In vitro Anti oxidant and Anti-inflammatory activity of ethanolic extract of *Cissus quadrangularis*

By

NIVETHA C.K

(16PBC009)

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 BIOCHEMISTRY

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Signature of the
Head of the Department **Signature of the Supervisor**

ACKNOWLEDGEMENT

Itis with the choice of blessings and the divine grace of **LORD ALMIGHTY** that any human endeavour is achieved. In addition to the will of supreme divinity, the willingness of many subject experts and erudite scholars to extend their assistance and help for the completion of a work plays, indeed a vitally important role.

I take the opportunity of expressing my sincere thanks to **Dr**. **(Thiru) P.R. KrishnaKumar**, chancellor, Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore, for providing the opportunity and infrastructure to undertake this investigation.

I immensely thank **Dr. PremavathyVijayan**, Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the entire facilities essential to carry out and complete the study.

I record my sincere thanks to **Dr. S. Kousalya**, Registrar, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for timely help rendered to carry out the work.

My sincere thanks to **Dr. A. Parvathi**, Dean, Faculty of Science, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for her guidance and affectionate support, expert suggestions throughout the project.

I express my special gratitude to **Dr. S. Annapurani,** former professor and Head of the Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her sustained support throughout the study.

I record my sincere thanks to **Dr. P.R. Padma**, Professor and Head of the Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity and timely help rendered to carry out the work successfully.

I owe my indebtedness, profound and deepest thanks to my guide**Dr. B. Suganthi,** Professor, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her excellent guidance, immense tolerance, meticulous care, good support, creative influence, thoughtful advice, steady encouragement, motherly love throughout the research and motivation right from the selection of topic to the compilation and completion of the work efficiently and effectively.

My special thanks to **Dr. AnithaSubash,** Tutor and Professor, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, who helped me to complete the work successfully and brought out the good ideas in me.

I submit my sincere thanks to all **TheStaff Members**, of department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for lending a helping hand and invaluable guidance during the course of this thesis work.

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1.0 INTRODUCTION

Medicinal plants and herbs have been proved to be of great importance to the health of the individuals and communities. In recent years, many scientific investigations of traditional herbal remedies for several diseases have been carried out and this has lead to the development of alternative drug and therapeutic strategy. Since the consumption of medicinal plants is increasing, it is interesting to use these plants as a supplement in food taking into account that these plants can present a significant amount of trace elements and other nutrients. (Saranya *et al.,* 2017).

Herbs had been praised for their medicinal, flavouring and aromatic qualities for centuries. The synthetic products of the modern age surpassed their importance for a while. The important advantage of medicinal plants in various treatments is their safety besides being less expensive, efficacy and availability throughout the world. The Ayurvedic system of medicine uses about 700 species of Unani, 700 of Sidda and modern medicine around 30 species. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower and seeds. Some drugs are prepared from excretory plant product such as gum, resins and latex. Even the Allopathic system of medicine has adopted a number of plant-derived drugs from an important segment of the modern pharmacopoeia. Some important chemical intermediates needed for manufacturing the modern drugs are also obtained from plants (Srivastava *et al.*, 2013).

Several natural products, mostly of plant origin have been shown to possess promising activities that could assist in the prevention and/or amelioration of diseases such as human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS), malaria, tuberculosis, among others. Many of these agents have other medicinal values as well, which afford them further prospective as novel, which leads to the development of new drugs that could deal with both viruses and other diseases (Adebayo and Kretti, 2011).

In the traditional system of medicine, various plants parts such as stem back, root bark, aerial root, vegetative bud, leaves, fruits and latex are used in the treatment of variety of ailments.Most of the clinical drugs that are currently in use were derived from plants and developed because of their usage in traditional medicine. Aspirin (anti-pyretic), atropine, digoxin, morphine (pain killer), quinine and so on, were discovered through the study of ethno-botany (Enechi *et al.*, 2013).

Phytochemicals are natural bioactive compounds widely distributed in plants, animals, microbes, and other forms of life. Plant-produced chemical compounds like alkaloids, glycosides, flavonoids, volatile oils, tannins, resins have been used in a wide range of commercial and industrial applications such as flavors, aromas and fragrances, enzymes, preservatives, cosmetics, bio based fuels and plastics, natural pigments and bioactive compounds. The research on phytochemicals and use of phytochemicals is increasing more because of the harmful side effects of the synthetic compounds. (Paritala Vikram, *et al.*, 2014).

A large number of plants worldwide have been found to have both strong antioxidant activity (Baratto *et al.*, 2003) and powerful scavenger activity against free radicals. (Kumaran and Karunakaran, 2007). There is now increasing increasing interest in antioxidant activity of phytochemicals present in our diet.

Living cells continually generate free radicals or reactive oxygen species (ROS) through the respiratory chain during energetic metabolism. They can either be harmful or play important physiological roles in our body. Besides being produced during normal cell metabolism there are numerous exogenous factors, such as irradiation by UV light, X-rays, gamma-rays, and atmospheric pollutants which may lead to generation of reactive oxygen species. Human body has various intrinsic mechanisms to counteract oxidative stress by producing antioxidants, or through externally derived foods and/or supplements. However whenever there is excess of free radicals their accumulation in the body generates a phenomenon called oxidative stress. (Neeti sharma, 2014).

Free radicals are chemical species that contain one or more unpaired electrons. They are produced when oxidation reactions occur in cells but excess production starts chain reactions and causes serious damage to biological macromolecules. Antioxidants terminate these chain reactions by removing the excess free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Natural compounds with anti-oxidative properties include flavonoids, lignans, tannins, essential oils and terpenoids. As a result of prevalence of oxidative stress, studies involving antioxidant chemistry is gaining much relevance in recent times (Taiye *et al.*, 2011).

Antioxidants are compounds or systems that delay autoxidation by inhibiting formation of freeradicals or by interrupting propagation of the free radical by one (or more) of several mechanisms namely,scavenging species that initiate peroxidation, chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, quenching O_2- preventing formation of peroxides, breaking the autoxidative chain reaction and/or reducing localized O_2 concentrations (Nawar 1996).

Antiinflammatory drugs are among the more commonly used drugs worldwide. Besides their high costs, severe adverse reactions and toxicity are the greatest disadvantages. (Srinivasan *et al.*,2011). Therefore,the screening and development of drugs for their anti-inflammatory activities from the indigenous medicinal plants are more important.

Cissus quadrangularis L. is a succulent plant of family Vitaceae commonly known as Asthisamhari found in tropical and subtropical xeric wood. The stem juice of plant is used to treat scurvy, menstrual disorders, otorrhoea and epistaxis. The plant has been documented in Ayurveda for the treatment of osteoarthritis, rheumatoid arthritis and osteoporosis. A paste of stem is given in asthma, burns and wounds, bites of poisonous insects and for saddle sores of horses and camels. (Mohammed , 2015).

The scrutiny of literature revealed some notable pharmacological activities of the plant such as antioxidant, free radical scavenging, analgesic, antipyretic, anti microbial, bone healing and diuretics. (U.shah, 2011).

Many studies have been carried out on the free radical scavenging activity of the plant *Cissus quadrangularis* selected for the study.However, not much work has been done especially on the enzymatic and non enzymatic antioxidant status and anti-inflammatory activity.

Hence the present study entitled, "***In vitro*** **antioxidant and anti-inflammatory activity of ethanolic extract of *Cissus quadrangularis*"** was undertaken.

The main objectives of the study were as follows:

- To screen the phytochemicals present in the candidate plant by qualitative analysis.
- To assess the enzymatic and non enzymatic antioxidant status of the plant stem.
- To determine the free radical scavenging activity of the plant sample against oxidative injury.
- To study the *invitro* anti-inflammatory activity of the selected plant .

2.0 REVIEW OF LITERATURE

The World Health Organization estimates that 80 percent of the world's inhabitants rely mainly on traditional medicines for their health care. In ancient times, medicinal plants have been used all over the world as unique sources of medicines and may constitute the most common human use of biodiversity (Hiremath and Taranath, 2010). Herbs contain some of the most powerful natural antioxidants and are highly prized for their antioxidant and antiageing effects. Natural products offer diverse chemical structures. These compounds often serve as lead molecules, the activities of which can be enhanced by chemical manipulation and by de novo synthesis. To date, many medicinal plants have proved successful in combating various ailments, leading to mass screening for their therapeutic components.(Mehar *et al.*, 2010).

Phytochemicals are bioactive compounds found in plants that work with nutrients anddietary fibre to protect against diseases. They are non-nutritive compounds (secondary metabolites) that contribute to flavor and colour. Many phytochemicals have antioxidant activity and reduce the risk of many diseases, for example, alkyl sulfide (found in onions and garlic), carotenoids (from carrots), and flavonoids (present in fruits and vegetables). ROS have been implicated in many diseases and in aging process. These free radicals, which cause tissue damage via oxidative stress, are generated by aerobic respiration, inflammation, and LPO. (Agbafor and Nwachukwu, 2011).

Many medicinal plants have proved to successfully aid in various ailments leading to mass screening for their therapeutic components. Today the search for natural compounds rich in antioxidant, anticancer, antimicrobial and antiinflammatory properties is escalating due to their medicinal importance in controlling many chronic disorders such as cancer and cardiovascular disease. Antioxidants aid in the prevention by scavenging the excess free radicals in the body (Lai *et al.*, 2010).

The review of literature related to the study ***" In vitro* Antioxidant and Anti-inflammatory activity of ethanolic extract of *Cissus quadrangularis*"** is discussed under the following headings:

2.1 Reactive Oxygen species and Free radicals

2.2 Oxidative stress

2.3 Medicinal value of plants

2.4 Phytochemical screening

2.5 Antioxidants

2.6 Inflammatory response

2.7 Medicinal Properties of *Cissus quadrangularis*

2.1 REACTIVE OXYGEN SPECIES AND FREE RADICALS

Free radicals are highly reactive species that have been implicated in the pathogenesis of many diseases (Shukula, 2010). Free radicals andReactive oxygen species (ROS), including the hydroxyl radical ($HO\cdot$), superoxide radical ($O2\cdot-$), peroxyl radical ($ROO\cdot$) and non-radical species such as hydrogen peroxide ($H2O2$), hypochlorus acid ($HOCl$) and Reactive Nitrogen Species (RNS) include free radicals like nitric oxide ($NO\cdot$) and nitrogen dioxide ($NO2\cdot$) are highly reactive, as the result of the presence of unpaired valence shell electrons. Reactive oxygen species form as natural byproducts from normal oxygen metabolism and perform important functions in cell signaling (Heo and Jeon, 2008).

Free radicals are created when cells use oxygen to generate energy. These by-products are generally ROS such as super oxide anion, hydroxyl radical and hydrogen peroxide that result from the cellular redox process (Mohammad *et al.*, 2010). At low or moderate concentrations, ROS exert beneficial effects on cellular responses and immune function but at high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage cell structures, including lipids, proteins, and DNA (Huy *et al.*, 2008).

The amount of free radical production is determined by the balance of many factors and ROS are produced both endogenously and exogenously. The endogenous sources of ROS include mitochondria, cytochrome P450 metabolism, peroxisomes and inflammatory cell activation. Molecular oxygen is an essential component of aerobic life and of our metabolism. Thus, radicals are produced either naturally or by some biological dysfunction.

ROS or RNS and their excess have harmful effects, such as the peroxidation of the membrane lipids, aggression to tissue proteins and membranes, damage to DNA and enzymes (Aiyegoro and Okoh, 2009).

The potentially reactive derivatives of oxygen, attributed as ROS, are continuously generated inside the human body. The generated ROS are detoxified by the antioxidants present in the body. However, overproduction of ROS and/or inadequate antioxidant defense can easily affect and persuade oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA (Patel *et al.*, 2010).

2.2 OXIDATIVE STRESS

Oxidative stress can arise from an imbalance between the generation and elimination of reactive oxygen species leading to the excess levels, which in turn cause various diseases and cell death. Many human diseases are caused by oxidative stress that results from the formation and neutralization of pro-oxidants (Hazra *et al.,* 2008).

Oxidative stress initiated by free radicals, such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite, play a vital role in damaging various cellular macromolecules. These include DNA molecules, proteins along with lipid peroxidation. This damage may result into many diseases, including diabetes mellitus, atherosclerosis, myocardial infarction, arthritis, anaemia, asthma, inflammation, neurodegenerative diseases and carcinogenesis. However, human cells have an array of protecting mechanisms to prevent the production of free radicals and oxidative damage (Oyedemi *et al.,* 2010). The need therefore for systematic screening of medicinal plants for antioxidant activity cannot be overemphasized.

2.3 MEDICINAL VALUE OF PLANTS

Plants have been used for treating various diseases of human beings and animals since time immemorial. They maintain the health and vitality of individuals, and also cure diseases, including cancer without causing toxicity. Plant molecules, their semi-synthetic derivatives are important sources of antitumor drugs (Mesquita *et al.*, 2009).

Several Indian medicinal plants are known for their beneficial therapeutic effects which also might have antioxidant properties (Valko *et al.*, 2007). Herbal drugs are rapidly becoming popular in recent years as an alternative therapy. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity. Plants contain a wide variety of free radical scavenging molecules, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids (including carotenoids) and some other endogenous metabolites, which are rich in antioxidant activity (Lana and Tijskens, 2006). Many pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis and quinine (WHO, 2008). Pravin (2006) reported that about 70 per cent of the human population is dependent (wholly or partially) on plant-based medicines.

Researchers have estimated that every serving increase in fruit and vegetable consumption reduces the risk of cancer. This has been confirmed by epidemiological studies(Gupta and Prakash, 2008). Luo *et al.* (2004) reported that antioxidants from water extractsof *Lycium barburum* fruits and polysaccharides may play a synergistic role in their hypolipidemic effect. Gupta *et al.* (2005) have reported that several green leafy vegetables are rich in antioxidant vitamins. Brahmi (*Centella asiatica*), curry leaf (*Murraya koenigii*), fenugreek (*Trigonella foenum graecum*) and keerae (*Amaranthus sp.*) are used in Indian culinary and are also known for their medicinal value.

Many herbal plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxy nitrite. Natural antioxidants present in food of plant origin protect against these radicals and are therefore important tools in obtaining and preserving good health. Several natural products such as vitamin C, vitamin E, _-carotene and curcumin have been found to show excellent antioxidant activity (Sharma and Gupta, 2008).

Plants have provided a source of inspiration of novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is two-fold namely: they provide key chemical structure for the development of new antimicrobial drugs and also as a phytomedicine to be used for the treatment of disease (Abukakar *et al.*, 2008).

2.4 PHYTOCHEMICAL SCREENING

There is ample literature on preliminary phytochemical surveys and the knowledge of the chemical constituents of plant is desirable to understand herbal drugs and their preparations. Most importantly, these studies will be helpful to isolate and characterize the chemical constituents present in those plant extracts. In addition, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies (Venkata *et al.*, 2010).

Phytochemical evaluation is one of the tools for quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques. Use of chromatography for standardization of plant products was introduced by the WHO and is accepted as a strategy for identification and evaluation of the quality of plant medicines (Sazada *et al.*, 2009). Plant extact is the best source of phytochemicals. (Suriyavathana et al., 2010).

2.4 ANTIOXIDANTS

Antioxidants are substances that can delay or restrict oxidative cellular oxidazable substrates (Lin *et al.*, 2010). Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

The intake of natural antioxidants has been associated with the reduced risks of cancer, cardiovascular diseases, diabetes and other diseases associated with ageing. In recent years oxidative stress has been implicated in a variety of degenerative processes, diseases and syndromes. Many studies evidenced that plants containing high content of antioxidant phytochemicals can provide protection against various such diseases (Amudha and Shanthi, 2011).

Antioxidant compounds play an important role in various fields such as medical field (to treat cancers, cardiovascular disorders, and chronic inflammations), cosmetics (anti-ageing process), food industries (food preservative) and others. Over the years, the search for new antioxidant compounds from natural products has mounted. This is due to health concerns regarding the potential toxic and side effects generated from synthetic antioxidants, as well as changes in consumer preferences for natural products. Many commercialized synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are used under strict regulation in certain countries because of their potential health hazards.
(Nurul *et al.*, 2011).

2.5.1 ENZYMIC ANTIOXIDANTS

2.5.1.1 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 turnover 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 concentration 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).

2.5.1.2 PEROXIDASE

Peroxidases belong to the important enzymes removing active oxygen species in plants. Plant peroxidases have been studied for important role in lignifications, active participation in the formation of diphenyl bridges, cross-linking of hydroxyl proline rich proteins in the cell wall matrix and control function of redox state in apoplast(Luhova *et al.*, 2006).

In the presence of peroxide, peroxidases from plant tissues are able to oxidize a wide range of phenolic compounds such as pyrogallol, chlorogenic acid, catechin and catechol (Onsa *et al.*, 2004). Peroxidase activity is correlated with wide range of physiological processes, including somatic embryogenesis and auxin metabolism (Zhang *et al.*, 2008).

2.5.1.3 SUPEROXIDE DISMUTASE

Superoxide dismutase is ubiquitous metalloenzyme that detoxify the highly reactive superoxide anions to hydrogen peroxide and molecular oxygen. Superoxide dismutase exists in several isoforms, which differ in the nature of active metal centre, aminoacid composition, co-factors and other features. There are three forms of superoxide dismutase present in humans: cytosolic Cu, Zn- SOD, mitochondrial Mn-SOD and extracellular-SOD (Kang *et al.*, 2008).

Superoxide dismutase plays an important role in the protection of all aerobic life systems against oxygen toxicity and the free radicals derived from oxygen. As an enzyme, SOD has particular value as an antioxidant that can help to protect against cell destruction. It has the clear ability to neutralize superoxide, one of the most damaging free radical substances in nature (Kim *et al.*, 2008).

2.5.1.4 GLUTATHIONE-S-TRANSFERASE

Glutathione-S-transferase (GST) catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. Their reactive metabolites formed via the cytochrome P450 monooxygenase system (Sinha *et al.*, 2007).

GST is known primarily as a detoxification enzyme. It catalyzes the conjugation of reduced glutathione with reactive electrophilic and hydrophobic molecules, making these compounds inactive and immobile. In plants, major role of GST is the detoxification of herbicidal compounds (Lim *et al.*, 2005).

2.5.1.5 GLUTATHIONE PEROXIDASE

Glutathione is an enzyme that reduces glutathione disulfide to the sulfhydryl form of reduced glutathione which is an important cellular antioxidant. Glutathione reductase plays an important role in protecting haemoglobin and biological cell membranes against oxidative damage by increasing the level of reduced glutathione in the process of aerobic glycolysis (Rajathi *et al.*, 2011)

2.5.2 NON-ENZYMIC ANTIOXIDANTS

Plants possess a complex battery of enzymic and non-enzymic antioxidants that protect cells from oxidative damage by scavenging ROS. Non-enzymic antioxidant includes vitamins such as vitamin A, vitamin C and vitamin E found in the everyday diet. These vitamins directly detoxify free radicals (Yim *et al.*, 2007).

Non-enzymic antioxidants include vitamin E and C, thiol antioxidants , melatonin, carotenoids, natural flavonoids and other compounds. Some antioxidants can interact with other antioxidants regenerating their original properties and this mechanism is often referred to as the "antioxidant network". Vitamin C, vitamin E and carotenoids are excellent antioxidants that protect cells from damage by oxidants. (Wang *et al.*, 2007).

2.5.2.1 α-TOCOPHEROL

α -Tocopherol interact with the polyunsaturated acyl groups of lipids, stabilize membranes, scavenge and quench various ROS and lipid soluble by-products of oxidative stress (Zhao *et al.*, 2008). Oxidative stress activates the expression of gene responsible for the synthesis of tocopherols in plants (Zong *et al.*, 2008).

This is a fat soluble vitamin existing in eight different forms. In humans, _-tocopherol is the most active form, and is the major powerful membrane bound antioxidant employed by the cell. The main function of vitamin E is to protect against lipid peroxidation and there is also evidence to suggest that _-tocopherol and ascorbic acid function together in a cyclic process (Ormeno *et al.*, 2007).

2.5.2.2 ASCORBIC ACID

Ascorbate is one of the most extensively studied antioxidants and has been detected in the majority of plant cell types, organelles and apoplast (Jaleel *et al.*, 2008). This is an important and powerful water-soluble antioxidant and thus works in aqueous environments of the body. Its primary antioxidant partners are vitamin E and carotenoids. Vitamin C cooperates with Vitamin E to regenerate _-tocopherol radicals in membranes and lipoproteins.

Ascorbate also raises intracellular glutathione levels thus playing an important role in protein thiol group protection against oxidation (Naziroglu and Butterworth, 2005).

2.5.2.3 REDUCED GLUTATHIONE

Glutathione (GSH) is a multifunctional intracellular antioxidant and is considered to be the major thiol-disulphide redox buffer of the cell. It is abundant in cytosol, nuclei and mitochondria and is the major soluble antioxidant in these cell compartments. Oxidized glutathione (GSSG) is accumulated inside the cells and the ratio of GSH and GSSG is a good measure of oxidative stress of an organism (Masella *et al.*, 2005).

2.5.2.4 POLYPHENOLS

Polyphenols are common secondary metabolites, which are abundant in terrestrialplants such as fruits, vegetables and medicinal plants and marine plants such as seaweeds.

Those substances have received the greatest attention and have been investigated extensively since they are high free radical scavengers and less side effects than synthetic antioxidants such as BHA and BHT (Loots *et al.*, 2008; Jung *et al.*, 2008). Plant phenolic compounds have been found to possess potent antioxidant, antimicrobial and anticancer activities (Adesegun *et al.*, 2009).

2.5.2.5 FLAVONOIDS

Flavonoids are a group of natural compounds widely found in the plant kingdom. Biological and pharmacological activities of these compounds have been reported. Flavonoids may inhibit many enzymes, including thyroid peroxidase (TPO) and 5'-deiodinase, key enzymes for thyroid hormone synthesis and metabolism (Ferreira *et al.*, 2006).

Flavonoids and other phenolic compounds (hydroxyl, cinnamic derivatives and catechins) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation (Sundararajan *et al.*, 2006). They appear to possess a variety of biological activities, including antioxidant, anti-inflammatory and vasodilatory actions (Lin *et al.*, 2008).

2.6 INFLAMMATORY RESPONSE

Inflammation is a severe response by living tissue to any kind of injury. Inflammation is either acute or chronic. The development of variety of human cancers such as oesophagal, stomach, colon, bladder, and prostate, in many cases it can be attributed to chronic inflammation.(Hofseth and ying, 2006). Novel anti-inflammatory agents can be effective in the treatment of these type of cancers. Anti-inflammatory drugs can reduce the pain and inflammation of joints. (Afsar et al., 2012).

However, all the currently available anti-inflammatory drugs have few vital shortcomings, there is a need for the new safe, potent, non toxic or less toxic anti-inflammatory drugs from natural sources. (Sunitha verma, 2016)

2.7 MEDICINAL PROPERTIES OF *CISSUS QUADRANGULARIS*

Cissus quadrangularis (Linn) has been used by common man in India for promotion of fracture healing and well known as "Hadjod". (Mishra et al.,2010). It is a common perennial climber, which is distributed throughout India particularly in tropical regions.

CLASSIFICATION:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Vitales
Family : Vitaceae
Genus : Cissus
Species : quadrangularis

STEM AND LEAVES OF *CISSUS QUADRANGULARIS*

It has been prescribed in Ayurveda as an alternative, anthelmintic, dyspeptic, digestive, tonic, analgesic in eye and ear diseases, and in the treatment of irregular menstruation and asthma. In some parts of world, the whole plant is used in oral re-hydration, while the leaf, stem, and root extracts of this plant are important in the management of various ailments.(Jainu M., et al.,2004).

In current scenario formulations now contain extracts of Cissus quadrangularis in combination with other active ingredients, used for the purpose of management of overweight and obesity, as well as complications resulting from these conditions, specifically metabolic syndrome.(Oben J et al., 2006).

The stout, fleshy quadrangular stem is traditionally used for the treatment of gastritis, bone fractures, skin infections, constipations, eye diseases, piles, anemia, asthma, irregular menstruation, burns and wounds. The leaves and young shoots are powerful alteratives. Powder is administered in treatment of hemorrhoids and certain bowl infections. The juice of stem is useful in scurvy and in irregular menstruation whereas the stem paste boiled in lime water is given in asthma. *Cissus quadrangularis Linn.* has potent fracture healing property and antimicrobial, antiulcer, antioxidative, antiosteoporotic, gastroprotective, cholinergic activity as well as beneficial effects on cardiovascular diseases.(Devi CS., *et al*.,2003).

Stem paste of the plant may also be useful for muscular pains, burns, wounds, bites of poisonous insects and for saddle sores of horses and camels. The powder of dry shoots is given in digestive troubles. A decoction of the shoots with dry ginger and black pepper is given for body pains. The infusion of the plant is anthelmintic. Young shoots are used in dyspepsia and indigestion. The powdered stem is mixed with pulses and fried in sesame oil, used as a remedy for several vata diseases. It is also used in skin diseases Leprosy, Cough, Epilepsy and Convulsions. (Mallika J, et.al., 2005).

Cissus quadrangularis constitutes one of the ingredients of an Ayurvedic preparation, 'Laksha Gogglu', which has been proved to be highly effective in relieving pain, reduction of swelling and promoting the process of healing of the simple fractures as well as in curing the allied disorders associated with fractures. It acts by preventing the conversion of arachidonic acid to inflammatory prostaglandins. (Mehta M, et al., 2001).

3.0 EXPERIMENTAL PROCEDURE

The methodology adopted for the present study entitled " ***In vitro* Antioxidant and Anti-inflammatory activity of *Cissus quadrangularis*"** is discussed under the following headings.

3.1 Collection of the plant material

3.2 Preparation of the extract

3.3 Phytochemical analysis

3.4 Determination of the levels of antioxidants

  3.4.1 Enzymatic antioxidants

  3.4.2 Nonenzymatic antioxidants

3.5 Assessment of free radical scavenging activity

3.6 Invitro Anti-inflammatory activity.

3.1 COLLECTION OF THE PLANT MATERIAL

The fresh plant of *Cissus quadrangularis*was collected from Coimbatore district, Tamilnadu. The taxonomic identification of the plants was confirmed by Tamilnadu Agricultural University, Coimbatore. Fresh plant stems were cleaned to remove dust and debris. Then the stems were spread evenly and shade dried for 10 to 14 days. The dried stems were then coarsely powdered and they were used for the experiments.

3.2 PREPARATION OF THE EXTRACT

5g of the powdered plant sample was extracted with ethanol and kept in the shaker at room temperature for 48 hours. The extract obtained with solvent was subjected to evaporation at 60°C to give a gummy solid residue. This crude extract was stored and used for the experiments.

3.3 PHYTOCHEMICAL ANALYSIS

The ethanolic stem extract of *Cissus quadrangularis* was screened for the presence of phytochemicals such as carbohydrates, glycosides, proteins, alkaloids, flavanoids, steroids, terpenes, tannins, saponins and phenols. The details of the procedure is given in Appendix – I.

3.4 DETERMINATION OF THE ANTIOXIDANTS

3.4.1 ENZYMATIC ANTIOXIDANTS

The stem extracts of *Cissus quadrangularis* were analysed for the enzymatic antioxidants such as catalase, peroxidase, superoxide dismutase, glutathione-s -transferase and glutathione peroxidase.

3.4.1.1 ESTIMATION OF CATALASE

Catalase activity was estimated by the method of Luck *et al.*, (1974), and the detailed procedure is given in Appendix II.

3.4.1.2 ESTIMATION OF PEROXIDASE

Peroxidase activity was estimated by the method of Reddy *et al.*, (1995), and the detailed procedure is given in Appendix III.

3.4.1.3 ESTIMATION OF SUPEROXIDE DISMUTASE

Superoxide dismutase activity was estimated by the method of Misra and fridovich *et al.*, (1972), and the detailed procedure is given in Appendix IV.

3.4.1.4 ESTIMATION OF GLUTATHIONE PEROXIDASE

Glutathione peroxidase activity was estimated by the method of Rotruct *et al.,* (1973), and the detailed procedure is given in Appendix V.

3.4.1.5 ESTIMATION OF GLUTATHIONE S – TRANSFERASE

Glutathione s – transferase activity was estimated by the method of Habig *et al.,* (1974), and the detailed procedure is given in Appendix VI.

3.4.2 NON ENZYMATIC ANTIOXIDANTS

The stem extracts of *Cissus quadrangularis* were analysed for the Non enzymatic antioxidants such as Ascorbic acid, α-Tocopherol, polyphenol, Flavonoid and Reduced glutathione.

3.4.2.1 ESTIMATION OF ASCORBIC ACID

Ascorbic acid was estimated by the method of Roe and Kuether (1953), and the detailed procedure is given in Appendix VII.

3.4.2.2 ESTIMATION OF α-TOCOPHEROL

α-Tocopherol was estimated by the method of Rosenburg (1992), and the detailed procedure is given in Appendix VIII.

3.4.2.3 ESTIMATION OF POLYPHENOL

Polyphenol content in the stem extracts of *Cissus quadrangularis* was estimated by the method of Malick and Singh (1980) and the detailed procedure is given in Appendix IX.

3.4.2.4 ESTIMATION OF REDUCED GLUTATHIONE

Reduced glutathione content was estimated by the method of Moron *et al.,* (1979), and the detailed procedure is given in Appendix X.

3.4.2.5 ESTIMATION OFFLAVONOID

Flavonoid content was estimated by the method of Cameroon *et al.,* (1943), and the detailed procedure is given in Appendix XI.

3.5 ASSESSMENT OF FREE RADICAL SCAVENGING ACTIVITY

The free radical scavenging capacities of ethanolic stem extract of *Cissus quadrangularis* was evaluated by the following assays.

3.5.1 DETERMINATION OF 1,1-DIPHENYL-2-PICRYL-HYDRAZYL (DPPH) SCAVENGING ACTIVITY

DPPH scavenging activity was performed by the method of Mensor *et al.,* (2001), and the procedure is explained in Appendix XII.

3.5.2 INHIBITION OF SUPEROXIDE RADICAL GENERATION

Inhibition of superoxide generation was measured by the method of Winterbourn *et al.,* (1969), and the procedure is explained in Appendix XIII.

3.5.3 DETERMINATION OF 2, 2-AZINOBIS-(3-ETHYL-BENZOTHIAZOLINE-6-SULFURIC ACID) (ABTS) RADICAL SCAVENGING ACTIVITY

ABTS radical scavenging activity was measured by the method of Shriwaikar *et al.,* (2006), and the procedure is explained in Appendix XIV.

3.5.4 DETERMINATION OF NITRIC OXIDE SCAVENGING ACTIVITY

Inhibition of nitric oxidegeneration was measured by the method of Green and Hill (1984), and the procedure is explained in Appendix XV.

3.5.5 DETERMINATION OF HYDROGEN PEROXIDE SCAVENGING ACTIVITY

Hydrogen peroxidescavenging activity was measured by the method of Ruch *et al.,*(1989), and the procedure is explained in Appendix XVI.

3.6 INVITRO ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory activity of *Cissus quadrangularis* was studied by using the following assays.

3.6.1 INHIBITION OF ALBUMIN DENATURATION

The inhibition of albumin denaturation assay was performed according to the method of Menon *et al.,*(2011). The detailed procedure is given in Appendix XVII.

3.6.2 PROTEINASE INHIBITORY ACTION

The test for antiproteinase action was performed according to the method of Oyedepo *et al.,*(1995). The detailed procedure is given in Appendix XVIII.

4.0 RESULTS AND DISCUSSION

The medicinal value of a plant lies in its phytochemical (bioactive) constituents which show various physiological effects on human body. Therefore, through phytochemical screening one could detect the various important compounds which may be used as the base of modern drugs for curing various diseases. Chemical compounds produced as a result of metabolic reaction during plant growth are known as phytochemicals or secondary metabolites. (Ezeonu *et al.*, 2016).

Medicinal plants have been used to treat human diseases for thousands of years because they have fast and diverse assortment of organic compounds that can produce a definite physiological action on the human body. Most important of such compounds are alkaloids, tannins, flavonoids, terpenoids, saponins and phenolics. Pharmacists are interested in these compounds because of their therapeutic performance and low toxicity (Akhtar *et al.*, 2015).

The crude extracts and pure natural components have been reported which have potent antioxidant potential. However there is still a need to find out more effective antioxidants having lesser side effects from natural sources (Siddique *et al.*, 2010).

Cissus quadrangularis exhibits excellent medicinal properties such as antimicrobial, antiulcer, antioxidative, antiosteoporotic and gastroprotective. Hence the present study entitled "**Invitro Antioxidant and Anti-inflammatory activity of ethanolic extract of *Cissus quadrangularis*"** was carried out and the results are presented and discussed as follows:

4.1 Preliminary phytochemical screening

4.2 Antioxidant assays in the stem sample of *Cissus quadrangularis*

4.2.1 ENZYMIC ANTIOXIDANTS
4.2.1.1 Catalase
4.2.1.2 Peroxidase
4.2.1.3 Superoxide dismutase
4.2.1.4 Glutathione-S-transferase
4.2.1.5 Glutathione peroxidase
4.2.2 NON-ENZYMIC ANTIOXIDANTS
4.2.2.1 Ascorbic acid
4.2.2.2 α-Tocopherol
s4.2.2.3 Flavonoids
4.2.2.4 Reduced Glutathione
4.2.2.5 Polyphenols

4.3 Free radical scavenging activity

4.3.1 DPPH Radical scavenging activity
4.3.2 ABTS Radical scavenging activity
4.3.3 Inhibition of Superoxide Radical generation
4.3.4 Nitric oxide scavenging activity
4.3.5 Hydrogen peroxide scavenging activity

4.4 *In vitro* Anti-inflammatory activity

4.4.1 Proteinase inhibitory assay
4.4.2 Bovine serum albumin denaturation assay

4.1 PRELIMINARY PHYTOCHEMICAL SCREENING

The qualitative phytochemical analysis of various extracts of the *Cissus quadrangularis* stemis presented in Table 1.

Table 1

Qualitative analysis of the phytochemical constituents of ***Cissus quadrangularis***stem

***Cissus quadrangularis* stem**			
Parameters	Ethanolic extract of fresh stem	Ethanolic extract of dried stem	Crude stem extract
Alkaloids	+	+	+
Phenols	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Glycosides	+	+	+
Terpenoids	-	+	-
Steroids	-	+	-
Saponins	+	+	+
Carbohydrates	+	+	+
Proteins	+	+	+

+ indicates presence, - indicates absence

From Table 1, it is evident that the *Cissus quadrangularis* stem showed the presence of various versatile constituents such as glycosides, alkaloids, flavonoids, tannins, saponins, phenols, carbohydrates and proteins in all the extracts studied. Among the various extracts analysed, the ethanolic extract of the dried stem sample indicated the presence of all the phytochemical components, whereas that of fresh and crude stem extract did not show the presence of steroids and terpenoids.

Netaji *et al.*, (2015) has also reported that the stem portion of *Cissus quadrangularis* revealed the presence of glycosides, alkaloids, flavonoids, tannins, saponins, phenols, carbohydrates and proteins.

In the present study, phytochemical analysis revealed the presence of alkaloids, flavonoids and other phytochemical components in all the three extracts of the stem, which might be responsible for their antioxidant ability and free radical scavenging activity.

4.2 ANTIOXIDANT ASSAYS IN THE STEM SAMPLE OF *Cissus quadrangularis*

Antioxidants can scavenge the active forms of oxygen involved in the initiation step of oxidation or can break the oxidative chain reaction by reacting with the fatty acid peroxy radicals to form stable antioxidant radicals, which are either too unreactive for further reactions or form non-radical products (Basha *et al.*, 2014).

4.2.1 ENZYMATIC ANTIOXIDANTS

The activity of the enzymatic antioxidants such as peroxidase, superoxide dismutase glutathione peroxidase, catalase and glutathione-S-transferase in the stem extracts of ***Cissus quadrangularis*** was determined and the results are presented in Table 2.

Table 2

Enzymatic antioxidant activity of *Cissus quadrangularis* stem extracts

S.no	**Enzymatic antioxidants**	**Ethanolic extract of fresh stem**	**Ethanolic extract of dried stem**	**Crude stem extract**
1	Catalase(U/g)	4.07±0.62	30.5±1.89	4.92±0.53
2	Peroxidase(U/g)	0.518±0.061	0.838±0.062	0.410±0.057
3	Superoxide dismutase(U/g)	58.37±1.32	92.37±1.70	11.69±1.38
4	Glutathione-S-transferase(U/g)	3.503±0.56	6.443±0.49	5.193±0.55
5	Glutathione peroxidase(U/g)	10.04±0.59	10.99±0.87	15.75±0.70

The values are Mean ± SD of triplicate

Units:

1. Catalase = Amount of enzyme required to decrease the absorbance by 0.5 units at 240nm
2. Peroxidase = change of absorbance/minute at 430nm
3. Superoxide dismutase = Amount of enzyme that causes 50% reduction in NBT
4. Glutathione-S-Transferase = µmoles of CDNB conjugated / minute oxidation
5. Glutathione peroxidase = µg GSH oxidised/minute/mg

4.2.1.1. CATALASE

Catalase is an important endogenous enzymatic antioxidant responsible for protection of the cell from oxidative damage induced by Reactive oxygen species.Catalase deficiency leads to oxidative stress by increasing lipid peroxidation.
(Sarawoot *et al.*, 2014).

From Table 2, it is evident that the catalase activity was found to be maximum in the ethanolic extract of dried stem sample(30.5±1.89 U/g).

Maximum catalase activity was recorded in the aqueous extract of *Acanthopanax senticosus* stem sample as reported by Sanghyun Lee *et al.*, (2003).

4.2.1.2. PEROXIDASE

Peroxidase is widely distributed in higher plants, which plays a major role in plant's resistance against diseases and insects (Yamasaki *et al.*, 2008). The main function of peroxidase is to reduce hydrogen peroxide ($H_2 O_2$) intowater ($H_2 O$) and oxygen (O_2). (Lin *et al.*, 2015)

From Table 2, it is clear that the ethanolic extract of dried stem sample had recorded the highest peroxidase activity (0.838±0.062U/g) when compared to the other extracts analysed.

Fatemeh *et al.*, (2016) have reported that the crude ethanolic extract of *Calamintha officinalis* has showed a significant peroxidase activity(28.84 U/100g).
Vitis vinifera which is also a member of *vitaceae* familylike that of
Cissus quadrangularis exhibited appreciable catalase and peroxides activities. (Gacche *et al.*, 2010).

4.2.1.3 SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD) is one of the most effective intracellular enzymatic antioxidants and it catalyses the conversion of superoxide anions to oxygen and hydrogen peroxide. (Rahman *et al.*, 2007).

From Table 2, the ethanolic extract of dried stem sample recorded a maximum superoxide dismutase activity (92.37±1.70 U/g) when compared with the other extracts.

4.2.1.4. GLUTATHIONE-S-TRANSFERASE

Gluthathione-S-transferases (GST) are enzymes that catalyse the reaction of glutathione with electrophiles of both endogenous and xenobiotic origins. The main biological roles of glutathione-S-transferases encompass detoxification and protection against oxidative stress. From Table 2, it is clear that the activity of Glutathione-S-transferase was found to be maximum in the ethanolic extract of dried stem. (6.443±0.49U/g).

4.2.1.5. GLUTATHIONE 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. (Fatiha *et al*., 2007)

Among the three extracts, the highest glutathione peroxidase activity (15.75±0.70 U/g) was exhibited by crude stem extract as indicated in Table 2.

Rani *et al.,*(2014) have observed higher activity of glutathione peroxidase in grapes (*Vitis vinifera)* when compared to that in orange and tomatoes.

Thus, the stem extracts of *Cissus quadrangularis* exhibits significant role of enzymic antioxidant, which in turn may reduce the risk of serious diseases caused by reactive oxygen species such as malignancy, cardiovascular diseases and severe neural diseases. Among the three extracts tested the ethanolic extract was found to be superior with highest enzymic antioxidant activity except Gluthathione peroxidase.

4.2.2. NON-ENZYMIC ANTIOXIDANT

Many plants contain substantial amounts of antioxidants such as ascorbic acid, tannins, α-tocopherol, carotenoids, and flavonoids that can be utilized to scavenge excess free radicals from the human body (Ranjbar *et al.,* 2009). The free radical scavenging potential of natural antioxidants varies among diseases and types of antioxidants (Janniti *et al.,* 2009).

Table 3 represents the levels of non-enzymic antioxidants in the stem extracts of *Cissus quadrangularis* .

Table 3

Non-enzymatic antioxidants of the stem extracts of *Cissus quadrangularis*

S.no	**Non-enzymatic antioxidants**	**Ethanolic extract of fresh stem**	**Ethanolic extract of dried stem**	**Crude stem extract**
1	Ascorbic acid (mg/g)	13.45±0.15	19.52±0.25	7.535±0.73
2	α–Tocopherol (µg/g)	0.816±0.67	1.254±2.50	0.632±0.81
3	Reduced glutathione (n mol/g)	69.29±0.68	84.18±1.03	133.61±0.94
4	Flavonoids (mg/g)	1.516±0.76	1.754±0.90	0.923±0.78
5	Polyphenols (mg/g)	79.29±0.98	94.18±1.93	63.61±0.97

The values are Mean ± SD of triplicate

4.2.2.1. ASCORBIC ACID

Several oxidase enzymes were reported to participate mainly in oxidation reduction reactions involving vitamin C. Vitamin C is the most important vitamin in fruits and vegetables. It is the major water soluble anti – oxidant within the body. Ascorbic acid is the principal biologically active form but L-dehydroascorbic acid, an oxidation product also shows significant biological activity. (Deekshika *et al.*, 2015)

From Table 3, it is evident that the ascorbic acid content of the ethanolic extract of dried stem sample (19.52±0.25mg/g) was maximum when compared to the other two extracts.

The ascorbic acid content of *Cissus quadrangularis* stem is comparable with that of *Vitis vinifera* (Green grape) which recorded a value of
(22.52 ± 2.43 mg/g). (Deekshika *et al.*, 2015)

4.2.2.2. α-TOCOPHEROL

Tocopherols are lipid-soluble antioxidant synthesized by all plants. They protect lipids and other membrane components by scavenging and quenching various ROS and lipid by-products of oxidative stress. (Racchi, 2013)

From Table 3, it is evident that the α-tocopherol content of the ethanolic extract of dried stem sample (1.254±2.50 µg/g) was maximum when compared to the other two extracts.

4.2.2.3. REDUCED GLUTATHIONE

Reduced glutathione is the most abundant thiol in mammalian tissues involved in the protection of cell against damage from electrophiles, freeradicals and ROS formed during xenobiotic metabolism. (Kanchana *et al.*, 2011)

Reduced glutathione content was found to be maximum in the crude stem extract (133.61±0.94 n mol/g) while the other two extracts recorded lesser value.

4.2.2.4. FLAVONOIDS

Flavonoids are a ubiquitous group of naturally occurring polyphenolic compounds characterized by the flavan nucleus and represent one of the most prevalent classes of compounds in fruits, vegetables and plant-derived beverages. (Dongmin *et al.*, 2009). Flavonoids have been shown to possess antimalignant and anti-inflammatory activity. (Anbu *et al.*, 2011).

From Table 2, flavonoids was found to be maximum in the ethanolic extract of dried stem. (1.754±0.90 mg/g).

Vitis vinifera, which also belongs to vitaceae family like that of *Cissus quadrangularis* has also recorded appreciable quantity of flavonoids.(Tripati *et al.*, 2017).

4.2.2.5. POLYPHENOLS

The antioxidant properties of polyphenols depend mainly upon factors such as metal reducing potential, chelating behaviour, pH and solubility characteristics.

Polyphenols was found to be maximum in the ethanolic extract of dried stem sample. (94.18±1.93 mg/g) while the other two extracts are comparatively lesser.

4.3 FREE RADICAL SCAVENGING ACTIVITY

Free radical scavenging assay is a quick, sensitive and widely used method to measure antioxidant activity of several compounds.

In the present study, stem extracts of *Cissus quadrangularis* were evaluated for their various radical scavenging activities namely DPPH, superoxide anion, ABTS, nitric oxide and hydrogen peroxide and the results are presented in Figures 1 to 5.

4.3.1 DPPH SCAVENGING ACTIVITY

Figure 1

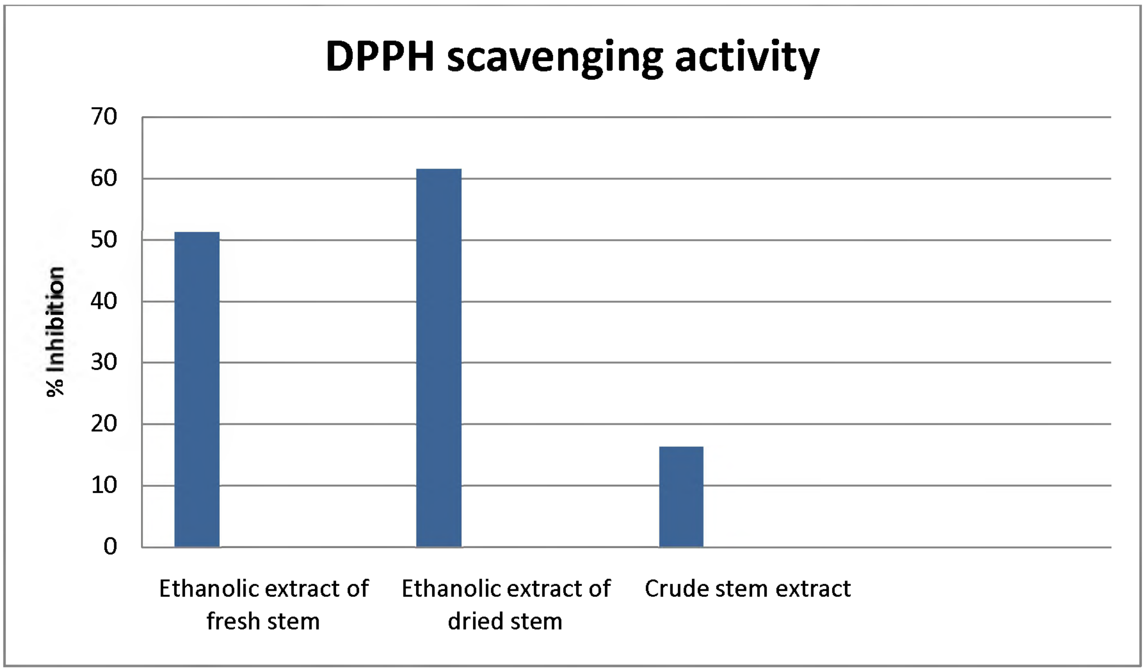


Figure 1 shows that the DPPH scavenging effect of the ethanolic extract of the dried stem was higher than the other two extracts of the stem.

Murthy *et al*., 2003 has also reported about 18.7% antioxidant activity at 100 ppm in the methanolic extract of dry stem of *Cissus quadrangularis*.

4.3.2 ABTS SCAVENGING ACTIVITY

Figure 2

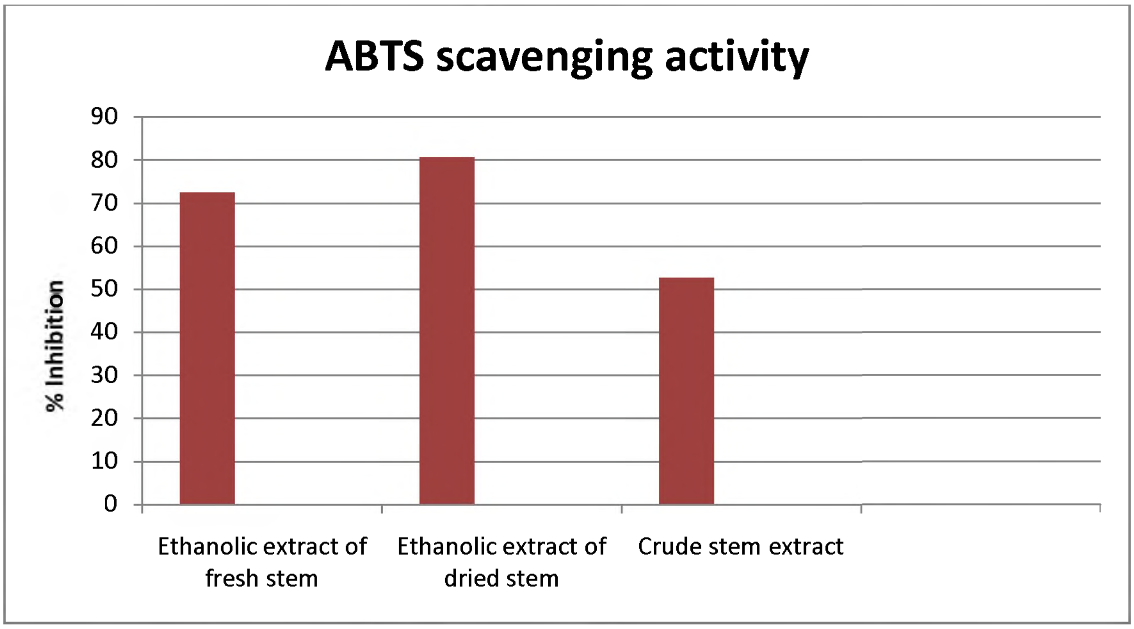
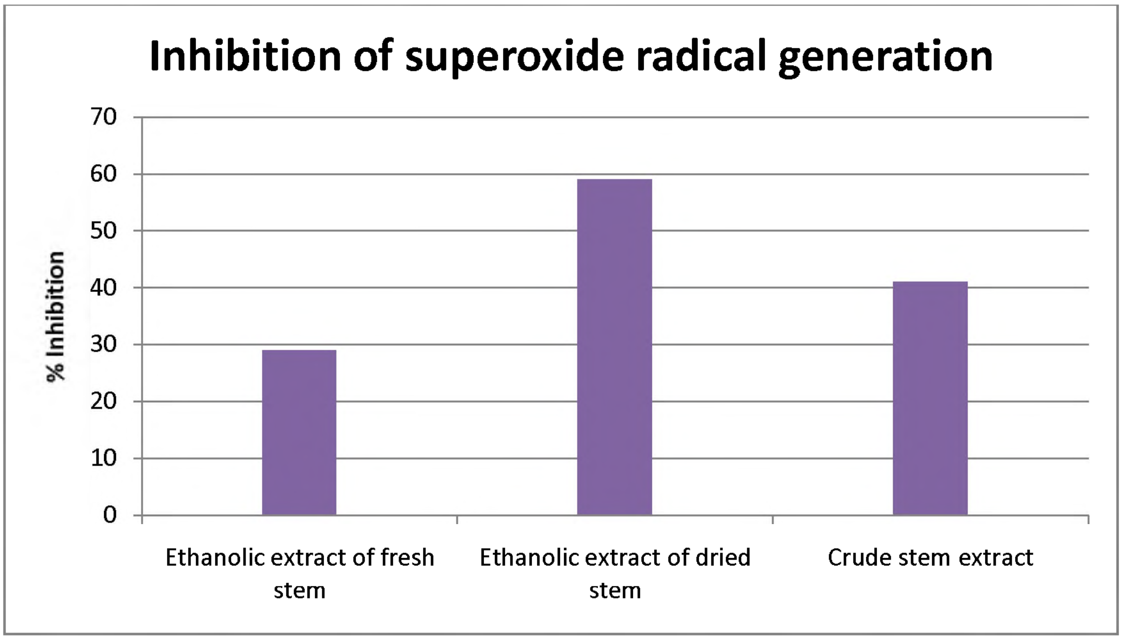


Figure 2 depicts that the ethanolic stem extract of dried stem sample showed the maximum percent of inhibition whereas both the fresh and crude extracts also showed good scavenging effect.

4.3.3 INHIBITION OF SUPEROXIDE RADICAL GENERATION

Figure 3

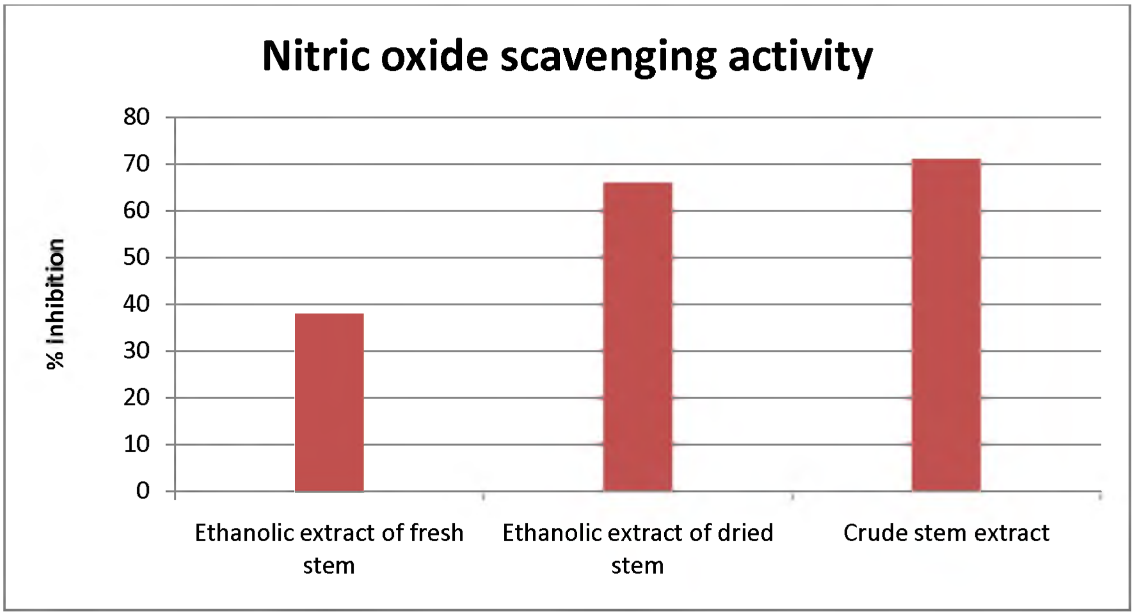


Among the three extracts of *Cissus quadrangularis,* the ethanolic extract of dried stem showed maximum percent of inhibition of superoxide radical generation and a minimum was recorded by the ethanolic extract of the fresh stem as depicted in Figure3

The ethanolic extracts of *Striga orobanchiodes* exhibited higher antioxidant and free radical scavenging activity as reported by Shrishailappa *et al.*,(2003).

4.3.4 NITRIC OXIDE SCAVENGING ACTIVITY

Figure 4

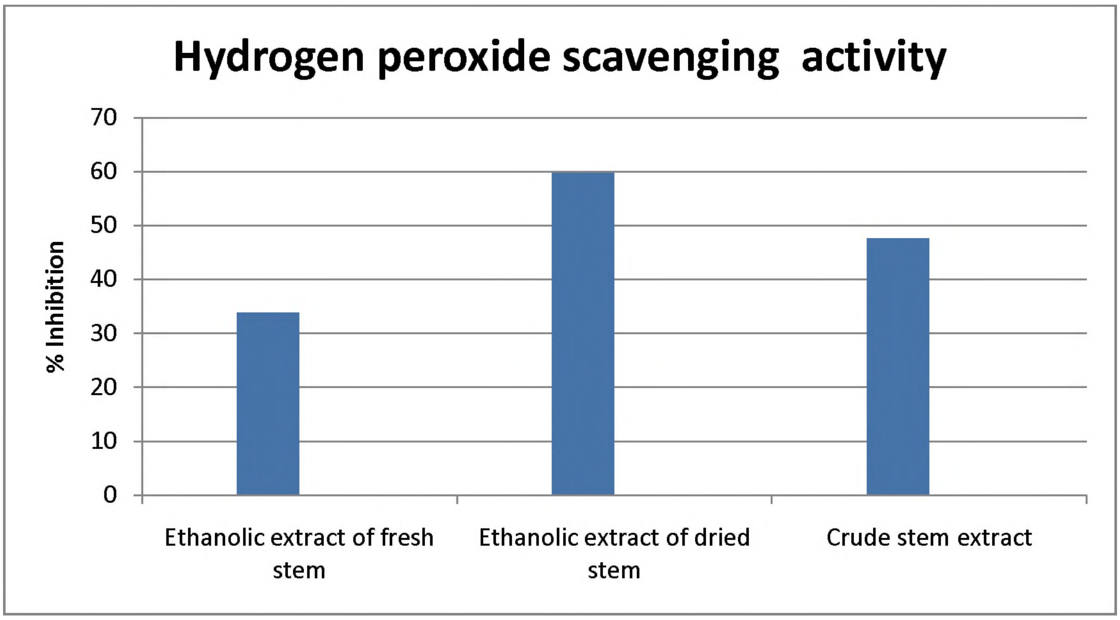


It is evident from figure 4, that the percent inhibition of nitric oxide radical generation of ethanolic extract of dried stem was found to be higher when compared to the other two extracts.

Ethanolic extracts of *Ocimum basilicum* showed significant nitric oxide radical scavenging activity. (Meera *et al.*, 2009).

4.3.5 HYDROGEN PEROXIDE SCAVENGING ACTIVITY

Figure 5



It is evident from figure 5 that the hydrogen peroxide scavenging activity of the ethanolic extract of dried stem was found to be higher than the crude and fresh stem extracts.

The hydrogen peroxide scavenging activity of ethanolic extract of *Cassia occidentalis* was 53% at 50 µg/ml. (Michael *et al.*,2013).

From the results obtained, it is clear that the ethanolic extract of dried stem exhibits maximum free radical scavenging effect, whereas that of fresh and crude stem extracts also registered appreciable levels of free radicals scavenging effect.

4.4 *IN VITRO* ANTI-INFLAMMATORY ACTIVITY

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is characterized by redness, swollen joints, joint pain, stiffness and loss of joint function. Currently it is treated by NSAIDS (Non steroidal anti inflammatory drugs) (Kumar *et al.*, 2013).

The *in vitro* anti inflammatory activity of *Cissus quadrangularis* was carried out by albumin denaturation assay and proteinase inhibitory assay and the results are presented in Table 4, 5 and Figure 6.

4.4.1 INHIBITION OF ALBUMIN DENATURATION

Table 4

Percent inhibition of albumin denaturation

S.no	**Aspirin standard (500 µg/ml)**	**Ethanolic extract of fresh stem**	**Ethanolic extract of dried stem**	**Crude stem extract**
1	97.02±0.55	60.06±1.5	73.0±3.05	53.0±2.0

Denaturation of protein is a well documented cause of inflammation. As part of the investigation on the anti-inflammatory activity, ability of plant extract to inhibit protein denaturation was studied.

From Table 4, it is clear that the stem extracts of *Cissus quadrangularis* are effective in inhibiting heat induced albumin denaturation. Maximum inhibition of 73% was observed in the ethanolic extract of dried stem followed by that of fresh stem and crude extracts. Aspirin, a standard anti-inflammatory drug showed 97.2% inhibition.

4.4.2 PROTEINASE INHIBITORY ASSAY

Table 5

Percent inhibition of proteinase activity

S.no	**Aspirin standard (500 µg/ml)**	**Ethanolic extract of fresh stem**	**Ethanolic extract of dried stem**	**Crude stem extract**
1	92.87±0.76	35±1.0	76.3±1.5	26±1.0

From Table 2, it was observed that that the ethanolic extract of dried stem showed the maximum percent of proteinase inhibition.

Figure 6

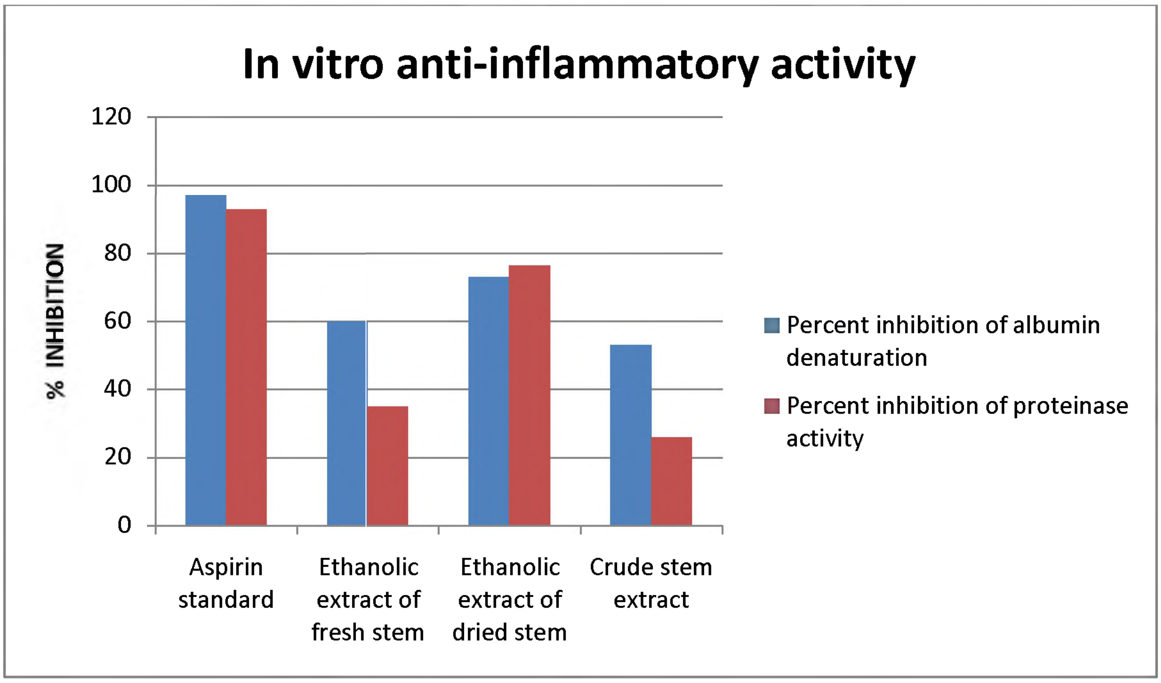


Figure 6 shows that the ethanolic extract of dried stem sample depicts significant effect of anti inflammatory activity than that of crude and fresh stem extracts.

The anti-inflammatory activity of *Cissus quadrangularis* may be due to the presence of phytochemicals, antioxidants and flavonoids in the sample.

5.0 SUMMARY AND CONCLUSION

There has been a resurgence of interest in natural products with a focus on plants as an alternative source of therapeutic compounds. Plants produce a vast array of secondary metabolites as defence against environmental stress or other factors like pest attacks, wounds or injuries. These secondary metabolites have found various therapeutic uses in medicine from time immemorial.

Oxidation process is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen centred free radicals and other reactive oxygen species (ROS) which are produced *in vivo* results in cell death and tissue damage. The most common ROS include superoxide anion, hydrogen peroxide, nitric oxide and hydroxyl radicals.

Antioxidants are believed to play a very important role in the body defense system against reactive oxygen species (ROS), which are the harmful by products generated during normal cell respiration.

In the present study the ethanolic extracts of fresh and dried stem samples and also the crude extract of the plant *Cissus quadrangularis* were analysed for their Phytochemical constituents, enzymatic and non enzymatic antioxidants, free radical scavenging effect and *in vitro*anti-inflammatory activity.

The findings of the present study are summarised as follows:

The qualitative Phytochemical screening revealed the presence of phytochemicals namely glycosides, alkaloids, phenols, saponins, carbohydrates and proteins in all the extracts of the stem.

The stem extracts were found to exhibit activities for all the enzymatic antioxidants studied in which the ethanolic extract of dried stem recorded higher activity than the fresh and crude stem extracts except for glutathione peroxidase which exhibited the maximum activity in the crude stem extract. The non enzymatic antioxidants such as ascorbic acid, tocopherol, flavonoids, polyphenols were also found to be higher in the ethanol extract of dried stem than in the other two extracts.

DPPH and ABTS radical scavenging effect of the ethanolic extract of the dried stem was higher than the other two extracts of the stem. The ethanolic extract of dried stem sample showed the maximum percent of inhibition whereas both the fresh and crude extracts also showed good scavenging effect.

Among the three stem extracts of *Cissus quadrangularis,* the dried stem extract showed maximum percent of inhibition of superoxide and nitric oxide radical generation and a minimum was recorded by the ethanolic extract of the fresh stem.

The hydrogen peroxide scavenging activity of the ethanolic extract of dried stem was found to be higher than the crude and fresh stem extracts.

The stem extracts of *Cissus quadrangularis* were found to be effective in inhibiting heat induced albumin denaturation. Maximum inhibition of 73% was observed in the ethanolic extract of dried stem followed by that of fresh stem and crude extracts.

It was also observed that the ethanolic extract of dried stem showed the maximum percent of proteinase inhibition indicating a significant anti inflammatory activity than that of crude and fresh stem extracts.

Hence from the present study it can be concluded that all the three stem extracts of *Cissus quadrangularis* namely ethanolic extracts of dried and fresh stem and the crude stem extract have many beneficial effects with respect to the presence of various phytoconstituents, enzymatic and non enzymatic antioxidants , free radical scavenging capacity and anti-inflammatory activity which are likely to combat many diseases and boost the immune system. However, the investigation reveals that the ethanolic extract of dried stem possessed maximum activity in all the parameters studied.

Therefore the results obtained support that the ethanolic extract of dried stem sample of *Cissus quadrangularis* is a potential source of antioxidant compounds and anti-inflammatory agents that may be utilized as a source of therapeutics.

Future studies:

- Isolation and identification of the active components responsible for the free radical scavenging and anti inflammatory activity may be carried out.

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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 PROTEINS:

To 2 ml of each extract, 1 ml of 40% sodium hydroxide and few drops of 1% copper sulphate were added; formation of 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 the side of the tubes. The mixture was then allowed to stand for 2 to 3 minutes. Formation of red or dull violet colour indicates the presence of carbohydrates in the sample extract.

APPENDIX II

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 III

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

APPENDIX IV

ESTIMATION OF SUPEROXIDE DISMUTASE

(Misra and Fridovich, 1972)

PRINCIPLE

The assay of SOD is based on the inhibition of formation of NADHphenazine methosulphate-nitroblue tetrazolium formazon, the extent of which can 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

Gmelina arborea leaves (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 30°C 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 V

ESTIMATION OD GLUTATHIONE PEROXIDASE ACTIVITY

(Rotruck *et al.*, 1977)

Principle

A known amount of enzyme preparation was used to react with hydrogen peroxide in the presence of GSH for a specified time pereiod when the screening was measured by the method of Ellman

$$2GSH + H_2O_2 \xrightarrow{Se - GPx} GSSH + 2H_2O$$

Reagents

1. 0.4M Tris Buffer
2. 10μM sodium azide
3. 10% TCA
4. 0.4μM EDTA
5. 19μM hydrogen peroxide

Procedure

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of plant extract were added following by 0.1ml hydrogen peroxide were added to the mixture, mixed well and incubated at 37°C for 10 minutes along with the tube containing all the reagent expect sample. After 10 min the reaction was arrested by the addition of 0.5ml of 10% TCA centrifuged and supernatant was assayed for glutathione by the method of Ellman

The activities are expressed as μg GSH consumed/min/mg protein

APPENDIX VI

ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

(Habig *et al.,* 1974)

PRINCIPLE

GST conjugates with GSH and CDNB and the extent of conjugation causes a proportionate change in the absorption at 340nm, which can be followed spectrophotometrically.

REAGENTS

1. 1mM – 1-chloro 2,4-dinitrobenzene (CDNB) in ethanol
2. 1mM - Glutathione
3. 0.1M Phosphate buffer
4. 50mM Tris –HCl buffer (pH 7.2)

PROCEDURE

ENZYME EXTRACT

The sample was homogenized with Tris–HCl buffer (pH7.2). The homogenated sample was centrifuged at 4°C for 30 minutes at 8500rpm.The supernatant was used as the enzyme source.

The estimation was done at 25°C under condition giving activities linear with respects to incubation time and protein concentration for at least 3 minutes.

The enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer. 0.1ml of both substrates GSH and CDNB was taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9ml. The reaction was started by the addition of 0.1ml of sample to this mixture; the readings were recorded against distilled water blank for a minimum of three minutes. The complete assay mixture without the sample served as the control to monitor non-specific binding of the substrate. It was taken to ensure that final concentration of ethanol in the mixture was always less than 4%.

APPENDIX VII

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 VIII

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 IX

ESTIMATION OF POLYPHENOLS

(Mallick and Shingh 1980)

PRINCIPLE

Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium to produce a blue coloured complex (molybdenum blue) which can be estimated spectrophotometrically at 650 nm.

REAGENTS

1. Ethanol (80%)
2. Folin-ciocalteau reagent (1N)
3. Sodium carbonate
4. Standard solution – 10 mg catechol in 100 ml of distilled water

PROCEDURE

The homogenate was prepared in 0.5 g of the plant sample in 10X volumes of 89% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes. The residue was re-extracted with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water. Different aliquots (0.2 to 2ml) were pipetted out into test tubes. The volume in each tube was made upto 3.0 ml with water. To all the tubes, 0.5 ml of Folin-ciocalteau reagent was added and mixed.

After 3 minutes, 2.0 ml 0f 20% Sodium carbonate solution was added to each tube. After mixing the tubes thoroughly, all the tubes were kept in a boiling water bath for exactly one minute, allowed to cool. The absorbance was measured at 650 nm against a reagent blank.

APPENDIX X

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 XI

ESTIMATION OF FLAVONOIDS

(Zhishen *et al.*, 1999)

PRINCIPLE

Flavonoids react with vanillin to produce a coloured product that can be measured spectrophotometrically at 360nm.

REAGENTS

1. Vanillin reagent (1% vanillin in 70% sulphuric acid)
2. Sulphuric acid (70%)
3. Methanol : water (2:1)
4. Methanol : water (1:1)
5. Hexane
6. Catechin standard (110µg/ 1ml)

PROCEDURE

PREPARATION OF PLANT EXTRACT

The leaves were weighed (0.5g) and divided equally into two parts. One part was extracted with methanol : water (2:1) and other part was extracted with methanol : water in the ratio of 1:1. These homogenates were allowed to stand over night. The next day, the two extracts were combined and evaporated to 1/3rd the original volume. The resultant aqueous extract was cleared of low polarity contaminants by extracting with hexane. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

ESTIMATION

An aliquot of the leaf extract was pipetted out and evaporated to dryness. Aliquots of the standard (0.2 to 1.0ml) were taken in test tubes and made up to 1.0ml with distilled water. Vanillin reagent (4ml) was added to all the test tubes and heated in a boiling water bath for 15 minutes. The absorbance was measured at 340nm after cooling. The values are expressed as mg flavonoids/g leaf.

APPENDIX XII

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;

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

APPENDIX XIII

INHIBITION OF SUPEROXIDE RADICAL GENERATION

(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,

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

APPENDIX XIV

ABTS RADICAL SCAVENGING EFFECTS

(Shirwaikar *et al.*, 2006)

PRINCIPLE

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation 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,

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

APPENDIX XV

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 25°C 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.

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

APPENDIX XVI

HYDROGEN PEROXIDE SCAVENGING ACTIVITY

(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 XVII

PROTEINASE INHIBITORY ACTION

Oyedopo *et al.,(*1995)

The reaction mixture (2 ml) was containing 0.06 mgtrypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1ml test sample of different concentrations (100 – 500µg/ml). The mixture was incubated at 37oC for 5 minand then 1 ml of 0.8% (w/v) casein was added. Themixture was incubated for an additional 20 min. 2 mlof 70% perchloric acid was added to arrest thereaction. Cloudy suspension was centrifuged and theabsorbance of the supernatant was read at 210 nmagainst buffer as blank. The experiment wasperformed in triplicate. The percentage inhibition ofproteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

APPENDIX XVIII

INHIBITION OF ALBUMIN DENATURATION

Menon *et al.*, (2011)

The reaction mixture consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 °C for 20 min and then heated to 51 ° C for 20 min, after cooling the samples the turbidity was measured at 660nm. The experiment was performed in triplicate.The Percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control

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Thesis submitted to
Avinashilingam Institute for Home Science and Higher Education for Women
Coimbatore- 641043.

In partial fulfillment of the requirement for the degree of
Master of Science in Biochemistry
April, 2018

10/4/18

Signature of the
Head of the Department

10/4/2018

Signature of the Supervisor