

PHYTOCHEMICAL ANALYSIS AND ANTIMICROBIAL EFFICACY OF LEAF EXTRACTS OF *PITHECELLOBIUM DULCE*

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ABSTRACT

The present study was designed to evaluate the antimicrobial activity of leaf of *Pithecellobium dulce* against twenty pathogenic microorganisms. Leaf extracts of *P. dulce* were prepared in distilled water and organic solvents, viz., benzene, chloroform, acetone and methanol in increasing and decreasing order of solvent polarity. Agar well diffusion technique was used to assess the antimicrobial activity of leaf extracts against five Gram-positive (*Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*), seven Gram-negative (*Aeromonas hydrophila*, *Alcaligenes faecalis*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacteria and eight fungi (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus*, *Alternaria alternata*, *Alternaria brasicola*, *Alternaria solani* and *Alternaria vitis*). The extracts showed variable inhibition zone (ranging between 7 to 27mm) against most of the tested microbes. Solvent extracts were found to be more effective than the aqueous extract. The most susceptible microorganism was *E. faecalis* exhibiting a zone of inhibition of 27mm. The broth microdilution assay gave minimum inhibitory concentration (MIC) values ranging from 200 to 1000 µg/ml. The lowest MIC values were obtained against *E. faecalis*, indicating the susceptibility of the strain for all the extracts. Preliminary phytochemical analysis of different extracts revealed the presence of alkaloids, anthraquinones, flavonoids, cardiac glycosides, proteins, tannins, sugars, and terpenoids. The results of the present study indicate that *Pithecellobium dulce* extracts possess bioactive compounds having significant antimicrobial properties. Hence, the plant extracts can be subjected to further isolation of therapeutic antimicrobial agents for pharmacological evaluation.

Keywords: *Pithecellobium dulce*, antimicrobial, antifungal, phytochemical analysis

INTRODUCTION

In the recent years, antimicrobial resistance has become a major global problem¹. The increased prevalence of antibiotic-resistant bacteria due to the extensive use of antibiotics has rendered the current antimicrobial agents inefficient to control several bacterial diseases^{2,3}. One of the measures to combat this problem is to have continuous investigations into new, safe and effective antimicrobials as alternative agents to substitute the present less effective ones. Plants have been traditionally proved to be a rich source of novel drug compounds, and the herbal mixtures have made large contributions to human health and well-being. The World Health Organization has also recommended the evaluation of the plants effective in conditions where safe modern drugs are lacking. Therefore, the search for drugs and dietary supplements derived from plants has been accelerated in recent years. Currently, a lot of research is being carried out to investigate ethnobotanical uses of plants prevailing among native people⁴. There are numerous reports evidencing the antimicrobial activity of plants against microorganisms^{2,5,6,7,8}. Plant derived natural products such as flavonoids, terpenoids and steroids have received considerable attention due to their diverse pharmacological properties including antibacterial, antifungal and antioxidant activities. Thus, it becomes necessary to analyze the vast untapped potential of the medicinal plants in combating the antibiotic resistant organisms. Hence, in the present investigation, *Pithecellobium dulce*, commonly known as *Jugli Jalebi* or *Manila Tamarind* has been tested for its efficacy to inhibit several pathogenic microorganisms.

Pithecellobium species belonging to the family Leguminosae and the subfamily Mimosoideae are widely distributed in the tropics, chiefly in Asia and America. *Pithecellobium dulce* Benth being one of the familiar species among them, is a small to medium-sized, evergreen spiny tree upto 18m height. The plant is reported to be a folk remedy for earache, leprosy, peptic ulcer and toothache. It also acts as emollient, anodyne and larvicidae in folk medicine. Infusions of different parts have been used traditionally to treat diseases, such as skin of the stem for dysentery, leaves for intestinal disorders, and seeds for ulcer, among others. Leaves can also be used as a plaster to allay pain from venereal sores, and can also relieve convulsions^{9,10,11}. Chemical investigations of the different parts of the plant have resulted in the isolation of a few novel and interesting metabolites, some of which have been screened for bioactivity^{12,13,14,15,16,17}. The plant is also reported to possess abortifacient¹⁸, anti-inflammatory

¹⁹, anti venom²⁰, protease inhibitory²¹, spermicidal²², antimicrobial and antitubercular activity^{23,24}.

Although the existing chemical, pharmacological and clinical literature on the plant is impressive, there are several aspects that remain unexplored. The current study is therefore an attempt to determine the antimicrobial activity of the crude extracts of this plant against a large number of pathogenic strains.

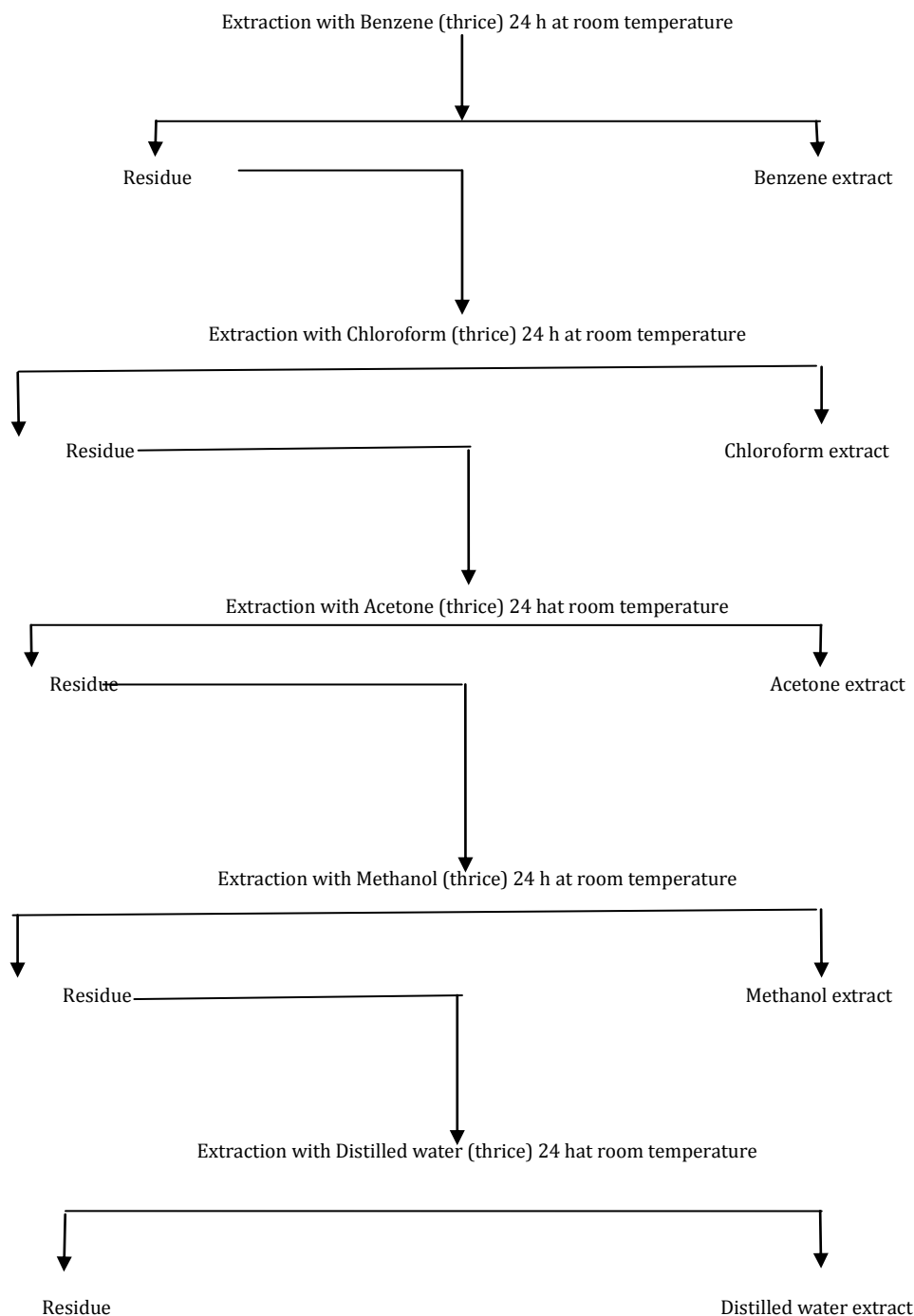
MATERIALS AND METHODS

Collection of plant material

The leaves of the plant, *Pithecellobium dulce* growing in the local areas of Rohtak district of Haryana State were collected during July 2010. The plant material was washed thoroughly, initially with tap water and then with distilled water to remove any debris or dust particles and was then allowed to dry in an oven at 40°C. The dried plant material was ground to a fine powder and stored at room temperature in airtight containers until used further.

Preparation of plant extract

To 500g of *Pithecellobium dulce* leaf powder, 1500 ml of each solvent, viz. benzene, chloroform, acetone, methanol and distilled water was added serially for preparing the extracts in increasing solvent polarity, and in reverse order for decreasing solvent polarity (flow chart-1). Extraction with each solvent was done for 24 h at room temperature, after which the supernatant of each solvent was recovered by filtering through Whatmann filter paper. This process was repeated thrice and the respective solvent from the supernatant was evaporated in a rotary vacuum evaporator to obtain the crude extract. These extracts (both increasing and decreasing polarity) were stored at 4°C until used for the evaluation of antimicrobial activity.

***Pithecellobium dulce* (Leaf powder)**

Flow chart 1: Schematic representation of the extraction procedure of leaves of *Pithecellobium dulce* prepared in increasing and decreasing order of solvent polarity.

Bacterial test organisms

A total of twelve bacterial (five Gram positive and seven Gram negative) and eight fungal strains were used for the evaluation of the antimicrobial activity.

Gram positive: (1) *Bacillus subtilis* (MTCC-1133), (2) *Enterococcus faecalis* (MTCC-2729), (3) *Micrococcus luteus* (MTCC-1809), (4) *Staphylococcus aureus* (MTCC-3160) and (5) *Staphylococcus epidermidis* (MTCC-3086).

Gram negative: (1) *Aeromonas hydrophila* (MTCC-1739), (2) *Alcaligenes faecalis* (MTCC-126), (3) *Enterobacter aerogenes* (MTCC-2823), (4) *Escherichia coli* (MTCC-294), (5) *Klebsiella pneumoniae* (MTCC-3384), (6) *Pseudomonas aeruginosa* (MTCC-1035) and (7) *Salmonella typhimurium* (MTCC-1253),

Fungal strains: (1) *Aspergillus flavus* (RKS- 108), (2) *Aspergillus niger* (RKS- 104), (3) *Aspergillus oryzae* (4655), (4) *Aspergillus terreus* (RKS- 124), (5) *Alternaria alternata* (6276), (6) *Alternaria brasicola* (1707), (7) *Alternaria solani* (4632) and (8) *Alternaria vitis* (4921).

All the bacterial strains were procured from Microbial Type Culture Collection (MTCC), Chandigarh. The fungal strains used were either the indigenous strains (RKS-108, RKS-104, and RKS-124) maintained in the Department of Biotechnology, CDLU, Sirsa, Haryana; or were procured from Indian Agricultural Research Institute (IARI), New Delhi (4655, 6276, 1707, 4632, and 4921).

Antimicrobial assay

The antimicrobial activity of different extracts was evaluated using agar well diffusion assay²⁵. In this method, 100 µl of 24 h old culture of the test organism was inoculated on the agar plates and then spread on to the surface of the agar with the help of a sterilized glass spreader. After 30 minutes of inoculation of the test microorganisms, wells (5mm diameter) were prepared with the help of sterilized steel cork borer. Out of five wells made in each plate, four wells were loaded with 60 µl of different test plant extracts. Extraction solvent was loaded in the fifth well to serve as the negative control. Sixty µl each of the standard antibiotics viz. ampicillin, penicillin, streptomycin, tetracycline, fluconazol, ketocanazole and miconazole loaded in different wells in a separate plate served as positive control. The plates were then aerobically incubated at 30°C for 24 h for bacterial and at 25°C for 48 h for fungal test microorganisms. Antimicrobial activity was determined by measuring the diameter of zone of inhibition. The diameter (in mm) of zone of inhibition was measured at cross angles and the mean of three independent measurements was taken. The efficacy of the plant extracts was compared by repeating the procedure with the standard antibiotics.

Minimum Inhibitory Concentration (MIC)

Defined as the lowest concentration of the test sample that results in a complete inhibition of visible growth, minimum inhibitory concentration (MIC) was determined by using the dilution method as recommended by the National Committee for Clinical Laboratory Standard²⁶. Different concentrations (ranging from 25 µg/ml to 1000 µg/ml) of all the extracts prepared both in increasing and decreasing order of solvent polarity were tested separately for each microorganism species. A stock solution of each active extract was appropriately diluted in 96-well microtiter plate with Mueller Hinton broth to obtain a concentration ranging from 25 µg/ml to 1000 µg/ml (with a gap interval of 25 µg/ml). A standardized inoculum for each bacterial strain was prepared so as to give an inoculum size of approximately 5x10⁶ CFU/ml in each well. Controls without plant extracts, and without bacterial inoculums were also included in the experiment. Microtiter plates were then kept at 30°C for an overnight incubation. Following incubation, the MIC was recorded as the lowest concentration of the extract inhibiting the visible growth of the bacterial strain. All experiments were carried out in triplicate.

Preliminary phytochemical analysis of the extracts

To assess the chemical composition of the various extracts qualitatively, a preliminary phytochemical analysis was conducted according to the standard methods^{27, 28}. Using these methods, the presence of several phytochemicals like sterols, tannins, proteins, sugars, alkaloids, flavonoids, saponins, anthraquinones, terpenoids, and cardiac glycosides was evaluated.

Test for sterols (Salkowski reaction): A few milligrams of the plant extract was dissolved in 2 ml chloroform and then 2 ml of conc. H₂SO₄ was added from the sides of the test tube. The test tube was shaken for a few minutes. Red colour development in the chloroform layer indicated the presence of sterols.

Test for tannins (Ferric chloride reagent test): The test sample of each extract was taken separately in water, warmed and filtered. To a small volume of this filtrate, a few drops of 5 % w/v solution of ferric chloride prepared in 90 % alcohol were added. Appearance of a dark green or deep blue colour indicated the presence of tannins.

Test for proteins (Xanthoproteic test): The extract (few mg) was dissolved in 2 ml water and then 0.5 ml of conc. HNO₃ was added in it. Yellow colour indicated the presence of proteins.

Test for sugars (Fehling's test for free reducing sugar): About 0.5 g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.

Test for flavonoids (Ferric chloride test): About 0.5g of each extract was boiled with 5 ml of distilled water and then filtered. To 2 ml of this filtrate, a few drops of 10% ferric chloride solution was added. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group.

Test for Saponins: One gram of each extract was boiled with 5 ml of distilled water and filtered. To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins.

Test for anthraquinones: An aliquot of 0.5 g of the extract was boiled with 10 ml of sulphuric acid (H₂SO₄) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for terpenoids (Salkowski test): To 0.5 g of each extract, 2 ml of chloroform was added, followed by a further addition of 3ml of concentrated H₂SO₄ to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test for cardiac glycosides (Keller-Killiani test): To 0.5 g of the extract diluted to 5 ml in water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlaid with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

RESULTS AND DISCUSSION

Antimicrobial assay

The results of the antimicrobial response shown by different extracts are summarized in figures 1 to 8. All the extracts prepared exhibited variable degree of antimicrobial activity against the tested microorganisms. However, the data indicates that the extracts prepared in organic solvents consistently displayed better antimicrobial activity than that of aqueous extracts. The aqueous leaf extracts prepared both in increasing and decreasing order of polarity exhibited almost no antimicrobial activity against all the tested microorganisms. On the other hand, the organic solvent extracts showed a remarkable activity against most of the tested microorganisms. Among the four organic solvent extracts, chloroform and benzene fractions showed a higher zone of inhibition against the bacterial strains; whereas, the fungal strains were found to be more susceptible to the acetone and chloroform fractions. Least activity was exhibited by the methanol fractions. The antimicrobial response of the extracts prepared in both increasing and decreasing order of solvent polarity was found to be almost comparable.

Benzene and chloroform extracts exhibited a strong antimicrobial activity against all bacterial (Gram +ve and Gram -ve) strains tested (Figs. 1, 2, 3, and 4). The benzene extract showed antimicrobial activity against all the twelve bacterial strains tested, exhibiting a zone of inhibition in the range of 6-20 mm against the Gram +ve bacterial strains and between 6-19 mm against Gram -ve bacterial strains. Benzene extracts prepared in increasing order of solvent polarity were found to be more effective than the extracts prepared in decreasing order of solvent polarity. Chloroform extracts showed a slightly higher response forming an inhibition zone in the range of 6-21 mm against Gram +ve and 6-23 mm against Gram -ve bacteria. Acetone extracts prepared in increasing order of solvent polarity formed a higher inhibition zone (7-27 mm) as compared to the extracts prepared in decreasing order of solvent polarity (8-14 mm). Gram +ve bacteria were found to be more susceptible than the Gram -ve bacteria. Methanol extracts were found to be least effective

showing antimicrobial response against only four Gram +ve and four Gram -ve bacterial strains. Contrary to the other solvent extracts, methanol extracts prepared in decreasing order were more effective than the extracts prepared in increasing order of solvent polarity. All the four extracts also showed a good antimicrobial response against all the eight fungal strains tested (Figs. 5 & 6), exhibiting a zone of inhibition in the range of 6-20 mm (Figs. 5 & 6); however, maximum activity was exhibited by the acetone extracts followed by the benzene, chloroform and methanol extract.

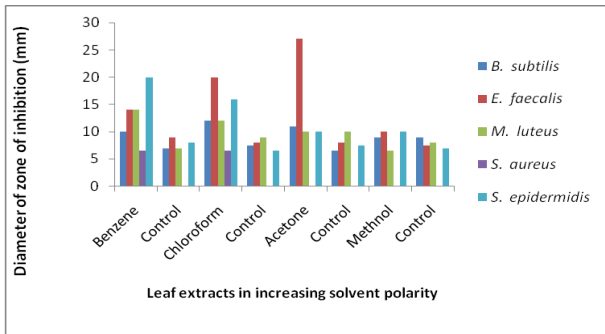


Figure 1: Antibacterial activity of various leaf extracts of *P. dulce* prepared in the order of increasing solvent polarity against gram positive bacteria.

No activity was found in the aqueous extract.

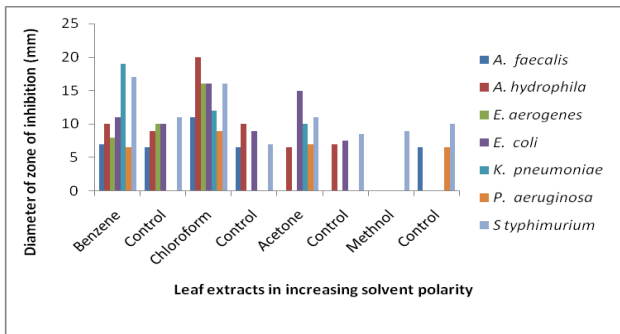


Figure 2: Antibacterial activity of various leaf extracts of *P. dulce* prepared in the order of increasing solvent polarity against gram negative bacteria.

No activity was found in the aqueous extract.

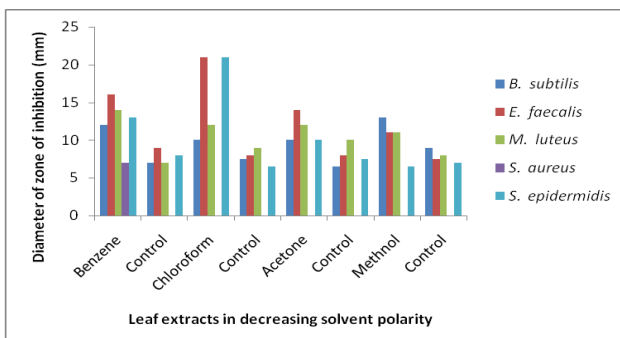


Figure 3: Antibacterial activity of various leaf extracts of *P. dulce* prepared in the order of decreasing solvent polarity against gram positive bacteria.

No activity was found in the aqueous extract.

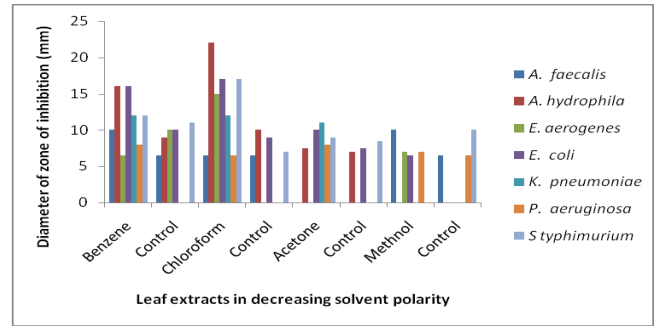


Figure 4: Antibacterial activity of various leaf extracts of *P. dulce* prepared in the order of decreasing solvent polarity against gram negative bacteria.

No activity was found in the aqueous extract.

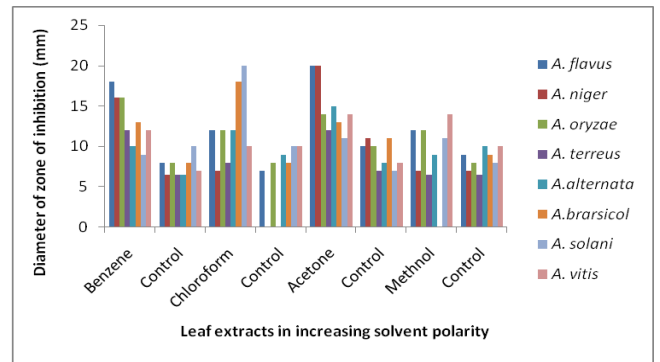


Figure -5: Antifungal activity of various leaf extracts of *P. dulce* prepared in the order of increasing solvent polarity.

No activity was found in the aqueous extract.

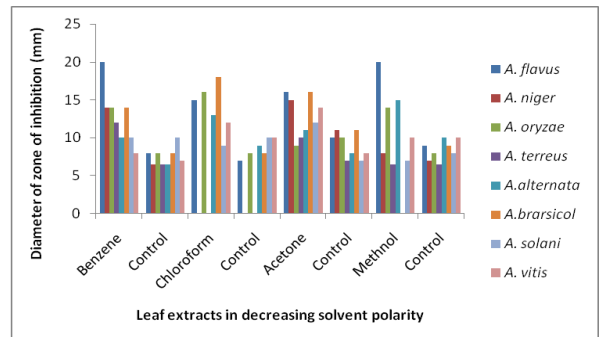


Figure 6:Antifungal activity of various leaf extracts of *P. dulce* prepared in decreasing order of solvent polarity.

No activity was found in aqueous extract.

Among the bacterial strains tested, *S. aureus* was found to be the most resistant Gram +ve strain (forming a smaller zone of inhibition and against the least number of extracts), whereas, *E. faecalis* was the most sensitive strain (exhibiting a larger zone of inhibition against most of the extracts). Among the Gram -ve bacteria, the most resistant strain was *A. faecalis* followed by *E. aerogenes* and *P. aeruginosa*; whereas, the most sensitive organism was *A. hydrophila* followed by *K. pneumoniae*, *E.coli* and *S. typhimurium*. Among the fungal strains, most of the fungi showed a fairly good antimicrobial activity; however, *A. flavus*, *A. niger*, and *A. solani* were more sensitive as compared to the other strains. *A. terreus* was the most resistant fungi, showing least activity with all the extracts. All the microorganisms were more sensitive to the benzene and chloroform extracts as compared to the other extracts.

The effectiveness of the extracts against the various microorganisms was compared with the antibacterial response shown by the antibiotics, viz., ampicillin, penicillin, streptomycin, tetracycline,

fluconazole, ketoconazole and miconazole used as standard drugs (figs. 1 to 6 compared with figs 7 & 8). All the four organic solvent extracts showed a higher activity against most of the microorganisms when compared with ampicillin and penicillin, and with streptomycin to some extent. As compared to the response exhibited by tetracycline, only the chloramphenicol and acetone extracts were found to be slightly better in case of some microorganisms. Among the three antifungal drugs tested, ketoconazole was found to be least effective, followed by a slightly higher response by fluconazole, and the maximum activity shown by miconazole. Compared with the zone of inhibition formed by the standard drugs, all the solvent extracts of *P. dulce* showed an appreciably greater zone than that formed by ketoconazole. The antifungal response exhibited by the ethyl acetate, chloroform, methanol and acetone extracts against some of the fungi was also comparable to that of the other two standard drugs (fluconazole and miconazole). Thus, the results in the present study show that *P. dulce* extracts exhibited significant activity against most of the tested microorganisms which was comparable to that of the standard drugs.

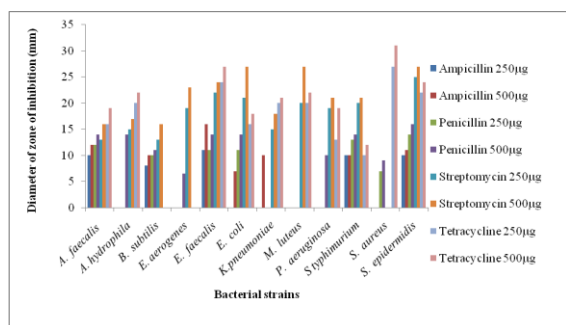


Figure 7: Antibacterial activity of standard antibiotics.

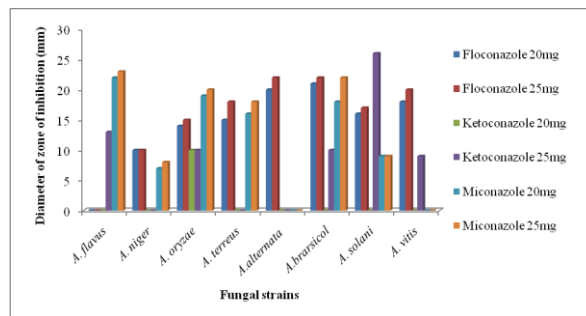


Figure 8: Antifungal activity of standard antibiotics.

Minimum Inhibitory Concentration (MIC)

All the active extracts (leaf extracts prepared in organic solvents) were further subjected to determination of minimum inhibitory concentration, the results being shown in Table 1. Lower MIC values were exhibited by the benzene extracts (200-500 µg/ml) against most of the bacterial strains, except against *P. aeruginosa*, *S. aureus*, *E. aerogenes* and *A. faecalis* where it was slightly higher; followed by the chloroform (between 200-500 µg/ml for majority of the microorganisms) and acetone (between 200-500 µg/ml for 50% of the microorganisms) extracts. Methanol extracts exhibited comparatively higher MIC, indicating less effectiveness of these extracts. Among the various bacterial strains tested, lowest MIC values were obtained for *E. faecalis*, followed by *S. epidermidis* and *A. hydrophila*, indicating that these bacteria were most sensitive to the *P. dulce* leaf extracts. Among the various fungi, the most sensitive was *A. flavus* (MIC values in the range of 200-475 µg/ml), followed by *A. solani* (MIC values in the range of 225-475 µg/ml) and *A. alternata* (MIC values in the range of 200-500 µg/ml). The results of MIC assay confirmed the findings of antimicrobial assays, wherein it was reported that benzene and chloroform extracts were more potent inhibitors of the microorganisms tested; and that *E. faecalis*, *A. hydrophila*, *A. solani* and *A. oryzae* were among the most sensitive strains.

Table 1: Minimum inhibitory concentration (MIC) values of different leaf extracts of *P. dulce* against the tested bacterial strains.

S. No	Microorganisms	Minimum inhibitory concentration (µg/ml)							
		Leaf Extract (<i>Pithecellobium dulce</i>)							
		Benzene (I)	Benzene (D)	Chloroform (I)	Chloroform (D)	Acetone (I)	Acetone (D)	Methanol (I)	Methanol (D)
A									
Bacterial Strains									
1.	<i>A. faecalis</i>	875	325	375	925	-	-	700	725
2.	<i>A. hydrophila</i>	225	400	200	250	250	450	-	-
3.	<i>B. subtilis</i>	250	200	850	750	900	350	850	850
4.	<i>E. aerogenes</i>	1000	1000	275	250	-	-	-	375
5.	<i>E. faecalis</i>	200	250	200	200	250	250	250	425
6.	<i>E. coli</i>	475	400	750	225	325	400	-	1000
7.	<i>K. pneumoniae</i>	250	250	550	500	425	475	-	-
8.	<i>M. luteus</i>	500	300	400	425	250	225	750	550
9.	<i>P. aeruginosa</i>	1000	1000	475	750	900	975	-	1000
10.	<i>S. typhimurium</i>	225	250	750	250	500	475	900	-
11.	<i>S. aureus</i>	1000	1000	1000	-	-	-	-	-
12.	<i>S. epidermidis</i>	200	250	250	225	375	250	275	925
B									
Fungal Strains									
13.	<i>A. flavus</i>	200	400	225	225	300	250	475	400
14.	<i>A. niger</i>	225	225	825	-	200	200	500	750
15.	<i>A. oryzae</i>	200	200	750	375	250	500	-	-
16.	<i>A. terreus</i>	500	550	475	-	400	400	850	850
17.	<i>A. alternata</i>	450	450	200	225	500	475	500	500
18.	<i>A. brasicae</i>	950	975	250	275	500	1000	-	-
19.	<i>A. solani</i>	450	475	225	225	250	250	250	350
20.	<i>A. vitis</i>	250	500	200	750	200	200	250	450

All the values are an average of three determinations.

- : No activity.

(I) : Increasing order of solvent polarity.

(D) : Decreasing order of solvent polarity.

Phytochemical analysis of plant extracts

Preliminary phytochemical analysis of leaf extracts (benzene, chloroform, acetone and methanol) showed the presence of alkaloids, anthraquinones, cardiac glycosides, proteins, tannins, terpenoids, and sugars (table 2). Saponins were found to be absent in all the tested extracts; while flavonoids and sterols were found to be present in 50% of the extracts, acetone & methanol and benzene & methanol, respectively. The relative antimicrobial activity of leaf extracts may not be easily correlated with any individual component but with a mixture of compounds present in these extracts. There

are reports showing that alkaloids are responsible for the antifungal activity in higher plants²⁹. It has also been suggested that the antimicrobial activity is mainly due to the presence of alkaloids, terpenoids and other natural polyphenolic compounds or due to free hydroxyl groups³⁰. Moreover, secondary metabolites such as tannins and other compounds of phenolic nature are also classified as antimicrobial compounds. Therefore, the presence of these phytochemicals (alkaloids, anthraquinones, cardiac glycosides, proteins, tannins, terpenoids, and sugars) could to some extent justify the observed antimicrobial activity in the current study.

Table -2: Preliminary phytochemical analysis of leaf extracts of *P. dulce*.

Phytochemicals tested	Test used	Leaf Extracts			
		Benzene	Chloroform	Acetone	Methanol
Alkaloids	Wagner's test	++	+	+	+
Anthraquinones		+	++	+++	+++
Cardiac glycosides	Keller-Killiani test	+	+	++	-
Flavonoids	Ferric chloride test	-	-	+++	++
Proteins	Xanthoproteic test	+	+	+	+++
Tannins	Ferric chloride reagent test	+	++	+++	++
Terpenoids	Salkowski test	++	+++	+	++
Saponins	Foam test	-	-	-	-
Sterols	Salkowaski test	++	-	-	++
Sugars	Fehling's solution test	++	+++	++	++

CONCLUSION

The present study scientifically validates the antimicrobial potential of the traditionally important plant, *Pithecellobium dulce*. The results provide an important basis for the use of benzene and chloroform extracts of the tested plant species for the treatment of infections associated with the pathogens used in this study, which could be useful for the development of new antimicrobial drugs. With an aim to identify the chemical nature of compounds responsible for the antimicrobial response, the study also involved a preliminary phytochemical analysis of the crude extracts. However, further studies related to the isolation and identification of the particular compounds responsible for the antimicrobial activity are underway. The antimicrobial mechanisms associated to each group of chemicals to which the isolated compounds belong, may explain the inhibition potency of the tested samples. Present results allow us to conclude that the crude extracts of *P. dulce* exhibit significant antimicrobial activity and properties that support folkloric use of this plant; corroborating the importance of ethno pharmacological surveys in the selection of plants for bioactivity screening.

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