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Phytochemical analysis and comparison of in *vitro* antibacterial activities of the leaf extracts of *Calotropis gigantea* and *Ficus religiosa*

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Abstract

Calotropis gigantea and *Ficus religiosa* leaves were collected and the antibacterial activity was determined against some human pathogens. The leaves of *Calotropis gigantean* and *Ficus religiosa* were extracted with aqueous, ethanol and methanol by using solvent extraction method. The human pathogens were isolated from wound samples and the isolates were characterized morphologically and biochemically and identified. They were identified as *E.coli, Bacillus subtilis Enterbacter aerogenes, pseudomonas aeruginosa*. The antibacterial activity of the solvent leaf extracts of *C. gigantean* and *F. religiosa* as determined by using agar well diffusion method. The aqueous extract of *C. gigantea* inhibited the growth of bacterial pathogens effectively at 100µl/ml concentration. The phytochemcial analysis of the plants showed the presence of alkaloids, flavonoids, terpenoids, glycosides, phenols, steroids, saponin and resins, and the results are discussed.

Key words: *Calotropis gigantea* , *Ficus religiosa* , leaves, solvent extraction, Agar well Diffusion, Phytochemical screening analysis.

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INTRODUCTION

Nature has provided a valuable source of medicine and has helped human in the maintenance of his health since time immemorial. The world has rich wealth of medicinal plants. Without the plant kingdom humans cannot survive on this earth because the plant products and their active constituents play an important role on their survival. The plants and their parts are used in various systems of medicine such as Chinese, Ayurveda, Siddha, Unani and Tibetan. Ancient literature such as Rigveda, Yajurveda, Atharvaveda, Charak Samhita and Sushrut Samhita also describe the use of plants for the treatment of various health problems (Balunas *et al.,*2005). Plants produce a diverse range of bioactive molecules, which constitute a rich source of different types of medicines. The green medicines are healthier and safer than synthetic ones. A number of herbal medicines are used for the management of various diseases. They are cost effective, pharmacologically active and provide an easy remedy for many human ailments as compared to the synthetic drugs with minimal toxicity and side effects (Saet *et al.,* 2007).

Medicinal plants are considered as the important source of new chemical substances with potential therapeutic effects and can be used to treat chronic and infectious diseases. Natural products from plants can be templates for new drug development and have many interesting biological activities like anti diabetic, antioxidant, antibacterial, anti-inflammatory, antipyretic, gastro protective effects, etc. All parts of the plant like leaf, stem, flower, bark, fruit, peel, rhizome, essential oil, latex, bud, etc. can be used as herbal medicine. The medicinal values of plants lie in phytochemicals present in them which produce a definite physiological action on the human body. Plants synthesize and accumulate in their cells a great variety of phytochemicals like tannins, flavonoids, phenolic compounds, glycosides, steroids, saponins, etc. Most of these are potent bioactive compounds that can be used for the synthesis of useful drugs. Phytochemicals regulate, protect and control many of the diseases in human beings, though the active principles differ from plant to plant because of their diverse biochemical nature. (Hill,1952).

Calotropis is a small genus with about 6 species of shrubs or small trees, distributed in tropical and subtropical Africa, Asia and central and South America. In India it is represented only by two species namely *Calotropis procera* and *Calotropis gigantea* linn. Both the species closely resemble each other in structure and find similar uses (Kirtikar *et al.,* 1994). *C. gigantea* is a glabrous or hoary, laticiferous shrubs or small trees, grows to a height of 3-4 m and commonly known as the swallow-wort or milkweed. Its stems are

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erect, up to 20 cm in diameter. The leaves are broadly elliptical to oblong-obovate in shape, with the size of 9-20 cm x 6-12.5 cm but sub sessile. The cymes are 5-12.5 cm in diameter. The inflorescence stalk is between 5-12 cm long, the stalk of an individual flower is 2.5-4 cm long. Sepal lobes are broadly egg shaped with a size of 4-6 mm x 2-3 mm. Petal is 2.5-4cm in diameter. It has clusters of waxy flowers 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 stem (Carol *et al*., 2012).

Ficus religiosa Linn is commonly known as 'peepal' tree and belonged to the family Moraceae. In India, since ancient time, it has got great mythological, religious and medicinal importance. This is considered as the oldest tree in Indian art literature. There are many chemical compounds which have been extracted out from different plants as they have very important use in the medicinal field. These compounds are beneficial in the treatment of many diseases such as diabetes, skin diseases, respiratory disorders, central nervous system disorder, gastric problems, etc. (Sirisha *et al.,*2010; Vinutha *et al.,*2007). The present article deals with the presence of major phytochemcial compounds in the different solvent extracts of the leaves of *Calotropis gigantea* and *Ficus religiosa*, and their potential role in the antimicrobial activity.

MATERIALS AND METHODS

Sample collection

Wound samples (specimen) were collected with the help of specialist using sterile swabs at Government Hospital, Mannargudi ,Thirurvarur District, Tamil Nadu. These swabs were immediately immersed into normal saline and transported to the laboratory.

Isolation and identification of microorganisms

The pathogenic microorganisms were isolated by swab method. Then the isolated colonies were identified by Gram's staining and Biochemical tests.

Collection of plant

The leaves, of *Calotropis gigantea* and *Ficus religiosa* were collected from the Herbal Garden at S.T.E.T Women's College, Sundarakkottai, Mannargudi.

Plant powder and extract preparation

500gm of the leaves were weighed and dried under shade for 15 days. The dried plant material was crushed into fine powder with the help of grinder and stored for required purpose. 5gm of the powder was dissolved in 45ml of solvent (Ethanol, Methanol and water) separately to prepare 10% extract in 200 ml flasks. The flasks were covered with the aluminum foil and kept on rotating shaker (120 rpm) for 2 days. The solution was filtered twice, first with cheese cloth (four fold) and then with Whatman's No.1 filter paper. The filtrates were collected in Falcon tubes and were concentrated to dryness by keeping it in incubator at 35ºC. The stock solution of each extract was prepared in Dimethyl sulfoxide (DMSO).

Phytochemical screening

Specific qualitative tests were performed to identify the bioactive compounds of pharmacological importance by using standard methods (Trease and Evans, 1989).

Test for Alkaloids

2.0 ml of the extract was measured in a test tube and picric acid solution was added. The formation of orange colouration indicated the presence of alkaloids (Julian Preston *et al.,* 2005).

Test for cardiac glycosides

5ml of the leaf extract of each plant was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. A brown ring at the interface indicated a deoxysugar characteristic of cardenolides. The presence of cardiac glycosides was inferred on the basis of the gradual formation of violet ring below the brown ring and a greenish ring in the acetic acid layer.

Test for anthraquinines

Sodium hydroxide was added to the leaf extracts. Formation of blue green or red colour indicated the presence of anthraquinone.

Test for tannins

The extracts were mixed with basic lead acetate solution. Formation of white precipitate indicated the presence of tannins

Test for Saponins

Froth test for saponins was used. 1g of the sample was weighed into a conical flask, 10ml of sterile distilled water was added and boiled for 5min. The mixture was filtered and 2.5ml of the filtrate was added to 10ml of sterile distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

Test for Flavonoids

5 ml of dilute ammonia solution added to a portion of the aqueous extract of each plant and then concentrated H_2SO_4 was added. Formation of yellow colour indicated the presence of flavonoids.

Test for steroids

One gram of the test substance (plant extracts) was dissolved in few drops of acetic acid. It was gently warmed and cooled under the tap water and a drop of concentrated sulphuric acid was added along the sides of the test tube. Appearance of green colour indicated the presence of steroids.

Test for Terpenoids

5ml of the leaf extract of each plant was mixed with 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. Formation of reddish brown colour at the interface showed the presence of terpenoids.

Test for reducing sugar

One gram of the aqueous extract was weighed and transferred into a test tube. This was diluted using 10 ml of de-ionised distilled water. Then Fehling's solution was added. The mixture was warmed to 40 ºC in water bath. Development of brick-red precipitate at the bottom of the test tube indicated the presence of reducing sugar. Same procedure was repeated using dimethylsulphoxide (DMSO) as the diluent for the ethanolic extract.

Test for resins

Two grams of the ethanolic extract was dissolved in 10 ml of acetic anhydride. A drop of concentrated sulphuric acid was added. Appearance of purple colour, which rapidly changed to violet, was indicative of the presence of resins. Same procedure was repeated using the aqueous extract of the plant material.

Test for Carbohydrate

To 2ml of the leaf extract 1ml molisch's reagent and few drops of concentrated sulphuric acid were added .Formation of purple or reddish ring indicated the presence of carbohydrate (Meera and Nagarjuna, 2009).

Test for Phenols

Small quantities of ethanol and aqueous extracts were taken separately and tested for the presence of phenolic compounds and tannins with 10% acetate solution. Appearance of white colour indicated the presence of phenolic compounds**.**

Assay for Antibacterial activity

Determination of concentration of test strains

The total count (concentration) of test bacterial strains was made by using McFarland Standard scale. The standard tubes were prepared by mixing varying amount of 1% $BaCl_2$ and 1% H_2SO_4 in air tight tubes.

Bacterial culture preparation and determination of Zone of inhibition (ZOI)

The isolated pathogenic organisms such as *B. subtilis, E. coli*, *Enterobacter aerogenes* and *P.aeroginosa* were used as test organisms.

The test strains were sub-cultured in Muller Hinton Agar (MHA) (Himedia). The well-grown bacterial colonies in MHA plate were picked and sub-cultured in Nutrient broth (Muller Hinton Broth) and incubated for 24 hrs at 37 ^{0}C and stored at 40 ^{0}C for further use.

The Kirby's Disc Diffusion method was used to determine the antibacterial efficacy of the selected plant extract. MHA 200ml was prepared and poured into sterile Petri plates for each test strain. Each Petri plate was inoculated with bacterial strains by sterile streaking loop method. Circular wells were prepared in each plate with the help of corkborer (diameter 6mm) and 20µl of each solvent leaf extract of each plant was added. The Petri plates were inoculated and incubated at 37 ^{0}C, and the antibacterial efficacy [expressed as mean value of inhibition diameter (mm)] of the two leaf extracts with different solvents was determined. The experiment was performed in triplicates.

RESULT AND DISCUSSION

The present study was carried out to determine the antibacterial activity of the solvent extracts of the leaves of two different plants against human pathogens.

Identification of Bacterial pathogens

The Petri plates containing nutrient agar showed the development of different colonies. The Gram staining showed, the presence of Gram positive cocci and Gram negative rod shaped bacteria. It was confirmed by various biochemical tests. The results are shown in Table 1. The identified organisms included *E.coli.*

Table.1. Morphological and Biochemical Characteristics of isolated Bacteria

Characteristics	*E.coli*	*B.subtilis*	*E.aerogens*	*P.aeruginosa*
Gram staining	-	+	+	-
Motility	+	+	+	+
Shape	Rod	Rod	Rod	Rod
Indole	+	-	-	-
MR	+	+	-	-
VP	-	+	+	-
Citrate	-	-	+	+
Urease	-	-	-	+
TSI	A/A	-	A/A	A/A
Catalase	+	+	+	-

Note : (+) Indicate Positive (-) Indicate Negative : A / A-Acid / Alkaline

Bacillus subtilis, Enterobacter aerogenes, and *Pseudomonas aeruginosa. E.coli* produced metallic sheen colour colones on EMB agar. *Pseudomonas* appeared as white mucoid colonies over the agar, and *Enterobacter aerogenes* and *Bacillus subtils* produced specific white colonies on Nutrient agar medium.

Phytochemical screening of plant extracts

Calotropis gigantea

The preliminary phytochemical screening of the aqueous, ethanol and methanol extract of *Calotropics gigantea* was made. The aqueous extracts showed the presence of alkaloids, flavonoids, glycoside, tannins, phenolic, saponin, terpennoids, steroids and resins but did not show anthraquinone. Methanol extract did not show carbohydrates, saponin, resins and anthraquinone but it contained alkaloids, flavonoids, tannis and phenolic compounds. Ethanol extract did not contained carbohydrates, resins and anthrquione but it showed the presence of alkaloids, flavoinoids, tannins, phenoilic and terpenoids (Table 2). The antibacterial activity has been attributed to the presence of some active constituents in the extracts. This antibacterial activity of the plant extracts lends validation to the folk medicine which could be used effectively as modem medicine to combat pathogenic microorganisms. The core use of the plants in folk medicine suggests that they represent and economic and safe alternative to treat infectious diseases. The flower of the plant contains the cardiac glycosides, calotropin, uscharin, calotoxin, calactin, uscharidin and gigantin. The flowers also contain the protease,

Table.2. Phytochemical analysis of *Calotropis gigantea* leaf extract

Phyto chemicals	Aqueous	Methanol extract	Eathnol extract
Alkaloids	+	+	+
Carbohydrates	_	_	_
Reducing sugar's	_	+	+
Flavonoids	+	+	+
Glycoside	+	+	+
Tannins	+	+	+
Saponin	+	_	+
Terpenoids	+	+	+
Steroids	+	+	+
Resins	+	_	_
Anthraquinone	_	_	_
phenolic	+	+	+

Note:(+) indicate Present (-) indicate Absent

calotropin DI and DII and calotropin FI and FII (Dhivya and Manimegalai, 2013).These findings support the traditional knowledge and also suggest that these plants could be potentially used to cure diseases in human beings (Eruteya *et al.,* 2009). The leaves of *Calotropis gigantea* are used traditionally for the treatment of abdominal tumors, boils, syphilis, leprosy, skin diseases, piles, insect bites and phylariasis.

Ficus religiosa

The phytochemical analyses of the leaf extracts of *Ficus religiosa* showed the presence of alkaloids, carbohydrates, glycosides, terpenoids, saponins, phenols xanthoproteic, flavonoids and tannins. The aqueous and methanolic extracts showed the presence of all the above mentioned phytochemical compounds. But the ethanol extract showed all the compounds other than carbohydrates (Table 3). Dhanalakshmi *et al.,* (2011) showed the presence of flavonoids and terpenoids in the ethanol extract of *Ficus religiosa*. Phytochemicals or secondary metabolites usually occur in different plant organs and stages of development. The bark and leaves of this group are used as astringent, haemostatic, anti-inflammatory and anti-septic; and in the treatment of diarrhea and dysentery, and also in the treatment of skin diseases such as ulcers, vaginal disorders, leucorrhoea, menorrhagia and deficient lactation.

Table.3. Phytochemical analysis of *Ficus religiosa* leaf extract

Phytochemicals	Aqueous	Methnol	Ethanol
Alkaloids	+	+	+
Carbohydrate	+	+	_
Glycoside	+	+	+
Terpenoids	+	+	+
Saponins	+	+	+
Phenols	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+

Note:(+) indicate Present (-) indicate Absent

Antibacterial activity

Agar well diffusion method

Calotropis gigantea

The antibacterial activity of the extracts was increased with increase in the concentration, for instance the aqueous extracts showed antibacterial activity against *Bacillus subtilis* which showed zone of inhibition high (24 mm) followed by *E.coli* (20 mm), *Enterobacter aerogenes* and *Pseudomonas aeruginosa* (17 mm and 15.5

Table.4. Antibacterial activity of *Calotropis gigantea* by Agar well diffusion method

Test Organism	Aqueous				Ethanol				Methanol			
	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
E.coli	2.1mm	4 mm	17 mm	8.6mm	1.8mm	2.5mm	4.1mm	6mm	1.3mm	2.2mm	3.3mm	8.6mm
E.aerogenes	1.6 mm	7.3 mm	12.1mm	20mm	1mm	2mm	5.8mm	7.6mm	1mm	1.2mm	2.7mm	5mm
P.aeroginosa	1 mm	3.6 mm	10mm	15.5mm	10mm	1.7mm	3.1mm	4.7mm	0mm	1mm	1.1mm	3mm
B.subtilis	9.5 mm	15.5 mm	20mm	24mm	2.2mm	3mm	6.2mm	10.0mm	1.1mm	2mm	4mm	2mm

mm). The ethanol extract showed a degree of growth inhibition which was less when compared to the aqueous extract. The maximum inhibition was found at 100 µl against *Bacillus subtilis,* (10.1 mm), followed by *E.coli* (7.6 mm). The minimum activity was recorded against *Pseudomonas aeruginosa* (6 mm) and *Enterobacter aerogenes* (4.7 mm), (Table 4). It is interesting to note that the aqueous extract of the leaves of *Calotropis gigantea* could be used against *Bacillus subtilis*. An interesting observation is that the majority of the crude extracts and their fractions are almost equally active both against drug resistant and sensitive bacterial strains. Multi target based approaches of screening of medicinal plant extracts and herbal drugs are expected to yield novel activities (Zhu-Nian Wang and Mao-Yuan Wang, 2008). The antibacterial activity of *C. gigantea* extract against both Gram positive and Gram negative bacteria could be attributed to the presence of broad spectrum antibacterial components. This indicates that the plant could be an useful source for the development of novel antibiotics against pathogenic bacteria.

Ficus religiosa

The inhibitory effect however showed that the antibacterial activity of the extracts increased with increase in the concentration, for instance the activity of the aqueous extracts at the concentration of 100 µl against *Bacillus subtilis* showed zone of invitation high (28 mm), followed by *E.coli* (25.6 mm), *Enterobacter aerogenes* and *Pseudomonas aeruginosa* (25.3 mm and 19.6 mm respectively). The maximum inhibition was found at 100 µl against *E.coli* (8.9 mm) followed by *Bacillus subtilis* (6.2 mm). The minimum activity was found against *Enterobaucter aerougenes* (6 mm) and *Pseudomonas aeruginosa* (1.5 mm) (Table 5). The activities of some phytochemicals of compound nature with flavonoids, palmatic acid (hexadecanoic acid, ethyl ester and n-hexadecanoic acid) and unsaturated fatty acid ocatadecatrienonic acid might be antimicrobial against the pathogenic bacteria. All the test organisms were susceptible to plant aqueous extracts with various degree of sensitivity. Flavonoids from different plants have been reported for their antibacterial activities (Plate V). Microorganism acquired resistance against commercial drugs due to the reduction of enzymes, resistant plasmids, and alteration of metabolic pathways in the pathogens. Natural compounds of their extract could be used for the development of new drugs. The secondary metabolites are promising sources of preventive agents in the pathogenesis and the microbial diseases. The aqueous extract of *Calotropis gigantea* more effectively killed all the bacterial pathogen at 100µl/ml concentration than the extract of *Ficus religiosa*. Different parts of the plant have immense potential to cure various diseases and disorders. It is used in various polyherbal preparations. *Calotropis* is used alone and sometimes with other plants to cure variety of human and animals ailments. This research has established the antimicrobial potentialities of the plants used, which provides a new light for those who are attempted to develop new drugs using plant products. Thus *Calotropis gigantea* is being evidently directed as a boon for further investigation.

Table.5. Antibacterial activity of *Ficus religiosa by* Agar well diffusion method

Test organism	Aqueous				Ethanol				Methanol			
	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
E.coli	12mm	9.3mm	12.6mm	25.6mm	4mm	5.2mm	5.6mm	6.2mm	3.6mm	4.5mm	4mm	5.6mm
E.aerogenes	10.2mm	13.3mm	23.5mm	25.3mm	4mm	4.8mm	5.3mm	6mm	3mm	3.5mm	4.6mm	5mm
P.aeroginosa	10.3mm	13.6mm	15.6mm	19.6mm	0mm	1mm	1.4mm	1.5mm	1mm	1mm	1mm	1.2mm
B.subtilis	12.3mm	20mm	21mm	28mm	6.4mm	7.9mm	8.4mm	8.9mm	6mm	6mm	7.4mm	7.6mm

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