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Original Article

EVALUATION OF PHYTOCHEMICAL AND ANTIBACTERIAL PROPERTIES OF *CALOTROPIS PROCERA* (AIT) R. BR. LEAVES

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ABSTRACT

Objective: The correlation of phytochemical and antibacterial attributes of *Calotropis procera* leaves with an intent to understand its potential for application in different clinical investigations.

Methods: The different solvent extracts, methanol, ethyl acetate, ethanol, acetone and aqueous extract of the *C. procera* leaves were subjected to qualitative estimation of phytoconstituents. The antibacterial effect of these extracts was studied against human pathogenic bacterial strains viz., *Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis* and *Micrococcus aureus* in Mueller-Hinton agar using well-diffusion assay. The minimum inhibitory concentration (MIC) was equally determined using serial dilution method.

Results: The phytochemical studies revealed the presence of alkaloids, tannins, saponins, flavonoids, steroids, terpenoids, reducing sugars, glycosides. The extract showed significant antibacterial activity against all the tested organisms, though it inhibited the growth of *Micrococcus aureus* more effectively (maximum zone of inhibition: 21 mm); was least effective against *Pseudomonas aeruginosa* (minimum zone of inhibition: 12 mm). The minimum inhibitory concentration (MIC) values of aqueous and organic solvent extracts varied from 5-20 mg/ml.

Conclusion: The results suggest that since the leaves of *C. procera* possess significant antibacterial properties and contain phytoconstituents, it can be potentially exploited for the development of novel chemotherapeutic agents.

Keywords: *Calotropis procera,* Phytochemical, Antibacterial, Minimum Inhibitory Concentration.

INTRODUCTION

Indiscriminate and random use of the available chemotherapeutic agents against different pathogens has resulted in selection of resistant microorganisms which are alarming for medical practitioners. Consequently, it has become imperative to search for alternative chemical agents who would demonstrate very low toxicity to humans, but effective against different etiological agents of different diseases from natural sources such as plants, algae and animals [1]. Plants have co-evolved with pathogens; they understandably have also developed the chemical protection pathways against the parasitic organisms. Therefore, it is reasonable to expect a variety of plant compounds with specific as well as general antibacterial activity and antibiotic potentials [2]. India is among nations which possesses historical record on medicinal plants and has contributed to the knowledge of world's traditional medicine. Nevertheless, the lack of validation, analysis and method of replication has prevented real engagement of this knowledge in diverse applications [3]. More than 8000 species and 40, 000 herbal formulations are largely practiced in India. Thus, conservation of these plants contributes self reliance, for the nation's specific health needs [4]. Depending on World Health Organization (WHO) traditional medicines are relied upon by 65–80% of the world's population for their primary health care needs [5]. It has been estimated that in developed nations such as the United States, ethno pharmacological compounds constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, it is much as 80%. This is a clear indication that the economic importance of medicinal plants is more in rapidly developing nations such as India as compared to many nations of the world [6]. It is believed that plant based drugs does not show other harmful side effects when compared with synthetic antibiotics [7].

Calotropis procera belongs to the family Asclepiadaceae and is a soft wooded, evergreen perennial shrub. It is a xerophytic erect shrub, bearing purple spotted pink scented flowers [8]. It is noted in most parts of the tropical world, delivering dry, sandy and alkaline soils. It occurs frequently in Asian lands that include Indonesia, Malaysia, Thailand, China and the Indian subcontinent as wasteland weed [9]. The latex of *C. procera* is used as purgative, while the flower and dried leaves are considered as digestive aids, useful in cough, asthma and anorexia. The root bark is useful in treating skin diseases, intestinal worms, also possesses an analgesic, anticonvulsant, and sedative effect. It is highly recommended in leprosy and different hepatitis. Oil extracted from leaves is very efficacious in treating cases of paralysis. Powdered root bark gives relief in dysentery. Fresh leaves are utilized to relieve rheumatic pains and inflammation in joints [10]. *C. procera* exhibits the presence of alkaloids, cardiac glycosides, anthraquinone, tannins, saponins, flavonoids, steroids, terpenoids, reducing sugars, and resins which are supposed to have significant antibacterial activity. *C. procera* plant contains antidiabetic properties provide useful sources for the development of drugs for the treatment of diabetes mellitus from ancient times [11]. The extracts of *C. procera* extracts possess good larvicide activity against mosquitoes and more studies are indicated to extract the active compounds for future studies and use in mosquito control [12]. The phytochemicals present in *C. procera* extracts has been found to act as antioxidants by scavenging free radicals and thus serve as therapeutic potential [13, 14].

Based on folklore claims, the present study was done to assess the in vitro antibacterial activity of *C. procera* leaves extracts against prominent human pathogenic bacteria by well diffusion method. Besides, leaves extracts has also been qualitatively analyzed for the presence of different phytochemicals using standard test procedure.

MATERIALS AND METHODS

Plant material

C. procera (Ait). R. Br. Bl. (Family: Asclepiadaceae) leaves were harvested from wastelands of Sangli, Maharashtra. The plant material was identified at the field. A voucher specimen was lodged in our laboratory. The authenticity of the plant was tested at the Department of Botany, Smt. K. W. College, Sangli, Maharashtra.

Extraction of plant material

Freshly collected *C. procera* leaves were dried under shade and pulverized [15]. The material, thus obtained was macerated in

distilled water for aqueous extract and in organic solvents-ethanol, methanol, acetone, ethyl acetate in the ratio of 1:10 (w/v). The liquid extract was filtered through Whatman filter paper No. 1 and dried. The obtained mass was weighed, stored in an airtight container and used for further investigations.

Chemicals

Dimethyl sulfoxide (DMSO) along with other reagents of analytical grade was purchased from Merck Ind. Ltd. Nutrient broth (NB), Mueller-Hinton Agar (MHA), Tetracycline disc, Streptomycin disc, Polymyxin-B disc and Gentamycin disc were recruited from Hi-Media, Mumbai, India.

Phytochemical analysis

The general nature of the different phytochemicals like sterols, tannins, proteins, sugars, alkaloids, flavonoids, saponins, terpenoids, and cardiac glycosides was evaluated [16-18].

Test organisms

Six isolates of bacteria used in the study were, *B. subtilis* (NCIM 2045), *M. aureus* (NCIM 2802), *P. aeruginosa* (NCIM 2036), *and E. coli* (NCIM 2832). These cultures were obtained from the National Collection of Industrial Microorganism (NCIM), Pune, Maharashtra. All these cultures were maintained on nutrient agar plates at 4°C.

Positive and negative control

The antibiotic compounds are used as positive control were, Tetracycline (25 µg/disc) for *B. subtilis*, Streptomycin (10 µg/disc) for *M. aureus,* Polymyxin-B (10 µg/disc) for *E. coli* and Gentamycin (30 µg/disc) for *P. aeruginosa.* DMSO was used as a negative control.

Antibacterial assay

Sensitivity of different bacterial strains to various extracts was measured in terms of a zone of inhibition using agar well diffusion assay using Mueller-Hinton agar media [19, 20]. The plates containing Mueller-Hinton agar media were swabbed with 0.2 ml of the inoculums equivalent to McFarland 0.5 ($15x10^7$cfu/ml) turbidity standards by using sterilized cotton swabs. Agar surface was bored by using sterilized gel borer to make wells (7 mm diameter). DMSO was used as negative control.100 µl of the test extracts and 100 µl of negative control were poured in to separate wells. The standard antibiotic disc was placed on the agar surface as positive control. For each bacterial strain, controls were maintained utilizing pure solvents. Plates were incubated at 37 °C for 48 h. Inhibition zones (in mm) were measured after 24-48 h at 37 ^{0}C. Experiment was carried out in triplicates for each test organism and the mean values were computed.

Determination of minimum inhibitory concentration (MIC)

The MIC of extracts was determined by the agar well diffusion assay. Two fold serial dilution of the stock solution was prepared in sterilized distilled water to make a concentration range from 1-20 mg/ml. The zones of inhibition were measured and the result was recorded. The lowest concentration of the extract which showed 12 mm zones of inhibition against the respective organisms was taken as MIC. Experiment was performed in triplicates for each test organism.

Statistical analysis

Results of the experiments are expressed as mean±S. E. M. All experiments were repeated three times. Microsoft excel was used for statistical analysis.

RESULTS

The present investigation provides a comprehensive profile of the phytochemical analysis and antibacterial activity of extracts of an important medicinal plant, *C. procera.*

Phytochemistry

The phytochemical analysis of leaf extracts (methanol, ethyl acetate, ethanol, acetone and aqueous) reveals the presence of alkaloids, tannins, saponins, flavonoids, sterols, terpenoids, cardiac glycosides, proteins and sugars (table 1). Reducing sugars were considered to be absent in the acetone and aqueous extracts.

Table 1: Qualitative analysis of phytochemicals in *Calotropis procera* leaves extracts

Phytoconstituents	Test	Inference				
		Methanol Extract	Ethyl acetate Extract	Ethanol Extract	Acetone Extract	Aqueous Extract
Alkaloids	Mayer's	+	+	+	+	+
Tannins	Ferric chloride	+	+	+	+	+
Saponins	Foam	+	+	+	+	+
Flavonoids	Ferric chloride	+	+	+	+	+
Sterols	Salkowski	+	+	+	+	+
Terpenoids	Salkowski	+	+	+	+	+
Glycosides	Keller Killiani	+	+	+	+	+
Proteins	Xanthoproteic	+	+	+	+	+
Reducing sugars	Fehling's solution	+	+	+	-	-

Key: '+' indicates presence; '-' indicates absence

Table 2: Antibacterial activity of *Calotropis procera* leaves extracts

Bacteria	Zone of inhibition (in mm)					
	Methanol extract	Ethyl acetate extract	Ethanol extract	Acetone extract	Aqueous extract	Positive control
Gram negative						
E. coli	13.0±0.28	11.0±0.14	15.0±0.11	17.0±0.28	13.0±0.11	21±0.20 (Polymyxin)
P. aeruginosa	12.0±0.15	12.0±0.12	13.0±0.17	13.0±0.08	-	20±0.14 (Gentamycin)
Gram positive						
B. subtilis	14.0±0.11	12.0±0.08	13.0±0.11	19.0±0.12	12.0±0.14	24.0±0.17 (Tetracycline)
M. aureus	13.0±0.12	13.0±0.05	12.0±0.11	13.0±0.12	21.0±0.08	22.0±0.08 (Streptomycin)

Key: (-) Indicates no activity; Values are mean±S. E. M (n = 3)

Antibacterial efficacy

The antibacterial efficacy of different solvent extracts of *C. procera* leaves against different Gram positive and Gram negative bacterial strains were significant although the inhibitory activity was strain specific (table 2). Their antibacterial potency was assessed by the presence or absence of inhibition zones (12 mm and above).

The antibacterial studies revealed that, the aqueous extract of *C. procera* showed the highest activity against *M. aureus* (21 mm), indicating its high susceptibility followed by acetone extract against *B. subtilis* (19 mm). The lowest activity was observed against *P. aeruginosa* (12 mm) for ethyl acetate and methanol extract. The aqueous extract of *C. procera showed* the antibacterial activity against Gram positive bacteria like *B. subtilis and M. aureus as* well as Gram negative bacteria like *E.coli.* Acetone and ethanol extracts showed maximum antibacterial activity against both Gram positive and Gram negative bacteria. Ethyl acetate and methanol extracts showed moderate inhibitory effect.

Minimum Inhibitory Concentration (MIC)

All the active extracts were also subjected to determination of MIC. MIC values of different extract of *C. procera* showed varying results (table 3). MIC values were, solvent extracts and strain dependent. Lower MIC values were exhibited by the ethanol extracts against most of the microbial strains, followed by the acetone and methanol. Ethyl acetate extracts exhibited comparatively higher MIC values for 50% of the microorganisms, indicating less effectiveness of this extract. Among the various bacterial strains tested, lowest MIC values were obtained for *B. subtilis* and *E. coli*, indicating that these bacteria were most sensitive to the *C. procera* leaves extracts; followed by *M. aureus and P. aeruginosa.*

Table 3: Minimum Inhibitory concentration (MIC) values of *Calotropis procera* leaves extracts against test bacterial strains using agar well-diffusion method

Solvent	Minimum Inhibitory Concentration (mg/ml)			
	Gram negative bacteria		Gram positive bacteria	
	E. coli	*P. aeruginosa*	*B. subtilis*	*M. aureus*
Methanol	10.0±0.28	15.0±0.16	5.0±0.16	10.0±0.5
Ethyl acetate	10.0±0.16	10.0±0.5	20.0±0.2	20.0±0.28
Ethanol	5.0±0.17	10.0±0.44	5.0±0.2	7.50±0.27
Acetone	10.0±0.28	15.0±0.16	5.0±0.17	10.0±0.28
Aqueous	20.0±0.44	-	10.0±0.28	12.50±0.26

Key: (-) indicates no activity; Values are mean±S. E. M (n = 3)

DISCUSSION

Tannins have been proven to form irreversible complexes with proline rich protein [21] resulting in the inhibition of cell protein synthesis. It is reported that tannins are known to react with proteins to provide the typical effect which is important in the treatment of inflamed or ulcerated tissues [22]. These observations therefore support the use of *C. procera* latex in herbal cure remedies. Flavonoids are in a position to effectively scavenging the reactive O_2 species because of their phenolic hydroxyl groups and so they are potent antioxidants [23] and exhibit a wide range of biological activities like antibacterial, anti-inflammatory, analgesic, anti-allergic, cytostatic properties [24].

Moreover, secondary metabolites such as tannins and other compounds of phenolic nature are also listed as antibacterial compounds. Phenolic compounds are known as potent chain breaking antioxidant. Numerous studies have described the antioxidant properties of medicinal plants which are rich in phenolic compounds [25]. There are indications showing that alkaloids are responsible for the antibacterial activity in higher plants [26].

Consequently, the presence of these pharmacologically active photochemicals justifies the observed antibacterial activity. Low activity of *C. procera* leaves extract against *P. aeruginosa* may be partly excused by some earlier reports that *Pseudomonas species* exhibited strong resistance against a host of antibiotics including plant extracts [27, 28]. Both the assays, antibacterial and MIC supported the sensitivity of Gram positive bacteria. Similar results of susceptibility of Gram-positive bacteria to plant extracts have been already reported [29, 30]. It is expected that Gram positive bacteria are more sensitive to the extracts because of the absence of a lipid layer over the cell wall which is unlike with that of Gram negative bacteria [31, 32]. The high MIC values in the case of some extracts could be due to high resistance rate of the tested bacterial strains.

In case of *C. procera* variations are observed in potential of plant parts screened (leaves, latex, stem, flowers and roots) to inhibit bacterial growth. The antibacterial activity may differ from one plant part to another. The flowers also showed antibacterial activity [33]; followed by roots and stem extracts of *C. procera* [34].

CONCLUSION

This investigation highlights the antibacterial potential of the traditionally important plant, *C. procera*. The results provide an important basis for the use of different extracts of the tested plant species for the treatment of infections, which could be used as an important tool for further development of new antibacterial drugs. The antibacterial activity against both Gram positive as well as Gram negative bacteria, reveals the presence of broad spectrum antibacterial properties of different compounds in the leaves extracts of *C. procera*. The relative antibacterial activity of leaves extracts may not be easily correlated with an individual component but built with a mixture of compounds present in these extracts. The antibacterial mechanisms associated with each group of chemicals to which the isolated compounds belong, may explain the inhibition potency of the tested samples. The antibacterial properties against *M. aureus,* is very significant observation as it is a very common wound infecting organism including nosocomial infections and equally resistant to many of the common medicines used in chemotherapy due to the presence of penicillin-binding protein of high molecular weight and has very low affinity for β-lactam antibiotics. The next wound infecting organism but lesser observed is *P. aeruginosa* which is also notorious for its resistance to the medicines used to treat wounds is also seen to be susceptible to these extracts. An interesting observation is that most of the active crude extracts are almost equally active both against drug resistant and sensitive bacterial strains. Multi target based approaches of screening of medicinal plant extracts and herbal drug is thus expected to yield novel activities.

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CONFLICT OF INTERESTS

We declare that we do not have any conflict of interest.

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