Extraction, phytochemical analysis and Antioxidant activities of *Calatropis gigantea* extracts

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Abstract: The present investigation deals with the extraction, phytochemical constituent analysis and antioxidant activity analysis of *Calatropis gigantea extracts*. It was observed that the yields of extraction depend on nature of solvent. It was found that the water and ethanol are the best possible solvents for having higher yields compared to other solvents chosen of varied polarities. Polyphenol content determination indicates that the water and methanol extracts were found to have highest percent of polyphenols than other extracts. The Flavonoid estimation of the extracts indicate that ethanol extract possess highest amount of flavonoids among all the extracts of different solvents. The DPPH activity analysis indicates that ethanol extract evidenced to have potential antioxidant activity among all extracts of different solvents. This is probably due to the significantly high biologically potent phytochemicals compared to other extracts. This investigation points out that the *Calatropis gigantea* contains significant amounts of antioxidant compounds such as polyphenols and flavonoids. Consequently various extracts showed potential antioxidant activity. Hence it is worth to consider *Calatropis gigantea* for further exploration in utilizing it as rich antioxidant.

IndexTerms - Calatropis gigantea, **extraction, phytochemicals, antioxidant activity,**

I. Introduction

In developing countries, the majority of people living in rural areas almost exclusively are traditional medicines in treating all parts of disease including diarrhea. There are large number of epidemiological and experimental evidence pertaining to worldwide acute diarrhoeal disease, which is one of the principal causes of death in the infants, particularly in malnourished and which is of critical importance in developing countries (1,2). It thus becomes important to identify and evaluate commonly available natural drugs. As alternative to commonly available natural drugs. As alternative to currently and antidiarrhoeal drugs, which are not completely free from adverse effects (3). Several studies have evaluated the effectiveness of some traditional medicines in teating diarrhea in all different continents (4-7) India has a great environmental and biological diversity compared with the rest of the world. A range of medicinal plants with antidiarrhoeal properties has been widely used by the traditional healers However, the effectiveness of many of their anti-diarrhoeal traditional medicines has not been scientifically evaluated.

Calatropis gigantea is a species of *Calotropies* native to Cambodia, Indonesia, Malaysia, Philippines, Thailand, Srilanka, India and China. It is a large shrub qrowing to 4m tall, it has clusters of waxy flower that are either white or lavender in colour. Each flower consists of five pointed petals and a small, elegant "crown" rising from the centre, which holds the stamens. The plant has oval, light green leaves and milky steam.

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono phenols are weak antioxidants.

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. Antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic disease including heart disease and some cancers. The free radical scavenging activity of antioxidants in foods has been substantially investigated and reported in the literature by miller and Rigelhof et al. (1,2). Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells.

As oxidative stress appears to be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease.

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (3). These compounds may be synthesized in the body or obtained from the diet (4). The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors.

Antioxidants are used as food additives to help guard against food deterioration. However, as oxygen is also important for plant respiration, storing plant materials in anaerobic conditions produces unpleasant flavors and unappealing colors (5). Consequently, packaging of fresh fruits and vegetables contains an 8% oxygen atmosphere. Antioxidants are an especially important class of preservation as, unlike bacterial or fungal spoilage, oxidation reactions still occur relatively rapidly in frozen or refrigerated food (6). These preservatives include natural antioxidants such as ascorbic acid and tocopherols, as well as synthetic antioxidants such as propyl gallate, tertiary butylhydroquinone, butylated hydroxyanisole and butylated hydroxytoluene (7). The most common molecules attacked by oxidation are unsaturated fats; oxidation causes them to turn rancid (8). Oxidation is often catalyzed by metals, which is why fats such as butter should never be wrapped in aluminum foil or kept in metal containers. Some fatty foods such as olive oil are partially protected from oxidation by their natural content of antioxidants, but remain sensitive to photooxidation (9). Antioxidant preservatives are also added to fat-based cosmetics such as lipstick and moisturizers to prevent rancidity. Antioxidants are frequently added to industrial products. A common use is as stabilizers in fuels and lubricants to prevent oxidation, and in gasolines to prevent the polymerization that leads to the formation of engine-fouling residues (10). In 2007, the worldwide market for industrial antioxidants had a total volume of around 0.88 million tons. This created revenue of 3.7 billion US-dollars.

A polyphenol antioxidant is a type of antioxidant containing a polyphenolic substructure. Numbering over 4,000 distinct species, these compounds have antioxidant activity in vitro but are unlikely to have antioxidant roles in vivo (11). Rather, they may affect cell-to-cell signaling, receptor sensitivity, inflammatory enzyme activity or gene regulation (12).

Polyphenols are the most abundant antioxidants in the diet. Their total dietary intake could be as high as 1 g/d, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants. For perspective, this is 10 times higher than the intake of vitamin c and 100 times higher than the intakes of vitamin E and carotenoids (13). Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, chocolate, and dry legumes also contribute to the total polyphenol intake. Despite their wide distribution in plants, the health effects of dietary polyphenols have come to the attention of nutritionists only rather recently. Until the mid-1900s, the most widely studied antioxidants were antioxidant vitamins, carotenoids, and minerals.

Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus (14).

Flavonoids (specifically flavonoids such as the catechins) are “the most common group of polyphenolic compounds in the human diet and are found ubiquitously, but in lesser plants” (15). Flavonols, the original bioflavonoids such as quercetin, are also found ubiquitously, but in lesser quantities. Preliminary research indicates that flavonoids may modify allergens, viruses, and carcinogens, and so may be biological “response modifiers”. In vitro studies show that flavonoids also have anti-allergic, anti-inflammatory (16), anti-microbial (17, 18) and anti-cancer activities (19). Good sources of flavonoids include all citrus fruits, berries, ginkgo biloba, onions (particularly red onion), parsley, pulses, tea (especially white and green tea), red wine, seabuckthorn, and dark chocolate (with a cocoa content of seventy percent or greater)

II. Materials and methods:

1, 1 - Diphenyl – 2 - picrylhydrazyl was procured from Sigma-Aldrich India Company. Ascorbic acid, Gallic acid, Vanillin, Phloroglucinol and Methanol were purchased from S. D. Fine Chemicals. All other solvents are of AR grade and distilled before use. Distilled water was employed for all the experiments. Areca nut was collected in the month of August – September in Tumkur district, Karnataka. The sample shade dried and powdered into 100 mesh size and was stored at room temperature in an airtight container. Solvents Ethyl acetate, Ethanol, Methanol, Acetone, Water were of analytical grade and distilled before use.

Water extract preparation: ~ water soluble polar compounds can be extracted by this case, cold water insoluble compounds but soluble in hot water can be extracted. 1: 10 proportion of material to solvent was taken for extraction and the extraction is carried out at boiling temperature of water (100^0 C) with a reflux arrangement for 3 hours with constant stirring and the extract is filtered and centrifuged to remove any un-dissolved material. The extract is then concentrated to 1/5 volume on the concentrator and dried completely. Thus prepared extract is stored in airtight bottles.

Ethanol extract preparation: Ethanol-water soluble polar compounds can be extracted by this method while the proteins and polysaccharides get precipitated. Here too, 1: 10 proportion of material to solvent was taken for extraction and the extraction is carried out at boiling temperature of ethanol (65^0 C) with a reflux arrangement for 3 hours with constant stirring and the extract is filtered and centrifuged to remove any un-dissolved material. The extract is then concentrated to dryness. Thus prepared extract is stored in airtight bottles.

Methanol extracts preparation: methanol soluble polar compounds can be extracted by this method while the proteins and polysaccharides get precipitated. Here too, 1: 10 proportion of material to solvent was taken for extraction and the extraction is carried out at boiling temperature of methanol with a reflux arrangement for 3 hours with constant stirring and the extract is filtered and centrifuged to remove any un-dissolved material. The extract is then concentrated to dryness. Thus prepared extract is stored in airtight bottles.

Ethyl acetate extracts preparation: ethyl acetate soluble compounds can be extracted by this method while the proteins and polysaccharides get precipitated. Here also, 1: 10 proportion of material to solvent was taken for extraction and the extraction is carried out at boiling temperature of Ethyl acetate with a reflux arrangement for 3 hours with constant stirring and the extract is filtered and centrifuged to remove any un-dissolved material. The extract is then concentrated to dryness. Thus prepared extract is stored in airtight bottles.

DPPH Assay: 1,1 Dipheny 1-2 –picrylhydrazyl (Oxididized form) is a stable free radial with purple color .in the presence of an antioxidant which can donate an electron to DPPH, the purple color which is typical to free DPPH radical dacays , and the change in absorbance at 520 nm is followed which can be measured spectrophotometrically. Dissolved 39.4 mg of DPPH in 100ml of methanol to get concerntration of 1mM stock .stored in dark bottle at 4°c until its use. The working concentration of DPPH in the assay was 0.14 mM. Methanol (50%) was prepared by diluting methanol 1:1 with de-ionized water Ascorbic acid standard stock I (conc.200µg/ml) was prepared by dissolving 2mg of ascorbic acid and make up to a volume of 10ml with de-ionized water. For making stander graph of ascorbic acid 2, 4, 6, 8, 10µg/ml concentration range was used. The DPPH assay was carried out by using modified method Brand – Williams (20) in brief to a 860 µl of 50% methanol / ascorbic acid / test sample with various concentration, added 140µl of 1Mm DPPH, mixed and incubated at 37° C For 30min. Read the observance at 520 nm against 50% methanol blank by spectrophotometer, a control reaction is carried out by without test sample addition. Color –correction contains the same

concentration of the test sample in the methanol without DPPH. The anti-oxidant activity was measured with reference to the standard ascorbic acid observance values. The actual absorbance is taken as the absorbance difference of the control and the test sample and IC_{50} Values were determined.

Polyphenol Assay: Phenolic compounds in alkaline condition (sodium carbonate) dissociate to yield a proton and phenolate anion, which is capable of reducing Folin ciocalteu reagent. FC reagent is an oxidizing agent comprised of heteropolyphosphotungstate-molybdate.Sequence of one or two electron reduction reaction lead to blue color species. The blue coloured mixture of the 1-,2-,4-, and 6- electron reduction products in the tungstate series $P_2W_{18}O_{62}^{-7}$ $H_4P_2W_{18}O_{62}^{-8}$ to and the 2-,4- and 6-, electron reduction products in the molybdate series $H_2P_2Mo_{18}O_{62}^{-6}$ to $H_2P_2MO_{18}O_{62}$.Folin ciocalteu reagent(0.1N) was prepared by diluting 1:20 with commercially available FC reagent with distilled water to get the required concentration. Sodium carbonate (7.5%) was prepared by dissolving 7.5 gm sodium carbonate in 100 ml of de-ionised water. Gallic acid (standard) stock 1 (conc.0.1mg\ml) was prepared by dissolving 1mg of gallic acid in 10 ml with 50% methanol. For making standard graph of Gallic acid concentration range of 2-20µg/ml was used. The assay was carried out by using singleton, V., Rossi, J. A. jr method (21),in brief, to a 200µL of 50% Methanol/standard/test sample with various concentration, added 1000µL of FC reagent, mixed and incubated at RT for 5 min. added 800µL of 7.5% sodium carbonate mixed and incubated at RT for 30 min. Read the absorbance at 750 nm against blank by spectrophotometer, colour correction was given with the same concentration was given with the same concentration of the test sample in 50%Methanol without FC reagent.

Flavonoids assay: Vannillin, an aromatic aldehyde condenses with the Flavon -3-ols and oligomers to form soluble pigments in acidic medium with an absorbance maximum at 500 nm, which can be detected by UV-VIS spectrophotometer. Vanillin reagent (1%) was prepared by dissolving 1gm of crystallised vanillin in 100 ml of 70% conc. H_2SO_4 (prepared fresh). Conc H_2SO_4 (70%) was prepared by diluting 70 ml on conc. H_2SO_4 in 100 ml de-ionised water. Methanol 50% was prepared by diluting 1:1 with de-ionised water.

Phloroglucinol (standard) stock 1(conc 1mg/ml):Dissolved 10mg of phlorogluinol and made up to volume of 10 ml with 50% Methanol, Then centrifuge at 12,000 rpm for 10 min. Stock11: Diluted to conc, to yield 0.1mg/ml with 50% methanol.For making standard graph of phloroglucinol , 1-10 µg/µL concentration range was used.The flavonoid assay was carrid out by using Swain, T and Hillis, W. E method (22). In brief to a 400µL of distilled water/ positive control/ test sample with various concentration, added 800ML of 1% vanillin reagent, mixed and incubated at RT 15 minutes. Read the absorbance at 500nm against blank by spectrophotometer. Colour correction was given with the same concentration with the test sample in distilled water without vanillin reagent. The Flavonoid content in the phytoextracts was measured with reference to the standard Gallic acid values.

III. Results and discussion:

Free radicals were a major interest for early physicists and radiologists and much later found to be a product of normal metabolism. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a consequence, reactive oxygen species are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, cataracts, chronic inflammation, and neurodegenerative diseases. ROS and free radicals are also considered as inducers of lipid peroxidation and cause the deterioration of foods. Although organisms have endogenous antioxidant defenses produced during normal cell aerobic respiration against ROS, other antioxidants are taken from the diet, both from natural and synthetic origin. Antioxidants are widely used as ingredients in dietary supplements and have been investigated or the prevention of disease such as cancer coronary heart and even altitude sickness.

Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation is harmful. In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline.

Extraction yields

Leaves:

Sl. No.	Solvent	% yield
01	Water	25.50
02	Ethanol	15.40
03	Ethayl acetate	7.50
04	Methanol	16.40

Flowers:

Sl. No.	Solvent	% yield
01	Water	14.57
02	Ethanol	19.23
03	Ethyl acetate	5.72
04	Methanol	12.57

The solvent such as water, ethanol, ethyl acetate, Methanol were utilized optimum extraction process so as to arrive at extract with at extracts with higher yield and better antioxidant potency. It was observed that water in case of leaves and Ethanol in case of flowers are the solvents which provide highest yield among all the solvents.

Polyphenol content:

Leaves:

Sl. No.	Solvent	Polyphenol content
01	Water	19.60
02	Ethanol	22.47
03	Ethyl acetate	40
04	Methanol	19.60

Flowers:

Sl. No.	Solvent	Polyphenol content
01	Water	20.40
02	Ethanol	14.49
03	Ethyl acetate	20.20
04	Methanol	17.54

Polyphenol content was determined by singleton method. It was found that the water and methanol extracts were found to have highest percent of polyphenol .

Flavonoids content:

Sl. No.	Solvent	Flavonoid content
01	Water	24.39
02	Ethanol	30.30
03	Ethyl acetate	20
04	Methanol	28.16

Sl. No.	Solvent	Flavonoid content
01	Water	21.77
02	Ethanol	35.77
03	Ethyl acetate	20.83
04	Methanol	32.78

It is evident from above data that ethanol extracts are proved to have highest contents of flavonoids.

DPPH Assay:

Leaves:

Sl. No.	Solvent	IC_{50} Values
01	Water	220 µg/ml
02	Ethanol	125 µg/ml
03	Ethyl acetate	180 µg/ml
04	Methanol	275 µg/ml

Flowers:

Sl. No.	Solvent	IC_{50} Values
01	Water	220 µg/ml
02	Ethanol	140 µg/ml
03	Ethyl acetate	230 µg/ml
04	Methanol	295 µg/ml

DPPH a stable free radical with a characteristic absorption at 517-520 nm was used to study radical scavenging effects of extracts. It is observed that ethanol extract is the best extract at inhibiting the DPPH activity. This is followed by ethyl acetate, water and methanol extracts.

Conclusion:

The present investigation deals with the extraction, phytochemical constituent analysis and antioxidant activity analysis of *Calatropis gigantea extracts*. It was observed that the yields of extraction depend on nature of solvent. It was found that the water and ethanol are the best possible solvents for having higher yields compared to other solvents chosen of varied polarities.

Polyphenol content determination indicates that the water and methanol extracts were found to have highest percent of polyphenols than other extracts. However, the other extracts also contain significantly high amounts of the polyphenols. This indicates that all these extracts may have significant antioxidant activities thereby eliciting beneficial physiological effects.

The Flavonoid estimation of the extracts indicate that ethanol extract possess highest amount of flavonoids among all the extracts of different solvents. Other extracts also have substantial amounts of flavonoids. Since the flavonoid content is directly proportional to the antioxidant activity, these extract could exhibit potential antioxidant activities.

The DPPH activity analysis indicates that ethanol extract evidenced to have potential antioxidant activity among all extracts of different solvents. This is probably due to the significantly high biologically potent phytochemicals compared to other extracts. It is also clear that *Calatropis gigantea* seems to have highly soluble compounds in ethanol and methanol; hence the yield is also significantly high.

Unequivocally, ethanol and water extracts emerge out as the best possible extracts in terms of antioxidant activity. Additionally methanol and ethyl acetate extracts are shown to high higher yields. However, methanol and ethyl acetate are not suitable solvents for consumption and extraction as they may cause severe deleterious effects even at very low concentrations. Due to this, the regulatory bodies all across the globe have very stringent norms for using methanol and ethyl acetate as solvents of extraction. In lieu of these facts, the comparatively potent water and ethanol extract could be utilized as potential antioxidant extracts.

The *Calatropis gigantea* has extensively been used in most of the countries across the globe and especially in Indian subcontinent even though it has been widely used in India since very long time, its health beneficial effects have not been documented scientifically until recently. However for the past few years considerable number of studies has attempted to understand its nature and its bioactivity. This investigation points out that the *Calatropis gigantea* contains significant amounts of antioxidant compounds such as polyphenols and flavonoids. Consequently various extracts showed potential antioxidant activity. Hence it is worth to consider *Calatropis gigantea* for further exploration in utilizing it as rich antioxidant.

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