Antibacterial properties of Alkaloid rich fractions obtained from various parts of *Prosopis juliflora*

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Abstract

The alkaloid rich fraction obtained from various parts of *Prosopis juliflora* were assessed for their antibacterial property using disc diffusion method on several Gram-negative and Gram-positive bacterial strains like *E.coli, Staphylococcus aureus, Bacillus cereus, Psuedomonas putida, Klebsiella, Salmonella, Acinetobacter and Alcaligen.* Strong antibacterial effect was shown by leaf, pod and flower extract, with MIC value ranging between 25µg/ml-100µg/ml. The extracts of leaves showed highest activity among all the plant parts. *Klebsiella* was found to be the most susceptible bacteria, whereas *Acinetobacter* and *Alcaligen* were the least susceptible. A comparison of zone of inhibition created by alkaloid rich fractions with that of standard antibiotics, ampicillin, tetracycline, chloramphenicol, oflaxacin, refampin, streptomycin and sulfa drug showed a comparable zone of inhibition. Growth of *Acinetobacter* and *Alcaligen* which were not inhibited by antibiotics, showed inhibition by the alkaloidal extracts, similarly a known ampicillin resistant *E.coli* strain was found to be inhibited by the plant extracts. Alkaloids present in the extracts were analysed by DART-MS. DART-MS analysis of the alkaloid rich fractions showed the presence of piperidine alkaloids.

Keywords: *Prosopis juliflora*, antibacterial, DART-MS, piperidine alkaloids

1. Introduction

Incidents of epidemics due to drug resistant microorganisms, pose enormous threat to the human health (**1, 2**). The emergence of antibiotics resistance in microbes due to indiscriminate use of antibiotics, requires the need to look for alternative sources of antimicrobial agents. One of the possible strategies towards this objective involves the rational localization of bioactive phytochemicals having antibacterial activity.

Plants have always been a source of natural product for the treatment of various disease (**3, 4**). Plants develop unique family of chemicals to protect themselves from various microbes. Some of their extracts were used by early human civilization against many forms of disease and infection. Even today plants are the almost exclusive source of drugs for the majority of the world population. People in developing countries utilize traditional medicine for their primary health care needs, but despite of having a wide historical background there are only a handful of plants that have been exhaustively studied for their potential value as a source of drugs(**5**).

Many researchers reported that the concentration of secondary metabolites varies from plant to plant species and even in the different parts of the same species.(**6**), however very little information is available about the distribution of secondary metabolites. Therfore it becomes imperative to analyze different parts of the plant so that full pharmacological potential of the plant could be exploited.

Prosopis juliflora, a member of family Leguminosae, is found in arid and semi-arid regions of India. It has been used as a folk remedy for catarrh, cold, diarrhea, dysentery, excrescences, flu, hoarseness, inflammation, measles, sore throat and in healing of wounds (**7**). Decoction prepared from leaf and seed extracts are used in wound healing, as disinfectant and also to treat scury (**8**). *P. juliflora* syrup prepared from ground pods is given to children showing weight deficiency or retardation in motor development, the syrup is believed to increase lactation. Tea made from *P. juliflora* is thought to be good for digestive disturbances and skin lesions.

P.juliflora are rich source of piperidine alkaloids. Many alkaloids such as juliflorine, julifloricine and julifloridine (**9**), juliprosine (**10**), juliprosinene and juliflorinine (**11**), 3'-oxojuliprosopine, sceojuliprosopinol, 3-oxojuliprosine and 3'-oxo-juliprosine (**12**) have been isolated from leaves and have proven to be pharmacologically active (**13, 14**), however less work has been done to evaluate biological activity and chemical characterization of other parts of the plants. The present work aims at screening alkaloid rich

fractions obtained from various parts of *P.juliflora* against some wild type and drug resistance bacterial strains and compare their activity with standard antibiotics. Chemical fingerprint of the active fractions with the help of DART-MS were generated to identify the compounds.

2. Method

2.1. Plant Material

Plant material(leaf, pod, flower, root and stem) of *Prosopis juliflora* were collected from the Shekhawati regions of Rajasthan, and identified by the help of Department of Botany, Central Drug Research Institute(CDRI), Lucknow.

2.2. Crude extract preparation for preliminary screening

100 grams of dry powder was taken in beaker and ethanol was added to it so that the plant material got totally immersed in the solvent. This whole setup was kept for 48 hours with frequent shaking. It was first filtered with a muslin cloth, then with whatman filter paper (No.1) and finally centrifuged at 5000 rpm for 5 mins. Whole process was repeated 3 times and supernatant were collected and pooled together. The extract was concentrated to $1/20^{th}$ of there initial volume with the help of rotary evaporator (Buchi Rotavapor R-200/205) at 40°C. This extract was tested against antibacterial strains at concentration of 100mg/ml.

2.3. Alkaloid extraction

The alkaloid extract was obtained by an acid/basic extraction process as described by (**15**). 500g of dry powder was extracted with ethanol, this ethanol extract was fractionated with the help of petroleum ether and water. The aqueous layer was separated and again extracted three times with petroleum ether. The ether extract contained waxes, steroids, triterpenoids and other neutral and acidic compounds, whereas aqueous layer contained alkaloids, sugars, amino acids etc. Aqueous layer was stirred with 0.2N HCl for 16 h followed by filtration. The solution was shaken with chloroform to remove the non-basic material. The aqueous layer was basified with ammonium hydroxide until it reached pH 11, and then was extracted with chloroform. The chloroform phase was evaporated leading to the production of the Alkaloid rich fraction(ARF). It was stored at concentration of 10mg/ml. Presence of alkaloid was tested by Dragendorf's reagent.

2.4. Separation of ARF by Thin layer chromatography

ARF were further separated by thin layer chromatography. The chromatographic estimations were performed using following conditions: stationary phase, RP C_{18} TLC plates precoated silica gel 60 F_{254} aluminium plates (20 cm×20 cm×250 μm); mobile phase, chloroform:methanol (9:1) . Each separated spot was scratched and dissolved in small amount of solvent and tested for its bioactivity using the disc diffusion assay as mentioned below. They were also tested for the presence of alkaloids by Dragendorf's reagent (**16**).

2.5. Antibacterial assay

Bacterial bioassay was performed by disc diffusion method (**17**) on the following test bacteria, obtained from MTCC(IMTECH, Chandigarh), *Escherichia coli* (MTCC 40), *Staphylococcus aureus*(MTCC3160), *Bacillus cereus*(MTCC430), *Pseudomonas putida*(MTCC672), *Klebsiella pneumonia*(MTCC3384), *Salmonella* species(MTCC3215), *E.coli(amp R), Alcaligen sp. and Acinetobacter sp.*(local isolates).

For the agar disc diffusion method, ARF(10µl) was applied on the sterile whatman filter paper disco of 0.5cm size and then allowed to dry for 1h. Then the disc was introduced on the upper layer of media with the bacteria. The plates were incubated for 24h at 37°C. Antibacterial activity of the compound was determined by measuring the diameter of zone of inhibition of microbial growth. Standard antibiotics, chloramphenicol, ampicillin, tetracycline, streptomycin, rifampin, sulfa drug and oflaxacin (1mg/ml) were also tested for their antibacterial activity.

2.6. Minimum inhibitory concentration (MIC)

MIC of ARF was determined by two-fold serial dilution method. A serial dilution of various fractions was carried out to give final concentrations between 0.5-0.0025 mg/ml. 0.1 ml of varying concentrations of alkaloid fractions were added into the test tubes separately, containing 10 ml of standardized suspension of tested bacteria (10^8 cfu ml^{-1}). The test tubes were incubated at 37°C for 24 h. Controls were used with the test organisms, using distilled water instead of the plant extract. The least concentration of the samples with no visible growth was taken as the MIC (18).

2.7. Determination of compounds by DART -MS analysis

The DART-MS was recorded on a JEOL-AccuTOF JMS-T100LC Mass spectrometer having a DART (Direct Analysis n Real Time) source. The given samples were subjected to DART source. Extracts were analysed in positive ion mode.

3. Result and discussion

The ethanol fractions obtained from various parts of *P.juliflora* were tested for its antibacterial property against several Gram-negative and Gram-positive bacteria. Results show that leaf, pod and flower extracts were efficient in inhibiting growth of bacteria, whereas root and stem extracts did not show zone of inhibition against any of the tested bacteria (Table1).

Ethanol extract of leaf, pod and flower showing antibacterial activity were further subjected for alkaloid extraction and then tested against bacterial strains. Table 2 show that ARF of leaf, pod and flower have strong antibacterial activity. ARF obtained from leaf was comparatively more effective in inhibiting bacterial growth, as evident by a larger zone of inhibition. The result show that *E.coli, P.putida, B.cereus. Klebsiella sp. S.aureus, E.coli(ampR)* are susceptible to ARF of all the tested plant parts whereas *Salmonella sp*., *Acinetobacter sp*. and *Alcaligen ap*. are inhibited by leaf and pod extracts and are resistant towards flower extract.

A comparison of zone of inhibition of leaf, pod and flower extract with that of standard antibiotics showed almost comparable antimicrobial activity (Table2). It was observed that except Oflaxacin, none of the antibiotic is able to inhibit the growth of all the tested bacterial strain whereas leaf and pod extracts are effective in inhibiting the growth of all the tested strains.

Table 2 shows that few bacterial strains are multidrug resistant, *Acinetobacter* is resistant towards ampicillin, chloramphinecol, tetracycline, rifampin, and sulfa drug whereas *Alcaligen* is resistant towards ampicillin, chloramphinecol, tetracycline, rifampin, streptomycin and sulfa drug. A known *E.coli* ampicillin resistant strain is not only resistant towards ampicillin but also towards sulpha drug. The growth of these were found to be inhibited by alkaloid extracts.

The result of the MIC test (Table 3) showed that the the minimum inhibitory concentration required to inhibit the growth of bacteria ranged from 25µg/ml-100µg/ml with leaf ARF having lower MIC vaules as compared with pod and flower. The least MIC value for leaf extract was observed against *Klebsiella sp*.(25 µg/ml), followed by *E.coli, P.putida, B.cereus, S.aureus, E,coli*(amp R)(50 µg/ml), and then *Alcaligen sp.* (75 µg/ml), *Acinetobacter sp*. and *Salmonella* sp.(100 µg/ml). The pod extract showed least MIC for *Klebsiella* (50 µg/ml), followed by *E.coli, P.putida, E,coli*(amp R) (75 µg/ml), and then *B.cereus, S.aureus, Alcaligen sp., Acinetobacter sp*. and *Salmonella sp.* (100 µg/ml). The flower extract exhibited the least MIC for *Klebsiella* (50 µg/ml), followed by *E.coli, P.putida* (75 µg/ml) and then *B.cereus, S.aureus and E,coli*(amp R) (100 µg/ml), for *Alcaligen sp., Acinetobacter sp. and Salmonella sp*. none of the tested concentrations of flower ARF were effective in inhibiting their growth.

ARF obtained from *P.juliflora* were separated on TLC and after spraying dragondorff reagent it was observed that leaf contains highest number of alkaloid with 3 spots giving positive alkaloid test. Pod extract was separated into 2 spots and flower extract showed one continous band . Each spot was tested for antibacterial activity. Out of 3 spots obtained from TLC separartion of leaf ARF, 2 fractions were effective in inhibiting growth of all the tested bacterial strains. ARF of pod and flower showed two and one spot respectively, and all fractions of each group was effective in inhibiting bacterial growth.

Active fractions separated by TLC were subjected to DART – MS analysis for alkaloids identification. DART-MS analysis of first active spot obtained from leaf extract showed the presence of main alkaloid of *P.juliflora*, Juliflorine or Juliprosopine (m/z $[M+H]^+$ 630.5815), which was present in highest concentration, Other minor alkaloids identified were, Juliprosine(m/z $[M+H]^+$ 628.5639) and Juliprosinine(m/z $[M+H]^+$ 626.5474). Second active spot showed presence of 3 alkaloids, in which the compound present in highest concentration was identified as Julifloridine(m/z $[M+H]^+$ 300.2813), other minor alkaloid include projuline (m/z $[M+H]^+$ 421.3983) and prosafrinine (m/z $[M+H]^+$ 298.2674).

Chemical fingerprint of leaf was compared with pod and flower (Table 5,6). Juliprosopine was almost absent and observed in very small amount only in pod. Major alkaloid present was Julifloridine,

Chemical fingerprint of both the active fractions of pod showed presence of similar group of alkaloids, which include Juliprosopine(m/z $[M+H]^+$ 630.5841), Julifloridine(m/z $[M+H]^+$ 300.2804), and Prosafrinine(m/z $[M+H]^+$ 298.2672) along with some other chemicals . Flower extract showed presence of only one main alkaloid, Julifloridine(m/z $[M+H]^+$ 300.2825).

4. Discussion

Ability of the alkaloid rich fraction obtained from various parts of *P. juliflora* to inhibit growth of almost all the tested bacterial species revealed a broad spectrum antibacterial property of the extracts. This result supports the findings of Elisabetsky and Costa-Campos, 2006(**19**) that alkaloids are used by the plants in defence mechanism against pathogens and predators. The earlier reports of Kandasamy, 1989 (**20**) have indicated antibacterial properties of leaf extract of *P.juliflora*, but the present investigation shows that pod and flower, also have the potential to inhibit bacterial growth.

Infections caused by multi-drug resistance bacterial species are among the most difficult to treat with conventional antibiotics (**21, 22**). In our study, the growth of *Acinetobacter sp, Alcaligen* sp., *Salmonella sp*. and *E.coli* (ampicillin resistant), that were found to be resistant towards many standard antibiotics, was remarkably inhibited by the alkaloid fractions, this shows the potential of these plant parts to control the growth of drug resistant microbes. It could also be concluded that the antibacterial compound extracted from *P.juliflora* may inhibit bacteria by a different mechanism than that of currently used antibiotics.

In order to identify the alkaloids present in leaf, pod and flower extract that are responsible for the antibacterial activity DART-MS was done. It was observed that piperidine alkaloids are present in all the active fractions. Two groups of alkaloid were present, one with indolizidine ring in the centre of the molecule and other without indolizidine ring. Juliprosopine, Juliprosine and Juliprosinine belong to first group of alkaloids were as Julifloridine, Projuline and Prosafrinine belong to second group. Antibacterial activity of first group of alkaloids is reported by Ahmed etal. 1978, however the present research group showed that other group of alkaloids also have the potential to inhibit bacterial growth. DART-MS analysis also revealed that Juliprosopine and Julifloridine are present in highest concentrations in the active fraction, therefore it could be conclude that these alkaloids could be mainly responsible for the antibacterial activity of leaf, pod and flower.

Lower MIC value of ARF obtained from leaf, as compared with pod and flower against all of the tested bacterial strain, show more potential of leaf extract to inhibit bacterial growth. This result is in consistence with TLC and DART analysis of leaf, pod and flower extracts, showing more diversity of alkaloids in leaf extract, which could have caused more synergistic effect of leaf alkaloids in inhibiting bacterial growth as compared to pod and flower extracts.

5. Conclusion

Antibacterial test conducted in the present work shows that leaf, pod and flower extracts of *P.juliflora* have antibacterial property and also have the potential to inhibit antibiotic resistance bacterial strains. This property can be exploited to control drug resistant pathogenic bacteria. DART-MS analysis showed that *P.juliflora* , pod and flower are also rich source of piperidine alkaloid along with the leaf. Leaf contain more diverse group of alkaloids whereas pod and flower contains lesser number of alkaloids.

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Table 1. Zone of inhibition exhibited by ethanol extracts obtained from various parts of *P.juliflora*, on tested bacterial strain

	Zone of inhibition(mm)				
Microorganism	**Leaf extract**	**Stem extract**	**Pod extract**	**Flower Extract**	**Root extract**
E.coli	12.81±0.45	-	9.80±0.56	10.01±0.09	-
P.putida	11.40±0.56	-	10.35±0.12	9.36±0.67	-
B.cereus	13.12±0.37	-	10.160.78	10.17±0.45	-
Klebsiell sp.	11.83±0.88	-	11.23±0.88	11.77±0.83	-
S.aureus	12.72±0.67	-	11.23±0.54	10.40±0.56	-
Salmonella sp.	11.04±0.33	-	7.46±0.39	-	-
E.coli(Amp R)	13.23±0.45	-	10.56±0.12	10.63±0.54	-
Acinetobacter sp.	9.33±0.11	-	7.03±0.03	7.09±0.01	-
Alcaligen sp.	8.26±0.67	-	7.05±0.02	-	-

Extract concentration 100mg/ml

Table2. Comparitive analysis of the Antibacterial activity of ARF with that of standard antibiotics on tested bacterial strains

	Zone of Inhibition(mm)									
	Leaf	Pod	Flower	Ampilcillin	Chloramphi-necol	Tetracyclin	Rifampin	Streptomycin	Sulfa drug	Oflaxacin
E.coli	1.7±0.62	1.2±0.16	1.2±0.33	1.5±0.57	1.6±0.79	1.3±0.54.	1.1±0.03	2.8±0.44	-	3.0±0.68
P.putida	1.5±0.57	1.1±0.66	1.3±0.51	1.5±0.66	1.5±0.88	1.9±0.08	1.6±0.23	2.2±0.33	-	2.2±0.98
B.cereus	1.4±0.33	0.8±0.57	0.9±0.88	1.0±0.00	1.2±0.88	1.5±0.67	-	2.3±0.79	1.1±0.89	1.7±0.66
Klebsiella sp	2.0±0.79	1.6±0.66	1.5±0.79	3.0±0.89	1.5±0.16	2.6±0.66	2.8±0.00	1.6±0.57	2.2±0.51	3.2±0.44
S.aureus	1.8±0.57	1.0±0.79	1.1±0.79	1.7±0.66	1.8±0.57	3.0±0.51	3.5±0.54	1.2±0.03	1.3±0.07	2.1±0.88
Salmonella sp.	1.1±0.3	1.0±0.07	-	2.3±0.51	2.5±0.16	-	0.9±0.00	2.9±0.89	1.2±0.07	2.9±0.66
E.coli(amp R)	1.7±0.79	1.2±0.89	0.8±0.89		1.9±0.33	1.2±0.05	1.1±0.3	2.2±0.45	-	3.1±0.88
Acinetobacter sp.	1.0±0.89	0.8±0.66	-	-	-	-	-	1.7±0.67	-	2.7±0.51
Alcaligen sp.	1.2±0.88	0.9±0.00	-	-	-	-	-	-	-	2.5±0.16

Extract- 10mg/ml ; Antibiotic-1mg/ml

Table 3. Minimum concentration of the ARF required to inhibit the growth of tested bacterial strains

	MIC(µg/ml)		
Microorganism	Leaf	Pod	Flower
E.coli	50	75	75
Psuedomonas putida	50	75	75
Bacillus cereus	50	100	100
Klebsiella sp.	25	50	50
Staphylococcus aureus	50	100	100
Salmonella sp	100	100	> 100
E.coli (Amp. resistant)	50	75	100
Acinetobacter sp.	100	100	>100
Alcaligen sp.	75	100	>100

Table 4*: P.juliflora* leaf PI-DART Peak Measurements

Compound	**Mol.formula**	**Exact mass[M+H]$^+$**
Juliprosopine	$C_{40}H_{75}N_3O_2$	630.5815
Juliprosine	$C_{40}H_{73}N_3O_2$	628.5639
Juliprosinine	$C_{40}H_{71}N_3O_2$	626.5474
Julifloridine	$C_{18}H_{37}NO_2$	300.2813
Projuline	$C_{26}H_{48}N_2O_2$	421.3983
Prosafrinine	$C_{18}H_{35}NO_2$	298.2674

Table 5: *P.juliflora* pod PI-DART Peak Measurements

Compound	**Mol. Formula**	**Exact mass[M+H]$^+$**
Juliprosopine	$C_{40}H_{75}N_3O_2$	630.5841
Julifloridine	$C_{18}H_{37}NO_2$	300.2804
Prosafrinine	$C_{18}H_{35}NO_2$	298.2672

Table 6*: P.juliflora* Flower PI-DART Peak Measurements

Compound	**Mol. Formula**	**Exact mass[M+H]$^+$**
Julifloridine	$C_{18}H_{37}NO_2$	300.2825