Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research

J. Chem. Pharm. Res., 2011, 3(6):330-336

ISSN No: 0975-7384
CODEN(USA): JCPRC5

Antibacterial efficacy and Phytochemical analysis of organic solvent extracts of *Calotropis gigantea*

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ABSTRACT

The leaves extract of Calotropis gigantea were screened for its antibacterial and phytochemical activities. The solvents used for the leaves extraction were n-Hexane, Ethanol, Methanol, Chloroform, Water and Ethyl acetate. The extract was tested against Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella typhi and Micrococcus luteus for its antibacterial activity. Ethyl Acetate leaves extract exhibited maximum zone of inhibition. Ethyl Acetate leaves extract was found to be most effective with MIC value also ranging from 0.25 to 1.0 mg/ml. Aqueous leaves extract showed weak antibacterial activity. A small portion of the dry extract was used for the phytochemical tests for compounds which include alkaloids, cardic glycoside, anthraquinone, tannins, saponins, flavnoid, steriods, terpeniods, reducing sugars and resins in accordance with the methods.

Key words: Antibacterial activity, *Calotropis gigantea*, Minimum Inhibitory Concentration (MIC), *S. aureus, B. cereus.*

INTRODUCTION

The prevalence of invasive, opportunistic microbial and fungal infections has increased at an alarming rate especially in immune-compromised individuals. They cause a broad spectrum of infections ranging from systemic and potentially fatal diseases to localized cutaneous, subcutaneous or mucosal infections [1]. This trend has also been attributed to the increasing use of cytotoxic and immunosuppressive drugs to treat both malignant and nonmalignant diseases. Although it appears to be a great array of antimicrobial and antifungal drugs, there is at present a

quest for new generations of antimicrobial and antifungal compounds due to the low efficacy, side effects or resistance associated to the existing drugs. Based on the knowledge that plants develop their own defense against microbial and fungal pathogens [2], they appear as an interesting source for antimicrobial and antifungal compounds.

Medicinal plants are the 'back bone' of traditional remedy. The traditional medicine related to treatment of both human and animal diseases with plant-derived preparations is considered a valuable knowledge for the discovery of new antimicrobial and antifungal drugs [3]. Ethno medical literature contains a large number of plants including, *Calotropis gigantea* that can be used against diseases, like diabetes, atherosclerosis, ischeamic heart disease, disorders induced by free radicals and other reactive oxygen species. India is very rich in natural resources and the knowledge of traditional medicine and the use of plants as source of new drugs is an innate and very important component drug discovery. *Calotropis gigantea* is a xerophytic, erect shrub, growing widely throughout the tropical and subtropical regions of Asia and Africa. Plants contain many biologically active molecules with different medicinal properties [4,5]. It is popularly known because it produces large quantity of latex and known as milkweed or swallowwort. Latexes are source of various biologically active compounds, including glycosides, tannins and many proteins, among others [6]. Plants endowed of antidiabetic properties provide useful sources for the development of drugs in the treatment of diabetes mellitus from ancient times; *Swarnabhasma*, an Ayurvedic preparation containing *Calotropis gigantea* is extensively used by Ayurvedic physicians for treatment of disorders such as diabetes mellitus, bronchial asthma, rheumatoid arthritis, and nervous disorders. Tribal people were using this plan parts to cure several illnesses such as toothache, earache, sprain, anxiety, pain, epilepsy, diarrhoea and mental disorders. However, very little information is available about many useful medicinal herbs as experimental data. Previous work, using different parts of the plant has advocated its use for a variety of disease conditions in addition to the application as an antidote for snake poisoning. *Calotropis gigantea* has the following potential pharmacological properties; wound healing [7], antidiarrhoeal [8], CNS depressant activity [9], antipyretic and analgesic [10,11], anti-inflammatory [12], analgesic activity in Eddy's hot plate and acetic acid-induced writhings [13]. The alcohol extract of the flower of *Calotropis gigantea* reported analgesic activity in chemical and thermal models in mice. The roots of *Calotropis gigantea* have been used in leprosy, eczema, syphilis, elephantiasis, ulceration, and cough in the Indian system of traditional medicine. It contains alkaloids, tannins, phenols and resins [14]. Tetra and pentacyclic triterpenoids, cardiac glycosides [15]. Its use in hepatitis has been illustrated in Indian System of Medicine. The folks and Vaidyas have clinically used it successfully. But no studies have been reported on phytochemical analysis and antimicrobial efficacy yet. This motivated the biological evaluation of antimicrobial activity of crude plant extracts.

EXPERIMENTAL SECTION

Collection of plant:

The leaves, roots, root bark and fruits of *Calotropis gigantea* were collected from Madhav Institute of Technology and Science, Gwalior, campus. They were further identified for physical characteristics of flower, leaf, root, and fruit morphology in Department of Botany, Jiwaji University, Gwalior (India).

Plant powder and extract preparation:
500gm of leaves, roots, root bark and fruits were taken and dried under shade for 15days. The dried plant material was crushed into fine powder by help of grinder and stored for required purpose. 5gm of the plant parts powder was dissolved in 45ml of solvent (n-hexane, ethanol, methanol, petroleum ether, distilled water, chloroform and ethyl acetate) to prepare 10% extract in 200 ml flask. The flask was covered with the aluminum foil and kept on rotating shaker (120 rpm) for 2 days. The solution was filtered twice, firstly with cheese cloth (four fold) and then with Whatman's filters paper. The filtrates were collected in Falcon tubes and were concentrated upto dryness by keeping it in incubator at 35ºC**.** The stock solution of each extract was prepared in Dimethylesulfoxide (DMSO).

Phytochemical screening:
Specific qualitative tests were performed to identify bioactive compounds of pharmacological importance through standard methods.

Test for Alkaloids (Mayer's test): 2.0ml of extract was measured in a test tube to which picric acid solution was added. The formation of orange coloration indicated the presence of alkaloids.

Test for Cardiac glycosides (Keller-Killani test): 5ml of each plant part extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer which shows the presence of Cardiac glycosides.

Test for anthraquinines: To the test substance, sodium hydroxide was added. Blue green or red color indicates the presence of Anthraquinone.

Test for tannins: The substance (extracts) mixed with basic lead acetate solution. Formation of white precipitate indicates the presence of Tannins.

Test for Saponins: Froth test for saponins was used. 1g of the sample was weighed into a conical flask in which 10ml of sterile distilled water was added and boiled for 5 min. 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 stopped and shaken vigorously for about 30 second. 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 were added to a portion of the aqueous filtrate of each plant part extract followed by addition of concentrated H_2SO_4. Formation of yellow color observed in each extract indicated the presence of flavonoids.

Test for steroids: One gram of the test substance (plant extracts) was dissolved in a 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 indicates the presence of Steroids.

Test for Terpenoids (Salkowski test): 5ml of each plant part extract was mixed in 2 ml of chloroform, and concentrated H_2SO4 (3 ml) was carefully added to form a layer. Formation of reddish brown coloration at the interface shows the positive results for presence of terpenoids.

Test for reducing sugars: One gram of the aqueous extract was weighed and placed into a test tube. This was diluted using 10 ml of de-ionised distilled water. This was fallowed by the addition of Fehling's solution. The mixture warmed to 40ºC in water bath. Development of brick-red precipitate at the bottom of the test tube was indicative of the presence of a reducing sugar. Same procedure was repeated using dimethylsulphoroxide (DMSO) as the diluent for the ethanolic extract.

Test for resins: Two grams of the ethanolic extract was dissolved in 10ml 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.

Assay for Antibacterial activity

Determination of concentration of test strains: The total count (concentration) of test bacteria strains were determined 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 as:

Scale	1% $BaCl_2$ (ml)	1% H_2SO_4 (ml)	No. of bacterial value *10^7
1.	0.1	9.9	300
2.	0.2	9.8	600
3.	0.3	9.7	900
4.	0.4	9.6	1200
5.	0.5	9.5	1500
6.	0.6	9.4	1800
7.	0.7	9.3	2100

The turbidity of overnight broth culture of test organism was compared with McFarland Standard scale tubes against white background and concentration was approximated as per the above table.

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

B. cereus, *B. subtilis*, *E. coli*, *K. pneumonia*, *M. luteus*, *S. aureus* and *S. typhi* were used as test organisms. The strain cultures were collected from Department of Biotechnology, Jiwaji University Gwalior. The test strains were sub-cultured in Muller Hinton Agar (MHA) (Himedia). The well-grown bacterial colony in MHA plate were picked and sub-cultured in Nutrient broth (Muller Hinton Broth) and incubated for 24 hrs at 37^0C and further stored at 4^0C for required purpose.

The Kirby's Disc Diffusion method was used to find antibacterial efficacy of selected plant. 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. The circular wells were prepared in each plate with the help of microtip (diameter 6mm) and 20µl of each solvent extract (prepared in DMSO) of plant were added. The petri-plates were inoculated and incubated at 37^0C and observed for possible antibacterial efficacy [expressed as mean value of inhibition

diameter (mm)] of various plant extracts prepared in organic/inorganic solvents. The experiment was performed in triplicate.

Minimum inhibitory concentration:
Minimum inhibitory concentration (MIC) is defined as the lowest concentration of phytochemical (or drug) which inhibits the visible growth of bacterium in liquid medium. The MIC values were determined according to the NCCL guidelines by double dilution method in the range of 2-0.25mg/ml plant extracts.

RESULTS AND DISCUSSION

Phytochemical analysis of bioactive compounds in different solvent extracts of *Calotropis gigantean:*
The plant extracts in different solvents were screened for the presence of various bioactive phytochemicals. The analysis revealed the presence of cardic glycosides, saponins, flavonoids, steroids and terpenoids in most prominent amount while alkaloids and tannins in less amount. Anthraquinone is not present in none of the extracts in various solvents *viz.* n-hexane, ethanol, methanol, water, chloroform and ethyl acetate of *Calotropis gigantea*. These were documented in Table 1. Resins present in aqueous solution only.

Antibacterial and Minimum inhibitory concentration efficacy analysis of different solvent extracts:
Antibacterial efficacy in terms of zone of inhibition (ZOI) of crude plant extracts (10mg/ml) in different solvents of *C. gigantea* tested against pathogenic organisms (Table 2). The antibacterial potency of *C. gigantean* extract was evaluated by the presence or absence of inhibition zones and zone diameters (mm). From the results, it is evident that the ethyl acetate extract of *C. gigantea* extract showed a maximum inhibitory zone. The bacterial strains *B. cereus* and *S. aureus* were susceptible to plant extracts with zone of inhibition (≥20 mm) diameter respectively. The minimum inhibitory concentrations (MIC) of the leaves extract 10mg/ml in different solvents on test organisms were shown in Tables 3. Results of MIC are reported in Table 3. The extract showed 0.25 to 1.0 mg/ml MIC value for ethyl acetate, but in case of the ethanol these vlues varies to 0.5 mg/ml to 2.0 mg/ml MI value for *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumonia*, *M. luteus*, *S. aureus* and *S. typhi* respectively. Methanol extract has value of MIC in range of 0.5 to 2.0 mg/ml.

Almost all the parts of *C. gigantea* have been documented to possess medicinal virtues in ethnobotanical surveys conducted by researchers in India. A critical analysis of literatures on *C. gigantea* have shown the interesting fact that crude latex and its various organic and aqueous extracts possesses an array of multidimensional pharmacological activities viz., antidiabetic, anti-inflammatory, antioxidant, anthelmintic, anticandidial, hepatoprotective, antiarthritic, wound healing and cytotoxic effects. Other parts of plant, roots and flowers have also shown promising biological activities such as hepatoprotective, anticonvulsant, antitumour, antiasthmatic and analgesic.

Table 1: Phytochemicals screening in the different organic plant extracts of *Calotropis gigantean*

Phytochemical Compound	Organic Solvent Extracts of plant					
	n-Hexane	Ethanol	Methanol	Water	Chloroform	Ethyl Acetate
Alkaloids	+	+	+	+	+	+
Cardic Glycoside	++	++	++	++	++	++
Anthraquinone	-	-	-	-	-	-
Tannins	+	+	+	+	+	+
Saponins	++	++	++	++	++	++
Flavnoid	++	++	++	++	++	++
Steriods	++	++	++	++	++	++
Terpeniods	++	++	++	++	++	++
Reducing Sugars	++	+	++	-	+	+
Resins	-	-	-	++	-	-

+ means Present; ++ means Most Prominent; - means absent

Table 2: Antibacterial efficacy (zone of inhibition) of the extract of *C. gigantea* on test organisms
(diameter in mm)

Test Organisms	Zone of Inhibition (mm, diameter)					
	Plant extraction solvents					
	n-Hexane	Ethanol	Methanol	Chloroform	Water	Ethyl acetate
Bacillus cereus	12	15	18	10	07	20
Bacillus subtilis	07	17	13	09	10	17
Escherichia coli	09	14	13	14	06	13
Klebsiella pneumoniae	06	11	10	10	08	19
Staphylococcus aureus	10	10	16	12	10	26
Salmonella typhi	06	12	15	10	06	19
Micrococcus luteus	07	10	09	09	10	21

Table 3: The minimum inhibitory concentrations (MIC) of Ethyl acetate extract (mg/ml) of *C. gigantea* on test organisms

Plant Extracts (mg/ml)	Minimum inhibitory concentrations (MIC) mg/ml						
	Bacillus cereus	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Salmonella typhi*	*Micrococcus luteus*
Ethyl acetate	0.5	1	1	1	0.25	1	0.5
Ethanol	0.5	1.5	1	1.5	2.0	1.5	2
Methanol	0.5	1.5	1	1.5	0.5	2	2

CONCLUSION

The phytochemical analysis revealed the bioactive compounds which are responsible for the In vitro antibacterial of *C. gigantea* over all bacteria strains in all extracts could be alkaloids, cardiac glycoside, tannins, saponins, flavnoid, steriods, terpeniods reducing sugars and resins.

A serious problem was observed to human health when the microorganisms were found to resistant to the antibiotics and recently, this problem has become more evident since most of the organisms exhibit some degree of resistance to the commonly available antimicrobial and chemotherapeutic agents. Moreover, powerful drugs against which antimicrobial resistance has not yet been developed are unavailable or very costly which is not affordable to mass people of developing countries including India So, there is a crying need for alternative treatments of different types of diseases.

Since other parts exhibits multidimensional pharmacological activities, in future, the study on leaves extract can reveal some of these properties and would be useful to mankind. The observed antibacterial activities of leaves extract in different solvents justify the traditional use of this plant against several antibacterial infections.

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