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Evaluation of antimicrobial efficacy of extracts from the bark of a semi arid plant *Albizia lebbeck* (L.) Benth

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In the present study, use of ethnomedicinal plant/ plant parts for the treatment of disease with little or no side effect is investigated against the pathogens related to infectious diseases. Bark of *Albizia lebbeck* (L.) Benth was collected and air dried and soxhlet extracted by using standard methods for flavonoid, alkaloid, steroid and different solvents. These extracts were then tested for antimicrobial activity using disc diffusion method. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and total activity (TA) were also calculated. Statistical analysis like mean value and standard deviation were calculated for the test bacteria and fungi. Data was analyzed by one-way analysis of variance and *P* values were considered significant at $P > 0.005$. Among the seven extracts, methanol extract exhibited highest inhibition zone (20 mm) against *R. planticola. P. aeruginosa, S. aureus*, and *R. planticola* were observed to be more susceptible pathogens in the investigation followed by *B. subtilis, E. aerogenes*, and *E. coli.* Bound flavonoid and methanolic extracts of the bark of *A. lebbeck* were found to be most bioactive. The range of MIC and MBC of extracts recorded was 0.625-0.078 mg/mL and 0.312- 0.039 mg/mL, respectively. The lowest MIC value 0.078 mg/mL was recorded against *R. planticola*, indicating significant antimicrobial potential of test extracts. High values of TA were observed against *R. planticola*, followed by *S. aureus*. Results of the present study revealed that the bark extracts of *A. lebbeck* showed great antimicrobial potential against the tested microorganisms and may be exploited for future antimicrobial drugs.

Keywords: Alkaloid, Antimicrobial, Flavonoid, Minimum bactericidal concentration, Mininum inhibitory concentration, Steroid, Total activity.

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Introduction

Our country has diverse climate, which supports growth of varieties of plants. Plants are used as medicine from ancient time and even now these herbal medicines are in continuous demand. Plants have become an essential and integral part in complementary and alternative medicine. Plants have the ability to form secondary metabolites like proteins, flavonoids, alkaloids, steroids, and phenolic substances, which are in turn used to restore health and heal many diseases[1].

Albizia lebbeck (L.) Benth known as *Shirisha* in *Ayurveda* is an important medicinal plant belonging to the family Fabaceae and a member of subfamily Mimosaceae. The bark is dark brown to greenish black, rough, with longitudinal and transverse fissures on the outer surface; inner surface whitish with fine longitudinal stations. It is a tree of great importance in India and has traditionally been used as a cure for many harmful diseases. *A. lebbeck* bark has an acrid taste and is used for bronchitis, leprosy, paralysis, and helminth infections and is reported to have antidiarrhoeal activity[2]. Barks are mainly used in dental infections[3]. The bark of *A. lebbeck* is also used in the treatment of piles[4]. It also possesses anti-spermatogenic and anti-inflammatory activity[5,6].

In recent years, multiple drug resistance has been developed in pathogens of human and plant due to indiscriminate use of synthetic drugs especially in the developing countries[7]. Thus, a diverse arsenal of new antibacterial agents is urgently needed to combat the diminishing efficacy of existing antibiotics[8]. It is reported that *A. lebbeck* contains saponins, macrocyclic alkaloids, anthraquinone glycosides, tannins, and flavonols[9]. A novel phenolic glycoside, albizinin and four known flavanols, (-) epicatechin, procyanidin B-2, procyanidin B-5, and procyanidin C-1, also had been isolated from the acetone extract of the bark[10]. Phytochemical and therapeutic efficacy studies of *A. lebbeck* have been reported[11]. The present investigation was undertaken to find out

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the antibacterial potential of flavonoids, alkaloids, steroids, and extracts with different polarity of the bark of *A. lebbeck* against some Gram-positive and Gram-negative bacteria.

Escherichia coli is a frequent cause of life threatening bloodstream infections[12] and other common infections, such as urinary tract infections. Antibiotic resistance rates in *E. coli* are rapidly rising, especially with regard to fluoroquinolones, and third- and fourth-generation cephalosporins[13,14]. *Raoultella planticola* is a Gram negative, aerobic, nonmotile bacilli primarily considered to be an environmental bacteria. It rarely causes human infections[15]. However, an increasing number of cases of *R. planticola* infection, including bacteraemia, soft tissue infection pancreatitis and urinary tract infection have recently been reported[16]. *Pseudomonas aeruginosa* is one of the leading nosocomial pathogens worldwide[17]. It exhibits antibiotic resistance, and demonstrates practically all known enzymic and mutational mechanisms of bacterial resistance[18]. *Enterobacter aerogenes* is a common agent of hospital-acquired infection. It exhibits a remarkable adaptive capability and easily acquires resistance to β-lactam antibiotics during therapy[19]. The existence of a prevalent resistant clone of *E. aerogenes* has been reported[20]. In addition, antibiotic resistance of *E. aerogenes* is associated with a high crude fatality rate in infected patients[21]. *Agrobacterium tumefaciens* is a tumor producing pathogenic bacteria and is a serious pathogen of walnuts, grape vines, and stone fruit. Pathogenic potential of *Bacillus subtilis* is generally described as low or absent[22]. In the literature, only a few cases of infections due to *B. subtilis* are reported[23] and only one retrospective study describes the isolation of antibiotic-resistant strains of *B. subtilis*[24]. *Staphylococcus aureus* is the most common hospital acquired pathogen and causes staph infections which is responsible for various diseases including mild skin infections (like folliculitis), invasive diseases (like wound infections and bacteremia), and toxin mediated diseases (like food poisoning, toxic shock syndrome and scaled skin syndrome). In infants, its infection can cause a severe disease called staphylococcal scalded skin syndrome[25]. Bacteremia due to *S. aureus* has been reported to be associated with mortality rates of 15–60 %[26,27]. Resistance to methicillin among *S. aureus* isolates is a growing problem: the prevalence of nosocomial infections in patients increased from 12.5 % in 1999 to 20.8 % in 2004[28].

Materials and Methods

Collection of plant material

Bark of *A. lebbeck*, was collected from Jaipur located in the eastern region of Rajasthan in June 2014. Plant was identified by a senior taxonomist, Dr. Sudhakar Mishra of the Department of Botany, University of Rajasthan and a voucher specimen (RUBL211460) was deposited in the Herbarium of the same Department of Botany.

Selected test pathogens

Seven pathogens, namely *Escherichia coli* (MTCC no. 46), *Pseudomonas aeruginosa* (MTCC no. 1934), *Raoultella planticola* (MTCC 2272), *Enterobacter aerogenes* (MTCC no. 2822), *Bacillus subtilis* (MTCC no. 121), *Agrobacterium tumefaciens* (MTCC no. 431), and *Staphylococcus aureus* (MTCC no. 87) were selected for the present study and were procured from CSIR-IMTECH, Chandigarh, India. The bacterial strains were grown and maintained on Mueller-Hinton agar medium.

Preparation of extracts

Flavonoid extraction[29]

Bark of *A. lebbeck* was collected; shade dried, finely powdered and extracted using the method of Subramanian & Nagarjan. Finely powdered sample (100 g) was soxhlet extracted with 80 % hot methanol (500 mL) on a water bath for 24 h and filtered. Using separating funnel the filtrate obtained was re-extracted successively with petroleum ether (fraction I), ethyl ether (fraction II), and ethyl acetate (fraction III). Fraction of petroleum ether was discarded due to being rich in fatty substances, where as fractions of ethyl ether and ethyl acetate were further analyzed for free and bound flavonoids, respectively. Using 7 % H_2SO_4, ethyl acetate fraction of the sample was refluxed for the hydrolysis for 2 h (for removal of bounded sugars) and again filtrate was extracted in separating funnel with ethyl acetate. Ethyl acetate extract thus obtained was washed with distilled water to neutrality. Ethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vacuum and weighed. The extracts were stored at 4 ^oC and were re-suspended in acetone to get 10 mg/mL concentration for antimicrobial assay.

Alkaloids extraction[30]

Finely powered sample (100 g) was extracted in 20 mL methanol after shaking for 15 min. After

filtration, filtrate was dried in vacuum and then the residual mass was treated with 1 % H_2SO_4 (5 mL twice). Extraction was then done in 10 mL chloroform ($CHCl_3$) by using separating funnel. Organic layer of chloroform was rejected and aqueous layer was basified with 30 % NH_4OH (PH=9-10). Once again, extraction was done in 10 mL chloroform and the organic layer of chloroform (lower layer) was collected in a flask and the above steps were repeated with fresh chloroform. Extract was then dried in vacuum for further use.

***Steroid extraction*[31]**

Finely powdered sample (100 g) was extracted in petroleum ether for 2-4 h. After filtration, residual mass was treated with 15 % ethanolic HCl for 4 h. Extraction was then done in ethyl acetate followed by washing in distilled water to neutralize the extract. The neutral extract was then passed over sodium sulphate to remove moisture contents and was dried in vacuum. Chloroform was used for reconstitution of extract, filtered, and dried for further use.

Extraction in different polar solvents

Powdered bark (20 g) was taken in each flask and water, methanol, and petroleum ether were used as the solvent. Dried material and solvents were taken in 1:10 ratio. Those were kept at soxhlet unit for 24 h. The obtained extracts were filtered by using Whatman No. 1 filter paper and the filtrate was subjected to evaporation to obtain dried extract. The percentage yield of each dried plant extract was calculated. The residual extracts were stored in a refrigerator at 4 °C in small and sterile glass bottles.

Antimicrobial susceptibility testing

Disc diffusion assay[32] was performed for screening. Mueller- Hinton agar base plates were seeded with the bacterial inoculums of size 1×10^8 CFU/mL. Sterile filter paper discs of Whatman no.1 (6 mm diam.) were impregnated with 100 µL of each of the extract (concentration- 10 mg/mL) to give a final concentration of 1 mg/disc. Discs were left to dry in vacuum, so as to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in triplicate along with streptomycin (1 mg/disc) as a standard for bacteria. The plates were kept at 4 °C for diffusion of extract, thereafter were incubated at 37 °C for 24 h. Activity index for each extract was calculated by the following formula:

Activity index = IZ produced by extract/ IZ produced by standard

Where, IZ = inhibition zone.

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test pathogens. Broth micro dilution method[33] was followed for determination of MIC values. Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10 mg/mL final concentration. Two fold serially diluted extracts were added to broth media of 96-wells of micro titer plates. Thereafter, 100 µL inoculum (1×10^8 CFU/mL) was added to each well. Bacterial suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titer plates were then incubated at 37 °C for 24 h. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another was kept at 4 °C for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of a micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum bactericidal concentration (MBC) was determined by sub culturing 50 µL from each well showing no apparent growth. Least concentration of extract showing no visible growth on sub culturing was taken as MBC.

Total activity determination[34]

Total activity (TA) is the volume at which the test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g of plant material by the MIC of the same extract or compound isolated and is expressed in mL/g.

Results

The result of the assessment of antimicrobial potency of flavonoids (bound and free), alkaloid, steroids, and extract obtained with different solvents (water, methanol, and petroleum ether) showed significant activity against most of the tested pathogens (Table 1-3). In the present investigation, results revealed that out of total seven extracts tested against seven different pathogens, alkaloid extract of bark remained inactive against all pathogens.

Among the seven extracts, methanol extract exhibited highest inhibition zone (20 mm) against *R. planticola*. *P. aeruginosa*, *S. aureus*, and *R. planticola* were observed to be more susceptible pathogens in the investigation followed by *B. subtilis, E. aerogenes* and *E.coli*. Significant activity index was observed for bound flavonoid and petroleum ether extract against *P. aeruginosa* and *B. subtilis*, respectively. Bound flavonoid and methanolic extracts of the bark of *A. lebbeck* were found to be the most bioactive

Table 1—Antimicrobial activity of extracts of *Albizia lebbeck* against some pathogenic bacteria

Extract	Microorganisms													
	E. coli		*P. aeruginosa*		*R. planticola*		*E. aerogenes*		*B. subtilis*		*A. tumefaciens*		*S. aureus*	
	IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI
F1	12	16	1.23±0.022			11	0.	12						
F2	-	8	0.615±0.068			9	0.6±0.055						9	0.333±0.025
A	-	-	-											
S	-	10	0.769±0.062	10	0.588±0.059								10	0.37±0.014
W	-	12	0.705±0.056	-										
M	-	12	0.923±0.023	20	176±0.016 13	13	0.86±0.445	15	1.153±0.025				16	0.592±0.088
P	-	15	1.153±0.052	12	0.705±0.032			16	1.23±0.016				12	0.444±0.033

values are Mean±SEM, IZ- Inhibition zone in mm (including 6 mm diam. of disc); F1- Bound flavonoids; F2- Free flavonoids; A- Alkaloids; S- Steroids; W- Water extract; M- Methanol extract; P- Petroleum ether extract; (-)- no activity.

Table 2—MIC and MBC of active extracts of *Albizia lebbeck* against different pathogens

Microorganisms	Extracts	F1	F2	A	S	W	M	P
E. coli	MIC	0.312	-	-	-	-	-	-
	MBC	0.156	-	-	-	-	-	-
P. aeruginosa	MIC	0.156	0.625	-	0.625	-	0.312	0.312
	MBC	0.078	0.312	-	0.312	-	0.156	0.156
R. planticola	MIC	-	-	-	0.625	0.312	0.078	0.312
	MBC	-	-	-	0.312	0.156	0.039	0.156
E aerogenes	MIC	0.312	0.625	-	-	-	0.312	-
	MBC	0.156	0.312	-	-	-	0.156	-
B.subtilis	MIC	0.312	-	-	-	-	0.312	0.156
	MBC	0.312	-	-	-	-	0.156	0.078
A.tumefaciens	MIC	0.312	-	-	-	-	-	-
	MBC	0.156	-	-	-	-	-	-
S.aureus	MIC	-	0.625	-	0.625	-	0.156	0.312
	MBC	-	0.312	-	0.312	-	0.078	0.156

F1- Bound flavonoids; F2- Free flavonoids; A- Alkaloids; S- Steroids; W-Water extract; M- Methanol extract; P- Petroleum ether extract; MIC- Minimum inhibitory concentration in mg/mL; MBC- Minimum bactericidal concentration in mg/mL; (-)- no activity

Table 3—Quantity and total activity of extracts of *Albizia lebbeck*

Extracts	Qantity of extract mg/g dwt	Total activity (mg/mL)						
		E. coli	*P. aeruginosa*	*R. planticola*	*E. aerogenes*	*B. subtilis*	*A. tumefaciens*	*S. aureus*
F1	7.5	24.03	48.07	-	24.03	24.03	24.03	-
F2	4.0	-	6.4	-	6.4	-	-	6.4
A	65.0	-	-	-	-	-	-	-
S	9.0	-	14.4	14.4	-	-	-	14.4
W	11.0	-	-	35.25	-	-	-	-
M	192.0	-	615.4	2462	615.4	615.4	-	1231
P	1.0	-	3.205	3.205	-	6.41	-	3.205

F- Bound flavonoids; F2- Free flavonoids; A- Alkaloids; S- Steroids; W- Water extract; M- Methanol extract; P- Petroleum ether extract; TA- Total activity (extract per g dried plant part/MIC of extract)

metabolite, as activity was observed against most of the tested pathogens. MIC and MBC values (Table 2) were evaluated for plant extracts, which had shown activity in diffusion assay. The range of MIC and MBC of extracts recorded was 0.625-0.078 mg/mL and 0.312- 0.039 mg/mL, respectively. In the present investigation, lowest MIC value of 0.078 mg/mL was recorded against *R. planticola*, indicating a significant antimicrobial potential of test extracts. The quantity of extracts per gram of plant material and TA calculated was recorded (Table 3). High values of TA were observed against *R. planticola* followed by *S. aureus.*

Discussion

In the recent years, infections have increased to a great extent and the antibiotics resistance has become an ever-increasing therapeutic problem[35]. In India, medicinal plants are widely used directly as folk remedies or indirectly in the pharmaceutical preparations. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic medicines[36]. Hence, continous investigations related to medicinal plants are required for the discovery of new drugs. The present study was an effort to investigate the medicinal properties of *A. lebbeck*, which has not yet been carried out in the earlier studies. In an earlier study, it was reported that the hydroalcoholic extract of *A. lebbeck* exhibited antibacterial activity[37], and the preliminary phytochemical screening of the ethanolic extract of the bark showed the presence of alkaloids, glycosides, flavanoids, saponins, tannins, carbohydrates and reducing sugar, while the ethanolic extract of the pods showed the presence of proteins, glycosides, flavanoids, saponins, tannins, carbohydrates and reducing sugar, which suggests that the plant may possess various pharmacological activities[38]. The methanol and the chloroform extracts of the seeds of *A. lebbeck* have been reported to inhibit the growth of pathogens. *B. subtilis* and *Aspergillus fumigatus* have been reported to be highly sensitive towards chloroform extract of seeds and methanol extract of seeds showed good activity against *B. subtilis* and *Candida albicans*. Chloroform extract has been reported to be more effective against *B. subtilis* than methanol extract residue[39]. A study by Srinivasan *et al.*[40] showed that the aqueous extract possesses antibacterial activity against *E. coli* and *Salmonella* spp. Although, the plant has been studied previously for its antimicrobial activity, but so far, it has not been investigated for bark flavonoids, alkaloids, steroids and extracts obtained in different polar and non-polar solvents. Mostly, the crude extracts of leaf, stem, flower, and root without MIC, MBC, and TA determination have been screened. Such studies could only indicate their antimicrobial potential, but are not helpful in establishing them as an antibiotic, hence can't replace the existing antibiotics. In the present investigation, IZ, AI, MIC, MBC, and TA have been evaluated for each extract. Extracts recorded for low MIC values indicate strong bioefficacy of the plant. The findings of the present investigation offer a scientific evidence to support the ethnomedicinal use of the plants as an alternative medicine.

Conclusion

A. lebbeck, a traditional medicinal plant of India, is a rich source of bioactive compound. As of now, little work has been done on the biological activity and hence, extensive investigation is needed to exploit the bioactive compounds for medicinal purpose. The results of the above study revealed that the extracts of *A. lebbeck* exhibits antibacterial activity, which might be helpful in preventing the progress of various diseases and can be used in the alternative system of medicine.

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