenols, terpenoids, tannins, glycosides, volatile oils, saponins and carbohydrates. The preliminary screening tests are useful in the detection of these bioactive constituents. In this phytochemical screening, secondary metabolites such as alkaloids, phenolics, flavonoids, tannins, glycosides, terpenoids, steroids and saponins have screened in petroleum ether, chloroform, hydroethanol, and aqueous leaf extracts of *Tabebuia rosea.*

The results reveal that all the different solvent extracts possess all the phytochemicals tested except volatile oils. Thus the results of phytochemical analysis provide justification for the use of the plants in folk medicine to treat various diseases.Hence *Tabebuia rosea* leaf extract can very well be considered for the formulation of pharmaceutical products to be used for the treatment of various diseases and disorders.

One of the biological mechanisms to combat oxidative stress is by producing antioxidants. Thus the harmful effect of oxidative stress is counteracted by the action of both antioxidant enzymes and non-enzymatic antioxidants. Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to Hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in presence of cofactors such as copper, zinc, manganese, and iron. Non-enzymatic antioxidants work by interrupting free radical chain reactions. Few examples of the non-enzymatic antioxidants are vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione.

Enzymatic antioxidant activities determined in the leaves of *Tabebuia rosea* revealed that the leaf extracts exhibited considerable enzymatic activity including the activities of catalase, peroxidase and superoxide dismutase. The considerable enzymatic antioxidant potential of *Tabebuia rosea* shown in the present study could alleviate the toxicity rendered by reactive species.

Non-enzymatic antioxidants such as Ascorbic acid, α-Tocopherol and Reduced glutathione were assessed in the leaves of *Tabebuia rosea.* The results indicate that the leaves of *Tabebuia rosea* is found to be a good source of non-enzymatic antioxidants as they exhibit a moderate levels of non-enzymatic antioxidants such as ascorbic acid, tocopherol and reduced glutathione.

Free radicals that are generated in several biochemical reactions in the body have been implicated as mediators of many diseases, including cancer, and several diseases and disorders. Although these free radicals can be scavenged by the *in vivo* produced antioxidant compounds, the endogenous antioxidants are insufficient to completely remove them and maintain a balance and hence, the analysis and measurement of radical quenching ability by these dietary or medicinal plants becomes necessary.

The results of radical scavenging assays reveal that the leaf extracts of *Tabebuia rosea* could scavenge the battery of radicals such as DPPH, ABTS, Hydroxyl radical and hydrogen peroxide tested in the present study to a better extent indicating that the considerable antioxidant potential of the plant extract might be responsible for their scavenging properties which further enable them to be used in pharmaceutical preparations to alleviate free radical and oxidative stress mediated diseases.

The inhibitory efficiency of various solvent extracts of the leaves of *T.rosea* revealed that both Nitric oxide and superoxide radical's generation were inhibited to a greater extent proving their therapeutic efficacy.

Thus it can be concluded that the leaves of *Tabebuia rosea* exhibited a good antioxidant potential and could scavenge the radicals readily which might be due to the presence of various phytoconstituents in the leaves of the candidate plant. More *in vivo* and *in vitro* studies along with detailed spectrophotometric and chromatographic investigations are needed in the hope to make use of these plant extracts in the prevention and therapies of diseases in which oxidants or free radicals are involved.

APPENDIX I

QUALITATIVE PHYTOCHEMICAL ANALYSIS

DETECTION OF ALKALOIDS (Raaman, 2006)

Mayer's test

A fraction of the extract was treated with Mayer's reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100ml of distilled water) and observed for the formation of crean coloured precipitate.

Dragenodorff's test

An aliquot of the extract was tested with Dragendorff's reagent (solution A: Bismuth nitrate (0.7g) in glacial aceticacid (2ml) and distilled water (8ml) Solution B: potassium iodide (4g) in glacial aceticacid (10ml) in water (20ml), mix Solution A and B dilute to 100ml with distilled water) and observed for the formation of rediish orange precipitate

Wagner's test

A fration of the extract was treated with Wagner;s reagent (1.2g of iodine and 2g potassium iodide in 100ml of distiied water) and observed for the formation of reddish brown coloured precipitate.

Hager's test

To afew ml of filtrate 1 or 2 ml of hager's reagent (Saturated aqueous solution of picric acid) were added. A prominent yellow precipitate indicated the test as positive

DETECTION OF PHENOLS (Raaman, 2006)

Ferric chloride test

To a fraction of theextract, 5% $Fecl_3$ solution was added and obseved for the formation of deep blue colour

Lead acetate test

A fraction of the extract was treated with 10% lead acetate soution and observed for the formation of white precipitate

DETECTION OF FLAVANOIDS

Aqueous NaOH test

To a fraction of the extract, a drop of 1N aqueous NaOH solution was added and observed for the formation of yellow orange colouration

Alkaline reagent test

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavanoids.

DETECTION OF TANNIN

For detection of treated with a few drops of lead solution. The formation of whit precipitate indicates the presence of tannin

IDENTIFICATION OF GLYCOSIDES (Raaman, 2006)

For detection of glycosides, 50mg of extract was hydrolysed with concentrated hydrochloric acid for 2hrs on water both, filtrated and the hydrolysete was subjected to the following tests.

Borntrager's test

To 2ml of filtrated hydrolysate, 3ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it pink colour indicated the presence of glycosides

DETECTION FOR SAPONIN (Raaman, 2006)

Sodium bicarbonate test

In a teat tube,he presence of about 5ml of extract was added and a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. The formation of a honey comb like froth showed of saponins

DETECTION FOR TERPENOIDS (Siddiqui and Ali, 1997)

5ml of extract was mixed with 2ml of chloroform and conc.H_2SO_4 (3m) was carefully added to form of layer. A reddish brown colouration of the interface formed to show positive for the presence of terpenoids.

DETECTION FOR STEROIDS (Siddiqui and Ali, 1997)

Liebermann Burchard reaction

A portion of the extract was treated with 10ml chloroform and filtrated. To 2m of fitrate was treate with 2ml of acetic anhydride and conc. Sulpuric acid. Blue, green ring indicates presence of steroids

TEST FOR PHYTOSTEROLS

To 1ml of plant extract, equal volume of choloroform and 3 drops of concentrated sulpuric acid were added. Formation of rown ring indicates the presence of steroids and formation of bluish green colour indicated the presence of phytosterols

TEST FOR PROTEINS:

To 2 ml of each extract, 1 ml of 40% sodium hydroxide and few drops of 1% copper sulphate were added; formationof violet colour indicates the presence of peptide linkage molecules in the sample extract.

TEST FOR CARBOHYDRATES*:*

Take 1 ml of extract, add few drops of Molisch's reagent and then add 1 ml of concentrated sulphuric acid at theside of the tubes. The mixture was then allowed to stand for 2 to 3 minutes. Formation of red or dull violet colourindicates the presence of carbohydrates in the sample extract.

APPENDIX II

ESTIMATION OF SUPEROXIDE DISMUTASE

(Misra and Fridovich, 1972)

PRINCIPLE

The assay of SOD is based on the inhibition of formation of NADHphenazinemethosulphate-nitroblue tetrazolium formazon, the extent of whichcan be assayed spectrophotometrically at 560nm.

REAGENTS

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186µM)
3. Nitroblue tetrazolium (NBT)) (300µM)
4. NADH (700µM)
5. Glacial acetic acid
6. n-butanol

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Plant sample (0.5g) were ground with 3.0ml of sodium pyrophosphate buffer, centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

ASSAY

The assay mixture contained in a total volume of 3.0ml, 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of enzyme preparations and 1.0ml of water. NADH (0.2ml) was added to start the reaction.

The assay mixture was incubated at 30ºC for 90 seconds and the reaction was stopped by the addition of 1.0ml of glacial acetic acid. n-butanol (4ml) was added to the above mixture, allowed to stand for 10 minutes and then centrifuged at 2000g for 5 minutes. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol as blank. The system devoid of enzyme served as control. One unit of enzyme activity is defined as the amount of enzyme causing a 50% reduction in NBT oxidation/minute.

APPENDIX III

ESTIMATION OF CATALASE ACTIVITY

(Luck, 1974)

PRINCIPLE

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease.

REAGENTS

1. Phosphate buffer (0.067M PH 7.0)

   Dissolved 3.522g of $KH_2PO_4$and 7.268g of $KHPO_4.2H_2O$ in distilled water and made up the volume to one litre.

2. Hydrogen peroxide – Phosphate buffer

   Dissolved 0.16 ml of H_2O_2 (10% W/V) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240nm with 1 cm light path.

PROCEDURE

ENZYME EXTRACT

The sample was homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay. Used fresh extract for assay.

ASSAY

Read against a control cuvette 3ml of H_2O_2 containing the enzyme solution as in the phosphate buffer (M/15). Pipetted into the experimental cuvette 3ml of H_2O_2 phosphate buffer. Mixed in 0.01-0.04ml sample with the glass or plastic rod flattened at one end. Noted the time it required for a decrease in absorbance from 0.45-0.4. This value was used for calculations. If ‘t’ was more than 60 seconds, repeated the measurement with more concentrated solution of the sample.

APPENDIX IV

ESTIMATION OF PEROXIDASE ACTIVITY

(Reddy *et al.*, 1995)

PRINCIPLE

In the presence of the hydrogen donor pyrogallol, peroxidase converts H2O2 to water and oxygen. The oxidation of pyrogallol to the coloured product purpurogalli can be quantified spectrophotometrically at 430nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

$$\text{Pyrogallol} + H_2O_2 \longrightarrow \text{Oxidized pyrogallol} + H_2O$$

REAGENTS

1. Pyrogallol(0.05M); Phoshphate buffer (pH 6.5): 630 mg of pyrogallol in 100ml of 0.1M Phosphate buffer.
2. Hydrogen peroxide (1%)

PROCEDURE

Macerated one gram of the sample with 5 ml (w/v) 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuged the homogenate at 300 g for 15 min. Used the supernatant as the enzyme source. All procedure were carried out at 0-5° C.

Pipetted out 3ml of 0.05 M pyrogallol solution and 0.5 to 1.0 ml of enzyme extract in a test tube.Adjusted the spectrophotometer to read '0' at 400 nm.Added 0.5 ml of 1% hydrogen peroxide in the test cuvette. Recorded the change in the absorbance every 30 seconds upto 3 minutes.E

CALCULATION

Change in absorbance / min = X

Weight of the plant material taken = 300 mg

Volume of the extract taken for the assay = 0.02 ml

Change in absorbance for 1.5 ml extract = (X / 0.02) x 1.5 – Y

(i.e) Peroxidase activity in 300 mg plant tissue = Y

Peroxidase activity / g plant tissue = Yx (1000/300) Units

One unit of glutathionereductase is defined as the μmoles of NADPH oxidized/minute.

APPENDIX V

ESTIMATION OF α-TOCOPHEROL

(Emmerie-Engel method, 1938 as described by Rosenberg, 1992)

PRINCIPLE

Tocopherol can be estimated using Emmerie – Engel reaction which based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2, 2'-dipyridyl. Tocopherol and carotenes are first extracted with xylene and the extraction read at 460nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520nm.

REAGENTS

1. Absolute alcohol
2. Xylene
3. 2, 2'- dipyridyl
4. Standard solution:

Dissolved 10mg/ 10ml of α-tocopherol in absolute alcohol 91mg of œ- tocopherol is equivalent to 100mg of tocopherol acetate.

EXTRACTION OF PLANT TISSUE

The sample was homogenized with water in a blender. Weighed accurately 2.5g of the homogenized sample into a conical flask.Added 50ml of 0.1N H_2SO_4 slowly without shaking. Stoppered and allowed to stand overnight. The next day contents of the flask were shaken vigorously and filtered through whatman No.1 filter paper, discarding the initial 10-15ml of filtrate. Aliquots of the filtrate were used for the estimation.

PROCEDURE

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetteed out 1.5ml of extract, 1.5ml of standard, 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard,added 1.5ml of water. Added 1.5ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0ml of 2, 2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33ml of ferric chloride solution.

The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols in } \mu g = \frac{\text{Reading at 520nm} - \text{Reading at 460nm}}{\text{Reading of standard at 520nm} \times 0.24 \times 15}$$

APPENDIX VI

ESTIMATION OF ASCORBIC ACID

(Roe and Kuether, 1953)

PRINCIPLE

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal and bromine. Dehydroascorbic acid then reacts with 2, 4- dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution whose absorbance can be measured spectrophotomerically at 540nm.

REAGENTS

1. 4%TCA
2. 9N H_2SO_4
3. 2% 2, 4-dinitrophenyl hydrazine: Dissolved 2g of DNPH in 100ml of 9N H_2SO_4
4. 10% thiourea
5. 80% sulpuric acid
6. Stock standard solution: Dissolved 100mg of ascorbic acid in 100ml of 4%TCA
7. Working standard: Diluted 10ml of the stock solution to 100ml with 4%TCA

PROCEDURE

About 1g of the sample was homogenized in 4% TCA up to 10ml. Centrifuged at 2000rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. Centrifuged once again and removed the charcoal residue. The volume of the clear supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay.

The assay volumes were made up 2.0ml with 4%TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0ml with 4%TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 hours.

The osazones formed were dissolved in 2.5ml of 85% sulpuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and

thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX VII

ESTIMATION OF REDUCED GLUTATHIONE

Moron *et al*., (1979)

PRINCIPLE

Reduced glutathione (GSH) was measured by its reaction with 5,5'- dithiobis-2-nitrobenzoic acid (DTNB) (Ellman's reaction) to give a yellow coloured compound that absorbs at 412nm.

REAGENTS

1. TCA (5%)
2. TCA (25%)
3. Sodium phosphate buffer (0.2M, pH 8.0)
4. DTNB (0.6M in 0.2M sodium phosphate buffer)

PROCEDURE

PREPARATION OF PLANT EXTRACT

The leaves of *Gmelina arborea* (0.5g) were ground with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and the supernatant (0.1ml) was taken for the estimation of GSH.

ESTIMATION

The volume of different aliquots (0.2 to 1.0ml) was made up to 1ml with phosphate buffer. Freshly prepared DTNB (2ml) was added to the tubes and the intensity of the yellow colour was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2 to 10nmoles of GSH. The concentration of GSH in the samples was calculated from this and the result were expressed as nmoles GSH/g leaf.

APPENDIX VIII

DPPH RADICAL SCAVENGING ACTIVITY

(Mensor *et al*., 2001)

PRINCIPLE

Antioxidants react with DPPH and convert it to diphenyl-picryl hydrazine by donating its OH group. The degree of discoloration from purple to yellow colour can be measured at 519nm, which is a measure of the radical scavenging potential of the extracts.

REAGENTS

1. DPPH (0.3mM in methanol)
2. Methanol

PROCEDURE

The different solvent extracts and crude aqueous extract (5µl) was added with 0.5ml of methanolic solution of DPPH and 0.495ml of methanol. The mixture was then allowed to stand at room temperature for 30 minutes. DPPH methanol solution was used as positive control and methanol alone acted as blank. After incubation, the conversion of purple colour to yellow colour was read at 518nm in a spectrophotometer. The per cent inhibition was calculated using the following formula;

$$\text{Scavenging activity (\%)} = 100 - \frac{\text{A (Control)}}{\text{A(Control) - A(Sample)}} \times 100$$

APPENDIX IX

ABTS RADICAL SCAVENGING EFFECTS

(Shirwaikar *et al*., 2006)

PRINCIPLE

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation de-colourisation assay was employed to assess the radical-scavenging effect of the leaf extracts of the candidate plant. ABTS is a chromogen, which changes into a coloured mono-cation radical form (ABTS+) in the presence of oxidative agent and the ABTS+ has an absorption peak at 750nm. Antioxidants will reduce ABTS+ into its colourless form and the extent of decolourisation corresponds to the per cent reduction of ABTS+.

REAGENTS

1. Ethanol
2. ABTS solution (7mM ABTS with 2.45mM ammonium persulfate).

The solution was incubated at room temperature for 12-16 hours before use.

PROCEDURE

The six different extracts (100µl each) were added to ABTS solution (300µl) and the final volume of each was made up to 1ml with ethanol. The absorbance was read at 745nm and the percentage inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = \frac{\text{A (Control) - A(Sample)}}{\text{A (Control)}} \text{ X } 100$$

APPENDIX X

HYDROXYL RADICAL SCAVENGING EFFECTS

(Elizabeth and Rao 1990).

PRINCIPLE

Hydroxyl radicals are generated from a Fe_2+/ascorbate/EDTA/H_2O_2 system, which attacks deoxyribose and eventually produces thiobarbituric acid reactive substances (TBARS). The ability of the plant extracts to inhibit TBARS formation is measured spectrophotometrically at 532nm.

REAGENT

1. Deoxyribose (28mM)
2. $FeCl_3$ (1mM)
3. EDTA (1mM)
4. H_2O_2 (10mM)
5. Ascorbate (1mM)
6. KH_2PO_4-KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1%)
8. HCl (25%)

PROCEDURE

The reaction mixture contained deoxyribose (0.1ml), $FeCl_3$ (0.1ml), H_2O_2 (0.1ml), ascorbate (0.1ml), buffer (0.1ml) and 20µl of leaf extracts which corresponded to 10mg concentration. The total volume was made up to 1ml with water. The tubes were capped tightly and incubated in a water bath at 37ºC for one hour. The reaction was terminated by the addition of TBA (0.5ml) and HCl (0.5ml). The tubes were heated in a boiling water bath for 20 minutes for colour development. The intensity of the pink colour formed, as the indication of TBARS formation, was measured at 532nm. The per cent TBARS produced for positive

control (H_2O_2) was fixed as 100% and the relative per cent TBARS was calculated for the extract treated groups.

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX XI

HYDROGEN PEROXIDE SCAVENGING EFFECTS

(Ruch *et al*., 1989)

PRINCIPLE

H_2O_2 scavenging activity was measured in terms of a decrease in the absorbance at 230nm spectrophotometrically.

REAGENTS

1. H_2O_2 (40mM in 0.1M phosphate buffer)
2. Phosphate buffer (0.1M, pH 7.4)

PROCEDURE

The plant extract was diluted to a concentration of 10mg in 10µl. This extract (10µl corresponding to 10mg) was added to 0.6ml of H_2O_2 solution and the final volume was made up to 3ml with the same buffer. After 10 minutes, the absorbance values at 230nm of the reaction mixtures were recorded against a blank containing phosphate buffer without H2O2 for each sample. The percent inhibition was calculated using the formula,

$$H_2O_2 \text{ scavenging activity (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX XII

NITRIC OXIDE SCAVENGING ACTIVITY

(Green and Hill, 1984)

PRINCIPLE

An aqueous solution of sodium nitroprusside spontaneously generates nitric oxide at physiological pH, which interacts with oxygen to produce nitrite ions, which is measured at 546nm.

REAGENTS

1. Sodium nitroprusside (100mM)

2. Phosphate buffered saline (PBS) (pH 7.4)

3. Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride)

PROCEDURE

Sodium nitroprusside (2.0ml), phosphate buffered saline (0.5ml) and each of the six different plant extracts (0.5ml) were mixed and incubated at 25ºC for 30 minutes. Griess reagent (0.5ml) was added and allowed to stand for 30 minutes. The control tube was prepared without leaf extracts. The absorbance of the pink coloured chromogen was read at 546nm against a reagent blank.

$$\text{Nitric oxide scavenging activity (\%)} = \frac{A \text{ (Sample)}}{A \text{ (Control)}} \times 100$$

APPENDIX XIII

SUPEROXIDE GENERATION SCAVENGING ACTIVITY

(Winterbourn *et al*., 1975)

PRINCIPLE

The extent of superoxide generation was studied on the basis of inhibition of the production of nitroblue tetrazolium formazon of the superoxide ion by the plant extracts and is measured spectrophotometrically at 560nm.

REAGENTS

1. EDTA (0.1M containing 1.5mg of sodium cyanide/10 ml)
2. Nitroblue tetrazolium (NBT) (1.5mM)
3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)
5. Dimethyl sulfoxide (DMSO)

PROCEDURE

The assay mixture contained 0.02ml of plant (solvent and crude aqueous) extracts with 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.63ml of phosphate buffer. DMSO, instead of plant extract, was considered as control. All tubes were vortexed and the initial absorbance was read at 560nm. The tubes were illuminated uniformly using a

fluorescent lamp for 30 minutes. The absorbance was read again at 560nm. The difference in optical density before and after illumination is the measure of superoxide generation and the percentage inhibition was calculated using the formula,

$$\% \text{ Superoxide Scavenging} = \frac{A \text{ (After illumination)} - A \text{ (Reference)}}{A \text{ (Control)}} \times 100$$

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