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Phytochemical Screening and Antibacterial Potential of Methanol, Ethanol and Aqueous Extracts from Seed, Bark and Leaf of *Bauhinia tomentosa* L.

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ABSTRACT

Background: *Bauhinia tomentosa* L. leaves, flower buds or root had been reported to possess anti-diabetic, anti-pyretic, antioxidant, anti-proliferative properties. The seeds and bark of the plant had not still studied for their anti-bacterial properties despite their uses in traditional medicines.

Methods: Solvent extraction method was used to prepare crude aqueous, methanol and ethanol extracts of leaf, seed and bark, used for phytochemical screening to determine the classes of metabolites present. Anti-bacterial activity of leaf, seed and bark extracts was evaluated using agar well diffusion assay. Minimum inhibitory concentration (MIC) of extracts was examined using broth dilution assay.

Result: Qualitative phytochemical analysis of aqueous seed extract revealed the presence of maximum number of phytochemicals (alkaloids, glycosides, flavonoids, phenols, saponins, tannins, terpenoids and amino acids). Aqueous and methanol seed extracts were observed to be effective against all the tested bacteria *viz*. *Enterobacillus*, *Micrococcus*, *Klebsiella pneumoniae*, *Streptococcus thermophilus* and *Haemophilus influenza*. On the other hand, all the leaf extracts (ethanol, methanol and aqueous) showed inhibition against *Enterobacillus*, *Micrococcus, S. thermophilus* and *H. influenzae* except *K. pneumoniae*. The aqueous extracts of seed, leaf and bark was observed to be more potent against all the Gram positive and Gram negative bacteria followed by methanol extracts of leaf and seed.

Key words: Antibacterial activity, Bauhinia, MIC, Phytochemicals.

INTRODUCTION

Since ages, plants have been used as a source of therapeutic agents for treating several intractable human ailments by the tribals. These medicinal plants consist of very diverse phytochemicals which can be exploited to synthesize various drugs for human use (Oladeji *et al.* 2019). According to WHO, nearly 80% of the world's population continues to divulge in traditional medicines to meet their primary health-care needs.

The phyto-constituents responsible for imparting medicinal values to plants fall in the category of secondary metabolites, present in root, stem, leaves, fruit or bark in varied amount (Rani *et al.* 2017). These secondary metabolites belong to various classes such as alkaloids, flavonoids, saponins, terpenoids, tannins and provide the plants antioxidant, anti-inflammatory, anti-microbial and anti-carcinogenic properties (Batiha *et al.* 2020). These natural plant products have been proved beneficial, compared to allopathic medicines for their minimal side-effects and general health.

India is one of the mega-biodiversity regions of the world with more than 6000 plants being used in folk, herbal and traditional health-care system (Rani *et al.* 2017). A tree among these plants, belonging to the genus *Bauhinia* (family Fabaceae) with 12 species, is one of the important Ayurvedic plants. *B. variegata* (Kachnar), *B. purpurea* (Orchid tree) and *B. rufescens* (Kharoub) are the most exploited species for phytochemical constitutions and biological properties. All the plant parts *viz.* flower buds, flowers, bark, root, stem and leaves have been medicinally important for curing piles, oedema, dysentery, skin diseases, goitre, liver disease and other ailments (Mahajan *et al.* 2007; Singh *et al.* 2019). Further, these *Bauhinia* species possess antioxidant, anti-diabetic, anti-cancer and anti-allergic properties (Gunalan *et al.* 2016; Osman *et al.* 2020).

Bauhinia tomentosa L. (yellow bell orchid tree), is cultivated as an ornamental shrub, is a minor tree with a maximum height of 4m endemic to India, Nepal, Tropical Africa, South Africa, Sri Lanka and Zimbabwe. The tree has smooth, greyish, and occasionally hairy bark with drooping

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slender branches. Flowers have a typical dark maroon patch at the base of petals, form ing bell shape. The fruit is a pod. Different parts of the plant like leaves, flowers, and buds are recommended for use in treating headache, diarrhoea, malaria and dysentery as per Ayurveda system. The fruit is diuretic and seeds are used as aphrodisiac agents (Singh and Panda, 2005). The anti-microbial potential is not well known, although roots have been shown to exhibit anti-microbial activity towards Gram +ve bacteria (Dugasani *et al.* 2010). The seeds and bark had not been studied for their anti-microbial properties in the literature, but survey has revealed their uses in traditional medicines.

MATERIALS AND METHODS

The present study was carried out on the crude extracts of leaves, seeds and bark of the *B. tomentosa* plant in 2021-22 at Akal University, Talwandi Sabo. The authentication of the selected plant species was confirmed by Curator, Herbarium Punjabi University, Patiala (PUN). Reference microbial strains were obtained from the Botany Department of Punjabi University, which included two Gram +ve bacteria (*Streptococcus thermophilus* and *Micrococcus*); three Gram -ve bacteria (*Haemophilus influenzae, Klebsiella pneumoniae* and *Enterobacillus*). These strains were kept at 4°C on agar slant and sub-cultured at 38°C for 24h on nutrient agar for further experiments.

The collected plant material was washed properly (to remove dust particles), air dried at room temperature and crushed to fine powder for further experimental work. The three solvents *viz*. methanol, ethanol and water, based on their polarity index were selected for the extraction purpose. 10g powdered plant sample (in triplicate for each leaf, seeds and bark) was soaked separately in 100mL of different solvents each. The solutions were shaken well and left undisturbed for 72 hours at room temperature and then filtered with the help of Whatsman filter paper. The filtrate of each sample was divided into two equal parts. One part for phytochemical analysis and *in-vitro* anti-bacterial activities and another rest was lyophilized.

Phytochemical screening of each extract was done employing different standard methods as follows

Wagner's test for alkaloids

2 mL of each extract was treated with 1 mL of Wagner's reagent (1.27g Iodine and 2g KI in 100mL distilled water) and observed for the formation of reddish-brown precipitation (Kokate *et al.* 2001).

Keller Kelliani's test for glycosides

Extracts (2 mL) were treated with 2 mL of glacial acetic acid and few drops of 5% ferric chloride solution followed by addition of 1 mL of concentrated H_2SO_4. Formation of reddish-brown ring at the interface indicated the presence of glycosides (Kaur *et al.* 2013).

Alkaline reagent test for flavonoids

Extracts (2 mL) were treated with few drops of 20% NaOH solution resulted in acute yellow coloration which disappeared on addition of dilute HCl and it indicated the presence of flavonoids (Khandewal, 2008).

Ferric chloride test for phenols

2 mL extract of each sample was treated with 0.5 mL of 5% aqueous ferric chloride and observed for the formation of deep blue\black color which confirmed the presence of phenols (Hema *et al.* 2012).

Foam test for saponins

6 mL distilled water was added in each extract (2 mL). The mixture was shaken vigorously for 10 minutes and observed for the formation of constant foam to confirm the presence of saponins (Dubey and Sushma, 2014).

Braymer's test for tannins

2 mL of 10% ferric chloride solution was added to each extract (2 mL) and observed for the formation of bluish or greenish color in the solution.

Salkowki's test for terpenoids

Chloroform (2 mL) was added to 2 mL of each extract and added a few drops of concentrated H_2SO_4. The mixture was shaken properly and observed for the presence of reddish-brown precipitates immediately after shaking.

Test for quinones

Extracts (2 mL) were added with 4-5 drops of concentrated HCl and the formation of yellow color\precipitate indicated the presence of quinones (Ugochukwu *et al.* 2013).

Ninhydrin test for amino acids and proteins

2-5 drops of 1% ninhydrin were added into 2 mL of each extract and placed in a hot water bath at 100^0C for 1-2 minutes. The formation of purple color indicated the presence of proteins (Singh *et al.* 2013).

Agar well diffusion assay was used for testing the anti-bacterial potential of all the extracts prepared from leaves, seeds and bark in different solvents. 25 g of nutrient agar (Hi-Media) was dissolved in 1 liter of boiling distilled water. It was then autoclaved at 121°C for 15 minutes at 20 psi and left to cool at room temperature. After cooling, it was poured into petri dishes and each petri dish was left for 30-40 minutes to get the medium solidify. 25 µL of the overnight grown culture was spread onto 20 mL of sterile agar plates by using L-rod to get an even culture all over the plates. Agar plates were allowed to dry for about 5 minutes and wells were punched over the plates using sterile gel puncher. The extracts were added at various concentrations (50, 75 and 100 mg/100 mL of DMSO). 100 µL of each extract to be tested was added by the sterile syringe into the wells. Amikacin at the concentration of 2mg/10mL of DMSO was used as positive control while DMSO was used as negative control. The plates were incubated for 24 hours at 37°C. Triplicates were maintained and experiment was repeated thrice. The diameter of inhibitory zones formed around each well was measured in mm and recorded.

Broth dilution assay was done to determine the Minimum Inhibitory Concentration (MIC) of all the extracts against bacteria by serial dilution method as described by Rai *et al*., (2010) with some modifications. Serial dilutions of all extracts were made to obtain concentrations ranging from 250 mg to 10 mg/mL. All the tests were carried out in tryptic soya broth (TSB). The plant extracts were dissolved in TSB. Selected colonies of the test bacteria were picked off to a fresh isolation plate and suspended in corresponding tubes containing 5 mL of the broth with plant extracts. Test tubes were incubated for 24 h at 37°C until there was visible growth. Mc Farland No.5 standard and Phosphate Buffer Saline (PBS) were used to adjust the turbidity to get a final density of 5×10^5 CFU/mL and confirmed by viable count. After the incubation, the tubes showing no visible growth were considered as the inhibition of bacteria which represent MIC values of the respective concentration of the extract.
CPCS-1 software was used to analyze the data. ANOVA was performed according to the randomized block design layout with three replicates. Each zone of inhibition experiment had three replicates and the mean of three replicates was reported.

RESULTS AND DISCUSSION

Phytochemical analysis

The phytochemical screening of different plant extracts revealed a varied combination of these phytochemicals (Table 1) in each plant part. The qualitative analysis of phytochemicals in leaves has shown the presence of alkaloids, phenols and tannins in the extracts derived from methanol, ethanol and water. Flavonoids were screened in aqueous extract only. Glycosides and saponins were present in the methanol and aqueous extracts. Similarly, amino acids, flavonoids and quinones were detected in the aqueous extract of leaves only. The aqueous extract of seed was tested positive for all the phytochemicals except quinones. On the other hand, both the methanol and ethanol seed extracts revealed the presence of alkaloids, glycosides, phenols, tannins and terpenoids only; amino acids, flavonoids and saponins were not detected in these extracts. The bark aqueous extract revealed the presence of the seven phytochemicals *viz*. phenols, saponins, tannins, terpenoids, glycosides and amino acids as compared to two (alkaloids and glycosides) in ethanol and methanol extracts.

With increasing awareness among the people towards herbal medicines and side effects of allopathic medicines. a number of experimental studies to explore and select the natural sources of compounds have been carried out in past (Palhares *et al.* 2015). In the present study, qualitative analysis of the leaf, seed and bark extracts of *B. tomentosa* prepared using three different solvents (methanol, ethanol and water) demonstrated the presence of a good number of active constituents in them overall; however, these extracts from different tissues, when compared, revealed remarkable variation in their phytochemical constitution (Table 1). Maximum of the metabolites could be extracted using water from all the plant parts under study followed by methanol and ethanol. Further, bark extracts were seen to constitute least number of phytochemicals using latter two solvents; however, with water it revealed similar composition like leaf and seed extracts. So, it could be stated that the polarity of a solvent as well as the nature of the metabolites present in a tissue determine the number and amount of the phytochemicals extracted from the plant part. Moreover, the maximum number of active constituents extracted using aqueous conditions, in all the plant parts, indicates towards their highly polar nature as per our study. Further, the presence of flavonoids and amino acids only in the water extracts strengthen the above statement. Our results corroborated the finding of Wen *et al*. (2007) and Thenmozhi *et al*. (2012) who have also shown the highest amount of the phenolic constituents in the high polar solvents. Further, the selection of the better plant parts and optimization of the extraction and purification process could result in the optimum yield of these active constituent. Here, on the basis of our results, we may suggest the use of *B. tomentosa* leaves followed by seeds and bark and water as the solvent system to extract maximum number of phytochemicals for medicinal use.

Table 1: Phytochemical screening of different leaf, seed and bark extracts of *B. tomentosa.*

Phytochemical	Test	Ethanol extract			Methanol extract			Water extract		
		Leaf	Seed	Bark	Leaf	Seed	Bark	Leaf	Seed	Bark
Alkaloids	Wagner's test	+	+	+	+++	+	+	+	++	++
Glycosides	Keller Kelliani's test	-	+	+	++	+	+	+++	++	+
Flavonoids	Alkaline Reagent test	-	-	-	-	-	-	+	+	-
Phenols	Ferric chloride test	+	+	-	++	++	-	++	++	+
Saponins	Foam test	-	-	-	+	-	-	+++	++	+
Tannins	Braymer's test	+	+	-	+	+	-	+++	++	+
Terpenoids	Salkowski's test	+	+	-	+	+	-	_	++	+
Quinones	Hydrochloric acid test	-	+	-	-	+	-	+	-	-
Amino acids /proteins	1% ninhydrin test	-	-	-	-	-	-	+	++	+

'+' present at low intensity; '++' present at moderate intensity; '+++' present at high intensity; '-'.

Broth dilution assay and antimicrobial screening

MIC values calculated using broth dilution assay for all the extracts against the tested bacterial strains could be seen in the Table 3. Whereas the lowest MIC value (17.6 mg/mL) for leaf aqueous extract was observed for *Enterobacillus*, the highest for *K. pneumoniae* (87.4 mg/mL). The seed aqueous extract of revealed the lowest (18.4 mg/mL) and the highest MIC value (62.5 mg/mL) for *Enterobacillus* and *K. pneumoniae* respectively. On the other hand, ethanol seed extract was found to exhibit MIC values against *H. influenzae* (18.9 mg/mL) and *Enterobacillus* (112 mg/mL) only. Methanol seed extract showed the lowest MIC value against *S. thermophilus* and the highest against *Enterobacillus*. The bark methanol, aqueous and ethanol extracts were observed to be effective at the MIC values in the range of 65.1-170 mg/mL, 19.4-125 mg/mL and 17.6-160 mg/mL respectively for the tested bacteria. Further, a comparative analysis of the antimicrobial potential of the seed, bark and leaf extracts in different solvents could be seen in the Table 2 and Fig. 1a-1c.

Table 2: *In-vitro* anti-bacterial activity of leaf, seed and bark extracts of *B. tomentosa*.
Values are the average of triplicate experiments and expressed as mean.

		Zone of inhibition (diameter in mm)										
Bacterial Strain	Plant part	Positive control	Negative control	Methanol extract (Concentration in mg)			Water extract (Concentration in mg)			Ethanol extract (Concentration in mg)		
				50	75	100	50	75	100	50	75	100
Enterobacillus	Leaf	18	0	3.2	4.03	5	3	3.1	8	0.17	1.1	2
	Seed	13.6	0	0.2	0.23	5.6	3.23	3.17	10	-	-	3.07
	Bark	12	0	0.07	1.17	4.9	-	-	3.03	-	2.3	5
	Mean	14.5	0	1.61	1.81	5.1	2.07	2.1	7.02	0.05	1.15	3.37
	CD (p=0.05) Plant Part used = 0.127							C.V. = 11.21				
	Treatment (microbe) = 0.243											
	Plant part used X microbe = 0.422											
Micrococcus	Leaf	10.6	0	0.17	0.3	2.06	0.23	3.13	4.2	-	2.16	3
	Seed	14.3	0	-	1.17	4.97	0.26	3.4	4.2	-	-	-
	Bark	14	0	-	1.13	3	-	1.3	5	-	3.3	5.3
	Mean	13	0	0.05	0.87	3.34	0.17	2.61	4.47	-	1.83	2.78
	CD (p=0.05) Plant Part used = 0.112							C.V. = 14.43				
	Treatment (microbe) = 0.215											
	Plant part used X microbe = 0.373											
K. pneumonia	Leaf	14.6	0	1.4	2.13	4.17	-	-	-	-	1.23	3
	Seed	16	0	-	0.86	3.36	-	1.1	4.3	-	-	-
	Bark	13.6	0	-	-	1.4	-	0.23	2.27	-	1.17	1.6
	Mean	14.7	0	0.47	1	2.51	-	0.44	2.18	-	0.8	1.54
	CD (p=0.05) Plant Part used = 0.172							C.V. = 21.92				
	Treatment (microbe) = 0.329											
	Plant part used X microbe = 0.571											
S. thermophilus	Leaf	15.6	0	-	3.4	4.06	-	2.26	5.3	-	1.1	3.2
	Seed	10.3	0	-	4.46	5.16	1.16	2.06	4.16	-	-	-
	Bark	16.3	0	-	-	4.2	-	-	-	-	3.3	5
	Mean	14.1	0	-	2.62	4.5	0.39	1.44	3.16	-	1.48	2.77
	CD (p=0.05) Plant Part used = 0.122							C.V. = 15.35				
	Treatment (microbe) = 0.233											
	Plant part used X microbe = 0.404											
H. influenzae	Leaf	13.3	0	-	-	2.4	2.4	3.2	7.6	-	1.23	4.5
	Seed	16.3	0	-	3..63	5.26	-	-	4.1	-	1.23	4.5
	Bark	12.3	0	-	-	2.4	-	3.4	7.6	-	-	-
	Mean	14	0	-	1.21	1.38	0.81	2.22	6.38		0.41	2.34
	CD (p=0.05) Plant Part used = 0.148							C.V. = 11.21				
	Treatment (microbe) = 0.284											
	Plant part used X microbe = 0.492											

Antimicrobial potential of leaf extracts

Overall, all the leaf extracts showed significant zone of inhibition against all the tested bacterial strains at 75 and 100 mg concentrations except *K. pneumoniae* which was inhibited only by methanol and ethanol extracts at the same concentrations; however, at 50 mg concentration, only the *Enterobacillus* and *Micrococcus* were found to be inhibited by all the leaf extracts. Among the leaf extracts, water extracts presented the maximum zone of inhibition against *Enterobacillus* (8 mm) and *H. influenzae* (7.6 mm) with MIC values of 17.6 mg/mL and 21.5 mg/mL respectively at 100 mg concentration. The minimum was recorded in *Micrococcus* (2 mm; MIC value = 29.3 mg/mL) and *H. influenzae* (2.4 mm; MIC value = 125 mg /mL) with methanol leaf extract.

Anti-microbial potential of seed extracts

In case of seed, water and methanol extracts revealed their potential against *Enterobacillus*, *Micrococcus*, *K. pneumoniae*, S. *thermophilus* and *H. influenzae* at 75 and 100 mg concentrations. However, the ethanol extract of seed worked against only *Enterobacillus* at 100 mg concentration and against *H. influenzae* at both 75 and 100 mg

Table 3: The MIC of *B. tomentosa* extracts prepared from different plant parts against selected bacteria.

Plant part and extract type	Minimum Inhibitory concentration (MIC) *(mg/mL)*				
	Enterobacillus	*Micrococcus*	*K. pneumonia*	*S. thermophilus*	*H. influenzae*
Methanol					
Leaf	13.2	29.3	65.2	18.8	125
Seed	87.4	75.9	62.0	29.2	32.1
Bark	65.1	112	170	148	160
Mean	55.2	72.4	99.0	65.3	105.7
Aqueous					
Leaf	17.6	31.2	87.4	25.2	21.5
Seed	18.4	20.2	62.5	23.8	25.6
Bark	78.1	9.4	102	125	20.2
Mean	38.03	20.2	83.9	58	22.4
Ethanol					
Leaf	75.2	24.4	130	110	20.3
Seed	112	-	-	-	18.9
Bark	92.0	18.9	160	17.6	-
Mean	93.0	21.6	145	63.8	19.6

'- 'indicates not determined.

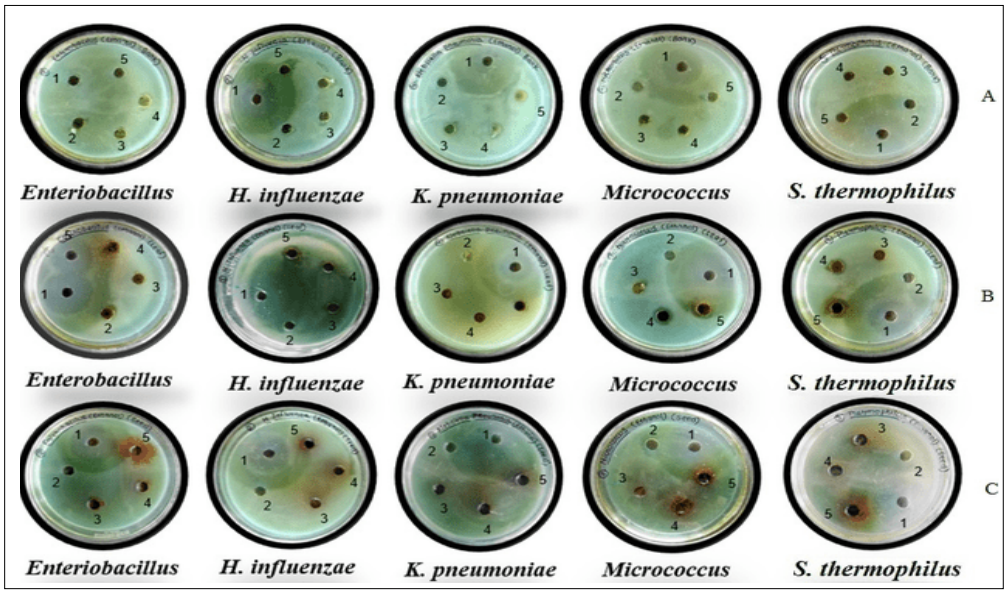

Fig 1a: Antibacterial activity of ethanol extract of A. Bark, B. Leaf and C. Seed of *B. tomentosa*.
1-Positive control 2-Negative control 3- at 50mg conc. 4- 75mg conc. 5- 100mg conc.

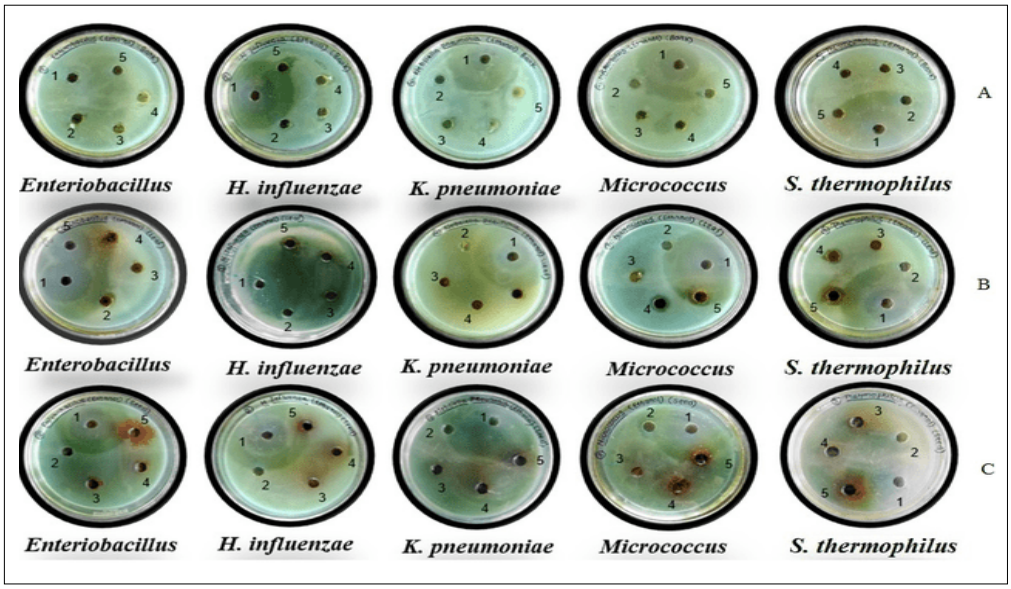

Fig 1b: Antibacterial activity of Methanol extract of A. Bark, B. Leaf and C. Seed of *B. tomentosa.* 1-Positive control 2-Negative control 3- at 50mg conc. 4- 75mg conc. 5- 100mg conc.

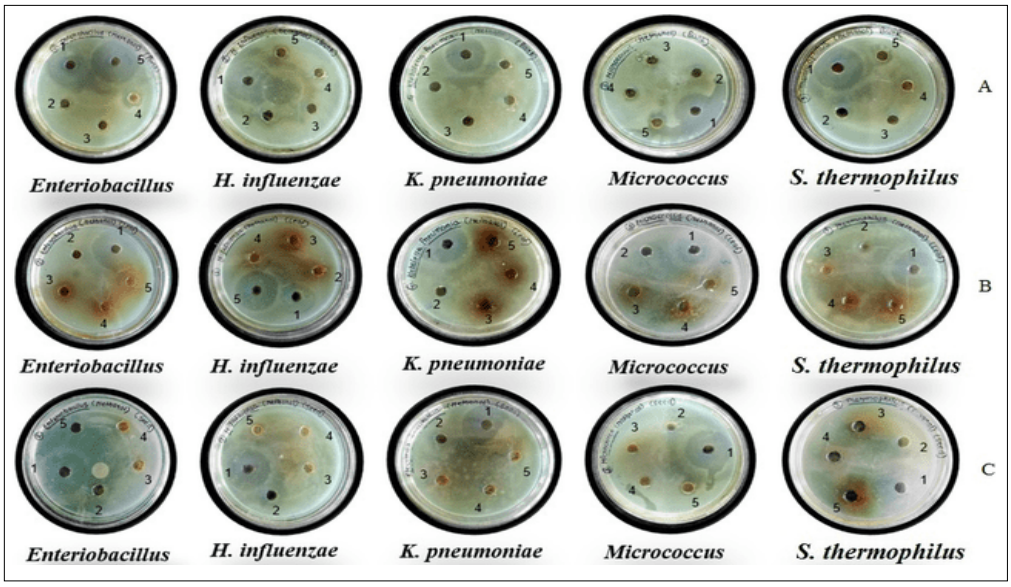

Fig 1c: Antibacterial activity of Aqueous extract of A. Bark, B. Leaf and C. Seed of *B. tomentosa.* 1-Positive control 2-Negative control 3- at 50mg conc. 4-75mg conc. 5-100mg conc.

concentrations. At 100 mg concentration, the maximum zone of inhibition (10 mm) was seen for *Enterobacillus* using aqueous extract (MIC of 18.4 mg/mL) and minimum zone of inhibition (3.07 mm) for *Enterobacillus* with ethanol extract (MIC of 112 mg/mL).

Anti-microbial potential of bark extracts

Extracts prepared in water showed the highest inhibition zone as compared to ethanol and methanol extracts for *S. thermophilus*, *Micrococcus*, *Enterobacillus* and *K. pneumoniae* at 75mg concentration and for *Enterobacillus*, *Micrococcus* and *S. thermophilus* at 100 mg concentration. Aqueous and ethanol extracts at 75 and 100 mg concentration revealed the highest zone of inhibition as compared to methanol extracts for *Micrococcus*. For *H. influenza,* water extract showed the maximum zone of inhibition (7.6 mm with MIC = 20.2 mg/mL) and the minimum inhibition zone was seen for *K. pneumoniae* (MIC value of 102 mg/mL) in water extract at 100 mg concentration.

Being the reservoir of pharmacologically important secondary metabolites, the plants have been exploited for the discovery of such natural compounds having potential in the health care industry for novel drug development properties (Djahida and Houcine, 2021). In the present study,

all the extracts from different plant parts *viz.* leaves, bark and seed revealed anti-bacterial potential against one or other kind of bacteria at different concentrations; however, comparatively more at higher concentration (100 mg). It marked towards the presence of these secondary metabolites in all the plant parts in variable amount. Different action mechanisms *viz.* cell lysis, interaction with genetic material and resulting in ineffective transcription and coagulation of cell contents after penetrating the cell wall have been suggested for their antimicrobial activities (da Silva *et al*. 2016; Hayek *et al*. 2013).

On comparison, overall effect of the aqueous extracts from all the plant parts was seen to be more pronounced on the three tested bacterial strains *viz. Enterobacillus* (Gram + ve)*, Micrococcus* (Gram + ve) and *H. influenzae* (Gram - ve) followed by methanol and ethanol extracts. In contrast, the two other strains *K. pneumonia* (Gram - ve) and *S. thermophilus* (Gram + ve) were inhibited more by the methanol extracts followed by aqueous and ethanol extracts. A number of other studies on the effects of different plant extracts for their antimicrobial potential demonstrated the both contradictory as well as similar results (Mishra *et al.* 2013; El-Moula *et al.* 2019; Chalghoumi *et al.* 2020). These differences in the antibacterial activities of the plant parts can be due to differences in the phytochemical composition of the part used and the solvents employed to extract these. The ability of showing very well antibacterial activities by crude aqueous leaf extract at different concentrations correspond to its good MIC values which made it more potent for bacterial inhibition in our results. However, in a report by da Silva *et al.* (2016), a weak positive relation was shown between MIC value and the bactericidal action of the plant extract.

Further, our study also revealed the Gram +ve bacteria to be more susceptible than Gram-ve at lower doses of the plant extracts (50 mg and 75 mg). But at higher doses, both strains were affected equally. Other studies have reported the former to be more susceptible than latter at higher concentrations of the extract used (Briers and Lavigne, 2015; da Silva *et al.* 2016). This intrinsic resistance in Gram-ve bacteria towards the lower doses of these plant extracts may be attributed to the fact that the multi-layered cell wall of the Gram -ve prevents the passage of active components inside it; moreover, the overexpression of efflux pumps (EPs) have also been reported to work efficiently in repelling the entry of these active compounds inside the cytoplasm (Venter *et al.* 2015).

CONCLUSION

The study confirmed the presence of the broad anti-microbial properties in the phytochemical contents of different parts of *B. tomentosa*. *Enterobacllus, H. influenza* and *Micrococcus* were found to be more affected by the aqueous extracts of leaf, seeds and bark; while *K. pneumoniae* and *S. themophilus* by the methanol extracts. Further research can be focused on isolation, identification and purification of the active principle in these extracts, which can be utilized as a novel source of anti-microbial drugs against particular bacteria.

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