Comparative studies on phytochemistry, antibacterial and antifungal properties of *Alstonia scholaris* and *Millettia pinnata*

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Abstract Phytochemical profiles and antimicrobial activities of *A. scholaris* and *M. pinnata* extracts have been assessed. Test microorganisms were evaluated for their susceptibility against aqueous, methanol, ethanol, n-hexane and chloroform extracts from leaves of both plant species. Antibacterial effects were evaluated against eight bacterial species (three gram positive and five gram negative) of high economic importance, whereas antifungal effects were evaluated against three devastating fungal pathogens. Maximum number of phytochemical groups was noted in methanol and aqueous extracts of both plant species which showed the best antibacterial activities. This indicates that methanol extract contained a wide range of antibiotic compounds, whereas ethanol extract was least effective against tested bacterial and fungal species. This study supports the use of plant origin pesticides against pathogenic microbes.

Keywords Antimycotic · Phytochemistry · Allelochemicals · Antibiotics

Plants have long been used as a source of antibiotics but the potential of plants as sources for new drugs is still generally

unexplored. Plant originated drugs also have the advantage of being less hazardous to environmental health (Bajwa et al. 2008; Shafique et al. 2011). Thus, the plant kingdom is being screened for new and effective chemotherapeutic agents (Shafique et al. 2013). Despite all efforts made by researchers, only a few plant species have been phytochemically investigated until now (Sabir et al. 2007). Plant extracts of many higher plants have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory trials (Akram et al. 2013; Bouamama et al. 2006). Among these plants, *Alstonia scholaris* is an evergreen tropical tree having latex rich in alkaloids (Hussain et al. 2010).

Another medicinally important plant, *Millettia pinnata* (Fabaceae), contains oils and juices with strong antiseptic and antimicrobial properties (Baswa et al. 2001). Considering plants as great sources of antibiotic agents, an investigation was performed to study the local flora for comparative phytochemistry, antibacterial and antifungal activity.

Leaves of *Alstonia* and *Millettia* plant species were collected from University of the Punjab, Lahore, Pakistan (31°17'34" N 74°10'29"E), in December 2012. Leaves of both plants were washed, dried and blended into fine powder separately. Aqueous, methanol, ethanol, n-hexane and chloroform extracts were prepared using the method of Azu and Onyeagba (2007). Extraction of plant biochemicals was performed at room temperature (25±2 °C) using solvents for plant biochemicals. Solvents of all extracts were removed using vacuum drier at 40 °C.

Different extracts were phytochemically analysed using the method of Brain and Turner (1975). Briefly, Mayer's reagent was used for alkaloids analysis; magnesium and acetone solutions were used for anthraquinones test; concentrated HCl and magnesium solutions were used to determine flavonoids; ferric chloride was used for phenols; thionyl chloride crystals were used in terpenoids test; lead acetate was used for

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Table 1 Observations of phytochemical screening

Phytochemical	<i>Alstonia scholaris</i>					<i>Millettia pinnata</i>				
	Aqu.	Eth.	Meth.	n-Hex.	Chlo.	Aqu.	Eth.	Meth.	n-Hex.	Chlo.
Anthraquinone	+	–	–	–	+	+	–	+	–	–
Alkaloids	+	+	+	–	–	+	+	+	–	–
Flavonoids	–	–	+	+	–	–	–	+	+	+
Phenolics	–	+	+	–	+	+	+	+	+	+
Saponins	–	–	–	–	+	–	+	+	+	–
Steroids	+	–	+	+	–	–	–	–	+	–
Tannins	+	+	+	+	–	+	–	+	–	–
Terpenoids	–	–	–	+	+	–	–	+	–	+

Aqu Aqueous, *Eth* Ethanol, *Meth* Methanol, *n-Hex* n-Hexane, *Chlo* Chloroform

determination of tannins and water was used to perceive the presence of saponins and formation of foam.

Bacterial cultures of *Bacillus subtilis* (FCBP-0174), *Bacillus fortis* (FCBP-0162), *Staphylococcus aureus* (FBT-0014), *Escherichia coli* (FCBP-0257), *Pseudomonas syringae* (FCBP-0275), *Pseudomonas putida* (FBT-0032), *Erwinia amylovora* (FBT-0053), *Burkholderia gladioli* (FBT-0034) and fungal cultures of *Alternaria alternata* (FCBP-0003), *Alternaria solani* (FCBP-0831) and *Fusarium oxysporum* (FCBP-0866) were obtained from the Culture Conservatory of the Institute of Agricultural Sciences, University of the

Punjab, Lahore Pakistan. Bacteria were cultured upon nutrient broth (NB) at 37 °C and fungal cultures were maintained on potato dextrose agar (PDA) at 28 °C.

Bacterial inoculum (10^6 cells/mL) was prepared by recording its absorbance at 550 nm (Sutton 2011) and fungal inoculum of 10^6 spores/mL was also prepared using a haemocytometer (Valgas et al. 2007). Both of the inocula were used in antibacterial and antifungal assays performed by disc diffusion method. Filter paper discs (0.5 cm diameter) were impregnated with 100 µg of 300, 200 and 100 µg/mL concentrations of extracts in a way that each disc contained 30, 20

Table 2 Antibacterial activity of extracts of *A. scholaris*

Extracts	Conc. in µg/disc	Zone of inhibition in mm							
		Gram positive			Gram negative				
		<i>B. subtilis</i>	<i>B. fortis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. syringae</i>	<i>P. putida</i>	<i>E. amylovora</i>	<i>B. gladioli</i>
Aqueous	10	4±0.8l	–j	15±0.5c	–l	11±1.8 g	–j	5±0.9 fg	7±0.4d
	20	7±0.6j	23±0.7d	26±0.9b	18±0.8c	20±0.6d	7±0.6i	11±0.3c	11±0.9c
	30	13±0.3 g	31±0.9b	34±1.6a	23±0.6b	26±0.3c	11±1.3 fg	14±0.4b	18±1.4b
Ethanol	10	5±0.1 k	–j	–i	–l	–k	5±0.1	–j	–h
	20	9±0.2i	10±0.5 h	–i	–l	8±0.2 h	6±0.1	3±0.4 h	–h
	30	13±0.5 g	16±0.4f	5±1.2 g	4±0.3 k	13±0.2 g	10±0.2 g	4±0.1 g	2±0.3 g
Methanol	10	4±0.1 l	7±0.7i	6±0.2 g	4±0.2 k	8±0.1 h	–j	–j	2±0.1 g
	20	9±0.3i	12±0.4 g	9±0.1e	6±0.1j	14±0.2f	11±0.3f	9±1.2d	3±0.2f
	30	15±0.3f	19±1.5e	11±0.6d	9±0.1 h	18±0.3e	19±1.6c	14±0.5b	7±0.2d
n-Hexane	10	–m	13±0.7 g	–i	11±0.4 g	–k	9±0.5 h	2±0.1i	–h
	20	16±0.1e	19±0.9e	3±0.3 h	14±0.3e	4±0.1j	13±0.4e	3±0.5 h	–h
	30	22±0.5c	25±1.4d	7±0.7f	19±0.3c	7±0.2i	17±0.2d	6±0.3f	3±0.3f
Chloroform	10	10±0.2 h	–j	3±0.3 h	8±0.1i	–k	13±1.2e	–	4±0.1e
	20	18±0.4d	18±0.8e	7±0.2f	13±0.5f	–k	20±1.5c	7±0.3e	9±1.3c
	30	26±1.5b	28±1.4c	11±0.5d	16±0.4d	31±1.2b	27±0.7b	13±1.2b	16±1.0b
Nystatin	10	29±0.2a	34±0.2a	35±1.2a	27±0.5a	33±0.1a	30±0.8a	28±0.4a	26±0.5a

Values were expressed as Mean ± Standard Error (S.E). Data were statistically analysed through Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. DMRT was performed using statistical package DSAASTAT (Onofri, Italy) and alphabetic letters showing significance of data follow each value. Values followed by similar letters showed no significant difference. Values followed by single consecutive letters showed significant difference; whereas, values followed by more than one letter showed no significant difference

and 10 µg extract, respectively. Discs impregnated with DMSO and Nystatin 100 µg were used as negative and positive controls, respectively. Activity was determined after 5 days of incubation.

Eight phytochemical classes (i.e. alkaloids, flavonoids, phenolics, steroids, tannins anthraquinone, saponins and terpenoids) were extracted using methanol as extraction solvent. Four different classes of biochemicals were observed in ethanol extract (Table 1), whereas five different phytochemical classes were observed in aqueous extract. Moreover, chloroform extract showed the presence of anthraquinones, flavonoids, phenolics, saponins and terpenoids. Presence of flavonoids, phenolics, saponins, steroids, tannins and terpenoids were observed in n-hexane extract (Table 1).

Maximum number of phytochemicals was observed in the methanol extract of *M. pinnata*; only steroids being absent. Aqueous extract contained anthraquinones, alkaloids, phenolics and tannins. Minimum number of phytochemical classes was observed in ethanol and chloroform extracts (Table 1).

All of the extracts presented varying antibacterial activity for tested bacterial strains at different concentrations. Leaf extracts of *A. scholaris* had antibacterial effects against different bacterial strains (Table 2). Methanol extract of *A. scholaris* presented the highest antibacterial activity. Methanol extract was able to inhibit growth of the most bacterial strains even at

the lowest concentration of 100 µg/mL. However, *P. putida* and *E. amylovora* were found resistant at the lower concentration of methanol extract. Ethanol extract was least effective against bacterial strains of both gram positive and gram negative types. Even at a concentration of 200 µg/mL, *S. aureus*, *E. coli* and *B. gladioli* were resistant against ethanol extract of *A. scholaris*. Maximum inhibitory zone diameter (34 mm) was observed in the aqueous extract of *A. scholaris* against *S. aureus* at the concentration of 300 µg/mL. The same bacterial strain was found to be resistant against n-hexane extract at the concentration of 100 µg/mL.

Methanol extract of *M. pinnata* was found effective against bacterial strains of both gram negative and gram positive types (Table 3). Three bacterial strains (i.e. *E. coli*, *P. putida* and *B. gladioli*) were found to be resistant against lower concentrations of ethanol extract. One bacterial species, *E. coli*, was found to be resistant against n-hexane extract at the concentration of 100 and 200 µg/mL. Bacterial species, *P. putida*, was also found resistant against chloroform extract of *M. pinnata* at lower concentrations but susceptible at higher concentrations. All other extracts represented average antibacterial activity at different concentrations.

Appreciable antifungal activity was observed in methanol extract of *M. pinnata* in which all fungal pathogens showed significant zones of inhibition at 100 µg/mL concentration.

Table 3 Antibacterial activity of extracts of *M. pinnata*

Extracts	Conc.in µg/disc	Zone of inhibition (mm)							
		Gram positive			Gram negative				
		<i>B. subtilis</i>	<i>B. fortis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. syringae</i>	<i>P. putida</i>	<i>E. amylovora</i>	<i>B. gladioli</i>
Aqueous	10	13±1.3e	16±0.4d	–j	3±0.5f	8±1.2 g	15±0.4 g	9±0.4 g	12±0.4f
	20	21±0.4c	22±1.2c	4±0.4i	8±0.4d	15±0.3d	17±0.2e	16±0.1e	18±0.6 cd
	30	32±1.7b	29±0.9b	7±1.3 g	11±0.6c	19±0.3b	23±0.8bc	20±0.6c	21±1.0b
Ethanol	10	–j	–k	5±0.4 h	–g	5±1.3 h	–j	4±0.9j	–l
	20	–j	4±0.8i	7±0.1 g	3±0.4f	11±0.7e	14±0.5 h	7±1.6hi	–l
	30	3±0.4i	10±0.2f	16±0.3e	7±0.6de	16±0.3c	20±0.5d	13±0.4f	6±0.4i
Methanol	10	–j	2±1.0j	7±0.8 g	–g	10±1.4f	11±0.5i	8±0.2 h	4±0.9 k
	20	2±0.1	6±0.3 h	15±0.9e	6±1.1e	14±0.3e	17±1.2ef	17±0.4d	10±1.3 g
	30	7±1.1 g	9±0.7 g	19±1.3d	13±1.0b	19±0.7b	22±0.6c	27±0.3b	17±0.5d
n-Hexane	10	–j	6±0.4 h	12±0.4f	–g	8±0.3 g	–j	6±0.1i	5±0.3j
	20	5±0.5 h	9±1.2fgh	17±0.8de	–g	11±0.4f	16±0.1f	9±0.6 g	8±0.5 h
	30	7±0.2 g	13±0.3e	25±1.3c	3±0.9f	17±1.3c	23±0.3b	13±0.5f	16±1.6d
Chloroform	10	3±1.3i	–k	7±1.2 g	–g	3±0.4i	–j	8±0.3 h	8±0.3 h
	20	9±0.7f	7±1.0 h	18±1.4d	4±1.0ef	6±0.7 h	–j	12±1.2f	13±0.2e
	30	17±0.6d	11±0.9f	31±1.3b	10±0.7c	10±1.0f	22±0.9bc	17±1.1de	19±0.7c
Nystatin	10	35±0.4a	34±0.5a	36±1.2a	26±0.7a	34±1.1a	24±0.1a	31±0.2a	23±0.1a

Values were expressed as Mean ± Standard Error (S.E). Data were statistically analysed through DMRT at $p \leq 0.05$. Statistical package DSASTAT (Onofri, Italy) for MS-Excel was used to perform the analysis and alphabetic letters following each value were used to mention significance of data. Values followed by similar letters showed no significant difference. Values followed by single consecutive letters showed significant difference; whereas, values followed by more than one letter showed no significant difference

Radius of inhibition zone directly increased with the increased concentration of plant extract which clearly showed that fungal growth inhibition was strictly concentration dependent. It was closely followed by methanol extract of *A. scholaris* for which the minimum concentration also significantly inhibited fungal growth on synthetic medium (Table 4).

Aqueous extract of *M. pinnata* had the second highest antifungal activity. However, *F. oxysporum* showed resistance to that extract and a very little inhibition zone (3 mm) was observed at the highest concentration. However, both species of *Alternaria* were efficiently controlled by a lower concentration of that extract. Every adjacent higher concentration brought significant increase in antifungal activity. Zones of inhibition produced by aqueous extract of *M. pinnata* against *A. solani* were 9, 16 and 27 mm at the concentrations of 100, 200 and 300 µg/mL, respectively. Two fungal species (*F. oxysporum* and *A. solani*) were resistant against aqueous extract of *A. scholaris* at 100 µg/mL concentration. However, at 200 µg/mL concentration, the extract controlled all three fungi efficiently.

Chloroform extract exhibited efficient antifungal activity at 200 µg/mL. At the lowest concentration, most of the fungal species were resistant to the chloroform extract. At 100 µg/mL concentration of both plant species, *F. oxysporum* was resistant, while *A. alternata* and *A. solani* were resistant to least

concentrations of *A. scholaris* and *M. pinnata*, respectively. Increased concentrations of these extracts significantly inhibited fungal growth up to 32 mm zone of inhibition in case of *A. alternata* with 300 µg/mL of *M. pinnata* extracts.

Antifungal potential was decreased from n-Hexane to ethanol extract. Only one fungal species was resistant to 200 µg/mL n-Hexane plant extract but this number was doubled in 200 µg/mL of ethanol extract of the same plants. A concentration dependent trend was also found in both n-Hexane and ethanol extracts.

In the qualitative phytochemical studies, it was observed that secondary metabolites of leaves of both plant species were more soluble in methanol suggesting that methanol is a good solvent for recovery of most of the phytochemicals from plants. Among both types of plants, ethanol extract was least effective against both bacteria and fungi. This can be attributed to this solvent being unable to dissolve many types of phytochemical groups.

These phytochemical constituents are valuable sources of antimicrobial agents (Bashir et al. 2013). Scalbert (1991) and Aboaba et al. (2006) reported antimicrobial properties of tannins in their investigations. Other compounds such as alkaloids (Damintoti et al. 2005), saponins (Hostettman and Nakanishi 1979), terpenoids and flavonoids (Leven et al. 1979) have also been investigated for their antimicrobial

Table 4 Antifungal activity of extracts of *A. scholaris* and *M. pinnata*

Extracts	Conc. in µg/disc	Zone of inhibition (mm)					
		<i>Alstonia scholaris</i>			<i>Millettia pinnata</i>		
		<i>A. alternata</i>	<i>A. solani</i>	<i>F. oxysporum</i>	<i>A. alternata</i>	<i>A. solani</i>	<i>F. oxysporum</i>
Aqueous	10	12±0.9e	–h	–g	13±1.4i	9±0.4f	–h
	20	19±1.2c	13±0.3e	12±0.6e	20±0.7f	16±0.2e	–h
	30	28±1.5b	19±0.5c	17±0.5c	29±1.5c	27±0.6b	11±0.4e
Ethanol	10	–g	–h	–g	7±0.6 k	–g	–h
	20	–g	9±1.1 fg	–g	12±0.6i	–g	–h
	30	13±0.4e	16±0.4d	15±0.8d	18±0.4 g	9±0.2	10±1.4ef
Methanol	10	8±0.9f	6±0.4d	7±0.7	10±0.8j	9±1.4f	6±0.6 g
	20	17±0.5d	10±0.6f	12±1.2e	16±0.5 h	17±1.1e	11±1.7de
	30	26±1.2b	17±0.8d	20±0.8b	25±1.3d	26±0.8bc	16±0.7b
n-Hexane	10	–g	–h	–g	–l	8±0.7f	–h
	20	13±0.2e	–h	10±0.7f	–l	15±1.5e	9±0.4f
	30	20±0.7c	17±0.9d	16±0.7 cd	30±0.4c	25±0.4c	14±0.6c
Chloroform	10	–g	8±1.1 g	–g	12±0.7i	–g	–h
	20	8±1.2f	16±1.2d	11±0.8ef	22±0.5e	15±1.4e	7±0.9 g
	30	13±1.1e	23±1.5b	17±0.5c	32±1.2b	22±1.0d	12±0.4d
Nystatin	10	31±1.0a	29±0.9a	31±0.4a	34±0.2a	33±0.8a	30±0.7a

Values were expressed as Mean ± S.E. Data were statistically analysed through DMRT at $p \leq 0.05$. DMRT was performed using statistical package DSAASAT (Onofri, Italy) and significance was mentioned using alphabetic letters following each value. Values followed by similar letters showed no significant difference. Values followed by single consecutive letters showed significant difference; whereas, values followed by more than one letter showed no significant difference

properties. This study indicates groups of metabolites extracted through different solvents, and it will help researchers for isolation of specific biochemical groups from plant material.

In this investigation we used the strains of fungi and bacteria of both gram positive and gram negative genera. They are important microorganisms due to their pathogenicity towards plants and humans. Methanol extract of leaves of both plant species comprised more antimicrobial properties as compared to other solvents (Ahmad et al. 2014). Therefore, methanol can be used to extract antimicrobial material from plants but cannot be encouraged for isolation of specific biochemical groups. The present study also justifies the use of phytochemicals against plant pathogenic fungi. All of the test fungal strains were susceptible to leaf extracts of both plant species. Based on all these results, use of leaf extracts of both these plant species as natural antibacterial agents is justified.

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