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Antibacterial, antifungal and insecticidal activities of the n-hexane and ethyl-acetate fractions of methanolic extract of the leaves of *Calotropis gigantea* Linn

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ABSTRACT

The study was conducted to evaluate the therapeutic potentials and insecticidal effects of *Calotropis gigantea* leaves against pathogenic bacteria, fungi, and insect by disc diffusion method. Five different species of Gram-positive and Gram-negative bacteria, six species of fungi, and *Tribolium castaneum* (Herbst) insect were used. No antibacterial and antifungal activities were observed for n-hexane fraction of the methanolic extract of *Calotropis gigantea* leaves but the ethyl-acetate fraction showed potential inhibitory effects on all tested bacteria and fungi except *Trichophyton rubrum*. The ethyl-acetate fraction showed significant inhibition of *Escherichia coli*, *Shigella flexneri* and *Shigella sonnei*. The best antifungal activity was recorded for the ethyl-acetate fraction against *Candida albicans*. In insecticidal activity assay, the ethyl-acetate fraction showed better activity with 80% mortality rate at a dose of 50 mg/ml in 48 hours whereas n-hexane fraction showed 40% mortality rate. Both fractions were almost nontoxic to tested insect up to 12 hours. The results established a good support for the use of *Calotropis gigantea* leaves in traditional medicine and insecticide.

Keywords: *Calotropis gigantea* Linn, *Tribolium castaneum* (Herbst), antibacterial, antifungal, insecticidal activity.

1. Introduction

The history of plants being used for medicinal purpose is probably as old as the history of mankind. Extraction and characterization of several active phytocompounds from these green factories have given birth to some high activity profile drugs [1]. Simultaneous with population explosion virulent strains of microorganisms become more common and their increased attack accounts for increased mortality [2]. Ethnopharmacologists, botanists, microbiologists and natural product chemists have been exploring the earth for phytochemicals and "leads" which could be developed for the treatment of infectious diseases. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids which have been found to have in vitro antimicrobial properties. Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians, several of them are already being tested in human and scientists realize that the effective life span of any antibiotic is limited. Second, the public are becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. The substances present in the plants serve as plant defense mechanism against predation by microorganisms, insects and herbivores [3]. Higher plants are rich source of novel natural substances that can be used to develop environmental safe methods for insect control [4]. *Tribolium castaneum* (Herbst) is considered to be a major pest of stored grains. In Bangladesh *Tribolium castaneum* is abundantly found in stored grains of different cereals. Control of these insects relies heavily on the use of synthetic insecticides and fumigants which led to problems such as disturbances of the environment, increasing cost of application, pest resurgence, resistant to pesticides and lethal effect on non-target organism in addition to direct toxicity to users [5-6]. Therefore, it is necessary to develop an alternative biopesticide from plant origin.

Calotropis gigantea Linn (Synonym- *Asclepias gigantea*) belonging to the family Asclepiadaceae is a moderate to large-sized perennial shrub 2 to 3 metres in height abounding in milky latex with opposite decussate oblong auriculate thick leaves, inodorous purplish white flowers and oblong follicles; available in the road in all the tropical and warmed part of India, Malaysia, Pakistan, Philippines, Myanmar, China, Indonesia, Sri Lanka, in the waste lands in all areas of Bangladesh. *Calotropis gigantea* is commonly known as giant milk weed and extracts of roots and leaves are used against abdominal tumors, cancer boils, syphilis, tuberculous leprosy, skin diseases, piles, wounds, rheumatism, and insect bites. Root bark is used in dysentery and as a purgative, alternative diaphoretic and emetic. Powdered flowers are useful in the treatment of colds, cough, asthma, catarrh, indigestion and loss of appetite. Latex is a violent purgative and abortifacient [7-9]. *Calotropis gigantea* is reported to possess alkaloids, cyanogenic glycosides, phenolic tannins, Cardenolides[10-11], flavonoids [12], [13-14], sterols [15], proteinases; calotropain-FI and Calotropain-FII[16], Calotropin- DI and Calotropin- DII[17] and non-protein amino acid Giganticine [18] as major phytochemical groups. So, for the further scientific investigation and for the development of effective therapeutic compounds, the present study was focused to determine the antibacterial, antifungal activity of n-hexane and ethyl-acetate extract of *Calotropis gigantea* Linn leaves against some pathogenic bacteria and fungi using disc diffusion method and insecticidal activity of the same extract against adult *Tribolium castaneum* (Herbst) by surface film treatment, respectively.

2. Materials and methods

2.1. Plant materials collection

The fresh leaves of *Calotropis gigantea* were collected in huge amount on July, 2009 from the village Duari of Rajshahi district of Bangladesh and identified by Md. Arshed Alom, Taxonomist, Department of Botany, University of Rajshahi, Bangladesh where a voucher specimen (No. 1, A. Alom, 15.08.2009) was recorded for future reference.

2.2. Extraction and fractionation

The collected leaves were sun-dried for 10-12 days and then kept in an electric oven for 72 hours at 40 °C. The dried leaves were then pulverized into coarse powder with the help of a grinding machine. The ground plant powder (800 gm) was extracted with methanol (4 litres) in an air tight clean flat bottomed container for 10 days at room temperature with occasional stirring and shaking [19]. The methanolic extract was filtered first through a fresh cotton plug and then through a whatman filters. The filtrate was evaporated to dryness in vacuo by a rotary evaporator at 40-50 °C to afford a blackish green mass (30 gm). Solvent-solvent partitioning was done using the protocol described elsewhere [20-21]. The crude extract was triturated with 90% methanol. The prepared solution was then fractionated successively using solvents of increasing polarity with n-hexane (100 ml×3) and ethyl-acetate (100 ml×3). All the fractions were evaporated to dryness and kept in air tight containers for further analysis.

2.3. Test organisms

Antibacterial activity of leaf extracts were determined against five Gram positive (*Staphylococcus aureus, Streptococcus agalactiae, Bacillus cereus, Bacillus megaterium* and *Bacillus subtilis*) *and* five Gram negative bacteria (*Pseudomonas aeruginosa, Escherichia coli, Shigella flexneri, Shigella dysenteriae, Shigella sonnei*). Antifungal activity was determined against six fungi (*Candida albicans, Aspergillus niger, Aspergillus ochraceus, Aspergillus ustus, Rhizopus oryzae* and *Trichophyton rubrum).* Test organisms were the laboratory stocks of Microbiology Lab, Pharmacy Department, Rajshahi University. Test insect *Tribolium castaneum* (Herbst) were collected from the Crop Protection and Toxicology Lab, University of Rajshahi.

2.4. Growth media and culture conditions

Nutrient agar media (Yeast extract 1%, Peptone 0.5%, NaCl 0.5%, agar 1.5%) purchased from Difco and Sabouraud Dextrose agar media (Enzymatic digest of casein 0.5%, enzymatic digest of animal tissue 0.5%, Dextrose 4%, Agar 1.5%) from Acumedia were used for antibacterial and antifungal activity assay, respectively. The strains were incubated at 37 °C for overnight as described elsewhere [22-23].

2.5. Test for antibacterial activity

In vitro antibacterial activity was carried out on nutrient agar plate by disc diffusion method [24-25]. Both the crude extracts were separately dissolved in 1 ml of their respective solvent and the filter paper discs (6 mm diameter) were impregnated with known amounts of test substances and prepared as 500 µg/disc. Discs were placed on agar plate culture of test organisms by sterilized forceps. Plates were then kept at low temperature (4 °C) for 24 hours to allow maximum diffusion. The plates were then allowed to incubate at 37 °C for overnight. After 18-20 h of incubation, the diameter (mm) of zone of inhibition for each extract against tested microorganisms was noted. Standard antibiotic disc of Kanamycin (30 µg/disc, Hi-media, India) was used as positive control and blank disc impregnated with solvent followed by drying off was used as negative control.

2.6. Test for antifungal activity

In vitro antifungal activity of crude extracts was carried out on Sabouraud Dextrose agar plate by disc diffusion method against six pathogenic fungi at a concentration of 500 µg/disc as described in antibacterial screening section. Standard disk of antifungal agent Clotrimazole (10 µg/disc, Hi-media, India) was used as positive control.

2.7. Insects

Red flour beetles *Tribolium castaneum* were used to test insecticidal activity of leaf extracts of *Calotropis gigantea* Linn. Insects were reared on a diet mixture of whole meal flour with Baker's yeast (19:1) [26]. At every three days the medium was replaced by a fresh one to avoid conditioning by the larvae [27].

2.8. Residual film method of insecticidal activity

For insecticidal activity residual film method was used [28-29]. A preliminary screening of different doses was performed on adult insects to obtain 0 to 100% mortalities. Then 50 mg of each extract was dissolved separately in 1 ml of corresponding solvent and was applied on petri dishes (9 cm diameter) in such a way that it made a uniform film over the petri dishes. For solvent evaporation, the petri dishes were air dried leaving the extract on it. After drying, 15 beetles were released in each petri dish with three replications. A control batch was also maintained with the same number of insects after preparing the petri dish by applying and evaporating the solvent only. The treated beetles were placed

in an incubator at the same temperature as reared in stock cultures and the mortality of the beetles was counted after 0.5, 12, 24, 36, and 48 hour post-exposure [30].

3. Results and discussion

3.1. Antibacterial activity

The crude extracts of n-hexane and ethyl-acetate of leaves of *Calotropis gigantea* were tested against ten pathogenic bacteria for their antibacterial activities at a dose of 500 µg/disc by disc diffusion method. No activity was observed in the case of n-hexane extract but ethyl-acetate extract showed distinct zone of inhibition, 11 to 14 mm for Gram-positive bacteria and 14 to 18 for Gram-negative bacteria (Table 1). The phytochemicals present in ethyl-acetate extract were more effective against Gram-negative bacteria than gram-positive bacteria. In contrast to standard antibiotic, Kanamycin, the ethyl-acetate extract showed a significant inhibition of bacteria. The ethyl-acetate extract showed the highest inhibition of *E. coli* among the tested organisms (Table 1).

Table 1: Antibacterial activities of n-hexane and ethyl-acetate extract of *Calotropis gigantea* leaves.

Bacterial strains tested	Diameter of zone of inhibition (mm)		
	Extract (500 µg/disc) of -		Kanamycin (30 µg/disc)
	n-hexane	Ethyl-acetate	
Gram (+ve)			
Staphylococcus aureus	-	14 ± 0.6	25 ± 1.2
Streptococcus agalactiae	-	12 ± 0.3	23 ± 1.3
Bacillus cereus		13 ± 0.7	23 ± 0.6
Bacillus megaterium	-	12 ± 0.7	23 ± 0.5
Bacillus subtilis	-	11 ± 0.5	22 ± 0.8
Gram (-ve)			
Pseudomonas aeruginosa	-	14 ± 1.0	22 ± 0.9
Shigella flexneri	-	16 ± 0.8	25 ± 1.5
Shigella dysenteriae	-	14 ± 1.1	23 ± 1.4
Escherichia coli	-	18 ± 0.8	25 ± 0.8
Shigella sonnei	-	15 ± 0.5	25 ± 1.1

(-) sign indicates no activity. Values were expressed as mean ± SD (n = 3).

3.2. Antifungal activity

Six fungi were used for antifungal activity test of the crude extracts. All tested fungi except *Trichophyton rubrum* were promisingly inhibited by the ethyl-acetate extract. On the contrary, no inhibition was found by n-hexane extract (Table 2). Among the tested fungi the ethyl-acetate extract was most effective against *Candida albicans*. However, both extracts were ineffective for the inhibition of *Trichophyton rubrum.*

Table 2. Antifungal activities of n-hexane and ethyl-acetate extract of *Calotropis gigantea* leaves.

Fungal strains tested	Diameter of zone of inhibition (mm)		
	Extract (500 µg/disc) of-		Clotrimazole 10 µg/disc
	n-hexane	Ethyl-acetate	
Candida albicans	-	14 ± 0.2	25 ± 0.5
Aspergillus niger	-	12 ± 0.3	23 ± 0.9
Aspergillus ochraceus	-	13 ± 1.0	23 ± 1.4
Aspergillus ustus	-	12 ± 0.8	23 ± 1.1
Rizopus oryzae	-	11 ± 0.6	22 ± 0.8
Trichophyton rubrum	-	-	20 ± 0.4

(-) sign indicates no activity. Values were expressed as mean ± SD (n = 3).

3.3. Insecticidal activity

The ethyl-acetate extract had shown 80% mortality rate of *Tribolium castaneum* (Herbst) at a dose of 50 mg/ml in 48 hours whereas at the same dose and exposure time n-hexane extract had shown 40% mortality rate (Table 3).

Table 3. Insecticidal activities of n-hexane and ethyl-acetate extract of *Calotropis gigantea* leaves.

Solvent used	Extract (mg/ml)	Number of insect used	Number of insects killed at exposure time (hr)					Mortality (%)
			0.5	12	24	36	48	
n-hexane	50	15	-	-	2	5	6	40%
Ethyl-acetate	50	15	-	1	5	10	12	80%

(-) sign indicates no activity.

No mortality was observed in 0.5 hr for both extracts and the substantial insecticidal activity was started after 12 hours. Moreover, like antibacterial and antifungal activities the ethyl-acetate fraction appeared as potential insecticide than n-hexane fraction. The overall results of this study indicated that the mortality caused by each sample was increased with the increasing of exposure time. This finding indicated that the exposure time played an important role in influencing susceptibility.

In this study the ethyl-acetate fraction of crude methanolic extract of *C. gigantea* leaves showed prominent inhibitory activity against all tested pathogenic bacteria whereas n-hexane fraction showed no antibacterial activity. Moreover, both the solvent fractions (n-hexane and ethyl-acetate) of crude methanolic extract had moderate antifungal activities. It was obvious that the components fractionated in the both extracts were active against tested organisms. Antimicrobial activities of tannins [31], flavonoids [32], saponins [33], terpenoids and alkaloids [34] from various plants have already been documented by many researchers. Our result corresponds to that of others. The antibacterial and antifungal activities of the leaves extract of *C. gigantea* should be due to the presence of these phytoconstituents. Killing larvae of *Tribolium castaneum* (Herbst) is a successful way of minimizing these insect densities before they reach adult stage. It extensively depends on the use of synthetic chemical insecticides. But the repeated use of those synthetic chemicals has made common environmental problems and widespread development of drug resistance. Plants offer an alternative source of insect-control agents since they contain a wide range of bioactive chemicals, many of which are selective and have little or no harmful effect on non-target organisms and environment. It is reported that the carbohydrates, saponins, phytosterols, phenols, flavonoids and tannins have mosquito larvicidal activity [35]. Therefore, the probable existence of flavonoids, terpenoids,

phytosterols, and tannins as well as other secondary metabolites in *C. gigantea* leaves can only elicit the toxic effects on the studied insects, *Tribolium castaneum* (Herbst). Here ethyl-acetate extract showed prominent antimicrobial activity rather than the n-hexane extract. However, the results of present investigation clearly indicate that the antimicrobial activities vary with the solvent used for extraction. Thus, the study ascertains the value of plants used in ayurveda, which could be of considerable interest to the development of new drugs.

4. Conclusion

The n-hexane and ethyl-acetate fractions of methanolic extract of *Calotropis gigantea* leaves showed a wide range of antimicrobial activities although some microorganisms were less sensitive against them in comparison with standard antibacterial and antifungal agents. Both extracts also showed insecticidal activities. This medicinal plant can be used for the remedy of infectious diseases caused by pathogenic bacteria and fungi as well as for the control of insect. The isolation of bioactive compound(s) in *Calotropis gigantea* leaves remain to be elucidated.

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