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In vitro studies on antibacterial, antifungal, and cytotoxic properties of *Leucas aspera*

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Abstract

The leaves of the plant *Leucas aspera* belonging to the family Lamiaceae was studied to determine the in vitro antibacterial, antifungal, and cytotoxic properties. All extracts showed remarkable antibacterial activity against all of the studied organisms except *Escherichia coli*. Methanol extract showed stronger activity compared to ethyl acetate and petroleum ether extracts. It showed highest activity against *Pseudomonas aeruginosa* with zone of inhibition of 15 mm. The standard chloramphenicol did not show any activity against *Shigella sonnei.* But all the extracts showed moderate activity against this pathogen with zone of inhibition ranging from 10 to 13 mm. None of the extracts has shown any significant antifungal activity against the fungi. Incase of brine shrimp lethality bioassay, methanol extract showed the strongest cytotoxic effect with LC_{50} value of 4.28 µg/ml which is followed by ethyl acetate extract with LC_{50} value of 5.36 µg/ml. Thus it can be inferred that this plant may be a potential source of novel bioactive compounds.

Keywords: *Leucas aspera*; antimicrobial; antifungal; brine shrimp lethality bioassay.

Introduction

In spite of having extensive advancement in scientific knowledge and medical technology, infectious disease remains the leading cause of morbidity and mortality worldwide (Moellering *et al.*, 2007). Pharmaceutical industries have produced a number of new antimicrobial drugs in recent years but the resistance of these drugs by microorganisms has also increased. Because generally, bacteria have the genetic ability to transmit and acquire resistance to drugs (Nascimento *et al.*, 2000).

Therefore, searching of new antimicrobial agents is more than urgent. Because the infectious diseases caused by bacteria and fungi are rampant and the causative agents are developing an increasing resistance against many of the commonly used antibiotics (Hart and Kariuki, 1998; Abebe *et al.*, 2003; Moellering, 2011). The antimicrobial properties of plants have been investigated by a number of studies worldwide (Iwu *et al.*, 1999; Nascimento *et al.*, 2000) and there has been a revitalization of interest in the development of drugs originated from plants. Different parts like roots, leaves, shoots, and bark could be the source of the plant derived substances. It has been reported that many plants are used in the form of crude extracts, infusions or plasters to treat common infections without any scientific evidence of efficacy (Noumi and Yomi, 2001).

Leucas aspera belongs to the family Lamiaceae is locally known as "Shwetadrone, Dronapushpi, dondocolos, Dulfi", etc. It has been reported to be distributed throughout the South Asia (India, Bangladesh, Nepal), Malaysia, and Mauritius (Press *et al.*, 2000). Traditionally, *L. aspera* has been reported to be used for coughs, cold, painful swelling, and in chronic skin eruptions (Kripa *et al.*, 2011). The leaves are applied to the bites of serpents, poisonous insects, and

scorpion sting. The leaves are also used as insecticides and mosquito repellent in rural area (Reddy *et al.*, 1993). Isolation of several compounds from different parts of *L. aspera* has been reported including a hydroxytetratriacontan-4-one, aliphatic ketones, nicotine, α-farnesene, α-thujene, menthol from leaf volatiles and amyl propionate, isoamyl propionate from flower volatiles, long chain aliphatic compounds, sterols, triterpenes, phenols, flavonoids such as leucasin from extract (Meghashri *et al.*, 2010).

The present study was designed to determine the antibacterial, antifungal, and brine lethality bioassay of the leaves of *L. aspera*.

Materials and Methods

Collection and extraction of sample

The plant *L. aspera* was identified by an expert taxonomist and the requisite plant part (leaf) was collected from Jahangirnagar University campus, Savar, Dhaka, Bangladesh and its surrounding areas. The samples were primarily dried in sunlight and finally dried in an oven at 60°C. The dried samples were ground to coarse powder with a mechanical grinder and then stored in a plastic container. 500 g of ground plant leaf was extracted using Soxhlet extractor at boiling point temperature of petroleum ether, ethyl acetate, and methanol as extraction solvents.

The extracts were passed through No. 1 Whatman filter paper (Whatman Ltd., UK). The filtrates obtained were concentrated under vacuum in a rotary evaporator at 40°C and stored at 4°C for further use. The three extracts were designated as PE (Petroleum ether extract), EA (ethyl acetate extract), and ME (methanol extract).

Organisms, media, and culture conditions

Both gram positive and gram negative bacteria were used. They were: *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella dysenteriae*, *Shigella sonnei*, and *Vibrio cholerae*. Nutrient agar medium was used to demonstrate the antimicrobial activity and for routine bacterial growth. For all experiments, aseptic techniques were followed.

Two yeasts were used in this study, one pathogenic (*Candida albicans*) and the other one is non-pathogenic (*Saccharomyces cerevisiae*). Five moulds, including one human pathogen (*Aspergillus fumigatus*) and four phytopathogens (*Botryodiplodia theobromae*, *Colletotrichum corchori, Curvularia lunata*, and *Fusarium equiseti*) were used for screening purposes. Potato Dextrose Agar (PDA) was used for growing and testing antifungal activity of moulds and yeasts.

Preparation of samples

Test samples were prepared by dissolving 50 mg of each of the PE, EA, and ME in 2 ml of respective solvents. The resulting solution contained 1 mg/40 µl. For antimicrobial screening, sample disc, standard disc, and blank disc were used. For preparation of sample disc, paper discs of 5 mm diameter were made from Whatman filter paper by punch machine and were autoclaved at 121°C for 15 min. 40 µl of extract was applied on the paper discs under aseptic conditions. Blank discs were also prepared using solvents only. Chloramphenicol discs (30 µg/disc) were used as standards.

Antibacterial test

For each of the test organisms, the preculture was taken from stock cultures and was grown in nutrient broth at 37°C for 24 h. 10^6 cells/ml of bacterial solution were spreaded on nutrient agar medium and then was incubated for 4 h at 37°C. Using sterile forceps, both the sample and blanks discs were placed on the marked positions on the seeded petri dishes maintaining an aseptic condition. The standard discs were placed separately onto another set of seeded petri dishes. The plates were kept at 4°C for 24 h to allow sufficient time for the test material to diffuse to a considerable area of the medium. Afterwards, they were incubated at 37°C for 24 h. The resulting clear zones were measured by a transparent scale.

Antifungal test

The anti-mould screening was done by poisoned food technique (Grover and Moore, 1962), while disc diffusion method was followed for anti-yeast screening. 100 mg of each extract was mixed uniformly with 100 ml of sterile PDA to get concentration of 1 mg/ml and immediately poured into petri dishes aseptically. After solidification, 10 mm agar blocks of the test mould were placed in the centre of the treated plate. For each of test moulds, one such plate was prepared. Similarly, another set of plates were prepared using a standard antibiotic clotrimazole at a concentration of 100 µg/ml. A set of control plates were

also prepared using PDA plates alone. All of the plates were incubated at 25°C for 5 days after which the radial growth of fungal colony was measured with a transparent scale in mm and the percentage of inhibition of mycelial growth was calculated using the following equation:

$$I = \frac{C - T}{C} \times 100$$

Here, I = percentage of inhibition, C = diameter of fungal colony in control, T = diameter of fungal colony in treatment.

For anti-yeast activity, same procedure as of antibacterial screening was followed with few exceptions: (i) PDA was used as medium, (ii) plates were incubated at 25°C, and (iii) the antibiotic clotrimazole was employed as standard for comparison.

Brine shrimp lethality bioassay

Shrimp nauplii were grown in seawater in a mini aquarium with standard procedures. The test samples PE, EA, and ME were prepared by dissolving 8 mg of each extract in 120 µl of DMSO to give a concentration of 1 mg/15 µl. 3 × 10 test tubes, each containing 10 ml of seawater and 10 nauplii, were taken for three samples. Serial dilutions were made to prepare 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.781 µg/ml of each extract. Tenth test tube was filled with 60 µl of DMSO, which served as control to observe the effect of DMSO. After 24 h incubation at room temperature, the test tubes were observed for number of dead nauplii. From this, the percentage of mortality of brine shrimp was calculated at each concentration of samples. The LC_{50} (the concentration at which 50% of nauplii died) was determined from the line by plotting the percentage of mortality against the logarithms of respective concentrations.

Results and Discussion

Antibacterial and antifungal activity

All the three extracts were tested for antibacterial activities against eleven pathogenic bacteria including four gram positive and seven gram negative following disc diffusion method. The results are shown in Table 1. In this case, clear zones more than 15 mm diameters were considered as highly susceptible, 8 to 15 moderately susceptible and less than 8 was considered as resistant. All the studied pathogens were found be moderately susceptible to ME extract with a zone of inhibition ranging from 8 to 15 mm. The highest activity was found against *P. aeruginosa* (with zone of inhibition of 15 mm). The EA extract also found to have potential antibacterial activity. It was active against all the bacteria except *E. coli* and *V. cholera*. The highest activity was found against *S. dysenteriae* (with zone of inhibition of 15 mm).

Table 1: In vitro antibacterial activities of *L. aspera* leaf extracts.

	Zones of inhibition (mm)			
Name of bacteria	**ME (1mg/disc)**	**EA (1mg/disc)**	**PE (1 mg/disc)**	**Chloramphenicol (30 µg/disc)**
Gram positive				
Bacillus cereus	10	11	12	35
Bacillus megaterium	13	13	12	43
Bacillus subtilis	12	14	12	34
Gram negative				
Escherichia coli	–	–	–	29
Pseudomonas aeruginosa	15	8	9	28
Salmonella paratyphi	13	11	12	28
Salmonella typhi	13	8	10	41
Shigella dysenteriae	14	15	14	44
Shigella sonnei	10	10	13	–
Staphylococcus aureus	12	9	12	29
Vibrio cholerae	8	–	–	43

–: No activity.

Table 2: In vitro antifungal activities of *L. aspera* leaf extracts.

	Zones of Inhibition (mm)			
Name of fungus	**ME (1 mg/disc)**	**EA (1 mg/disc)**	**PE (1 mg/disc)**	**Clotrimazole (100 µg/ml)**
Yeasts				
Candida albicans	0	0	0	51
Saccharomyces cerevisiae	0	0	0	43
Moulds				
Aspergillus fumigatus	0	6	33	89
Botryodiplodia theobromae	8	9	97	87
Colletotrichum corchori	7	13	2	82
Curvularia lunata	2	4	25	78
Fusarium equiseti	11	18	8	93

From above result, it is evident that methanol extract had a stronger antibacterial activity than the other two extracts. However, the standard antibiotic chloramphenicol showed very strong inhibition against almost all of the test bacteria.

All the three extracts were tested for antifungal activities against five moulds and two yeasts. However, none of the extracts showed any activity against the yeasts tested while the standard clotrimazole showed very strong activity against both yeasts and moulds (Table 2). In addition, the extracts rather stimulated the growth of some moulds.

In a previous study, the aqueous extract of the entire plant of *L. aspera* was evaluated for antimicrobial activity. It has been found that higher range of zone of inhibition was observed for *S. aureus*, *E. coli*, and *P. aeruginosa* (Srinivasan *et al.*, 2001). Except *E. coli*, this result is congruent with the zone of inhibition observed for ME extract of the present study. The difference of the result for *E. coli* may be due to the different solvent used for the extraction and also the fact of using whole plant and part of the plant.

Brine shrimp lethality bioassay

The brine shrimp lethality bioassay (BSLA) is routinely used in the primary screening of the extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay (Meyer *et al.*, 1982; Sam, 1993).

All three extracts were tested for cytotoxicity against brine shrimp nauplii. The LC_{50} values were calculated using the plot of percentage of mortality against log concentration of the extracts. The results are shown in Table 3. The result clearly shows that all of the extracts showed strong cytotoxic activity against brine shrimp nauplii. The methanol extract

Table 3: Brine shrimp lethality bioassay of *L. aspera* leaf extracts.

Sample concentration (µg/ml)	Log concentration	Percentage mortality			LC_{50} (µg/ml)		
		PE	**EA**	**ME**	**PE**	**EA**	**ME**
400	2.602	100	100	100	–	–	–
200	2.301	100	100	100	–	–	–
100	2.000	100	100	100	–	–	–
50	1.698	90	100	100	–	–	–
25	1.397	70	90	90	13.29	5.36	4.28
12.5	1.096	40	60	90	–	–	–
6.25	0.795	40	60	90	–	–	–
3.125	0.494	20	40	50	–	–	–
1.5625	0.193	0	40	10	–	–	–

–: No activity.

appeared to be the strongest one with (LC_{50} value of 4.28µg/ml), which was followed by the ethyl acetate extract (LC_{50} value of 5.36µg/ml) and petroleum ether extract (LC_{50} value of 13.29µg/ml). Standard vincristine sulphate had LC_{50} value of 0.53µg/ml.

Conflict of Interests

None declared.

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