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Antimicrobial activity of traditionally used medicinal plants against human pathogenic bacteria and fungi

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Abstract
Antibiotic treatment is a preferred choice to cure the microbial diseases. But long time consumption of antibiotics has been caused to formation of multi drug resistant microorganisms. The present study was aimed to determine the anti-microbial activity of methanol leaf extracts of five medicinal plants of Sri Lanka against the clinically isolated and confirmed human pathogens. A disc diffusion assay was conducted to determine minimum inhibitory concentration (MIC) and the synergetic antimicrobial activity. The MIC value for methanol extract of B. *ceylanica*, P. *pinnata* and C. *cauliflora* was within a range of 0.025g/ ml – 0.2g/ml. Among the plants tested, B. *ceylanica* was the most promising plant to inhibit the growth of MRSA (17.5±0.65 mm) and S. *aureus* (18.5±0.29 mm) and this was higher than the value of positive control. L. *zeylanica* had comparatively less antimicrobial potential than the B. *ceylanica* but, it showed variable antimicrobial activities against all selected human pathogens. There were tannins, phenols, flavonoids and alkaloids in various qualitative representations in all selected plants. The study showed a potent and diverse antimicrobial activities of the plants against selected human pathogens.

Keywords: medicinal plants, antibacterial activity, antifungal activity, MRSA

1. Introduction
In recent global medicines, the antibacterial therapy has been challenged due to the emergence and spread of multidrug-resistant (MDR) bacterial pathogens [1]. Hence, the MDR bacterial infections often resulted in high mortality and pre and post medical care with cost involving medical treatments [1, 2]. Because of that, the world recognized medical organizations face difficulties to overcome the problems of some selective human pathogen from their detrimental effects on human health [1, 3]. Among them, the bacterial agents including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and Methicillin Resistant S. *aureus* (MRSA) and *Candida albicans* are the sources of several human infections [4]. In addition, due to continuous consumptions of antibiotics, development of multi-drug resistance microorganisms is an unavoidable process to the available antimicrobial agents. Importantly, there are some adversative effects have been arising such as microorganisms have become resistant and some other contrary effects associated with the host such as hypersensitivity, allergic reactions, and immune suppressant. Very importantly, multi drug resistant microorganisms has created immense clinical problems during the treatments of infectious diseases [5]. Therefore, medical researchers are enforced to search for convenient, appropriate, less cost effective and minimal or no side effects containing medicines to treat the diseases caused by such bacteria.

Natural products are known to play an important role in human life. Various parts of the plants like root, bark, seed and leaves have been an important source of medicine since thousands of years. In recent years a predominant interest has been observed in evaluating different plant extracts for their antimicrobial properties against bacteria and fungi. The usefulness of plant extracts for antimicrobial therapy and/or other diseases have been observed to be promising remedies since ancient time [6]. As known fact, medicinal plants are rich in secondary metabolites which are found mostly in the form of flavonoid, tannin, terpenes and alkaloids. The presence of such compounds in these plants have open a way to search for alternative medical treatments using medicinal plants which consist important substances with pharmacological effects and can be used as natural composite sources that act as new anti-infectious agents [7]. Although, plants produce these substances for their natural defense mechanisms, these compounds are much related to antimicrobial potential and other plant related physiological activities. The present study was aimed to evaluate the *in vitro* antimicrobial activity of methanol extracts of traditionally used endemic and native medicinal plants (*Berberis ceylanica*, *Leucas zeylanica*, *Pongamia pinnata*, *Cynometra cauliflora* and *Morinda coreia*) of Sri Lanka against Gram-positive and negative bacterial strains such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and fungi *Candida albicans*.

2. Materials and Methods
2.1 Selection of test microorganisms
Stock cultures of the bacteria and fungi used were clinical isolates and confirmed. They were obtained from the microbiology laboratory, Ampara Base Hospital, Sri Lanka. Bacteria; *S. aureus* (ATCC 26923), *E. coli* (ATCC 29218), *P. aeruginosa* (ATCC 27853), and Methicillin Resistant S. aureus (MRSA) (ATCC25923) and fungi *Candida albicans* (ATCC 24433) were collected for the experiments. Cultures were maintained as nutrient agar slants in screw-capped bottles and stored at 4°C. All cultures were checked for

viability and purity by regular plating. Test cultures were prepared by transferring a loop full of bacteria from stock culture nutrient broth and incubated at 37°C for 24 h. Fungi were transferred into freshly prepared dextrose agar plates and incubated at 25°C for 3 days.

2.2 Selection of plant materials

The plants were randomly collected from densely populated areas of two districs of Sri Lanka. The four sample points were located in Parakaduwa which is in Rathnapura district (The average annual temperature is 27.1°C and the rainfall is around 3679 mm per year) in Sabaragamuwa province and one sample point was located in the Pattipola area which is in Nuwaraeliya district (The average annual temperature is 16.3°C and the rainfall here is around 2050 mm per year) in Central province. Disease-free fresh leaves of four selected medicinal plants were collected from Parakaduwa and stem of another one was collected from Pattipola as shown in the Table 1. All plants were identified and authenticated by using previous literature, ethnobotanical information and expert opinions that were obtained from ayurvedic doctors attached to the Pussella Ayurveda Hospital, Sri Lanka.

2.3 Preparation of crude extract

The collected plant parts were cleaned and shade dried for 2-3 weeks at room temperature (30°C) to obtain complete dryness. Dried plant materials were ground well using mechanical blender [8]. The resulting coarse powders were sieved through 212 microne sieve to get fine powders and stored properly in labeled air tight dark glass bottles at 4˚C until use. A 10 g of each powdered plant material was soaked in 50 ml of 80% (v/v) methanol and they were placed in a mechanical shaker at 350 rpm for overnight. This process was repeated until the residual material got exhaustively extracted and finally extracts were pooled. The pooled extracts of each medicinal plant were filtrated separately using Whatman filter paper No.1 and the filtered extract was placed in the rotary evaporator at 40°C to obtain a crude extract by evaporating the excess methanol [9, 10]. The crude extracts that obtained after rotary evaporation were dissolved in 4% dimethyl sulfoxide (DMSO) to get six different concentrations to be tested [11].

Table 1: Description of plants that were selected for the preparation of methanol extracts

Scientific name of plant	Local name	Endemic/ native	Conservation status	Part of plant used	Collection area
Leucas zeylanica	Gata thumba	Native	LC	Leaves	Parakaduwa
Pongamia pinnata	Mangul karanda	Native	LC	Leaves	Parakaduwa
Morinda coreia	Ahu	Native	NE	Leaves	Parakaduwa
Cynometra cauliflora	Naminam	Native	LC	Leaves	Parakaduwa
Berberis ceylanica)	Daruharidra	Endemic	LC	Stem	Pattipola

LC – Least Concern, NE – Not Evaluated

2.4 Anti-Bacterial assay

The antibacterial and antifungal activities were determined using the agar disk diffusion method [12, 13]. This method is highly effective for rapidly growing microorganisms, and the activities of the test extracts are expressed by measuring the diameter of the zone of inhibition. Sterilized filter paper disks (6mm in diameter) were purchased and impregnated in appropriate concentration of each plant extract before placing them on the agar plates. The disks were allowed to absorb the plant extracts as described by Mahasneh [13]. Plates of Mueller-Hinton sensitivity agar were aseptically inoculated with inoculums containing 1×10^5 CFU/ml of bacteria. A similar method was applied to the fungi *Candida albicans* using Sabouraud dextrose agar. The plates were allowed to dry for a few minutes.

The disks containing the plant extract were transferred using flamed but cooled forceps onto the surface of the seeded agar plates. They were sufficiently spaced to prevent the resulting zones of clearing from overlapping. The extractive solvent (methanol) was used as a negative control. Pre-impregnated sterilized discs with standard antibiotics, Gentamicin for *E. coli, MRSA, S. aureus* and Ceftazidime for *P. aeruginosa*, and fluconazole for *C. albicans* were used as positive control for comparison. The plates of bacterial cultures were then incubated at 37°C for 24 h and fungal cultures at 25°C for 3 days in an aerobic environment. After incubation, plates were observed for the zone of inhibition and the diameters of the inhibition zone in millimeters were measured using a scale. As the diameter of the disk was 6 mm, inhibition zones of less than 7mm were not evaluated [14, 15]. Each extract was tested three times and mean values were recorded.

2.5 Determination of Minimum inhibitory concentration (MIC)

The lowest concentration of the antimicrobial agent that inhibits the bacterial and fungal growth after 24hrs is called as MIC. MICs of bacteria and fungi isolates were determined using a broth micro dilution method proposed by Contreras-Lynch Sergio *et al*. [16].

2.6 Phytochemical screening

Crude extracts of both leaves and stem of all selected medicinal plants were subjected to a thorough phytochemical screening using standard methods as described below.

Phenols: A few drops of each crude extracts were mixed with 3-4 drops of 1% ferric chloride and the colour change of bluish black indicated the presence of phenols [17].

Tannins: A 3-5 drops of plant extract was diluted up to 10 mL by adding distilled water. Diluted sample was mixed well and boiled and then filtered through a filter paper (Whatman No. 40). 1% ferric chloride solution was added to the few drops of filtered extract and the presence of tannins was indicated by the development of brownish green or a blue-black colour change [18].

Flavonoids: A few drops of crude extract was added to 2 mL of NaOH (2%). The availability of flavonoids in the crude extract was confirmed from the disappearance of intense yellow colour on the addition of few drops of diluted HCl. Similarly, a white precipitation confirmed the occurrence of flavonoids while adding 1 mL of lead acetate (10%) to 1 mL of crude extract [18].

Alkaloids: Few drops of diluted HCl were added to 1ml of the plant extract and mixed well. One or two drops of

freshly prepared Mayer's reagent was added to it. Appearance of white precipitate indicated the presence of alkaloids [19].

2.7 Statistical analysis

The collected data was analyzed using the following statistical test (SPSS version 17, Inc. Chicago, USA)

1. Mean value and standard deviation
2. One-way analysis of variance
3. Turkey's pairwise comparison test to carry out multiple comparisons in bacterial and fungi inhibition zones between the groups ($P < 0.05$).

3. Results and Discussion

The antimicrobial activities of selected five medicinal plant species have been estimated against selected human pathogens including four bacterial strains and one fungal strain under sterilized conditions. According to the present study, most of the plant extracts of selected medicinal plants were exhibited broad spectrum of antimicrobial activity.

The Minimum Inhibitory Concentration (MIC) values for tested medicinal plants were demonstrated in Table 2. The MIC value for these five medicinal plants was within a range of 0.025g/ ml – 0.2g/ml against test microorganisms.

Table 2: Determination of minimum inhibitory concentration (MIC) of crude extracts of five selected plants.

Plant name	E. *coli*	S. *aureus*	MRSA	P. *aeruginosa*	*C. albicans*
C. *cauliflora*	0.2g/ml	0.05g/ml	0.025g/ml	0.2g/ml	-
M. *coreia*	-	-	-	-	0.2g/ml
P. *pinnata*	0.2g/ml	0.025g/ml	0.025g/ml	0.2g/ml	0.05g/ml
B. *ceylanica*	0.2g/ml	0.05g/ml	0.025g/ml	0.2g/ml	0.025g/ml
L. *zeylanica*	0.2g/ml	0.2g/ml	0.2g/ml	0.2g/ml	0.5g/ml

The MIC value of methanol extract of stem of B. *ceylanica* and leaves of P. *pinnata* and C. *cauliflora* showed the antimicrobial activity with in the same range (0.025g/ ml – 0.2g/ml) and considered to be a promising medicinal plants for controlling the growth of all microorganisms tested at the lowest concentration. However, the MIC value of the C. *cauliflora* against the fungi was unable to establish. Similarly, *M. coreia* could able to show the MIC value against the C. *albicans* only.

3.1 Comparative antimicrobial screening

Results obtained for the antimicrobial tests performed using methanol extracts of selected medicinal plants are presented in Table 3. Our results showed that the methanol extract of all plants had a broad spectrum of antimicrobial activity, being active to both Gram-positive, Gram-negative organisms and as well as to fungi. The zones of inhibition ranged from 7.25 mm – 18.5 mm (Table 3).

Table 3: Determination of zone of inhibition diameter values of methanol crude extracts against selected human pathogens.

Zone of inhibition (mm)					
Treatment	**E. *coli***	***MRSA***	**S. *aureus***	**P. *aeruginosa***	**C. *albicans***
Positive control*	18.33±0.33	18.66±0.33	18.33±0.33	26.66±0.33	20.33±1.20
C. *cauliflora*	11.25±0.63	13.50±0.96	8.75±0.48	8.75±0.49	-
B. *ceylanica*	8.25±0.25	17.50±0.65	18.50±0.29	8.75±0.49	8.75±0.25
P. *pinnata*	8.00±0.71	9.00±0.49	12.75±0.75	8.25±0.25	8.75±0.50
L. *zeylanica*	9.05±0.29	8.25±0.25	8.50±0.50	7.25±0.25	8.75±0.25
M. *coreia*	-	7.75±0.25	8.50±0.50	7.75±0.25	7.25±0.48
Negative control§	-	-	-	-	-

Mean ±standard error of mean (SEM) for triplicate readings.

*- Positive control- gentamycin for E. *coli*, *MRSA*, S. *aureus*, *C*eftazidime for P. *aeruginosa* and fluconazole for C. *albicans*.

§Negative control – methanol

According to the findings, B. *ceylanica* was the most effective plant to inhibit the growth of MRSA (17.50±0.65 mm) and S. aureus (18.50±0.29 mm and this was slightly higher than the value of positive control). L. *zeylanica* plant extract has comparatively less antimicrobial potential than the B. *ceylanica* plant extract but it showed variable antimicrobial activities against above all selected human pathogens. C. *cauliflora* showed only antibacterial activities against (MRSA, *S. aureus*, *E. coli* and *P. aeruginosa*) and not against the C. *albicans*. However, M. *coreia* did not show antibacterial against E. *coli*. When comparing the results, *Psuedomonas aeruginosa* exhibited resistant (inhibition zone was ranged from 7.25 mm to 8.75 mm) to all selected plants extracts and this research finding was in line with Gislene et.al [20]. According to the current study, S. *aureus* is the most susceptible to B. *ceylanica* and P. *pinnata* compared to all other selected plant extracts. Because S. *aureus is* gram positive bacteria which have not had outermost lipopolysaccharide layer. Therefore, plant extract can diffuse inside the cells quickly to destroy them. The resistance of E. *coli* and P. *aeruginosa* are relatively high since they are gram negative bacteria and, cells are covered from outermost lipid layer. The results of the current study were confirmed by the previous findings [8, 21, 22].

Vivek K. Bajpai *et al.* [23] have stated antimicrobial effect of P. *pinnata* plant extract against E. *coli* and S. *aureus* with zones of inhibition 9.00±0.2mm and 12.00±0.3mm respectively. Similarly, the present results also showed a closely same inhibition zone against the E. *coli* and S. *aureus* (8±0.75mm and 12.75±0.75mm respectively) by P. *pinnata* (Table 3). According to the previous study on different species of berberis plants, the antimicrobial effect of B. *ceylanica against* S. *aureus and* C. *albicans* had zones of inhibition; 15mm and 8mm [24]. However, the value of zone of inhibition according to the current study is higher against S. *aureus* (18.5±0.29mm) and similar with C. *albicans* (8.75±0.25mm). Although, the M. *coreia* leaf extracts didn't show antibacterial activity against E. *coli* compared with all other medicinal plants, the prospective activity of fruits aqueous extracts of M. *coreia* against E. *coli*, S. *aureus*, and P. *aeruginosa* has been reported.

Among all plant tested, B. *ceylanica* showed a significant antimicrobial potential ($p<0.05$) on selected human

pathogens as revealed in the Table 4 below. Antimicrobial effect of B. *ceylanica* plant extract exhibits relatively similar activity among E. *coli* and S. *aureus* but significantly different between MRSA and *S. aureus*. As well as antimicrobial effect of B. *ceylanica* plant extract is significantly different between S. *aureus* and P. *aeruginosa.* The antimicrobial potential of L. *zeylanica* plant extract significantly had an effect on selected human pathogens ($p<0.05$). Antimicrobial effect of L. *zeylanica* is similar among MRSA, S. *aureus* and P. *aeruginosa* except to the E. *coli*. The effect of above plant extracts didn't have an activity on C. *albicans*. According to the Turkey's pairwise comparison, the antimicrobial effect of M. *coriea* was similar among tested microorganisms except to the E. *coli*. Likewise, antibacterial activities of C. *cauliflora* was significantly different among all bacteria except to the C. *albicans* (Table 4).

Table 4: Results of Turkey's pairwise comparison of plant against the tested microorganisms

Plant Organism	C. *cauliflora*	B. *ceylanica*	L. *zeylanica*	P. *pinnata*	M. *coreia*
E. *coli*	11.25±0.63a	8.25±0.25a	9.50±0.29a	8.00±0.71a	-
MRSA	13.05±0.96ab	17.50±0.65a	8.25±0.25b	9.00±0.49a	7.75±0.25b
S. *aureus*	8.75±0.48bc	18.5±0.29b	8.50±0.50b	12.75±0.75a	8.50±0.50b
P. *aeruginosa*	8.750±0.49c	8.75±0.49c	7.25±0.25b	8.25±0.25a	7.75±0.25b
C. *albicans*	-	8.75±0.25	8.75 ±0.25 c	8.75±0.25a	7.25±0.48b

The summary of qualitative phytochemical screening of chemical constituents of plant extracts of the study has been illustrated in Table 5. There were tannins, phenols, flavonoids and alkaloids in various qualitative representation in all selected medicinal plants. The methanol extracts of plants including C. *cauliflora* possessed with high contain of phenol and tannin. The high presence of phenol and tannin have been proved by the study conducted by Rabeta *et al* [25] and in which it has been reported that C. *cauliflora* highly contained phenol. In addition, B. *ceylanica* and M. *coreia* plant extracts also qualitatively exhibited a large amount of phenol and alkaloid.

Table 5: Results of preliminary qualitative phytochemical screening of five medicinal plants

Plants Test name	C. *cauliflora*	P. *pinnata*	*B. ceylanica*	L. *zeylanica*	M. *Coreia*
Alkaloid	++	+	+++	+	+++
Phenol	+++	+++	+++	+	+++
Flavonoid	++	+++	+++	+	++
Tannin	+++	+	+	+	++

(+++ -highly presence, ++ - Moderately presence, + - slightly presence).

There are several medicinal properties of plants due to the presence of different classes of secondary metabolites (SMs) such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols, etc. Meenakshi Singh *et al*. [24] have reported that in Berberidaceae species has berberine and alkaloids. These have antimicrobial potential. Many studies have shown phenols and phenolic compounds are greatly used in skin infections, wound healing, inflammation; antioxidant, immune enhancers, anti-clotting and hormone modulators. P. *pinnata* plant highly contains phenol and tannin according to the current study (Table 5). It has been reported P. *pinnata* highly contain tannin, Flavonoid, alkaloid and other SMs [26]. *Leucas zeylanca* slightly contain phenol, alkaloid, flavonoid and tannin. Geethika *et al* [27] have reported phytochemical screening of six *Leucas* species. In which, all six *Leucas* members screened for qualitative phytochemical analysis were found to possess high amount of phenolic substances as well as flavonoids and minimum in qualitative presence of tannins in methanol extracts. Several studies have found that methanol extracts showed the presence of most of the phytochemicals analyzed i.e., alkaloids, flavonoids, tannins, phenolic, terpenoid, proteins, carbohydrates and glycosides, with the exception of saponin, proteins and amino acids. Tannins are one of the secondary metabolite presence in the plants and also polyphenolic compounds. Tannins bind to a proline rich protein that can interferes protein synthesis in microbes. Therefore, tannins have shown to have antimicrobial activity. Flavonoids are hydroxylated polyphenolic compounds which is produced by plants in response to microbial infections. This aspect has been broadly studied and found to have antimicrobial activity against an array of microorganisms in vitro [28]. Therefore, we can interpret, except L. *zeylanica*, all other four selected medicinal plants have antimicrobial activities and L. *zeylanica* had comparatively less antimicrobial activity.

4. Conclusions

The study showed potent and diverse antimicrobial activities of methanol extracts of B. *ceylanica*, L. *zeylanica*, P. *pinnata*, M. *coreia*, C. *cauliflora*. The findings of the study, therefore, may be used to develop alternative therapeutics in the management of methicillin resistant S. *aureus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and C. *albicans*.

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