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Study on Antibacterial Compounds from Methanolic Extract of Bark of Prosopis juliflora (Vilayati babhul)

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Abstract: ***This study was aimed at evaluating antibacterial potential of the Prosopis juliflora bark (Vilayati bhabul) in attempt to identify potential natural sources for synthesis of new drug to avoid the growing antibacterial resistance. The peals of bark were extracted by hot methanol by Soxhlet extraction method. Antibacterial activity of crude extract was determined by agar cup method on various human pathogens. Antibacterial susceptibility test of crude extract was performed and it showed promising activity against tested organisms. The crude extract was subjected for HPTLC analysis to separate phyto-compounds such as glycosides, tannins, saponins and alkaloids. The crude extract was subjected to Activity guided fractionation with different polarity solvents, showed varying levels of bactericidal activity. Fraction D and fraction F shows maximum activity. MIC of fraction D was 3.38mg/ml and 1.69mg/ml for Staphylococcus aureus and Escherichia coli respectively whereas MIC of fraction F for these two organisms was observed to be 2.39mg/ml and 4.77mg/ml respectively and also HPTLC and bioautography was performed. Phytochemical analysis was carried out by tube method and HPTLC plate. Preparative HPTLC was performed to obtained semi purified bioactive compound and separated band were subjected to AST, UV spectroscopy, GCMS, FTIR,CHNS(O) analysis and NMR spectroscopy.***

Keywords: Prosopis juliflora, Methanolic extract, AST, Fraction D (ethyl acetate), Fraction F (methanol), phytochemical analysis, HPTLC

Introduction:

A special feature of higher plants is their capacity to produce a large number of chemicals of high structural diversity. Collectively plant produce remarkably diverse array of over 1,00,000 low molecular mass natural products; also known as secondary metabolites. *Prossopis juliflora* commonly known as 'mesquite'. Secondary metabolites are present such as tannins, flavonoids and alkaloids. *P juliflora* is well known for its antibacterial and antioxidant properties.

Pharmacological properties under in vitro study shown that antimicrobial, antifungal, anti- inflammatory and hemolytic activities attributed to leaves extract of *Prosopis juliflora*. Additionally; cytotoxic, antitumoral activity against human epithelial tumor cell (HeLa), human hepatic tumor (HepG2) [7].

Prosopis juliflora DC. is native to tropical America, but is naturalized in many countries including Egypt and India. [8] The nutrient concentrations in components of *Prosopis juliflora* are quite high as compared to many temperate trees. Leaves and small branches together accounted for less than 29% of the biomass in small trees, they contained 60%, 57%, 63%, 31%, and 63% of the total tree N, P, K, Ca and Mg respectively. Fast growing legumes like *Prosopis juliflora*, have high in litter fall and fixes atmospheric nitrogen.[4]
It has bipinnately compound leaves, alternate in arrangement. The shape is belong with an entire margine, blun apex, obtuse base, glabrous surface, reticulate vanation, petiole. The leaf size is 2.5cm in length and 0.3cm in breath. Fresh leaves are green in colour, and are odourless with a less patalable taste.[9] It is an evergreen tree with a large canopy. *Prosopis juliflora* is considered as poor man's fuel wood, as it is the only fast growing fuel wood capable of growing in wide range of soil including problematic sites like eroded lands and salt affected soils. It is one of the most tolerant species for saline, alkaline soils and also capable of growing in waterlogged areas.[8]. There are benefits from this plants such as nitrogen fixation promotion, soil amelioration, livestock feeds, biopesticides, honey and wax etc. When antibacterial and antifungal activity of *P juliflora* and *P cineraria* were compared , *P juliflora* was found to be good antibacterial agent for *Bacillus subtilis*, *Escherichia coli*, *vibrio cholerae* and *Enterobacter aerogenes*.

Phytochemicals: Phytochemicals are a large group of plant derived compounds hypothesized to be responsible for the disease protection conferred from diet high in fruits, vegetables, beans, cereals**.**[5] phytochemicals are the bioactive, non-nutritive compound.[1]Secondary metabolites found in plants are alkaloids, tannins, saponins, glycosides, flavonoids, pheniloics etc. Two compounds are belong to alkaloids which was isolated from bark of *P juliflora* tree are 1,3-Oxojuliprosopae and Secojuliprosopinal. Most of alkaloids found in leaf and pods of *P juliflora*

Material and methods:

Plant materials: Plant material collected from Dighanchi, Maharashtra in May 2012. Plant was identified by botanist.

Extraction: Bark of *Prosopis juliflora* were dried under sunlight. Bark powder was made by grinder and stored in dry place. Extraction from bark were prepared by using Whatman 41 filter paper and in methanol solvent.

Activity guided Fractionation: Activity guided fractionation was performed with organic solvents in increasing order of polarity from Petroleum ether (fraction A)< Chloroform (fraction B)< Benzene (fraction C)< Ethyl acetate (fraction D)< Acetone (fraction E)< Methanol (fraction F)< Water (fraction G) of crude extract of bark so that the active components soluble in that particular solvent can be extracted and further assayed for their antimicrobial activity.

Media for bacterial growth: Nutrient agar slant was used to grow bacteria. Antibacterial studies were done by using Muller Hinton agar.

Bacterial strains used: 2 strains of gram positive were *Staphylococcus aureus* (MTCC 1144), *Streptococcus pyogenes* (Lab strain), and others were gram negative *Escherichia coli* (NCIM 2641), *Klebsiella pneuminiae* (MTCC 4032), *Salmonella typhimuriun* (NCIM 2501), *Salamonella paratyphi* B (MTCC 3220), *Shigella flexneri* (MTCC 1457), *Proteus mirabilis* (NCIM 2813), *Pseudomonas aeruginosa* (Lab strain).

Antibacterial assay: Antibacterial assay was carried out by disc method and agar cup method by using Muller Hinton agar.

Phytochemical analysis: Phytochemical analysis was preformed with different test to find the phyto constituents present in the fraction.

Result and Discussion:

1. Minimum inhibitory concentration: In order to determine the exact concentration of the extract that inhibits the growth of the test cultulres, a 96 well Microtiter plate assatay was performed using serial two fold dilutions of extract. Upon incubation 37^0c overnignt, colour change to pink was observed in the wells where the test cultures were growing and the rest of the wells showed no colour change indicating the inhibitory action of antibacterial extract.Minimum inhibitory concentration value attributed to the fact that the active components are present in low concetration or there is some antagonistic components present that serve as groth of bacteria, therby necessitating the presence of high amount of extra to inhibit the growth.

Table no 1: Minimum inhibitory concentration

Extract/ fraction	***E coli***		***S aureus***	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Crude extract	± 6.75	± 3.38	± 4.77	± 2.39

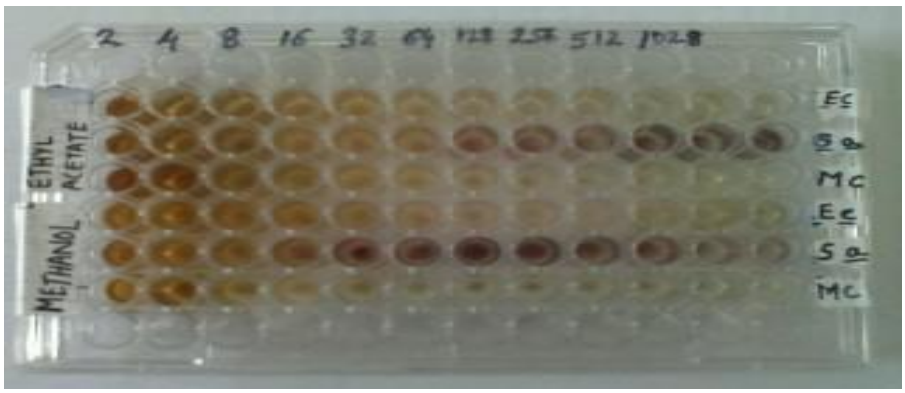


Fig 1: MIC of Ethyl acetate fraction and Methanol fraction for *Escherichia coli* NCIM 2641 & *Staphylococcus aureus* MTCC 1144

2. Antibacterial activities determination: The antibacterial activity of the extract and its potency was quantitatively assessed by the presence or absence of the zone of inhibitions and measurement of zone diameter. Hot extraction with methanol showed good inhibitory action on all tested organisms. Two methods used to check its bactericidal action that is Agar cup diffusion method and Paper disc diffusion method. On comparing, Agar cup diffusion method gave best results than paper disc method.

In this case, *Salmonella paratyphi B*(25mm), showed maximum activity while, the sensitivity of other strain showed inhibition: *Pseudomonas species* > *Klebsiella pneumoniae* MTCC 4032 > *Proteus mirabilis* NCIM 2813 > *Escherichia coli* NCIM 2641 > *Shigella* MTCC 1457 > *Streptococcus pyogens* > *Staphylococcus aureus* MTCC 1144. *Proteus mirabilis* NCIM 2813 exhibited highest antibacterial activity by disc diffusion method. Other strain showed inhibition: *Escherichia coli* NCIM 2641 > *Staphylococcus aureus* MTCC 1144 > *Shigella* MTCC 1457 > *Streptococcus pyogens* > *Salmonella paratyphi* B MTCC 3220. *Klebsiella pneuminiae* did not showed inhibition when checked by the same method.Therefore, in both cases methanolic extract of bark shows maximum activity against Gram negative and Gram positive organisms. Its Antibacterial activity thus served to be broad spectrum as its activity was independent of the organisms being Gram positive and Gram negative.

Table No. 2: AST of crude extract by Paper disc diffusion method.

Culture	Zone of inhibition (mm) Methanolic extract of bark		Antibiotic control (mm)
	Conc Extract	Extract (1:2)	
E cherichia coli NCIM 2641	13.5	-	15
Proteus mirabilis NCIM 2813	19	10	10
sedomonas aeruginosa (lab strain	10	-	10.5
ebsiella pneumoniae MTCC 4032	-	-	16
monella paratyphi B MTCC 3220	9.5	15	28
Shigella flexneri MTCC 1457	12	-	16
phylococcus aureus MTCC 1144	12	8	23
eptococcous pyogenes (lab strain)	11	9.5	20

Key : Antibiotic controls: For Gram -ve organisms= Tetracyclin (30µg)

For Gram +ve organism = Penicillin (10 units)

Graph 1: Graphical representation of AST of crude methanolic extract by paper disc diffusion method.

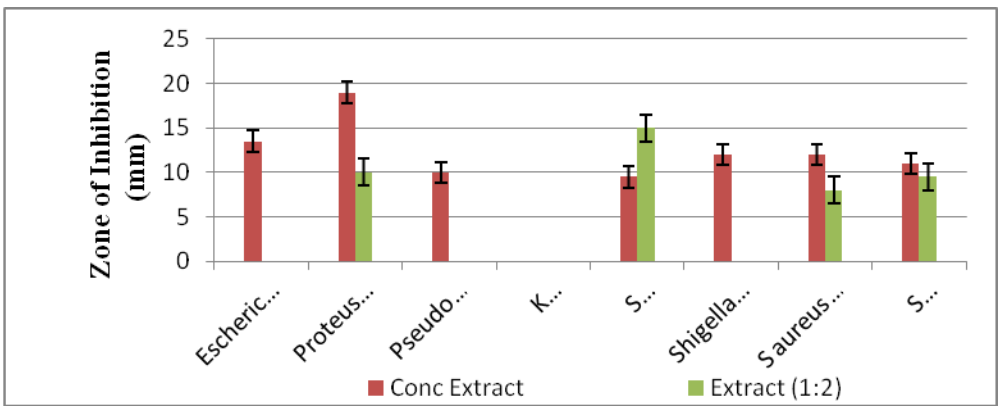


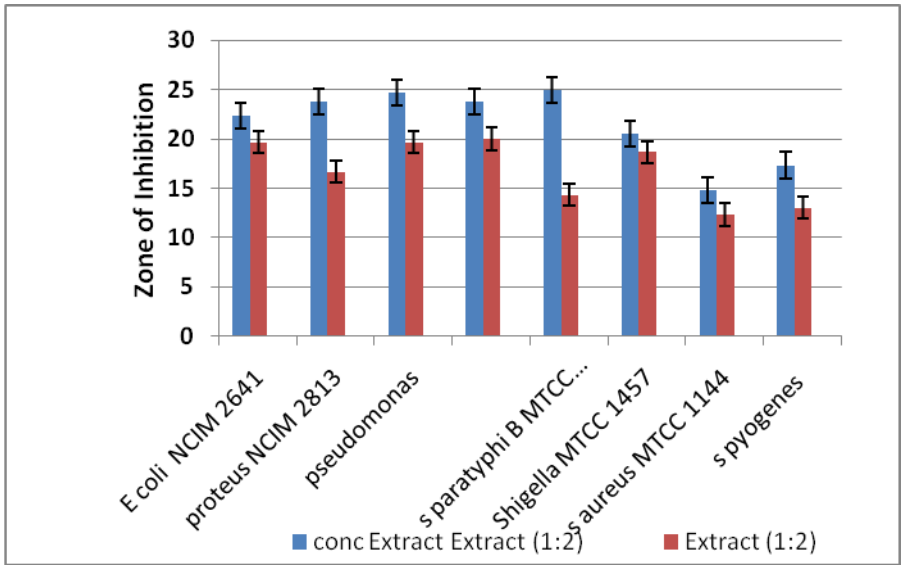
Table no. 3 : AST crude extract by Agar cup method on MHA plate

Cultures	Zone of inhibition(mm) Methanolic extract of bark		Antibiotic control (mm)
	Conc. Extract	Extract (1:2)	
Escherichia coli NCIM 2641	± 22.33	± 19.66	± 20
Proteus mirabilis NCIM 2813	± 23.83	± 16.66	± 16
PseaPP Psedomonas aeruginosa (lab strain)	± 24.66	± 19.66	± 12
Klebsiella pneumoniae MTCC 4032	± 23.83	± 20	± 23.5
Salmonella paratyphi B MTCC 3220	± 25	± 14.33	± 24
Shigella flexneri MTCC 1457	± 20.5	± 18.66	± 22.5
Staphylococcus aureus MTCC 1144	± 14.83	± 12.16	± 18.5
Streptococcous pyogenes (lab strain)	± 17.33	± 13	± 35

Keys : Antibiotic controls for Gram-ve organisms= Streptomycin (10µg)

For Gram+ve organisms= penicillin (2units)

Graph 2 : Graphical representation of AST by agar cup method of crude methanolic extract of bark.



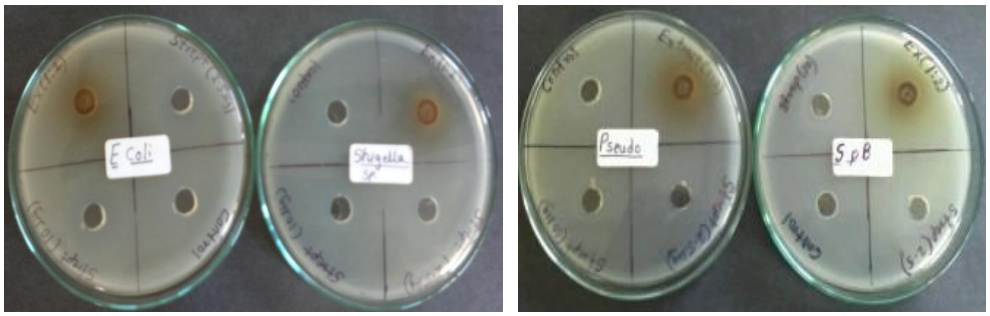


Fig 2 a) : AST by Agar cup method aginst *Escherichia coli* NCIM 2641 and *Shigella flexneri* MTCC 1457

Fig 2 b) : AST by Agar cup method aginst *Pesudomonas aeruginosa*(lab strain) and *Salmonella paratyphi* B MTCC 3220

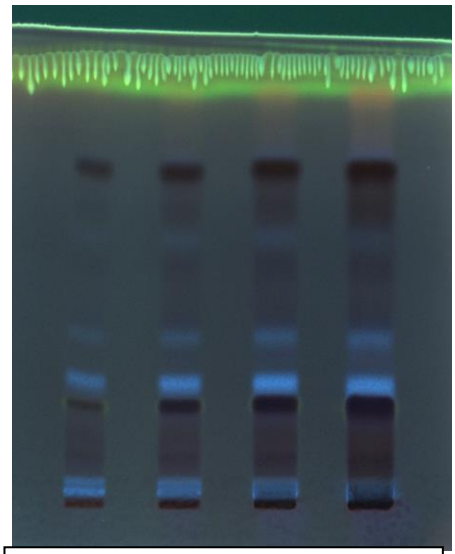


Fig 3: plate observed at 366nm for flavonoids

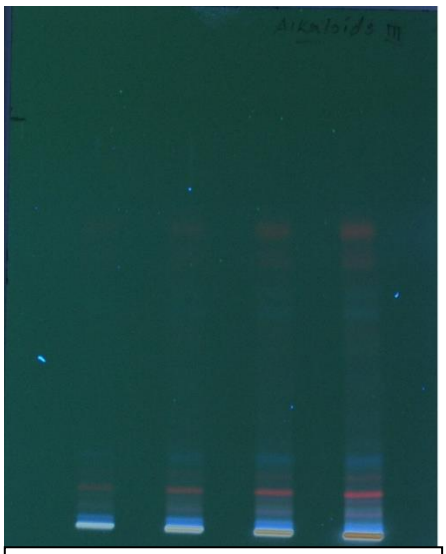


Fig 4 : plate observed at 254 nm for alkoloids

3. Phytochemical analysis by Qualitative method:

Table no.4: AST of Fraction against *E coli* and *S sureus*

Sr.No.	Solvent Fractions	Zone of inhibition (mm)	
		E coli	***S aureus***
1	Fraction A	± 15	± 12
2	Fraction B	± 12	± 12
3	Fraction C	± 10	± 11.5
4	**Fraction D**	**± 26**	**± 21**
5	Fraction E	± 17.5	± 19.5
6	**Fraction F**	**± 23**	**± 20**
7	Fraction G	-	-

4. HPTLC analysis of Fractions:

Table no. 5: HPTLC analysis of fraction and their bioautography

Sr no	Solvent fraction	HPTLC			Bioautography
		Solvent system	No. of peaks	End Rf value	Inhibition
1	Fraction D	Toluene: Chloroform: Ethanol (4:4:1:)	02	0.063 0.075	30 mm 15mm
2	Fraction F	Toluene: Chloroform: Ethanol (4:4:1:)	02	0.063 0.075	28mm 16mm

Fraction D and Fraction F showed positive test for alkaloids, saponins, tannins, glycosides while, flavonoids. Phytosterol, anthaquinone and coumarin were absent in both. From literature survey, 3''''-Oxojuliprosopine and Secjuliprosopinal are belong to class of alkaloids were isolated from methanolic extract of bark of *Prosopis julilfora*. To isolate such type of phytochemicals further classification and characterization must be done. [10]

5. **GCMS analysis:** The Gas chromatography Mass Spectroscopy analysis was carried out with the help of GCD 1800 A instrument which identify the compound presents in the sample. Fraction D (ethyl acetate) was send for GCMS analysis which was prepared in DMSO. One peak was detected of DMSO. 3 other peak was observed and 6 probable structures were detected through GCMS

library research having molecular weights of 795, 794, 717, 694, 774, 748 of pyridine (probability 30.4%), Indole (probability 29.2%), Myo- Inositol, 4-C-methyl (probability 32.6%), Myo-Inositol,2-C-methyl (probability 11.9%),N-β-Chloropropionyltryptamine (probability 60.2%) and3-(2-N-Acetyl- N-Methylaminoethyl)indol (probability 18.1%) respectively. 2 probable structures were identified N–β –Chloropropionyltryptamine (60.2%) and Myo-inositol-4C methyl.

6. FTIR Analysis: To know the possible functional group present in the compound, FTIR analysis was carried out with the help of FTIR analyzer MANGA 550. The result revealed that the unknown compound may be 'aromatic' in nature, probably contained 'amino' and 'chloro' group.

7. CHNS(O) Analysis : For identification of the compound, it is important to know the percentage of carbon, hydrogen, nitrogen, sulfur and oxygen to elucidate structure of the active components. Thus, CHNS(O) analysis were carried out on CHNS(O) analyzer FLASH EA 1112. From the results, it can be seen that, sulfur was absent in both the sample.

Table no.6: Presenting percentage of C, H, N, S and O.

Percent of	Fractions tested	
	Ethyl acetate	Methanol
Carbon	51.038 %	50.163 %
Hydrogen	5.35 %	5.815 %
Nitrogen	3.057 %	2.757 %
Oxygen	22.722	24.44

8. NMR Analysis: To know the structure of probable compound present in the sample can be detected in the NMR spectroscopy. On comparing with standard dell values, Fraction D showed probable compound may be **N-β-chloropropionyltryptamine**[3-N-ethyl(1Chloropropionamide)Benzoate] with probability 60.2% and Fraction F showed probable compound **Myo-inositol 4C methyl** with probability 36.7% as detected in GCMS analysis and its dell value matches with standard dell value. These peaks were matched with standard as well as GCMS results. The probable bioactive compound as **N-β-Chloropropionyltryptamine and Myo-inositol 4C methyl**

Conclusion: Plants are the largest biochemical and pharmaceutical stores ever known on our planet. These living stores are able to generate endless biochemical compounds. Medicinal plants are rich in a numerous variety of secondary metabolites of antimicrobial properties such as saponines, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpenes lactones, terpenoids and phorbol esters.

As people develop new drugs to fight the disease, those microorganisms develop new ways to strengthen themselves and live longer. However, plants are able to develop new, faster and natural antimicrobials and then man-made remedies and that is explaining why plants succeed in its fighting against microbes since millions of years while human failed. [2].

Therefore, a little contribution in isolation of bioactive compound from plant origin leads to 2 probable compound **N-β-Chloropropinoyltryptamine** and **Myo-Inositol, 4-C methyl**. These compounds can be used in formulating medicines after animal studies and Food and Drug Administration approved.

Future Prospect: *Prosopis juliflora* showed antimicrobial, antifungal, antioxidant, antitumor, antiplasmodial, antileishmanial and antitrypanosomal activities. Today, advanced tools are demanded to investigate the correct correlation between N and S fertilization and crop resistance management. It has shown that the N and S containing secondary metabolites are influenced by optimum supply of N and S and their good nutrition can enhance the capability of plant to cope with biotic and abiotic stress. Therefore, additional research in area of natural pesticide development is needed in current scenario[6].

Additionally, there is an urgent need to bridge the wide gap existing between phytochemists, animal scientist to establish and promote collaboration between them. This will give better opportunity in concerned subject. Further purification of extract may increase its activity proving to be more effective. However, several clinical and pharmacological tails have to be carried out for complete analysis of the active compounds in different parts of *Prosopis juliflora* [3].

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