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RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY OF *CALOTROPIS* ON THE RHIZOSPHERE BACTERIA

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ABSTRACT

Calotropis has been ethnomedicinally utilized from ancient times. *Calotropis* leaves were collected from the Campus Playground of Urumu Dhanalakshmi College, Trichy and authenticated through the Rapinat Herbarium, St.Joseph's college, Trichy. *Calotropis* leaves were collected dried, powdered and extracted with 80% methanol. The Rhizosphere bacteria of *Calotropis* was isolated and identified based on their morphological and biochemical characters. Plant extract was tested against the rhizosphere bacteria of *Calotropis* (*Pseudomonas aeruginosa*, *Eschercia coli*, *Staphylococcus aureus*, *Bacillus subtilis*) for the antimicrobial activity in Muller – Hinton agar medium at different concentrations.

INTRODUCTION

Plants, as extracts and in various other forms, are being used for centuries in different traditional systems of medicine for the treatment of human ailments, particularly those caused by pathogenic bacteria, fungi as well as viruses. Their use against plant pathogens, though a relatively recent practice, has gained momentum due to the well-known problems associated with the use of synthetic pesticides for the purpose. Use of plant products for the control of human and plant diseases has certain advantages, for instance, the plants are generally readily available, production costs are low, highly acceptable by people and above all the products are biodegradable. The effective plant constituents can combat human and plant pathogenic bacteria, fungi and viruses without toxic side effects and environmental hazards. It is because of these reasons that search for plant products having antimicrobial properties has intensified in recent years (Ray *et al*., 2004). Innumerable studies have been reported on various plants against different microbes. In nature there are large numbers of different types of antimicrobial compounds that play an important role in the natural defense of all kinds of living organisms. The antimicrobial compounds are generally secondary metabolites of various types and origin that are ubiquitous among higher plants (Jonathan and Walton, 2001). According to Oudhia *et al*. (1997) the *Calotropis* plants and their parts are used to treat many diseases. Similarly the leaf and stem extract of *Calotropis* affect germination and seedling vigor of many agricultural crops. Rasik *et al*. (1999) reported that *Calotropis* was used in ayurvedic system of medicine based on its wound healing potential. The latex augmented the wound healing process in guinea pig markedly increasing collagen, DNA and protein synthesis and epithelisation leading to reduction in wound area.

The plant root bark is an emetic, the flower a digestive, in a tonic and used for asthma and catarrh. Bark and wood stimulate lactation in cattle. Roots (extremely poisonous) are applied for snake bite. The milky sap is used as a rubefacient and is also strongly purgative and caustic. The latex is used for treating ringworm, guinea worm blisters, scorpion stings, venereal sores and ophthalmic disorders, also used as a laxative. Its use in India in the treatment of skin diseases has caused severe bullous dermatitis leading sometimes to hypertrophic scars. The local effect of the latex on the conjunctiva is congestion, epiphora and local anaesthesia. The twigs are utilized for the preparation of diuretics, stomach tonic, antidiarrhoetics and for asthma. The twigs also are used in abortion, as an anthelmintic, for colic, cough, whooping cough, dysentery, headache, lice treatment, jaundice, sore gums and mouth, toothache, sterility, swellings and ulcers. Rajesh *et al.* (2005) reported that the latex of *Calotropis* is a rich source of useful components, which have medicinal properties especially in controlling bleeding. The crude latex protein is having strong proteolytic activity. The extract hydrolyses casein to clot the bleeding. The latex promotes coagulation and wound healing process. Compounds derived from the plant have been found to have emetocathartic and digitalic properties. The principle active medicinals are

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asclepin and mudarin. Other compounds have been found to have bactericidal and vermicidal properties. The latex contains a proteolytic enzyme called calotropaine. An infusion of bark powder is used in the treatment and cure of leprosy and elephantiasis. It is inadvisable to use bark that has been kept for more than a year (Rasik *et al.,* 1999). But this study focuses only upon the recent investigations against the 4 microbial species *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa and Escherichia coli* that have been identified to be present in the rhizosphere of *Calotropis*.

MATERIALS AND METHODS

Isolation and Identification of Rhizosphere bacteria

The Rhizosphere soil sample of *Calotropis* was collected in a sterile polythene bag and brought to the laboratory. 10g of soil sample was weighed aseptically, added to 100ml of sterile distilled water, and was shaken for 15 minutes on a mechanical shaker [REMI-RS 24 BL]. After shaking, serial dilutions were prepared (10^{-2} to 10^{-6}), and 0.1 ml of each dilution was transferred to sterile Petri plates having nutrient agar medium in 6 replicates [Streak plate method] (Sabitharani *et al.,* 2003). After inoculation the plates were incubated at 37^0C for 24 hours. The bacterial colony morphology, consistency, pigmentation, margin and elevations were carefully examined. The plates were observed for the growth of pure colonies. Based on the morphological character and their biochemical reactions the bacterial genera were identified.

Plant material

The healthy mature *Calotropis* leaves were washed with tap water followed by distilled water several times, dried under shade and hot air. The air dried material was powdered and stored in a airtight glass container.

Preparation of extracts

The plant extract was prepared by the method of Alade and Irobi (1993) with minor modification. The air dried powdered leaf material was extracted with 80% methanol twice constantly. The extract was filtered through Whatman No.1 filter paper. The filtrate was further concentrated in a vacuum rotary evaporator. The residue was weighed and stored at 4^0C until further use.

Test organisms

Four test organisms *P.aeruginosa, E.coli, S.aureus and B.subtilis* were obtained from the rhizosphere area of *Calotropis* plant by serial dilution technique. The organisms were subcultured in their appropriate media. Based on the gram reaction and the biochemical tests the organisms were identified and confirmed.

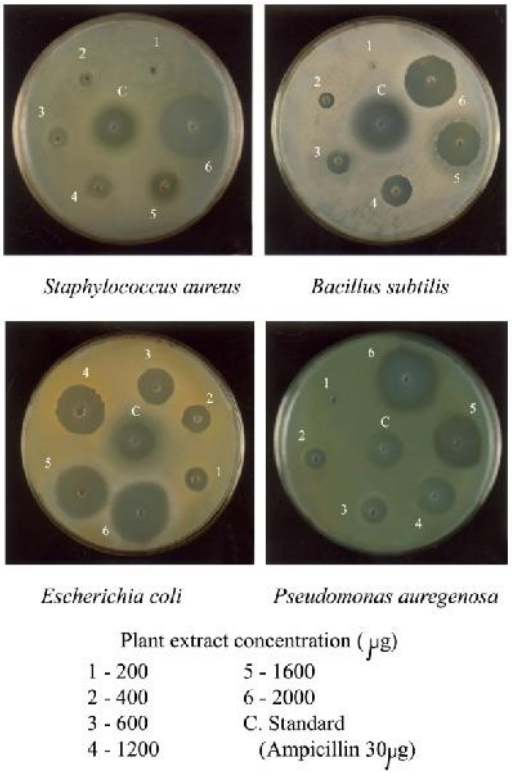
Culture media

The tested strains were subcultured twice for activation in Erlenmeyer flasks (125ml) containing 25ml of Muller Hinton broth (NCCLS, 1982) at 37^0C for 24 hours. Optical density was determined at 600nm. The culturing was stopped at 0.8 to 1.0 OD_{600} range (equivalent to 10(CFU) and used for antimicrobial testing).

Screening of antibacterial activity

The agar well diffusion method (Perez *et al.,* 1990) was adopted for this assay. An amount of 23g of Muller – Hinton agar was suspended in 1000ml of distilled water and dissolved completely. The medium was autoclaved at 15 psi for 20 minutes. The medium was poured into petri plates under aseptic conditions in a laminar air flow chamber and left to solidity. These petri plates were inoculated with 0.1 ml of 24 hour old cultures of concentrated test organisms. After inoculation cups were scooped out with 3mm wide sterile cork borer and the lids of the dishes were replaced. To each cup, 20µl sample (plant extract suspension) of different concentrations (200-2000 µg) was added (Oncar *et al.,* 1995). Antimicrobial activity was evaluated by measuring the zone of inhibition against each test organism, (Minimum inhibitory Concentration test (Rios *et al*., 1988)). The plates were incubated at 37^0C for 24-48 hours. After the incubation period, the diameter of the inhibition zone of each well was measured. Triplicates were maintained in each extract and the average values were calculated for the eventual antimicrobial activity. Simultaneously the standard antibiotics (Ampicillin 30 µg) were tested against the bacteria.

Plate: Antibacterial activity of *Calotropis gigantea* R.Br.



RESULTS AND DISCUSSION

Several medicinal plants have been tried against several pathogenic microorganisms to show their inhibitory activities. The medicinal properties of *Calotropis* have instigated the

determination of the antimicrobial efficacy on the 4 different rhizosphere microbial taxa. In the present study different concentrations of alcoholic *Calotropis* leaf extracts (200, 400, 600, 1200, 1600 and 2000 µg/ml) were tested against the microbial strains namely *S. aureus, B.subtilis*, *P. aeruginosa* and *E. coli* isolated from rhizosphere area of *Calotropis*. The zones of inhibition for the microorganisms by the plant extract were seen. In *S. aureus*, the low concentration of plant alcoholic extract (200µg/ml) did not create any zone of inhibition. As the concentration increased, the susceptibility of *S. aureus* also increased (i.e., the zone of inhibition increased). The maximum zone of inhibition (28mm) was observed in the highest experimental concentration (2000 µg/ ml) which is the only one mm above the effect of standard antibiotic (Figure 1). The susceptibility of *B. subtilis* to *Calotropis* extract was identical to that of standard antibiotic (ampicillin) at 1600 µg/ml (Figure 2). The higher experimental concentration (2000 µg/ml) produced a zone of inhibition which measured 22 mm in diameter. This is higher than that of the standard. Even low concentration of plant extract (200 µg/ml) inhibited the growth of *E. coli* (zone of inhibition 10 mm diameter). A maximum zone of inhibition of 32mm was observed at 2000 µg/ml (Figure 3). *P. aeruginosa* was susceptible to all the experimental concentrations of the plant extract. At 200µg/ml of extract the zone of inhibition was 5 mm. The highest response was observed in the highest concentration (2000µg/ml) (Figure 4).

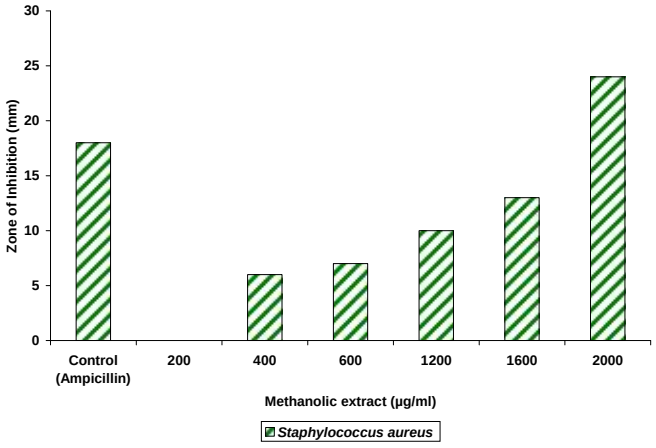


Fig. 1. Antibacterial activity of *Calotropis* on *Staphylococcus aureus*

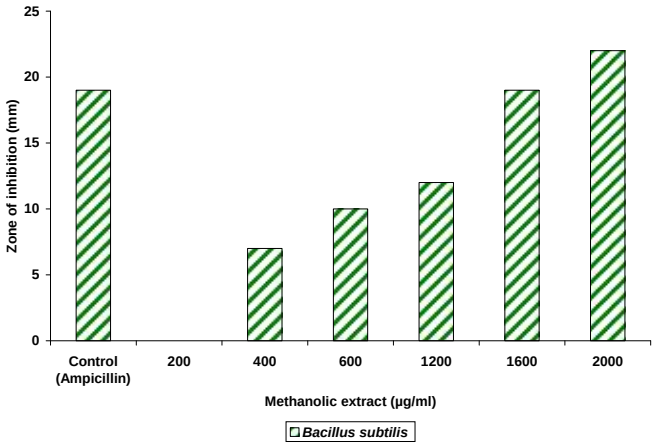


Fig. 2. Antibacterial activity of *Calotropis* on *Bacillus subtillis*

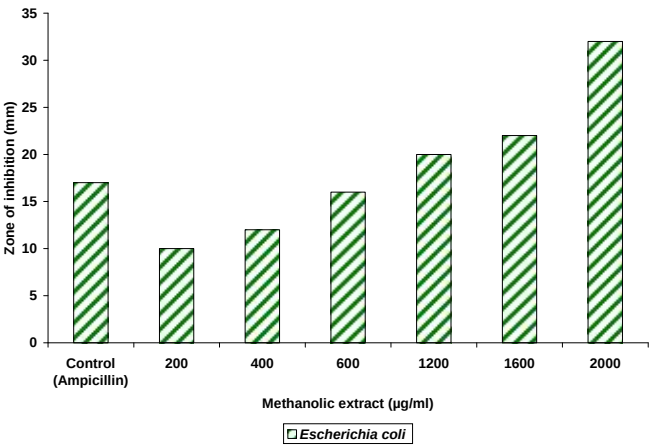


Fig. 3. Antibacterial activity of *Calotropis* on *Escherichia coli*

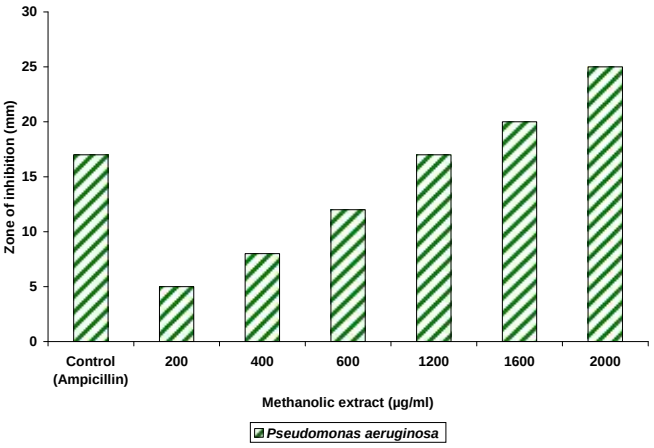


Fig. 4. Antibacterial activity of *Calotropis* on *Pseudomonas aureginosa*

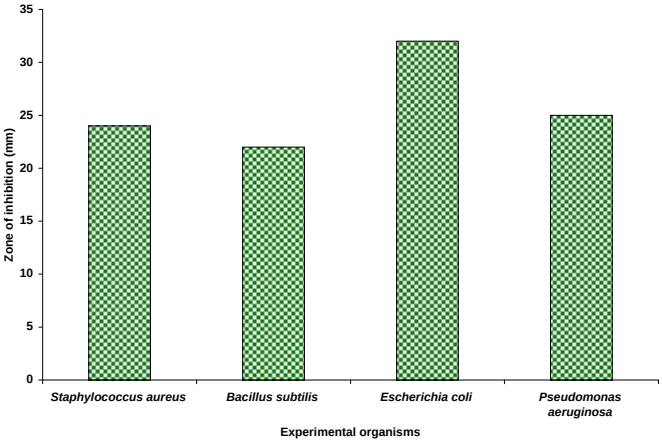


Fig. 5. Relative impact of the plant extract (Highest experimental concentration) on various bacteria

The relative impact of the plant extract at the highest concentration (2000 µg/ml) can be viewed in Figure 5. Out of 4 test organisms *E. coli* exhibited the highest zone of inhibition followed by *P. aeruginosa*. The minimum reaction was exhibited by *B. subtilis*. Hence the susceptibility of the experimental concentration is dissimilar.

In order to confirm the differential susceptibility of different bacterial taxa, the zones of inhibition were compared with that of the standard antibiotic (ampicillin). Table 1 provides a comparative analysis of a standard antibiotic with that of the higher reactive concentrations (1200-2000 µg/ml) of the plant extract. Only at the highest experimental concentration (2000 µg/ml) the impact of plant extract exceeded that of the standard antibiotic in *S. aureus*. The 1600 µg/ml concentration the impact was identical to that of the antibiotic and more at 2000 µg/ml in *B. subtilis.* The other two organisms exhibit equal and higher zone of inhibition even at 1200 µg/ml.Out of the two, *E. coli* is more susceptible than *P. aeruginosa*. Thus the susceptibility sequence may be *E. coli* > *E. aeruginosa* > *B. subtilis* > *S. aureus*. When a statistical correlation analysis was performed between the zone of inhibition and concentration of plant extract, variation is exhibited in the degree of correlation. The maximum correlation was observed in *P. aeruginosa* and minimum in *S. aureus*. Eventhough *E. coli* expressed maximum zone of inhibition all through the experimental concentrations, only *P. aeruginosa* exhibits a highest correlation value between the concentration and zone of inhibition. This could be because of the uneven jump in the values for *E. coli* when compared to those for *P. aeruginosa* (Table 2). The differential response of the four experimental microbial taxa could be because of the complexity in biochemicals.

Earlier Mueen Ahmed *et al*. (2005) have reported the presence of antimicrobial compounds *viz.*, frugoside, calotropin, uscharin, calotoxin and calactin in *Calotropis.* In this study catechin (flavanoid), γ-terpinene (terpenoid), stigmasterol (steroid) and histamine (alkaloid) have been detected in *Calotropis*. The impact of these different compounds could be quantitatively varying in the different bacterial taxa with different morphological and biochemical features. The morphological and biochemical variations of these four taxa are listed out in Tables 3 and 4. The antibacterial activity of *Tagetes miunta* flavonoids has been reported against *S. aureus* and *Staphylococcus epidermis* (Mori *et al.*, 1987). The flavonoids induce antioxidant enzymes to produces cytotoxic effects (Middleton and Kandasamy, 1993). Since *Calotropis* contains the flavonoid it could be responsible for the antibacterial activity. Similarly the roots of *Agrimonia polisa* possess the flavonoid catechin expressing antibacterial activity against *S. aureus* (Shizuo Kasai *et al.*, 1992). *Paullinia cupana* (Guarana) seed with catechin (flavonoid) also showed antibacterial activity against *E. coli*, *P. aeruginosa* and *B. subtilis* (Lucija Majhenic, 2007). *Calotropis* contains steroidal compounds *viz*, protosterol, and stigmasterol (Kahn and Malik, 1989). The root of *Salvia jaminiana* containing stigmasterol and β-sitosterol, remarkably inhibited the growth of *B. subtilis* and *S. aureus*. Similarly the *Calotropis* crude extract containing stigmasterol could have inhibited the growth of *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis.* Medeiros *et al*. (2003) reported, the presence of terpenoids in

Table 1. Relative impact of plant extract with reference to antibiotic (Ampicillin)

Sample (µg/ml)		Zone of inhibition (mm)			
		Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
Ampicillin	30	18	19	17	17
Plant crude extract concentration	1200	-	-	20	17
	1600	-	19	22	20
	2000	24	22	32	25

Table 2. Correlations with concentration of *Calotropis* extract

Organisms	Correlation value	Statistical inference	Significance
Staphylococcus	0.918	P<0.05 Significant	.028
Bacillus	0.975	P<0.01 Significant	.005
E.coli	0.968	P<0.01 Significant	.001
Pseudomonas	0.991	P<0.001 Significant	.000

Table 3. Morphological Characters of bacteria in the rhizosphere of *Calotropis*

S.No	Characters	Staphylococcus aureus	Bacillus subtilis	Escherichia coli	Pseudomonas aeruginosa
1	Gram Reaction	Gram positive	Gram positive	Gram Negative	Gram Negative
2	Morphology	Cocci	Rod	Rod	Rod
3	Motility	Non-motile	Motile	Motile	Motile
4	Spore	Non-sporulated	Spore producer	Non-sporulated	Non-sporulated
5	Capsule	Non-sporulated	Spore producer	Non-sporulated	Non-sporulated

Table 4. Biochemical characters of bacteria in the rhizosphere of *Calotropis*

S.No	Biochemical Reaction	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
1	Indole	-	-	+	-
2	Citrate	-	+	-	+
3	Urease	-	Variable	-	-
4	Triple sugar iron	Acid No gas	Acid, No gas	Acid + Gas producer	+
5	Methyl Red	+	-	+	-
6	Voges Proskauer	-	+	-	-
7	Oxidase	-	+	-	+
8	Coagulase	+	+	-	+
9	Catalase	+	-	+	+
10	Carbohydrate fermentation reaction	+	-	+	-

Hedychium gardneriamum and *Pittosporum undulatum* having a strong antibacterial activity against *S. aureus* and *P. aeruginosa*. The broad spectrum antibacterial activity of *Calotropis* would be because of its γ-terpinene. In *Calotropis* the presence of cardenolide, 'proceragenin' has been reported to express antibacterial activity by Larshini *et al*. (2001). Akhtar *et al.* (1992) reported that tobacco stem containing histamine inhibited *Micrococcus luteus* and *Campylobacter pylori*.

Conclusion

The Rhizosphere soil of Calotropis contains four different bacterial genera *Pseudomonas aeruginosa*, *Eschercia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and the susceptibility of the rhizosphere bacteria to the plant extract is as follows: *Eschercia coli* > *Pseudomonas aeruginosa* > *Bacillus subtilis* > *Staphylococcus aureus.*

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