ASSESSMENT OF ANTIOXIDANT STATUS AND FREE RADICAL SCAVENGING ABILITY OF *Crescentia cujete*

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1.0 INTRODUCTION

Medicinal Plants used in the traditional medicine are well-known significant sources of natural antioxidants. Medicinal plants-derived natural antioxidants, which are in the form of raw extracts and/or chemical constituents, are very efficient to block the process of oxidation by neutralizing free radicals. It is also commonly accepted that medicines taken from plant products are safer than their synthetic counterparts; however, the toxicity profile of most medicinal plants have not been comprehensively assessed (Janpur *et al.,* 2015).

In recent years, phytochemicals in medicinal plants have received a great deal of attention mainly on their role in preventing diseases caused as a result of oxidative stress which releases reactive oxygen species such as singlet oxygen and various radicals as a damaging side-effect of aerobic metabolism. These radicals are possibly involved in a number of disorders including cardiovascular malfunctions, tissue injury, DNA damage and tumour promotion. Several studies suggest that antioxidants could prevent accumulation of these reactive oxygen species and beneficial for treatment of different pathological conditions (Balaji *et al.,* 2011).

Increased consumption of fruit and vegetables is associated with a lower risk of all causes of mortality, and particularly Oxidative stress related disorders and disease. Inflammation plays a key role in the initiation and progression of cardiovascular disease. Atherosclerosis is a disease in which plaque builds up inside arteries. ROS/ RNS molecules are highly reactive and can interact with lipids, proteins, nucleic acids, carbohydrates, and small molecule metabolites. These biomolecules promote oxidation, nitrosation, and nitration which in turn provoke inflammatory responses. Lower concentrations of antioxidants are present in patients with cardiovascular disease, whereas markers of oxidative stress in serum are elevated due to increased demand from excessive generation of ROS by activated macrophages. Low intake of

antioxidant-rich diets may further contribute to the risk and extent of the pathology. Thus, increased intake of dietary antioxidants represents one strategy to decrease disease-related oxidative stress (Chingsuwanrote *et al*., 2016).

An increasing interest in the measurement and use of plant antioxidants for scientific research as well as industrial (nutrition, pharmaceutical and cosmetic) purposes. This is mainly due to their strong biological activity, exceeding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis. Therefore, the need exits for safe, economic, powerful, and natural antioxidants to replace these synthetic ones. Many 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, peroxynitrite which results in oxidative stress leading to cellular damage. Epidemiological studies have indicated the relationship between the plant antioxidants and reduction of chronic diseases. Therefore, in recent years it is considered to be an important task in evaluating plant antioxidant activity and their free radical quenching ability (Gopalakrishnan *et al.,* 2012).

Antioxidant properties of natural compounds are very important because of their uses in medicine, food and cosmetics. In living systems, various metabolic processes and environmental stresses generate various reactive species. These are free radicals and mainly ROS. Increased level of ROS can damage structure of biomolecules and modify their functions and lead to cellular dysfunction and even cell death. The cumulative effect of increased ROS can increase oxidative stress in systemic level and it is manifested in the form of a variety of health problems such as cancer, age related disease and cardiovascular diseases. Cellular ROS are regulated by interplay of complex antioxidant machineries in living systems. Nature has bestowed living systems with numerous antioxidant molecules. These natural antioxidants are known to minimise the adverse effects of free radicals in living system (Choudary *et al.,* 2012).

It is possible to reduce the risk of chronic diseases and prevent disease progression by either enhancing the body‘s natural antioxidant defenses or by supplementing with dietary antioxidants. Antioxidants offer resistance against oxidative stress by scavenging the free radi-cals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent the disease progression. The most commonly used synthetic antioxidants at present are buty-lated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tert-butyl hydroquinone. However, they are suspected of being responsible for liver damage and acting as carcinogens in laboratory animals. The search for new products with antioxidative properties and fewer side effects is very active domain of research. Therefore, the development and utilization of more effective antioxidants of natural origin is desirable (Vadivukarasi, 2015).

Under normal conditions the body‘s antioxidants convert ROS to prevent the over production of free radicals. Studies have shown that vitamin C and E effectively scavenge free radicals and decrease lipid peroxidation (Kendler, 2011) . Flavonoids are substance of vegetable origin are found to act as effective antioxidants (Narang *et al.,* 2011). Recently, natural foods and food derived antioxidants such as vitamins and phenolic phytochemicals, have received growing attention, because they are known to function as chemo preventive agents against oxidative damage and are considered beneficial for human health (Kumar *et al*., 2010).

Plant materials remain an important resource to combat serious diseases in the world. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries. The medicinal value of these plants lies in some chemical active substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannin, flavonoid and phenolic compounds (Mohamad *et al.,*2010).

Plants are the source of energy for the animal kingdom and are commonly used in treating or preventing specific ailments or diseases and are considered to play a beneficial role in health care. ROS such as superoxide anion ($O^{2\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen (O^2) and hydrogen peroxide(H2O2) have the damaging effects on cells. Antioxidant compounds present in many plants can protect cells against this damaging caused by ROS. Cancer, cardiovascular diseases, diabetes, inflammation, degenerative diseases, anemia, ageing, and ischemia are common complicated illnesses in human directly or indirectly affected by ROS (Parvin *et al*., 2014)

Recent studies have shown that the vegetative parts of several members of the family Bignoniaceae contain a wide variety of chemical compounds (amino acids, phenolics and alkaloids) The gourd tree *Crescentia cujete* (Bignoniaceae) is a perennial species widely distributed in tropical lowlands of Mesoamerica, commonly growing in savannahs and semi-evergreen tropical forests. It is also found in home gardens since its fruits are used for preparing containers and bowls commonly known as ‗jícaras' (Casas *et al.,*2012).

The leaves of *Crescentia cujete* Linn. is the result of this selection which is based primarily on the fact that not much experimentation has been done for this plant species. The fruit of *Crescentia cujete* Linn. also has a reputation of being used as traditional medicine to cure many diseases and other health related issues in the Philippines. Its effects include laxative, diuretic, purgative, anti-inflammatory, hypertension and diarrhea (Laciapag *et al*., 2017*)*

According to folk medicine, the fruit pulp is used for respiratory problems such as asthma and also used as laxative. The bark is used for mucoid diarrhea. Bark decoction used to clean wounds and pounded leaves used as poultice for headaches. Internally, leaves used as diuretic and also used to treat hematomas and tumors. Fruit decoction used to treat diarrhoea, stomachaches, cold, bronchitis, cough, asthma, and urethritis. The leaves are also used for hypertension . The juice from fruits mixed with

sugar and/or bee's honey and eaten for problems of the respiratory system (asthma, catarrh), the digestive system (stomach pains, intestinal parasites) and the female reproductive apparatus (infertility). Naphtoquinones, iridoid glycosides, aucubin, plumieride, and asperuloside have been reported as the constituents of the leaves of this plant (Islam *et al.,* 2014).

Objectives

The main objective of the study are as follows

- To assess the antioxidant status of different solvent extracts from the leaves of *Crescentia cujete.*
- To determine the free radical scavenging activity of different extracts of *Crescentia cujete.*
- To identify the bioactive constituents of different solvent extracts of *Crescentia cujete* by phytochemical analysis.

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

REVIEW OF LITERATURE

Medicinal plants used in the traditional medicine are well-known significant sources of natural antioxidants. Medicinal plants-derived natural antioxidants, which are in the form of raw extracts and/or chemical constituents, are very efficient to block the process of oxidation by neutralizing free radicals. It is also commonly accepted that medicines taken from plant products are safer than their synthetic counterparts; however, the toxicity profile of most medicinal plants have not been comprehensively assessed(Rezaeian *et* al.,2015).

The review of literature pertaining to the current topic **Assessment of antioxidant status and radical scavenging activity of *Crescentia cujete"*** under the following headings.

Free radicals
Sources of free radicals
Free radical generation
Oxidative stress
Stress mediated effects
Diseases mediated by free radicals
Free radicals and aging
Alzheimer's disease
Atherosclerosis
Cancer
Antioxidants
Enzymatic antioxidants
Non enzymatic antioxidants
Plants as antioxidants
Phytochemicals

Free radicals

Medicinal plants have been used in many domains including medicine, nutrition, flavoring, beverages, dyeing, repellents, fragrances, cosmetics, smoking, and other industrial purposes. Since the prehistoric era, medicinal plants have been the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century. The preservative effect of many plant spices and herbs suggests the presence of anti-oxidative and anti-cancerous constituents in their tissues. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity. Many medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases (Hayyan *et al.,* 2016).

There is an inverse relationship between the antioxidative status and occurrence of human diseases. In addition, antioxidant compounds which are responsible for such therpeutic activity, could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders. Therefore, research to identify antioxidative compounds is an important issue. Although it remains unclear which of the compounds, of medical plants are the active ones, polyphenols recently have received increasing attention because of some interesting new findings regarding their biological activities. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such as free radical scavenging and inhibition of lipid peroxidation, are the most crucial aspects of metabolism. Even though a variety of herbs are known to be sources of phenolic compounds, studies isolating polyphenols and evaluating their antioxidative effects have rarely been carried out. In living systems

various metabolic processes and environmental stresses generate various reactive species. These are free radicals and mainly Reactive Oxygen Species . Increased level of ROS can damage structure of biomolecules and modify their functions and lead to cellular dysfunction and even cell death. The cumulative effect of increased ROS can increase oxidative stress. Systemic level and it is manifested in the form of a variety of health problems such as cancer, age related disease and cardio vascular diseases. Cellular ROS are regulated by interplay of complex antioxidant machineries in living systems.. These natural antioxidants are known to minimise the adverse effects of free radicals in living system. Many of these naturally occurring antioxidants are now isolated, fully characterised, and available for various applications. The chemical structures of these pure compounds are very diverse in nature. Thus, it is expected that the correlation between structure and antioxidant properties will be a difficult preposition. Many of these natural antioxidants are actively considered as prophylactic and therapeutic agents for possible applications for radiation counter measures combating cancers and aged related disease (Mishra *et al*., 2011).

Sources of free radicals

Free radicals can be formed from both endogenous and exogenous substances. They are continuously formed in cells and cellular environment during metabolism. Different sources of free radicals are as follows

- Exposure to UV radiations, X-rays, gamma rays and microwave radiation.
- Metal-catalyzed reactions.
- Oxygen free radicals in the atmosphere considered as pollutants.
- Inflammation initiates neutrophils and macrophages to produce ROS and RNS.
- Neutrophils stimulated by exposure to microbes.
- ROS formed from several sources like mitochondrial cytochrome oxidase, xanthine oxidase, neutrophils and lipid peroxidation.
- ROS generated by the metabolism of arachidonic acid, platelets, macrophages and smooth muscles.
- Interaction with chemicals, automobile exhaust fumes and smoking of cigarette.

- Burning of organic matter during cooking, forest fires and volcanic activities.

- Industrial effluents, excess chemicals, alcoholic intake, certain drugs, asbestos, certain pesticide and herbicide. Some metal ions, fungal toxins and xenobiotics.
- Free radicals can adversely affect various important classes of biological molecules such as nucleic acid, lipids and proteins, thereby altering the normal redox status leading to increased oxidative stress (Latha *et al.,* 2015).
- Sources of ROS production include xanthine oxidase, lipooxygenase, mitochondrial respiration, cytochrome P450, cyclooxygenase NAD(P)H oxidase. These sources contribute to ROS formation (Greabu *et al*., 2007: Greabu *et al*., 2008).

Free radical generation

Free radicals generally involved in chain reactions, a series of reactions that leads to regeneration of radical that can begin a new cycle of reactions. Free radical reactions occurs in three distinct identifiable steps:

- Initiation step: formation of radicals
- Propagation test: In this step required free radical is generated repeatedly as a result of chain reaction, which would take the reaction to completion.
- Termination step: Destruction of radicals (Sen *et al.,* 2010).

Oxidative stress

Oxidative stress is a harmful condition that occurs when there is an excess of ROS and decrease in antioxidant levels, this may cause tissue damage by physical, chemical and physiological factors that lead to tissue injury in human and causes different diseases (Titan *et al.,* 2007). A disturbance in the balance between pro-oxidants and antioxidants in favor of the former, leading to oxidative damage gives rise to an increase in oxidative stress. Oxidative stress is the rate at which oxidative damage is generated. Implicating that oxidative stress is a continuous variable that is unlikely to

ever be exactly zero since pro-oxidants are continually produced and some oxidative damage is always generated (Almeida *et al.*, 2016)

Oxidative stress is imposed on cells as a result of one of three factors:

- An increase in oxidant generation and decrease in antioxidant level.
- A failure to repair oxidative damage (Saha and Tamrakar, 2011).

Stress mediated effects

Oxidative stress leads to

- DNA damage
- GSH depletion
- Direct damage to protein and rise in intracellular free Ca^{2+} that leads to cytoskeleton damage.
- Rise in intracellular free iron membrane peroxidation and destruction which leads to injury to adjacent cells
- Increased lipid peroxidation (Saha and Tamrakar, 2011)

Persistent oxidative stress may give rise to pathological conditions and is increasingly implicated as a contributing factor to several human pathologies (over 150 disorders), cellular senescence and aging (Furness and Speakman, 2008). Recent studies show that levels of pro – oxidant and antioxidants may also have relevant ecological and evolutionary roles may help understand functional interactions among life history traits (Constantini, 2008: Monaghan *et al.,* 2009).

Diseases mediated by free radicals

Oxygen derived free radical reactions have implicated in the pathogenesis of many human diseases including

- Neurodegenerative disorders like Alzhiemer‘s disease, Parkinson‘s disease, multiple sclerosis, amylotrophic lateral sclerosis, memory loss and depression.

- Cardiovascular disease like atherosclerosis, ischemic heart, cardiac hyperstrophy, hypertension, shock and trauma.
- Pulmonary disorders like inflammatory lung disease such as asthma and chronic obstructive pulmonary diseases.
- Diseases associated with premature infants, including bronchopulmonary, dysplasia, periventricular leukomalacia, intraventricular hemorrhage, retinopathy of prematurity and necrotizing enterocolitis.
- Autoimmune disease like Rheumatoid arthritis.
- Renal disorders like glomerulonephritis and tubulointerstitial nephritis, chronic renal failure, proteinuria and uremia.
- Gastrointestinal diseases like peptic ulcer, inflammatory bowel disease and colitis.
- Tumors and cancers like lung cancer, leukemia, breast cancer, rectum cancer (Valko *et al.,* 2007; Pham – Huy *et al., 2008;* Sen *et al.,* 2009).

Free radicals and ageing

The various pathological conditions have been implicated in human beings due to ROS and oxidative stress, involving cardiovascular disease, cancer, neurological disorders, diabetes and ageing . The process of ageing may be defined as a progressive decline in the physiological functions of an organisms after the reproductive phase of life. The major mechanism of ageing attributes to DNA or the accumulation of cellular and functional damage (Narang *et al*., 2011).

Alzhiemer's disease

Alzhiemer's disease (AD) is the most prevalent dementia subtype and accounts for about 60% of all cases (Vanmarum, 2008). The earliest symptoms of AD appear as subtle and intermittent deficits in the ability to remember minor events of everyday life. At later stages, AD gradually progresses to severe dementia, which affects multiple cognitive and functional behavioral functions. Fortunately, basic research has identified many of the pathways that contribute to this devasting disease, providing opportunities for the development of new treatments. Among the potential new treatment options are

certain naturally occurring phytochemicals (Singh *et al*., 2008). The initial source of oxidative stress in AD is unclear. Recent research reveals that dietary antioxidants may have promising therapeutic potential in delaying the onset and preventing the aging population with AD and its related complications (Narang *et al*., 2011).

Atherosclerosis

According to the theory of oxidative stress, atherosclerosis is the result of the oxidative modification of low density lipoproteins (LDL) in the arterial wall by reactive oxygen species. Evidence suggests that common risk factors for atherosclerosis increase the risk of the production of free the smooth muscle cells and the advential cells. Thus hypercholestrerolemia, diabetes mellitus (DM), arterial hypertension, smoking, age and nitrate intolerance increase the production of free ROS (Vogiatzi *et al.,* 2009).

Cancer

Cancer is the second leading cause of death worldwide. Although great advancements have been made in the treatment and control of cancer progression, significant deficiencies and room for improvement remain. Strong and consistent epidemiology evidence indicates a diet with high consumption of antioxidant rich fruits and vegetables significantly reduces the risk of many cancers, suggesting that certain dietary antioxidants could be effective agents for the prevention of cancer incidence and mortality. These agents present in the diet are a very promising group of compounds because of their safety, low toxicity and general acceptance (Fresco *et al.,* 2008).

Cancers are often described by the body part that they originated in. However, some body parts contain multiple types of tissue, so for greater precision, cancers are additionally classified by the type of cell that the tumor cells originated from. These types include:

- Carcinoma: Cancers derived from epithelial cells. This group includes many of the most common cancers, particularly in older adults. Nearly all cancers developing in the breast, prostate, lung, pancreas, and colon are carcinomas.

- Sarcoma: Cancers arising from connective tissue (i.e. bone, cartilage, fat, nerve), each of which develop from cells originating in mesenchymal cells outside the bone marrow.
- Lymphoma and leukemia: These two classes of cancer arise from cells that make blood. Leukemia is the most common type of cancer in children accounting for about 30%. However, far more adults develop lymphoma and leukemia.
- Germ cell tumor: Cancers derived from pluripotent cells, most often presenting in the testicles or the ovary (seminoma and dysgerminoma, respectively).
- Blastoma: Cancers derived from immature "precursor" cells or embryonic tissue. Blastomas are more common in children than in older adults (*Varricchio* and *Claudette, 2004).*

ANTIOXIDANTS

Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant constituents of the plant material act as radical scavengers, and helps in converting the radicals to less reactive species. A variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables and tea, etc. This review presents some information about the antioxidant/antiradicals and their role in our body and also their presence in spices and herbs (Adeulo *et al.,* 2008).

2.4.1 Enzymatic antioxidants

The antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase (SOD) metabolize oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for optimal catalytic activity (Sen *et al.,* 2010).

Catalase

Catalase is an enzyme found in nearly all living organism exposed to oxygen (such as bacteria, plants and animals). It catalyses the decomposition of hydrogen

peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by ROS. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can covert millions of hygrogen peroxide molecules to water and oxygen (Ellis *et al.,*2007)

Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Hydrogen peroxide is also damaging and is degraded by other enzymes such as catalase. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. One exception is *Lactobacillus plantarum* and related lactobacilli, which use a different mechanism to prevent damage from reactive (O_2^-) (Nashef *et al*., 2016).

Peroxidase

Peroxidase are a large family of enzymes that typically catalyze a reaction of the form: For many of these enzymes the opyimal substrate is hydrogen peroxide, but others are more active with organic hyperoxides such as lipid peroxides (Karthikeyan *et al.,* 2005)

Glutathione – S – transferase

Glutathione *S*-transferases (GSTs), previously known as ligandins, comprise a family of eukaryotic and prokaryotic phase II metabolic isozymes best known for their ability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. The GST family consists of three superfamilies: the cytosolic, mitochondrial, and microsomal — also known as MAPEG— proteins Members of the GST superfamily are extremely diverse in amino acid sequence, and a large fraction of the sequences deposited in public databases are of unknown function. The Enzyme Function Initiative (EFI) is using GSTs as a model superfamily to identify new GST functions (Akkinson and Babbit,, 2009).

Glutathione peroxidase

Glutathione peroxidase (GPx) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Mugesh, 2010).

2.5.2 Non -enzymatic antioxidant

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

Ascorbic acid

Ascorbate (VitaminC) is a most abundantpowerful and water soluble antioxidant, which occur in all plant tissues (high in photosynthetic cells and meristems). It provides protection by scavenging O2•– and OH free radicals (Chiu-ping *et al.,* 2016)

Reduced Glutathione

Glutathione (GSH) is an important antioxidant in plants, animals, fungi, and some bacteria and archaea. Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals. It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine, and the carboxyl group of cysteine is attached by normal peptide linkage to a glycine (Wu *et al.,* 2017).

α-Tocopherol

Lipid soluble antioxidant and are localized in thylakoid membrane of chlor oplast.Out of four isomers (α,β,γ,δ), α tocopherol has the highest antioxidative ac tivity and helps in the scavenging of O2 radical. the mono-methylated form ddd-gamma-tocopherol is the most prevalent form of vitamin E in oils, there is evidence that

rats can methylate this form to the preferred alpha-tocopherol, since several generations of rats retained alpha-tocopherol tissue levels, even when fed only gamma-tocopherol through their lives. (Guan *et al.,* 2018).

2.5. Plants as antioxidants

Only recently there has been an exponential growth in the field of herbal medicine and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less side effects. Ethnic people and tribals of India are endowed with a deep knowledge concerning the utilization of medicinal plants to cure various diseases.

Herbal and natural products of folk medicine are practiced for centuries in almost all cultures worldwide. However, in most of the world countries, especially those in the African continent, Asia and South and Central America, the majority of the population (roughly 80%) still relies to a great extent on herbal medicine for their primary healthcare. Moreover, in developing countries and rural societies,the use of medicinal plants is both a valuable resource and a necessity, and furthermore a real alternative for prevention of diseases. Despite the great progress in commercial drugs, the increasing confidence in alternative therapies (especially herbal therapies), even in western countries, stems from the fact that some of these remedies have so far proven to be very effective in countries like France and Germany. It is suggested that many conventional drugs prescribed worldwide are exclusively of plant based. Examples include salicylic acid, which upon acetylation produces aspirin, which is isolated from the bark of *Salix caroliniana*, anticancer drug Taxol isolated from *Taxus brevifolia*, the pain killer morphine and the anti-cough codeine from *Papaver somniferum* etc (Panda *et al.,*2016).

The *Biogonaceae* is one of the largest family of plant kingdom that consists of 250 genera and 1149 species are recognized as valid. This tree possesses stupendous biological activities such as expectorant, antitussive, laxative, ulcers, wound healing, anti-viral, cancer and stomach disorders. Traditionally, the leaves, fruits of *C*. *cujete*

practiced as a medicine in ancient times in Indian and Vietnamese folk medicine (Prabukumar *et al*., 2015)

Crescentia cujete, popularly known as the gourd tree or calabash tree, is a species of plant that is native to Central and South America. The fruit is the most striking feature, which is a stiff gourd, woody shell and a jelly-like pulp that are incorporated various seeds. Folk medicine cites the use of the fruit pulp to treat respiratory problems (asthma, for example) and also as laxative. The decoction of barks is commonly used for diarrhea and to clear wounds. The powdered leaves are used as a poultice for headaches and internally as a diuretic and in the treatment of hematomas and tumors. Fruit decoction can be used to treat bronchitis, diarrhea, stomachaches, urethritis, cold, cough and asthma. The leaves and barks have shown remarkable anti-inflammatory and antibacterial activities, as well as therapeutic potential on disease processes caused by destabilization of biological membranes. Naphtoquinones, iridoids and iridoid glycosides, aucubin, plumieride and asperuloside have already been reported as the constituents of the leaves of this species. In general, the chemical content of the plant comprises citric acid, crescentic acid, tartaric acids, tannins, β-sitosterol, stigmasterol, α- and β-amyrin, stearic acid, triacontanol, palmitic acid, flavonoids (quercetin, apigenin), 3-hydroxyoctanol glycosides and *p*-hydroxybenzoyloxy glucose (Almeida *et al*., 2016).

2.6 Phytochemicals

Several hundred scientific studies focused on the activity non – nutritional compounds present in the diet. This heterogenous class of molecules generally known as phytochemical includes vitamins (carotenoids) and food polyphenols such as flavonaoid, phytoalexins, phenolic acids, indoles and sulphur rich compounds (Chandra *et al.,*2016).

The present study aims to compare the free radical scavenging activity of different solvent extracts of *Crescentia cujete* leaves and to qualitatively determine the

phytochemical constituents in the leaves. The methodology adopted for the present study is discussed in the next chapter.

PLATE 1. *Crescentia cujete*

METHODOLOGY

Herbs are widely exploited in the traditional medicine and their curative potentials are well documented. Plants have been a rich source of medicines because they produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection. The use of medicinal plants is increasing worldwide (Mirunalini,K,K,S. 2012). Majapahit (*Crescentia cujete*) plant is one of such plants, whose leaves, barks, fruits, or roots have medicinal and preservative properties. Various parts of the plant have been used as a purgative, diuretic, analgesic, or anti-inflammatory drug possibly because they contain alkaloids, flavonoids, saponins, phenolics tannins, steroids, vitamins, carbohydrates, organic acids, amino acids and mineral 2,3,4,5 Specific bioactive compounds in the plant include β-sitosterol, estigmastrol, α- and β-amirina, apigenins, and carotenoids (Rahmaningsih *et al.,* 2017). The methodology adopted for the present study is discussed under the following headings.

Collection of the plant materials

Preparation of the extracts

Phytochemical analysis

3.3.1 Qualitative Analysis

Antioxidant activity

Assay of enzymatic antioxidant activity

Assay of non – enzymatic antioxidant activity

Free radical scavenging activity

DPPH scavenging activity

ABTS scavenging activity

H_2O_2 Scavenging activity

Hydroxyl radical scavenging activity

Inhibition of free radical generation

Measurement of inhibition of superoxide generation

Measurement of inhibition of Nitric oxide generation

COLLECTION OF PLANT MATERIALS

The *Crescentia cujete* leaf sample was collected in Coimbatore. The collected fresh sample was dried, ground, powdered and then stored in plastic bottles and used for the experiments of the present study.

PREPARATION OF THE EXTRACT

About 5g dried powdered plant material was extracted with solvents in Soxhlet extractor. The solvents used were petroleum ether, chloroform, hydroethanol and aqeous. 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.

QUALITATIVE ANALYSIS

The qualitative analysis is depicted in Appendix XIII

TABLE I

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

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 *Crescentia cujete* was arrested by determining enzymatic and non – enzymatic antioxidant status.

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 samples was 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 (Luck., 1974. The detailed procedure is explained in Appendix III.

PEROXIDASE

The peroxidase activity was estimated according to the procedure of the Raddy *et al,* 1995. The detailed procedure is depicted in Appendix IV

Non – enzymatic antioxidant activity

Non enzymatic antioxidant activity of *Crescentia cujete* leaf samples was determined by α – tocoperol, ascorbic acid and reduced glutathione methods.

α – TOCOPHEROL

Non – enzymatic activity of α – tocoperol of leaf sample is proposed by the Engel *et al.,* 1938. The full protocol was elloborately explained in Appendix V

ASCORBIC ACID

Ascorbic acid activity of leaf sample was determined by the Kuether *et al.,* 1943. This procedure was described in Appendix VI

REDUCED GLUTATHIONE

Reduced glutathione activity of *Crescentia cujete* leaf sample was done by Moron *et al.,* 1979 method. This method was explained in Appendix VII

FREE RADICAL SCAVENGING ACTIVITY

Free radicals scavenging activity on *Crescentia cujete* leaf sample was determined by four different methods such as DPPH, ABTS, hydroxyl radical, hydrogen peroxide scavenging activity.

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

ABTS RADICAL CATION 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 given by Appendix IX

HYDROGEN PEROXIDE SCAVENGING ACTIVITY

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

HYDROXYL SCAVENGING ACTIVITY

The hydroxyl scavenging activity was currently done by Elizabath *et al.,* 1990. The procedure was elloborately given in Appendix XI

Inhibition of free radical generation

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

NITRIC OXIDE GENERATION

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

SUPEROXIDE GENERATION

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

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

4.0 RESULTS AND DISCUSSION

Free radicals, which are generated in several biochemical reactions in the body, have been implicated as mediators of many diseases, including cancer, atherosclerosis and many diseases. Many of the ROS mediated responses actually protect the cells against oxidative stress and re-establish –redox homeostasis‖. On the other hand, over production of ROS has the potential to cause damage. In the recent decades, ROS has become a focus of research 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, ischemia or reperfusion injury, neurodegenerative disorders, hypertension and ageing (Gutowski and Kowalczyk, 2013). A great number of plants have been reported with medicinal value and are the rich sources of antioxidants. Antioxidants prevent or delay the oxidation of the substrate and their importance in the safeguarding of health, and the protection from several diseases including cancer, has been recently established, thus constituting them as functional food preservatives. Thus, it becomes imperative to study the phytoconstituents, antioxidant potential and radical quenching ability of the different solvent extracts of *Crescentia cujete* leaves.

Phytochemical Analysis of *Crescentia cujete*

Preliminary phytochemical screening was performed in the present study using the leaves of *Crescentia cujete.* Leaf extracts were prepared using different solvents in the order of increasing polarity includes petroleum ether, chloroform, hydroethanol and water. The desirable characteristics of edible plants and their use in the field of pharmaceutical preparations might be due to the presence of a variety of phytoconstituents such as carbohydrate, phenol, glycosides, flavonoids, alkaloids, terpenoids, tanins, steroids, volatile oils and saponins. The preliminary screening tests are useful in the detection of these bioactive constituents. The result of phytochemical screening carried out in the present study is depicted in Table I.

The results of various parameters such as analysis of phytoconstituents, determination of antioxidants including both enzymatic and non-enzymatic antioxidants and free radical scavenging ability of the different solvent extracts done are presented and discussed below.

Phytochemical Analysis of the leaf extracts of *Crescentia cujete*

Table 1

Secondary metabolites	Petroleum ether extract	Chloroform extract	Hydroethanol Extract	Aqueous Extract
Alkaloids	++	++	++	+
Flavonoids	++	++	-	-
Phytosterols	++	++	+	-
Reducing sugars	+	++	++	+
Saponins	+	+	-	-
Tannins	+	++	-	++
Terpenoids	++	-	-	++

(-) absence, (+) slight appearance, (++) moderate appearance

Table 1 shows that the different solvent extracts analysed for the presence of secondary metabolites indicate that all the extracts tested exhibits all the phytochemicals analysed namely alkaloids, flavonoids, phytosterols, reducing sugars, saponins, tannins, and terpenoids in slight and moderate appearances.

The leaf extracts showed varying results in terms of intensity of the secondary metabolites that are present. Although it is difficult to establish definitive relationship between structure and biological activity due to the occurrence of a vast number of phytochemicals with similar chemical structures, and to the complexity of physiological reactions, many papers describe some of the definitive functions of the phytochemicals present in plants. The presence of tannins indicate that the sample is a good potential

source of components having anthelmintic and antioxidant properties. The presence of phytosterols showed that the sample is a good cholesterol-lowering agent. The presence of alkaloids, saponins, flavonoids, and terpenoids showed that the *Crescentia* has antimicrobial as well as antioxidant effects. The presence of reducing sugars also has a hypoglycemic effect for the reason that the aldehyde, in the form of glucose in this case is oxidized into carbonyl group (Laciapag *et al*., 2017).

The earlier studies on preliminary analysis of MF-MeOH was positive for the presence of anthracene derivatives, flavonoids and tannins, lignans, mono and diterpenes, triterpenes and steroids, whereas for the presence of alkaloids, coumarins and naphtoquinones, the fraction was negative. This data shows the significant presence of phenolic compounds, such as anthracene derivatives, lignans and mainly flavonoids and tannins. Phenolics can be defined as aromatic hydroxylated compounds, having one or more aromatic rings with one or more hydroxyl groups, presenting a large structural diversity which is divided into different subclasses, such as flavonoids, phenolic acids, including hydroxybenzoic acids and hydroxycinnamic acids, tannins, oxidized polyphenols, stilbenes and lignans. Besides the protective actions in biological systems, like anti-inflammatory and antitumor properties, these phenolic compounds exhibit antioxidant activity and can be classified as free radical inhibitors, peroxide decomposers, metal inactivators or oxygen scavengers (Almeida *et al*., 2016).

The literature cited above added credit to the current findings on leaf extract of *Crescentia cujete* which carries a good source of pharmaceutically valuable components like alkaloids, flavonoids, phytosterols, terpenoids and tannins in the qualitative screening performed. Thus the *Crescentia cujete* leaf extract can very well be considered for the pharmaceutical preparations and can be validated for the use in the treatment of various diseases and disorders that are related with reactive species.

Antioxidant Activity of *Crescentia cujete*

An antioxidant can be defined as: —any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate‖. The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition (Young and Woodside, 2001).

Table 2

Antioxidant Potential of the Leaves of *Crescentia cujete*

S.No.	ENZYMATIC ANTIOXIDANTS	ACTIVITY	NON -ENZYMATIC ANTIOXIDANT	LEVELS
1	Catalase (U*/g)	0.42±0.02	Ascorbic acid (mg/g)	0.64±0.03
2	Peroxidase (U#/g)	0.63±0.03	Reduced glutathione (nmoles/g)	0.11±0.03
3	Superoxide dismutase (U@/g)	0.31±0.03	α-Tocopherol (µg/g)	0.81±0.03

The values are mean ± S.D. of triplicate

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

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

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

The activity of enzymatic antioxidants such as catalase, peroxidase and superoxide dismutase estimated indicate that the leaves of *Crescentia cujete* exhibited considerable activity of all the enzymes tested proving their use in traditional medicine as these enzymatic antioxidants are involved in metabolic processes of living system.

The study of enzymic antioxidants in the leaves of *Calotropis procera, Datura stramonium, Argemone mexicana, Withania somnifera* and *Solanum nigrum* indicated that among these medicinal plant leaves; the activities of enzymic antioxidants v*iz.* Catalase and glutathione reductase activities were highest in the leaves of *Withania somnifera* (Bind *et al.,* 2014).

The levels of non- enzymatic antioxidants such as ascorbic acid, reduced glutathione and α- Tocopherol exhibited in the leaves of *Crescentia cujete* is found to be 0.64±0.03, 0.11±0.03 and 0.81±0.03 indicating its nutrient antioxidant status.

The leaves of *Moringa oleifera* and *Centella asiatica* are good sources of non enzymatic antioxidants. The radical scavenging activity of different extracts may be due to the contribution of the phytonutrients rendering antioxidant property (Mrudula *et al*., 2014)

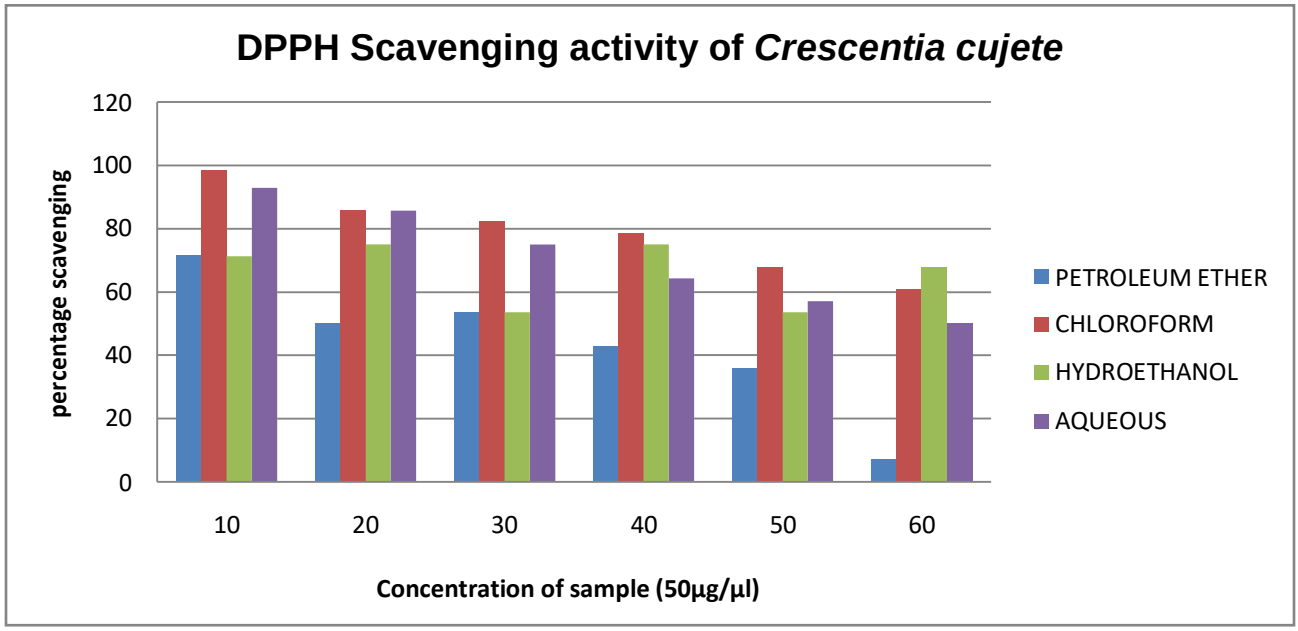
Thus, it can be inferred from the results of the antioxidant assessment that the leaves of *Crescentia cujete* can very well be used in the treatment of diseases associated with oxidants as the different solvent extracts were found to be a good source of both enzymatic and non-enzymatic antioxidants possessing nutritional as well as metabolic significance in elimination reactive species.

Free radical Scavenging Activity

The most phenolic compounds are the major components of plant 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.

DPPH Radical Scavenging Activity of the leaves of *Crescentia cujete*

Figure 1



From the Figure 1, it is evident that among the various extracts analysed, chloroform extracts shows maximum scavenging activity at a concentration followed by petroleum ether, hydroethanol and aqueous extract. The order of activity of DPPH of the leaves of *Crescentia cujete*was Chloroform> Petroleum ether>Hydroethanol> Aqueous extract.

DPPH radical scavenging assay is a widely used method to evaluate the free radical scavenging ability of natural compounds. This assay is based on the measurement of the scavenging ability of antioxidant substances toward the stable radical (Pavithra and Vadivukkarasi, 2015). DPPH assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds. Though this radical has limited similarities with peroxyl radicals, this assay is commonly used to measure the antioxidant content of wheat grain and bran, vegetables, conjugated linoleic acids, herbs, edible seed oils, and flours in different solvent systems including ethanol, aqueous acetone, methanol, aqueous alcohol, and benzene (Mishra *et al.,* 2012).

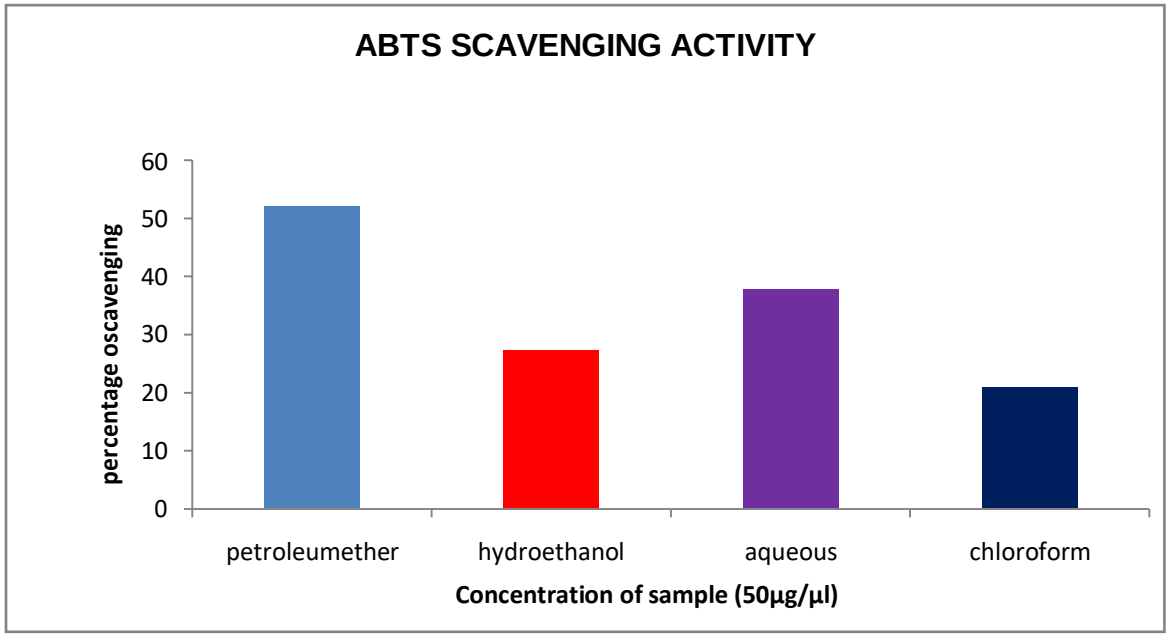
DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of the plant extracts. Scavenging of DPPH• radical is related to the inhibition of lipid peroxidation. DPPH• is usually used as a substance to evaluate the antioxidant activity. Antioxidants either transfer an electron or a hydrogen atom to DPPH•, thus neutralizing its free radical character. The DPPH• assay has been largely used as a quick, reliable and reproducible parameter to search the *in vitro* general antioxidant activity of pure compounds as well as plant extracts. The reducing capacity of compounds could serve as indicator of potential antioxidant property (Jamuna *et al.,* 2012).

The present study showed that all the extracts tested were found to be effective against DPPH free radicals, except aqueous extract.

Das *et al*., 2014 reported that the ethyl acetate extracts showed better radical scavenging activity rather than aqueous extracts followed by crude extracts of *C.cujete*.

Figure 2

ABTS Scavenging Activity of the Leaves of *Crescentia cujete*



The Figure 2 results showed that the ABTS scavenging activity were found to be maximum in petroleum ether extract followed by aqueous>hydroethanol>chloroform.

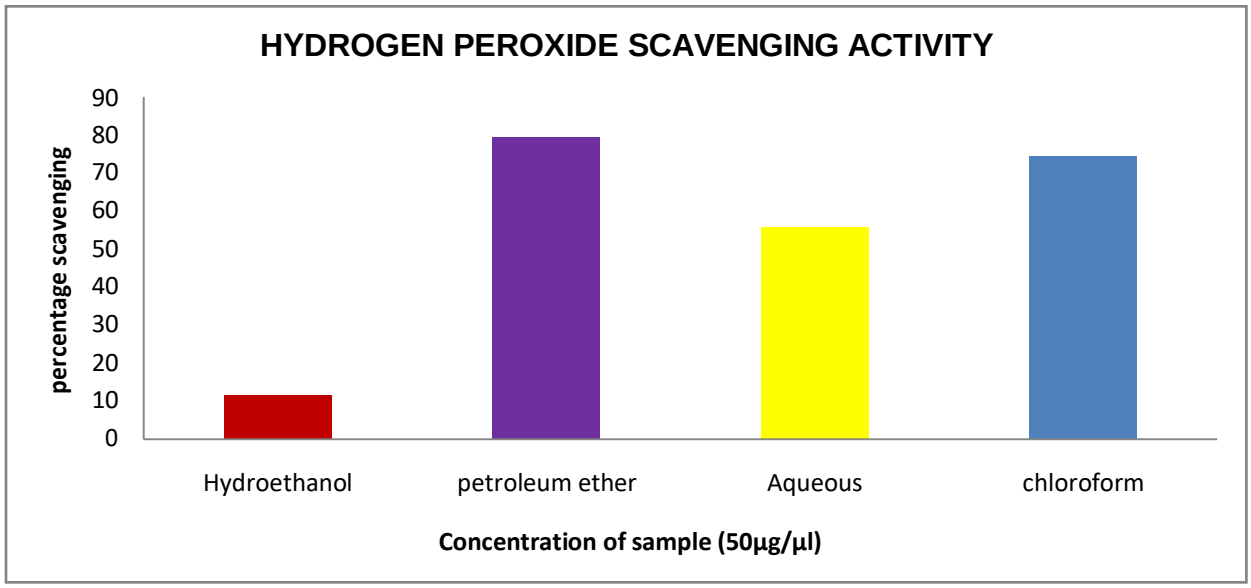
Several methods have been developed to assay free radical scavenging capacity and total antioxidant activity of plant extracts. The most common and reliable method involves the determination of the disappearance of free radicals using a spectrophotometer, such as 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic) acid radical (ABTS). Reduction of ABTS causes a loss of absorbance at 734 nm (Cai *et al.,* 2004).

ABTS can be oxidized by potassium per sulphate or manganese dioxide, giving rise to the ABTS cation radical (ABTS•+) whose absorbance diminution at 743 nm was monitored in the presence of Trolox, chosen as standard antioxidant (Pisoschi *et al.,* 2011).

ABTS radical cation decolourisation assay, showed quite similar results compared to those obtained in DPPH reaction. The extracts of *G. macrorrhizum* and *P. fruticosa* were the most active: they have completely scavenged ABTS+. In the ABTS assay, the activity of *S. officinalis* extracts was slightly lower. The extracts of *S. glutinosa*, *R. carthamoides* and *S*. *sclarea* also possessed strong scavenging activity (Miliauskas *et* al., 2004). Grape seeds extract also showed a considerable antioxidant activity in terms of ABTS radical (Babbar*et al.,* 2011).

Figure 3

Hydrogen peroxide Scavenging Activity of the Leaves of *Crescentia cujete*



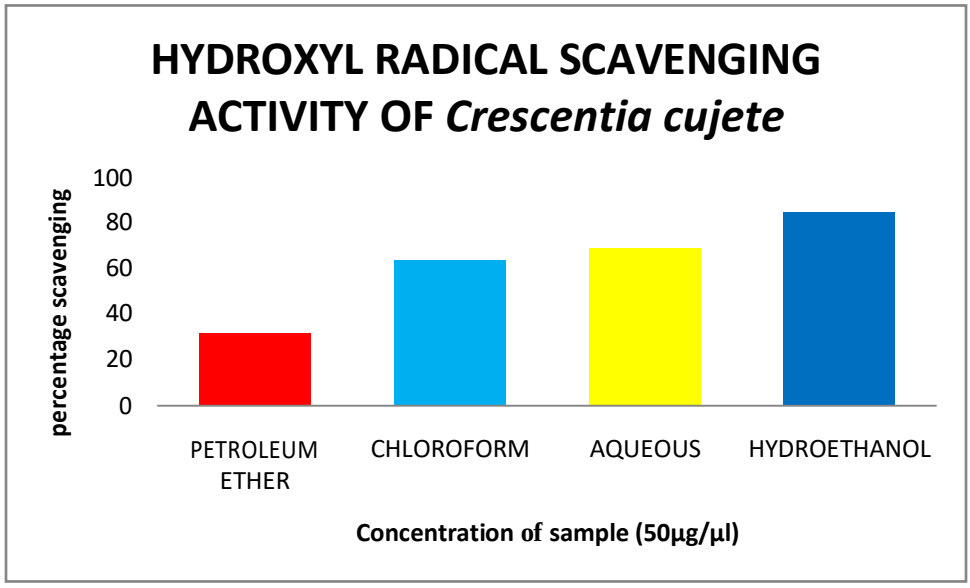
From the Figure 3, the percentage of hydrogen peroxide scavenging activity were found to be higher in the petroleum ether extract when compared to other extracts tested in the present study.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe2+, and possibly Cu2+ to form hydroxyl radical and this may be the origin of many of its toxic effects (Nishaa *et al.,* 2012)

M.arundinacea extract showed a maximum activity of 69.49 % inhibition very much comparable to that of BHT with an activity of 71% at the same concentration of 500µg/ml. The 30 mg/ml of Monodesmosides and crude extract of *Leontices mirnowii* tubers exhibited 85% and 79% scavenging activity on hydrogenperoxide, respectively, as reported by Nishaa *et al.,* 2012.

HYDROXYL RADICAL SCAVENGING ACTIVITY OF *Crescentia cujete*

Figure 4



From the Figure 4, the results revealed that hydroethanol have higher scavenging activity when compared with other extracts.

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage. The deoxyribose method is a simple assay to determine the rate constants for reactions of hydroxyl radicals (Baris*et al.,* 2011).

The methanolic and acetone extracts were shown better hydroxyl free radical scavenging activity than other solvent extracts, as reported by Chanda *et al.,* (2011).

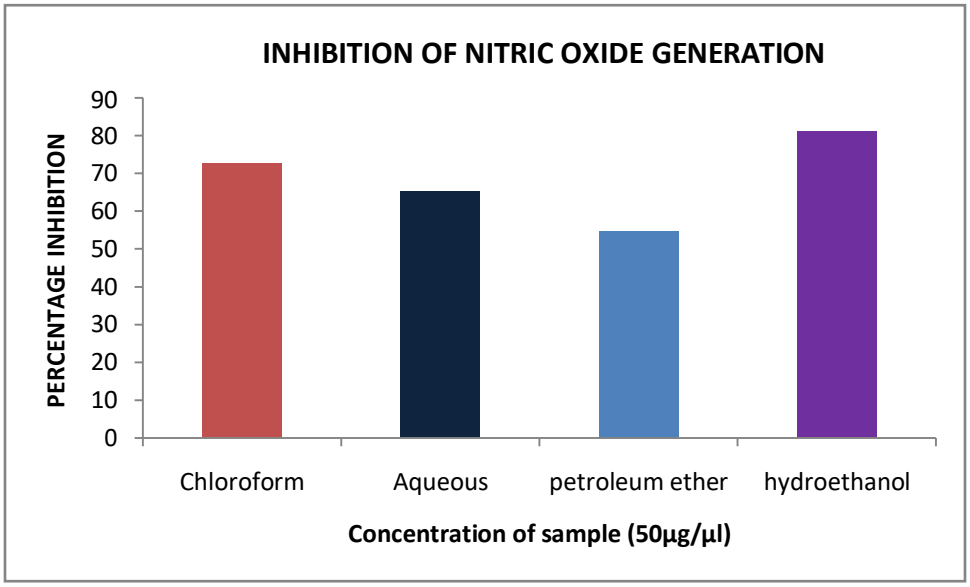
Balaji *et al.* (2011) showed that the maximum hydroxyl radical scavenging effect was found in the leaf extracts of *Ocimum tenuiflorum* where as the stem shows lesser activity. Hydroxyl radical is the major active oxygen centered radical formed from the reaction of various hydroperoxides with transition metal ions causing lipid peroxidation and biological damage. The inhibition effect of *O.tenuiflorum* leaf shows 69.2% were

found to be (BHT = 99.8% and ascorbic acid = 99.7%) indicating more radical scavenging activity than the stem.

The results of the scavenging assay proved that the leaf extracts of *Crescentia cujete* exhibit better scavenging ability against the team of radicals analysed in the present study including DPPH, ABTS, Hydrogen peroxide and hydroxyl radical indicating their potency in the use of medicine which might be attributed by the presence of phytochemicals and antioxidants.

Inhibition of Nitric oxide generation by *Crescentia cujete*

Figure 5

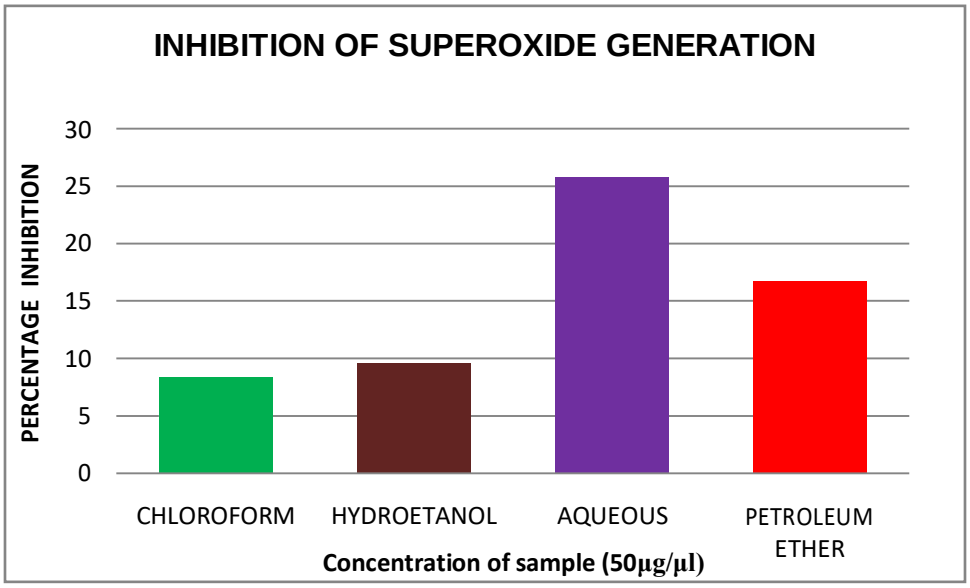


The results (Figure 5) revealed that hydroethanol have high inhibition towards nitric oxide generation. Nitric oxide is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays multiple roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumour activities. Suppression of released NO may be partially attributed to direct NO scavenging (Nishaa *et al.,* 2012).

Idamokoro *et al.,* 2017 reported that the acetone and aqueous extracts of *V. karroo* shows more inhibition against nitric oxide radicals than that of standard ascorbic acid and butylated hydroxyl toluene.

Inhibition of Superoxide generation by *Crescentia cujete*

Figure 6



The results revealed that the aqueous extract has high level of inhibition against superoxide free radicals rather than other solvent extracts.

The superoxide radicals can be converted into hydrogen peroxide by the action of superoxide dismutase and the H_2O_2 can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions (e.g., iron and copper), or by UV photolysis. Hydroxyl radicals can attack DNA molecules to cause strand scission (Shyur *et al.,* 2003).

The superoxide scavenging activity of ethanolic extract of *D. gangeticum* was comparable with that of the standard ascorbic acid as reported by Venkatachalam and Muthukrishnan, (2012). Thus, the results of inhibition of radical generation assay reveal that the inhibitory potential of various solvent extracts of the leaves of *C.cujete* indicate

that both superoxide and nitric oxide radical‘s generation were inhibited to a maximum extent proving their therapeutic efficacy as these are diffusible free radical that plays multiple roles as an effector molecule in diverse biological systems.

It can be concluded that the leaves of *Crescentia cujete* could serve as a potential source of secondary metabolites, antioxidants and in turn could scavenge the radicals that are produced during metabolic events and also further prevent the formation of radicals. Thereby, it could eliminate the toxicity and damage to the biomolecules caused by the reactive species and therefore alleviates diseases and disorders associated with free radicals and oxidative stress. The leaves can very well be incorporated in the formulations that are prepared for the treatment of oxidative stress mediated diseases.

5.0 SUMMARY AND CONCLUSION

In the recent decades, ROS has become a focus of research 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, ischemia or reperfusion injury, neurodegenerative disorders, hypertension and ageing (Gutowski and Kowalczyk, 2013). A great number of plants have been reported with medicinal value and are the rich sources of antioxidants. Antioxidants prevent or delay the oxidation of the substrate and their importance in the safeguarding of health, and the protection from several diseases including cancer, has been recently established, thus constituting them as functional food preservatives. Thus, it becomes imperative to study the phytoconstituents, antioxidant potential and radical quenching ability of the different solvent extracts of *Crescentia cujete* leaves.

Preliminary phytochemical screening was performed in the present study using the leaves of *Crescentia cujete.* Leaf extracts were prepared using different solvents in the order of increasing polarity includes petroleum ether, chloroform, hydroethanol and water. The desirable characteristics of edible plants and their use in the field of pharmaceutical preparations might be due to the presence of a variety of phytoconstituents such as carbohydrate, phenol, glycosides, flavonoids, alkaloids, terpenoids, tanins, steroids, volatile oils and saponins. The preliminary screening tests are useful in the detection of these bioactive constituents. The results revealed that that the different solvent extracts analysed for the presence of secondary metabolites indicate that all the extracts tested exhibits all the phytochemicals analysed namely alkaloids, flavonoids, phytosterols, reducing sugars, saponins, tannins, and terpenoids in slight and moderate appearances.

Thus it is evident from the results that *Crescentia cujete* carries a good source of pharmaceutically valuable components like alkaloids, flavonoids, phytosterols, terpenoids and tannins in the qualitative screening performed. Thus the *Crescentia cujete* leaf extract can very well be considered for the pharmaceutical preparations and can be validated for the use in the treatment of various diseases and disorders that are related with reactive species.

The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition. The activity of enzymatic antioxidants such as catalase, peroxidase and superoxide dismutase estimated indicate that the leaves of *Crescentia cujete* exhibited considerable activity of all the enzymes tested proving their use in traditional medicine as these enzymatic antioxidants are involved in metabolic processes of living system.

The non-enzymatic antioxidant content such as ascorbic acid, reduced glutathione and α- Tocopherol exhibited in the leaves of *Crescentia cujete* is found to be moderate indicating its nutrient antioxidant status. Thus, it can be inferred from the results of the antioxidant assessment that the leaves of *Crescentia cujete* can very well be used in the treatment of diseases associated with oxidants as the different solvent extracts were found to be a good source of both enzymatic and non-enzymatic antioxidants possessing nutritional as well as metabolic significance in elimination reactive species.

Several methods have been developed to assay free radical scavenging capacity and total antioxidant activity of plant extracts. DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of the plant extracts. The most common and reliable method involves the determination of the disappearance of free radicals using a spectrophotometer, such as 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic) acid radical (ABTS). Reduction of ABTS causes a loss of absorbance at 734 nm. The results of the scavenging assay proved that the leaf extracts of *Crescentia cujete* exhibit better scavenging ability against the team of radicals analysed in the present study including DPPH, ABTS, Hydrogen peroxide and hydroxyl radical indicating their potency in the use of medicine which might be attributed by the presence of phytochemicals and antioxidants.

Thus, the results of inhibition of radical generation assay reveal that the inhibitory potential of various solvent extracts of the leaves of *C.cujete* indicate that both superoxide and nitric oxide radical‘s generation were inhibited to a maximum extent proving their therapeutic efficacy as these are diffusible free radical that plays multiple roles as an effector molecule in diverse biological systems.

It can be concluded that the leaves of *Crescentia cujete* could serve as a potential source of secondary metabolites, antioxidants and in turn could scavenge the radicals that are produced during metabolic events and also further prevent the formation of radicals. Thereby, it could eliminate the toxicity and damage to the biomolecules caused by the reactive species and therefore alleviates diseases and disorders associated with free radicals and oxidative stress. The leaves can very well be incorporated in the formulations that are prepared for the treatment of oxidative stress mediated diseases.

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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 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;

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

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,

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

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,

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

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,

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