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RESEARCH ARTICLE

ANTIMICROBIAL EFFICACY OF *Azadirachta indica* AND *Leucas aspera* IN COMPARISON TO COMMERCIAL ANTIBIOTICS

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ABSTRACT

Infectious diseases are one of the major problems in developing as well as developed countries. Plants produce a diverse range of natural products that have long been providing important drug leads for infectious diseases. *Azadirachta indica* Linn. (Neem) is a tree which has been found to possess antibacterial, antifungal, anti-inflammatory, anti-tumour properties and is also used as a pesticide. *Leucas aspera* Link (Donokolosh) has also been found to have antioxidant, antibacterial and cytotoxic effect. The objective of the present study was to evaluate the antibacterial activity of *Azadirachta indica* and *Leucas aspera* by using disc diffusion method. Clinical bacterial isolates such as *Escherichia coli, Klebsiella sp.* and *Staphylococcus aureus* were used as test organisms. Acetone extract of *Azadirachta indica* and methanolic extract of *Leucas aspera* were used.

INTRODUCTION

Almost every people on earth have put their faith on the huge variety of plants for their therapeutic properties. The plant extracts used to combat microbial infection are reported in our ancient Ayurvedic compendium 'Charak Samhita' and 'Sushrat Samhita' (Chatterjee and Pakrashi, 1994). From the dawn of civilization medicinal plants have been used to combat diseases. Plants contain secondary metabolites such as tannins, phenolic compounds, steroids, etc. which have potent antimicrobial activities. Although their efficacy and mechanisms of action have not been tested scientifically in most cases, these simple medicinal preparations often mediate beneficial responses due to their active chemical constituents (Barnes *et al*., 2007). Since pathogenic microorganisms put up side effects and resistance against antibiotics, recently scientists started paying attention to herbal extracts and biologically active compounds from plant species used in herbal medicines (Sarac and Ugur, 2007). *Azadirachta indica* Linn. belonging to family Meliaceae has been used in traditional medicine as a source of many therapeutic agents in the Indian culture and the plant is prevalent in the tropical countries. For treatment of various diseases like eczema, ringworm, acne, anti-inflammatory activities neem leaves are used. Neem leaves are also very effective in treating chronic wounds, diabetic foot and gangrene. Moreover, it helps in blood purification through removal of toxins from the body (Tirupatirao *et al*., 2014). *Leucas aspera* belonging to the family Lamiaceae is a common aromatic herb found as weed in Asia-temperate, Africa and Asian tropical countries. They are of medicinal importance due to their better antibacterial activity against pathogens. Different parts of this plant such as root, flower, leaf and stem have been found to have antibacterial, antioxidant and cytotoxic activity (Chew *et al*., 2012). Antipyretic and insecticidal properties of *Leucas aspera* has been reported (Ilango *et al*., 2008). Leaf juice of *Leucas aspera* has also been described as an external agent for psoriasis, chronic skin eruption, and painful swelling (Ilango *et al*., 2008).

Microorganisms are getting resistant to multiple drugs which are stipulated to be a major problem in coming years. So in quest of new antimicrobial agents based on natural products would be beneficial and of utmost importance (Zgoda and Porter, 2001). Skin, saliva and urinary samples were taken as the source of microfloras that have the potential to cause infection. The aim of the present study was to investigate antimicrobial activity of *Azadirachta indica* A. Juss. and *Leucas aspera* Link against human pathogenic bacteria and human microflora, and also to compare their efficacy in comparison to some standard antibiotics.

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MATERIALS AND METHODS

Collection of plant material and extract preparation

The plant materials were collected from an undisturbed area of Cachar district of Assam from healthy and uninfected plants. The fresh plant parts were washed under running tap water to eliminate dust and other foreign particles. The plant materials were then dried in shade. The completely shade dried material was coarsely powdered using a grinder and stored in air dried container. The powdered materials were then used for further experiments. 100g of the powdered plant parts of *Azadirachta indica* were dissolved in 500ml of 80% methanol. 100g of the powdered plant parts of Leucas *aspera* were dissolved in 500ml acetone. Both the extracts were then kept separately in rotatory shaker for 48 hrs (Selvamohan *et al*., 2012). After soaking the extracts were filtered using Whatman filter paper no. 1. The filtrates were then used for antimicrobial testing.

Preparation of sterile discs

What man filter paper no.1 was punched using a punching machine to prepare discs of 6mm diameter. It was then sterilized under UV ray in laminar air flow. Each sterile disc was impregnated with 20 µl of the extract using micropipette taking care that the solvent extract do not overflow from the disc to the outer surface. This was done for all the five types of extracts viz., leaf and bark extract of *Azadirachta indica* and leaf, flower and root extract of *Leucas aspera*.

Microorganisms used

The test organisms *Escherichia coli*, *Klebsiella* sp. and *Staphylococcus aureus* were clinical isolates obtained from the Microbiology Department of Silchar Medical College and Hospital, Silchar, Assam, India which were previously identified and characterized. The strains were maintained on nutrient agar slants at 4°C.

Isolation of bacteria from body samples

0.1ml of body samples was taken in a test tube and 9 ml of distilled water was added to it. The test tube was shaken for 10 min. A series of dilution of the suspension was made by pipetting 1 ml aliquot into 9 ml sterilized distilled water. The serial dilution technique was performed up to 10^{-5} dilution. Samples (dilution factor 10^{-3}, 10^{-4} and 10^{-5}) were then inoculated onto petridish containing 18-20 ml Nutrient Agar (NA) media and kept in an incubator at 37^0C for 48 hrs.

Identification of bacteria

Fine isolated and distinct colonies were picked up and streaked freshly on NA plates and incubated at 37^0C. After the recovery isolates in pure form, they were identified on the basis of the standard protocol given by Cappuccino and Sherman (2005) and Dubey and Maheshwari (2011).

Morphological and biochemical characterization

All the body samples were processed through different biochemical tests viz., Catalase Test, Indole Production Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test, as described by Cappuccino and Sherman (2005). The samples were also tested by Gram's staining to identify the nature of bacteria.

Antibiotic sensitivity and resistance pattern of the bacterial isolates

The susceptibility of microorganisms against antibiotics was determined by Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966). A total of 8 antibiotics were used. The concentration of discs used were Amikacin (AK, 30 mcg), Gentamycin (HLG, 120 mcg), Streptomycin (S, 10 mcg), Rifampicin (RIF, 5 mcg), Vancomycin (VA, 5 mcg), Tetracycline (TE, 30 mcg), Polymyxin – B (PB, 300 mcg) and Penicillin (P, 10 mcg). By measuring the zones of inhibition, the sensitivity of antibiotics against the isolates was determined on Mueller Hinton Agar (MHA) medium (Cappuccino *et al*., 2005). The isolates were inoculated in Mueller Hinton Agar (MHA) plates and antibiotic disc were placed equidistantly on surface MHA plates. After incubation at 37ºC for 24 hrs, the zone of inhibition was measured to the nearest mm using inhibition zone scale and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method.

The antimicrobial activity of the plant extracts was determined by disc diffusion technique. The sterile media was poured into the petri plates. Then from the turbid broth 200 µl of the suspension was inoculated onto the surface of Mueller Hinton agar using a spreader. The plate was then allowed to dry. Whatman filter paper was punched using a punching machine to prepare discs of 6mm diameter. It was then sterilized. By using a sterilized forcep, sterile Whatman filter paper discs were transferred onto the agar media. The plates were incubated at 37ºC for 24 hrs. After the plates were incubated at specific temperature and time, the zone of inhibition was calculated using an inhibition zone scale.

RESULTS

The three body samples were subjected to biochemical testing and were suspected to be *Staphylococcus* sp. Gram positive *Staphylococcus* sp. was sensitive to all antibiotics except Penicillin-G. The highest zone of inhibition was shown by Gentamycin of 28 mm and lowest 13 mm by Polymyxin- B and Rifampicin. Gram negative *E.coli* and *Klebsiella sp*. were multidrug resistant. *E.coli* was resistant to Penicillin-G, Tetracycline, Rifampicin and Vancomycin. Amikacin showed highest zone of 20 mm while Streptomycin showed a lowest of 12mm zone against *E.coli* and *Klebsiella sp*. *Klebsiella sp.* was resistant to Rifampicin, Vancomycin and Penicillin-G. Gentamycin showed highest inhibition zone of 22mm against *Klebsiella sp* and *streptomycin* showed lowest activity of 10mm. The bacterial strain of skin flora was sensitive to all antibiotics except Penicillin-G. Highest activity was shown by Amikacin with a zone of 24 mm and lowest activity of 8 mm was shown by Rifampicin. The salivary bacterial strain also showed resistance to Penicillin-G. In case of saliva sample

Table 1. Morphological and biochemical testing of skin, saliva and urine sample

Body samples	Gram staining	Indole test	Methy-Red test	Voges-proskauer test	Citrate test	Catalase test	Suspected Organism
Skin	Cocci, +ve	-	+	-	-	+	*Staphylococcus* sp.
Saliva	Cocci, +ve	-	+	+	-	+	*Staphylococcus* sp.
Urine	Cocci, +ve	-	+	+	-	+	*Staphylococcus sp.*

Table 2. Antibiotic susceptibility and resistance array of *E.coli*, *Staphylococcus aureus*, *Klebsiella sp.*, skin, saliva and urine samples against standard antibiotics

Isolates	Amikacin AK 30	Polymyxin-B PB 300	Tetracycline TE 30	Penicillin-G P 10	Streptomycin S 10	Rifampicin RIF 5	Gentamycin HLG 120	Vancomycin VA 5
E.coli	20(S)	16(S)	8(R)	NZ	12(S)	11(R)	15(S)	10(R)
Staphylococcus aureus	23(S)	13(S)	24(S)	NZ	20(S)	13(R)	28(S)	18(S)
Klebsiella sp.	25(S)	11(S)	19(S)	NZ	10(S)	9(R)	22(S)	9(R)
Skin	24(S)	13(S)	11(I)	NZ	20(S)	8(R)	17(S)	19(S)
Saliva	22(S)	13(S)	18(S)	NZ	19(S)	12(R)	23(S)	11(R)
Urine	22(S)	14(S)	12(I)	16(S)	10(S)	17(R)	12(S)	18(S)

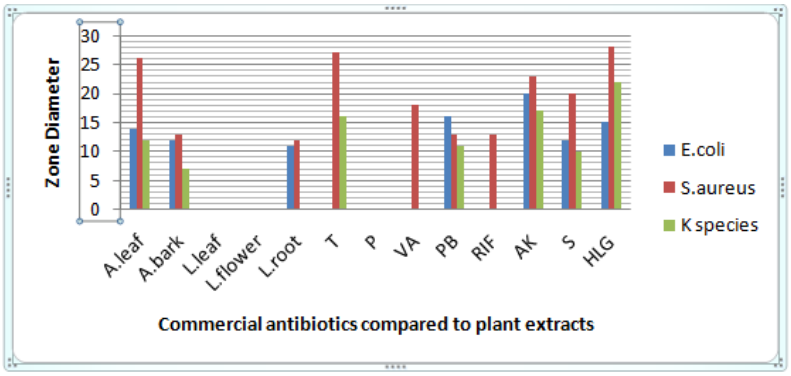
Letters indicates: NZ=No Zone, R=Resistance; I=Intermediate; S=Susceptible

Table 3. Antibiotic susceptibility and resistance assay of *E.coli, Staphylococcus aureus*, *Klebsiella sp.*, skin, saliva and urine samples against plant extracts

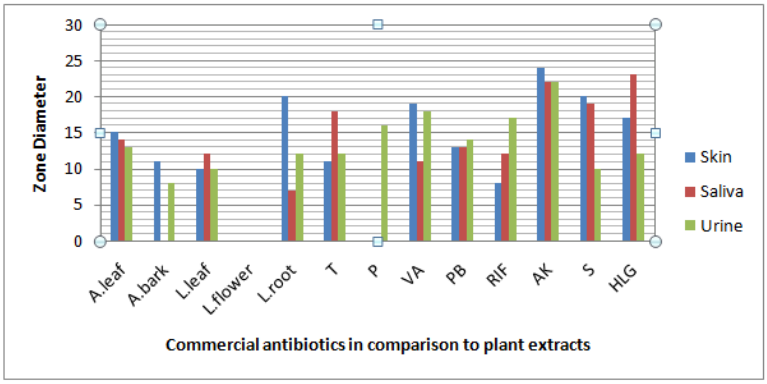
Isolates	*Azadirachta* indica leaf	*Azadirachta* indica bark	*Leucas aspera* leaf	*Leucas aspera* flower	*Leucas aspera* root
E.coli	14(S)	12(S)	NZ	NZ	11(S)
Staphylococcus aureus	26(S)	13(R)	NZ	NZ	12(S)
Klebsiella sp.	12(S)	7(R)	NZ	NZ	NZ
Skin	15(S)	11(I)	10(R)	NZ	20(S)
Saliva	14(S)	NZ	12(S)	NZ	7(R)
Urine	13(S)	8(R)	10(R)	NZ	12(S)

Letters indicates: NZ=No Zone, R=Resistance; I=Intermediate; S=Susceptible

Graph 1. The effect of commercial antibiotics and plant extracts to *E.coli, Staphylococcus aureus* and *Klebsiella sp.*



Graph 2. The effect of commercial antibiotics and plant extracts to Skin, Saliva and Urine sample suspected to be *Staphylococcus sp.*



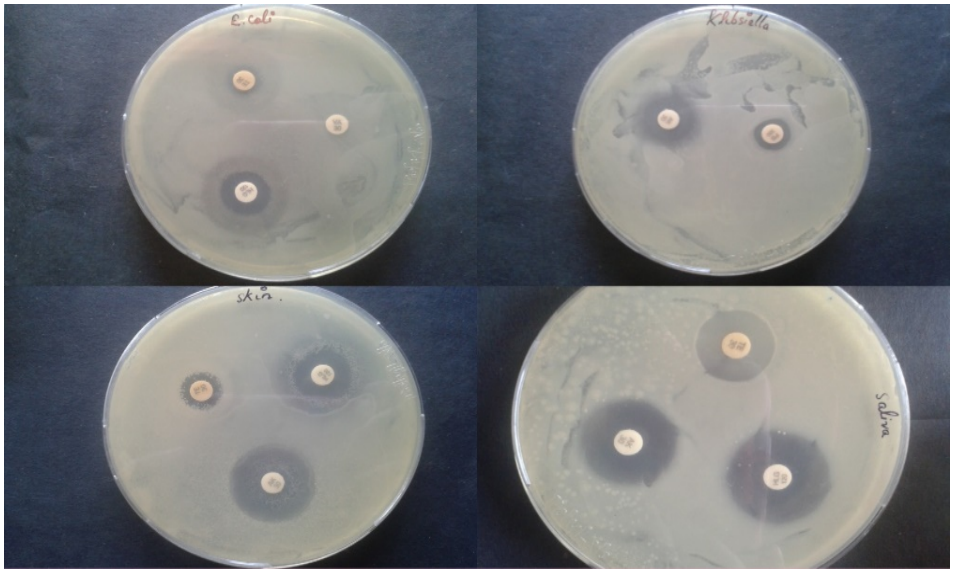


Fig. 1. Inhibition zones shown by antibiotics against *E.Coli, Klebsiella sp*., Skin and Saliva samples

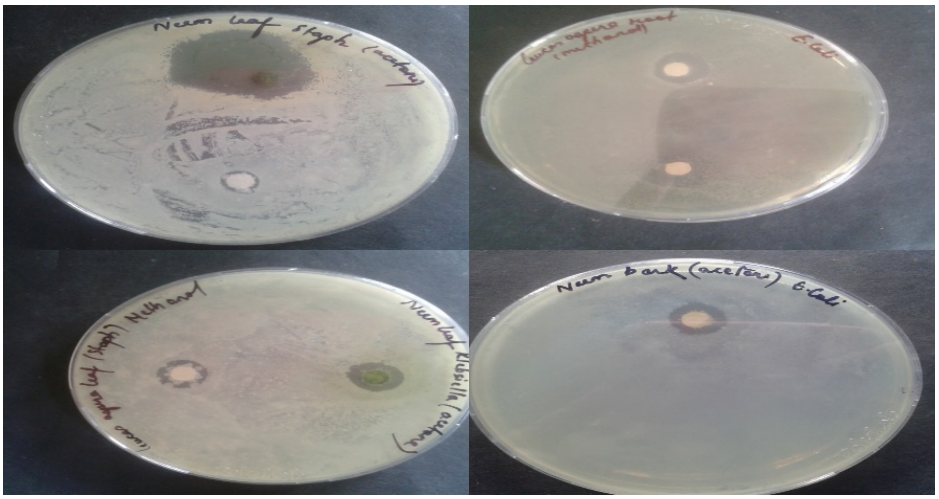


Fig. 2. Inhibition zone shown by plant extracts against *E.coli, Klebsiella sp.* and *Staphylococcus aureus*

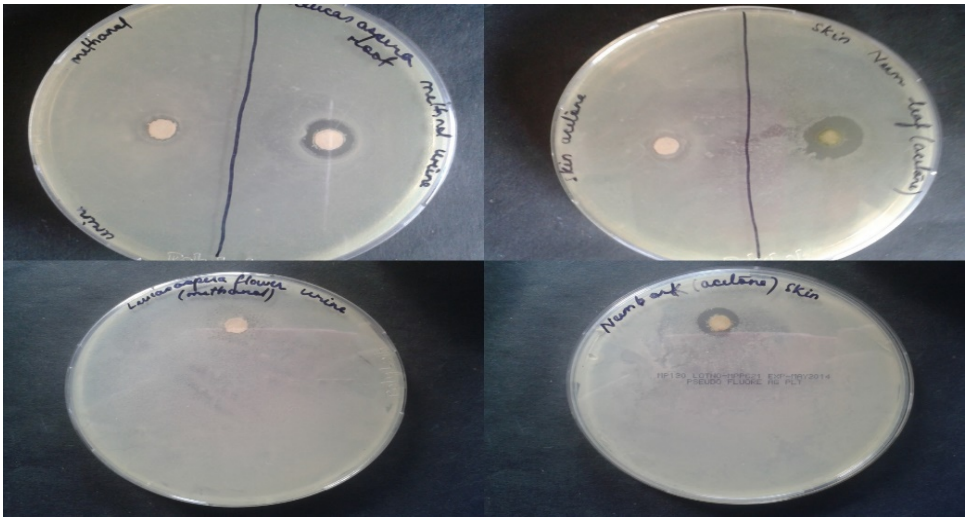


Fig. 3. Inhibition zones shown by plant extracts against skin, saliva and urine samples

Gentamycin showed highest zone of activity with 23 mm while Vancomycin showed the lowest activity of 11 mm. The bacterial strain of urinary flora was sensitive to all antibiotics with Amikacin showing highest 22 mm zone of inhibition and streptomycin showed lowest zone of inhibition of 10 mm.

Staphylococcus aureus, E.coli and *Klebsiella sp.* were resistant to both *Leucas aspera* leaf and flower extracts. *Klebsiella sp* was resistant to *Leucas aspera* root extract. Bacterial strains of skin, saliva and urine sample were sensitive to all the plant extracts. *Leucas aspera* flower extract did not show activity against any of the bacterial strains. *Azadirachta indica* leaf acetone extract showed activity against all of the samples. 80% methanol extract of *Leucas aspera* flower did not show activity against any of the bacterial strains. *Azadirachta indica* leaf had high antibacterial activity against *Staphylococcus aureus* with inhibition zone of 26mm and lowest against *Klebsiella sp*. with a zone of 12 mm. *Azadirachta indica* bark extract and *Leucas aspera* root extract also showed good results against the strains. *Leucas aspera* leaf extracts did not show any activity against *E. coli*, *Staphylococcus aureus* and *Klebsiella sp.* but showed inhibition zones against skin, saliva and urine samples. Against multidrug resistant *E. coli, Azadirachta indica* leaf extract showed highest activity with zone of 14mm and for *Klebsiella sp*. also *Azadirachta indica* showed highest zone of 12 mm. For the skin sample, *Leucas aspera* root extract showed a zone of 20 mm but the skin microflora was resistant to *Leucas aspera* leaf extract. In case of bacterial strain of saliva sample *Azadirachta indica* leaf showed highest 14 mm zone and *Leucas aspera* root showed 7 mm zone. For the urine sample highest 13mm zone was showed by *Azadirachta indica* leaf and lowest 8 mm by *Azadirachta indica* bark.

DISCUSSION

The drugs already in use to treat infectious disease are of concern because drug safety remains an enormous global issue. Most of the synthetic drugs cause side effects and also most of the microbes developed resistant against the synthetic drugs. To alleviate this problem, antimicrobial compounds from potential plants should be explored. These drugs from plants are less toxic, side effects are scanty and also cost effective. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. According to World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs. In the present work antimicrobial activity of *Azadirachta indica* and *Leucas aspera* were tested. *Azadirachta indica* leaves possess good antibacterial activity, confirming the great potential of bioactive compounds and emphasized the need for rationalizing the use of this plant in primary health care (Saradhajyothi and Subbarao, 2011). The bacterial strains *(E.coli, Staphylococcus aureus* and *Klebsiella sp.*) were chosen as test pathogenic microorganisms as they are important pathogens and rapidly develop antibiotic resistance. In disc diffusion technique, the mean zone of inhibition as shown by the commercial antibiotics was larger than those by the plant extracts. It may be attributed to the fact that the plant extracts being in crude form contains smaller concentration of the bioactive compounds. The type and level of biological activity exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture content, drying method, storage conditions, and post-harvest processing. In this study, the plant extracts were active against both gram positive and gram negative bacteria suggesting the presence of broad spectrum of antibiotic compounds.

No inhibitory action of methanol extract of *Leucas aspera* flower was observed against any of the bacterial strains. But according to Chew *et al.* (2011) methanol extract of *Leucas aspera* flower showed an inhibition zone of 7mm against both *E.coli* and *Staphylococcus aureus.* According to Dutta Choudhury *et al*. (2010) *Azadirachta indica* acetone extract showed an inhibition zone of 7 mm against *E.coli* and 8 mm against *Staphylococcus aureus* and 9.3 mm against *Klebsiella sp*. Vinoth *et al*. (2012) concluded that *Azadirachta indica* leaf acetone extract showed an inhibition zone of 17 mm and 18 mm against *E.coli* and *Staphylococcus aureus* respectively. Irshad *et al*. (2011) showed that *Azadirachta indica* leaf acetone extract were effective against *E.coli* and *Staphylococcus aureus* with a zone of 2 mm each. In this study *Azadirachta indica* .leaf acetone extract had an inhibition zone of 14mm, 26mm, 12mm against *Ecoli, Staphylococcus aureus* and *Klebsiella sp*. respectively.

According to Chew *et al*. (2012) *Leucas aspera* leaf 80% methanol extract showed 7mm of inhibition zone against *Staphylococcus aureus* but no zone against *E. coli*. The root extract showed zones of 9 mm against *E. coli* and 10mm against *Staphylococcus aureus* respectively. *Leucas aspera* flower showed an inhibition zone of 7 mm against both E. *coli* and *Staphylococcus aureus*. Britto *et al*. (2012) concluded that *Leucas aspera* leaf 95% methanol extract showed an inhibition zone of 7 mm, 8 mm and 8 mm against *Staphylococcus aureus, Klebsiella vulgaris* and *E. coli* respectively. In our study methanol extract of *Leucas aspera* leaf showed no zones of inhibition against any of the bacterial strains i.e., E. *coli*, *Staphylococcus aureus* and *Klebsiella* sp; *Leucas aspera* flower also did not show any zone of inhibition against these strains. However, *Leucas aspera* root extract showed 11 mm of inhibition against *E. coli* and 12 mm against *Staphylococcus aureus* but showed no zone against *Klebsiella sp.*

Conclusion

Microorganisms are developing resistance to available drugs more quickly than new drugs are being found. The future research in antimicrobial therapy should be aimed to overcome the resistance of microorganisms or to find an alternative improved therapeutic agent to treat infections. Various plant species have been investigated scientifically for their antimicrobial activity and a large number of plants have been shown to inhibit the growth of microorganisms. A number of these plant based bioactive compounds appear to have structures and modes of action that are distinct from those of the commercial antibiotics. So, it is worthwhile to explore plant based bioactive compounds against resistant pathogenic microorganisms.

Azadirachta indica has already been established to possess antimicrobial activity. In this study it was observed that

Azadirachta indica leaf and bark extracts showed highest activity against almost all of the bacterial strains and also *Leucas aspera* root extract showed good antibacterial activity against the bacterial strains. *Leucas aspera* leaf although did not show any zone of inhibition against *E. coli, Staphylococcus aureus and Klebsiella species*, but it showed good activity against the body samples. Hence, the leaf extract of *Leucas aspera* can be used to treat infections apart from *Azadirachta indica* leaf, and bark extracts and *Leucas aspera* root extract.

The present study suggests that acetone extract of *Azadirachta indica* leaf and bark and methanol extract of *Leucas aspera* root would be useful to develop new antimicrobial drugs. Although antibiotics Gentamycin and Amikacin showed better results than the above stated plant extracts but still these plant extracts have the potential to give new lead compounds to design better antimicrobial drugs as they showed good results against almost all of the given strains. These plant extracts can also be used synergistically along with the commercial antibiotics to treat diseases caused by bacteria, so that the risk of side effects of the available antibiotics is reduced without losing their efficacy. Further, in-vivo studies are needed to substantiate the finding.

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