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EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND ANTIBACTERIAL POTENTIAL OF *AVICENNIA MARINA* AND *RHIZOPHORA MUCRONATA* FROM INDUS DELTA OF PAKISTAN

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ABSTRACT

In the present study, phytoconstituents and antibacterial response of mangroves *Avicennia marina* and *Rhizophora mucronata* were evaluated. Various parts of both plants were collected from Indus delta, Thatta. Qualitatively test results confirmed that alkaloids, flavonoids, tannins, terpenoids, saponins and sterols are present in crude extracts of different parts of both plants. Total phenol contents, flavonoids and antioxidant activity were determined by spectrophotometer. Total phenol contents were varied from 10.7±0.5 to 29.85±1.2 mg/ml; total flavonoids from 2.37±0.42 to 16.53±0.19 mg/ml and antioxidants from 2.4±0.5 to 14.4±0.17 mg/ml in different parts of both plants. Leaf extracts of both mangrove species showed the highest amounts of total phenol, total flavonoids and antioxidant activity as 29.85±1.2 mg/ml, 16.53±0.19 mg/ml and 14.4±0.17 mg/ml respectively, while root extracts of both plants accumulated the lowest amount of total phenol, flavonoids and antioxidant activity. Crude ethanol extracts of *A. marina* and *R. mucronata* were also tested against three pathogenic bacterial species. T1 preparations (10% ethanol extracts) of leaves of both species showed a wide range of growth inhibitions 5.5±0.7 – 13.5±0.4 mm while T2 preparations (5% ethanol extract) showed poor response 3.0±0.65 - 7.6±0.2 mm against *E. coli*, *K. pneumoniae*, *S. aureus*.

KEY WORDS: Mangroves, Phenolic acids, Flavonoids, Antioxidants, and antibacterial activity

INTRODUCTION

Plants produce a diverse range of non-nutritive bioactive compounds that not only protect the plants but also have preventive properties for humans against different diseases (Shelar*et al*., 2012). Plant products have shown antimicrobial responses due to the presence of phytoconstituents such as alkaloids, essential oils, phenolic acids, flavonoids, quinines, tannins, terpenoids, etc (Edeoga *et al*., 2005; Ravi Kumar *et al*., 2010). The interest of microbiologists has increased towards the development of formulations of new antimicrobial agents from plants with an evaluation of their efficacies (Pandian *et al.,* 2006).

Mangroves are the salt tolerant group of plants of tropical and subtropical intertidal zones of the world restricted to between 30° north and south of the equator (Bandaranayake, 2002). Along with wood firewood and charcoal production (Tomlinson, 1994), mangrove plants have been used traditionally in medicines due to the presence of a wide range of novel bioactive agents with their pharmaceutical potential (Govindasamy and Kannan, 2012). These bioactive compounds have reported antidiabetic and antiviral responses (Chandrasekaran *et al*., 2009). Arivuselvan *et al*., (2011) also reported the antimicrobial activity of mangrove plants against human pathogens such as *K. pneumonia*, *S. aureus*, *P.aerunginosa*, *P. digitatum*, *V. cholera*.

The Indus delta of Pakistan comprises an area of 0.6 million hectares and ranked 6th in the world (Keerio, 2004). About 97% mangrove forests mainly confined in the Indus Delta and in few patches along the Balochistan coastal lines (Saifullah, 1997; Rasool and Saifullah, 2005). Currently, four mangrove species *Avicennia marina*, *Rhizophora* mucronata, *Ceriops tagal* and *Aegicerus corniculatum* are growing in this area. *A. marina* (Forsk) Vierhis the dominant species that occupies 95-97% of mangrove trees of Indus delta (Irfan and Khan, 2000). Sindh Forest Department and IUCN successfully planted and established *R. mucronata* species at Hajamiro creek, Kati Bander (IUCN Pakistan 2005). *A. marina* and *R. mucronata* are rich in bioactive compounds of pharmacological importance (Arivuselvan, *et al*., 2011). The extracts of *A. marina* are traditionally used for the treatment of ulcers and rheumatism (Bandaranayake, 2002). The aerial parts of *A. marina* possess anti-microbial activities (Bobbarala, *et al.,* 2009) and have been used for the treatment of rheumatism, small pox, ulcers (Bandaranayake, 1995). *Rhizophora mucronata* contains alkaloids, anthocyanidins, condensed and hydrolysable tannins, flavonoids, inositols, polyphenols, procyanidins, saponins, steroids, triterpenes, phenols and volatile oils (Padmakumar and Ayyakkannu, 1997; Sahoo, *et al*., 2012). *R. mucronata* also showed bioactivity against selected bacterial species (Gurudeeban, *et al*., 2013).

MATERIALS AND METHODS

Plant Materials: The samples of *Avicennia marina* (Forsk.) and *Rhizophora mucronata* plants

were collected from Hajamro Creek, Keti Bandar, district Thatta, Sindh, Pakistan during September-October 2013. Different parts of *A. marina* and *R. mucronata* including roots, bark, leaves, flowers and propagules were separated, washed thoroughly in tap water, rinsed by distilled water and then dried in an oven at 40°C for four days.

Extraction of Phytochemicals: For the preparation of 10% solvents extracts, 2.0gram of each dried sample was ground in pestle motor using 10.0ml of water,ethanol (70%), methanol (70%) and acetone (70%) separately as solvents and centrifuged at 6000rpm for 15 minutes at room temperature. The supernatant was collected in separate bottles and extraction process was repeated twice, finally, the volume of each extract was raised to 20.0ml by adding each solvent separately and stored at -40°C for further analysis.

Qualitative Screening of Phytochemicals: The extract of roots, leaves, bark, flowers and propagules of *A. marina* and *R. mucronata* were qualitatively screened for various phytochemicals such as alkaloids, saponins, tanins (Soni and Sosa, 2013), flavonoids (Njoku and Obi, 2009) and terpenoids (Edeoga, *et al*., 2005).

Quantification of Total Phenol Content: Total phenol content was quantified by Follin Ciocalteau test as reported by Chun, *et al*., (2003) with slight modifications using gallic acid as phenolic compound standard. Briefly, 1.0ml crude extract was mixed with 1.0 ml Folin Ciocalteu (10 fold diluted) and 0.8 ml of aqueous Na_2CO_3 mixed thoroughly and left for 30min at room temperature. The phenol contents were measured by spectrophotometer at 765nm. The calibration graph was prepared using gallic acid solutions (0.1–0.5 mg/ml) in 70% acetone.

Estimation of Total Flavonoid Contents: The total flavonoid contents were estimated spectrophotometrically by aluminum chloride method (Djeridane *et al*., 2006). Briefly, 0.1ml of each extract was added with 0.3 ml of 5% sodium nitrate. After 5 minutes 0.3 ml of aluminum chloride and 2.0 ml of 1M NaOH were added and wait for 10 min at room temperature. The flavornoid contents of the mixture were read against blank by spectrophotometer at 510 nm and total flavonoid contents were calculated as quercetin equivalence from standard graph of quercetin. The standard calibration graph was prepared in the same manner using 0.05– 0.25 mg/ml of quercetin solutions in methanol.

Determination of Antioxidant Activity: The antioxidant activity of mangrove extracts was measured according to Prieto, *et al*., (1999) with slight modifications. Briefly, 0.2 ml extract mixed with 2.0ml solution of 4.0mM ammonium molybdate, 28.0mM sodium phosphate and 600mM sulphuric acid in test tubes. The tubes were capped with aluminum foil and incubated in the boiling water bath at 95°C for 90 minutes and then cooled to room temperature. The absorbance of the mixture was measured 695 nm against blank and antioxidants were calculated as tocopherol equivalence from standard graph of tocopherol. The calibration curve was prepared in the same manner using 0.1–0.5mg/ml of tocopherol solutions.

Antibacterial Response of Extracts: The antibacterial activity of crude extracts was evaluated through agar well diffusion method. 0.2ml of each *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* species were inoculated and spread on sterilized LB media in Petri dishes. 5.0 mm^3 diameter wells were made in each seeded Petri plate and 25µl of T1 preparation (10% extract) and T2 preparation (5% extract) of *A. marina* and *R. mucronata* were added in labeled wells and incubated at 37°C. For negative control, 25µl of sterile distilled H_2O was added in the well. After 24 hours, bacterial growth inhibition was measured as mm of zones of inhibitions.

RESULTS

Qualitative Screening of Phytochemicals: According to qualitative results of phytochemicals (Table-1), alkaloids, flavonoids, tannins, terpenoids, saponins and sterols were detected in ethanol extracts of *A. marina* and *R. mucronata*. Root extracts of *A. marina* showed negative results for tannins, terpenoids, saponins and sterols. Similarly, saponins were not detected in flower and bark while terpenoids in flower and propagules of *A. marina*. The color intensity of each reaction mixture was different that showed that variable amount of phytochemicals were present in different parts of *A. marina* and *R. mucronata*. The color intensity was detected higher in leaves and bark extracts of both plants (Table 1).

Table-1: Qualitative screening of some phytochemicals in various parts of *A. marina* and *R. mucronata* from Keti Bandar

Phyto- chemicals	*A. marina*					*R. mucronata*				
	Flower	Leaf	Bark	Root	Propagule	Flower	Leaf	Bark	Root	Propagule
Alkaloids	+	++	+	+	+	+	++	+	+	+
Flavonoids	+	++	++	+	+	+	+++	++	+	+
Tannins	+	++	+++	-	+	+	++	++	-	+
Terpenoids	-	+	+	-	-	+	++	+	+	+
Saponins	-	+	-	-	+	+	++	++	+	+
Sterols	+	+	+	-	+	-	++	++	+	-

+ = Present - = Absent

Total Phenol Contents: The results obtained, total phenol contents were variable in different parts of both species and even same plant. Leaf extracts of both species showed higher total phenol contents followed by bark, propagules, flowers and roots extracts respectively. The highest amount of total phenol contents (22.29 ± 2.6 - 29.85±1.2mg/ml) were accumulated in leaves extracts of both plants while the lowest amount of total phenol contents (10.7±0.5 - 16.71 ±0.8mg/ml) were detected in root extracts of both plants. Ethanol extracts of leaves of *R. mucronata* and *A. marina* showed the highest amount of total phenol contents 29.85±1.2mg/ml and 26.93±2.1 mg/ml respectively (Figure 1 and 2).

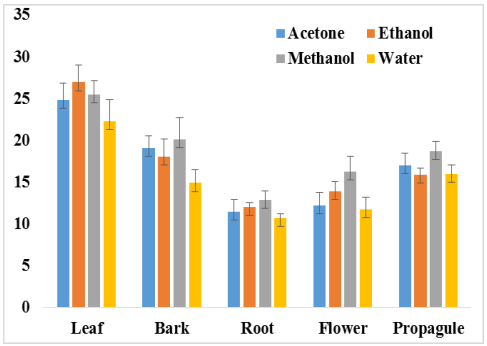

Figure-1: Total phenol contents in different parts of *A. marina* plants

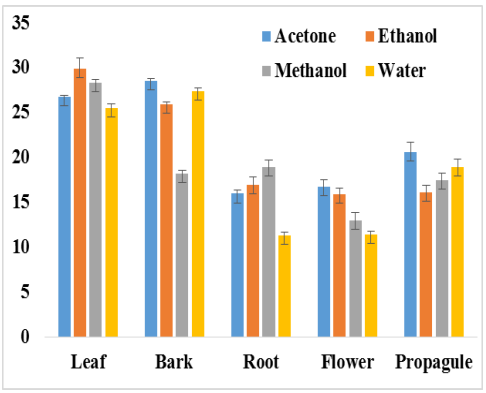

Figure-2: Total Phenol contents in different parts of *R. mucronata* plants

Total Flavonoid Contents: Extracts of *A. marina* and *R. mucronata* exhibited varying degrees of flavonoids. Total flavonoid contents were demonstrated in figures 3 and 4. The findings of leaves extracts revealed the highest amount of total flavonoid contents at 9.9±1.15 - 5.08±1.2mg/ml in *A. marina* and 10.99±0.3 – 16.53±0.19mg/ml in *R. mucronata* respectively. Similarly, the lowest amount of total flavonoid contents 2.37±0.42 – 4.7±0.8 mg/ml were accumulated in root extracts of *A. marina*.

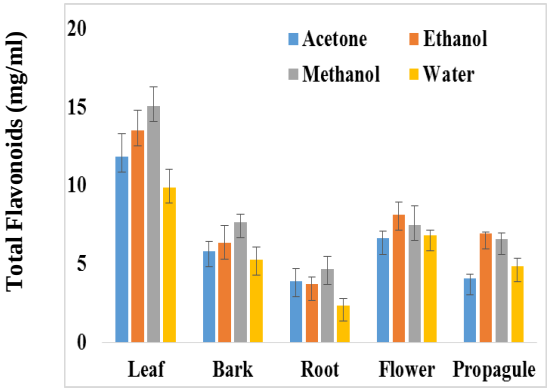

Figure-3: Total Flavonoids in different parts of *A. marina* plants from Keti Bander

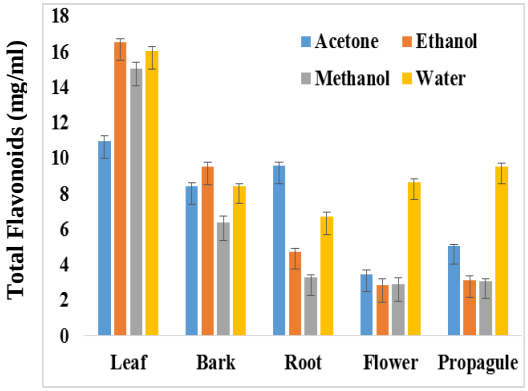

Figure-4: Total Flavonoids in different parts of *R. mucronata* plants from Keti Bandar

Antioxidant Activity: Total antioxidant capacity was assessed in solvent soluble extracts of *A. marina* and *R. mucronata*. The results of total antioxidant capacity are summarized in figures 5

and 6 showed that a wide range of antioxidant activity was exhibited by *A. marina* (2.4±0.5 - 10.48±0.6mg/ml) and *R. mucronata* (4.4±0.3 - 14.4±0.17mg/ml). The results indicated that leaf extracts of both species showed higher antioxidant activity (8.5±0.6 - 14.4±0.17mg/ml).

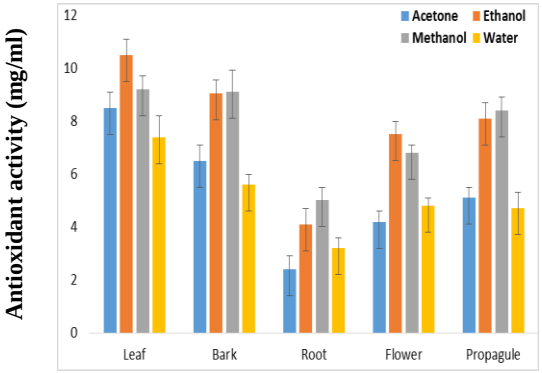

Figure-5: Antioxidant activity in different parts of *A. marina* plants from Keti Bandar

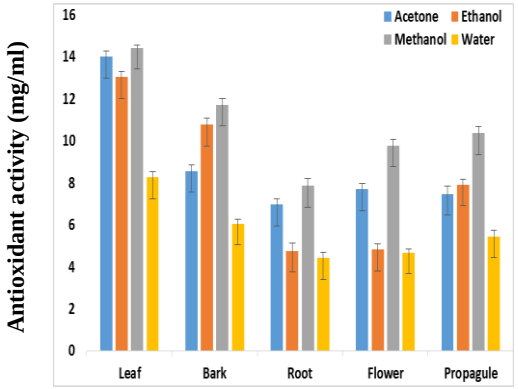

Figure-6: Antioxidant activity in different parts of *R. mucronata* plants from Keti Bandar

Antibacterial Response of Extracts: The results of antibacterial response of ethanol extracts of *A. marina* and *R. mucronata* against *E. coli*, *K. pneumoniae* and *S. aureus* are summarized in table 2. According to these results, different extracts showed a wide range of antibacterial response as 3.0±0.65 -13.5±0.4 mm zones of inhibitions were observed against three tested bacterial species. T1 preparations (10% extract) of both plant species showed better response as 4.0±0.2-13.5±0.4 mm zones of inhibitions were recorded against all species while T2 preparations (5% extract) showed poor response as 3.0±0.65 - 7.6±0.2 mm zones of inhibitions were observed. Similarly, growth was not inhibition against *K. pneumoniae*, *E. coli* and *S. aureus* when T2 preparations (5% extract) of bark and roots were applied in the wells.

DISCUSSION

Plants produce secondary metabolites under stress that play multiple role in the life of plants e.g. as allelopathic agents, provide defense against pathogens and predators, inhibiting insect growth and development, protection against ultraviolet radiation, etc. (Chaves, *et al*., 2010; Ikonen, *et al*., 2001; Ryan, *et al*., 2001; Zagrobelny, *et al*., 2004). Mangrove plants also contain a vast array of these substances which have ecological, pharmacological and toxicological importance. The qualitative screening of phytochemicals revealed that flavonoids, tannins, terpenoids, alkaloids, saponins and sterols are present in almost all parts of *A. marina* and *R. mucronata*.

Table-2: Inhibitory effect of Ethanol extracts of mangroves *A. marina* and *R. mucronata*against some pathogenic species of bacteria

Plant Parts	Bacterial Species	Zones of inhibitions in mm (Mean ± SD)			
		A. marina		*R. mucronata*	
		T – 1	T – 2	T – 1	T – 2
Leaf	*S. aureus*	8.0±0.3	6.5±0.2	7.0±0.5	5.5±0.4
	E.coli	13.5±0.4	7.5±0.3	8.0±0.3	5.5±1.15
	K. pneumoniae	6.5±0.2	4.0±0.2	5.5±0.7	3.0±0.65
Bark	*S. aureus*	7.5±0.5	7.0±0.4	6.5±0.5	5.0±0.5
	E.coli	11.0±0.4	3.2±0.4	5.0±0.3	3.0±0.25
	K. pneumoniae	7.5±0.3	5.0±0.26	5.5±0.4	-ve
Root	*S. aureus*	4.0±0.1	-ve	4.0±0.4	-ve
	E.coli	6.5±0.4	6.0±0.4	3.0±0.65	-ve
	K. pneumoniae	5.5±0.24	5.0±0.2	5.0±0.34	4.5±0.9
Flower	*S. aureus*	6.5±0.45	4.5±0.4	7.5±0.7	4.5±0.5
	E.coli	8.0±0.3	7.5±0.25	6.0±0.4	2.5±0.3
	K. pneumoniae	3.5±0.43	-ve	7.0±0.87	4.0±0.2
Propagule	*S. aureus*	8.0±0.2	7.6±0.2	6.5±0.4	4.5±0.5
	E.coli	8.5±0.24	3.0±0.2	7.5±0.3	5.5±0.5
	K. pneumoniae	5.0±0.4	3.0±0.5	8.0±0.4	6.5±0.65

However, the color intensity of positive samples was variable, which may be due to differrences in relative amounts of these compounds in different organs of plants. Such type of variability in alkaloids profile was also detected in *L. argenteus* plants originating from different or same site (Lee, *et al*., 2005; Gurib-Fakim, 2006). Anderson and Jordheim (2006) and Dinelli *et al*., (2006) reported that flavonoids contents varies from plant to plant or even in different organs of the same plant and geographical location.

Quantitative analysis of present study showed that total phenol contents, total flavonoids and anti-oxidant activity were detected in higher amounts in leaves extracts while lower in roots extracts of both plants. In previous reports, higher amounts of these compounds were also found in leaves of plants. This accumulation of secondary metabolites may be due to the early stage of growth and development. Krischik and Denno (1983) observed that water, protein and secondary metabolites usually, change during the development of an organ.

In present study, it was observed that total phenol contents, flavonoids and antioxidant activity were higher in leaves and other parts of *R. mucronata* as compared to *A. marina*. As *R. mucronata* species is not native to Indus delta and has been planted and established successfully recently at Hajamro creek, Kati Bander (IUCN, 2005). The presence of higher amount of these secondary compounds in *R. mucronata* may be due to new geographical location of the species. In present study, we observed variable amounts of secondary compounds in different parts or organs of *A. marina* and *R. mucronata*. These results are in agreement with other reports. Qualitative and quantitative variations occur in biosynthesis of secondary metabolites between different species, populations of the same species and even organs of an individual plant (Brenes-Arguedas and Coley, 2005; del Valle, *et al*., 2015; Masa, *et al*., 2016). Higher concentrations of antioxidants in the leaves of *A. marina* and *R. mucronata* may be abiotic stresses. Plants exposed to abiotic stress such as drought, high salinity and or mineral deficiencies result in the imbalance between the production of reactive oxygen species and the quenching activity of the antioxidants (Mittova, *et al*., 2004). Higher salts exposure increases antioxidant activity in rice and lentil (Lee, *et al*., 2001; Bandeolu, *et al*., 2004). Our results are in agreement with these reports as we obtained higher values of antioxidants in leaves of mangrove growing in salinity stress in Indus delta.

Mangrove plants have been reported to have a huge variety of phytochemicals which have various bioactivities such as antibacterial, antifungal, antiviral antioxidant, anthelmintic, anticancer etc (Arivuselvan et al., 2011; Bobbarala *et al.,* 2009; Chandrasekaran et al., 2009; Govindasamy and Kannan, 2012; Gurudeeban, et al., 2013). In present study, ethanol extracts T1 (10% extracts) of *A. marina* and *R. mucronata* showed a wide range of zones of inhibitions (4.0±0.2 - 13.5±0.4 mm) when tested against *E. coli*, *K. pneumoniae* and *S. aureus* in agar well diffusion method. T1 (10% extracts) preparation of *A. marina* showed 13.5±0.4 mm zone of inhibitions against *E. coli* (Tables-2). In many reports, antibacterial activities of mangrove extracts have shown against pathogenic bacterial species. Our report is in agreement with the results of Abeysinghe *et al.*, (2006) and Nayak, *et al.,* (2014) on various bacterial species using various solvent extracts of *A. marina*. These inhibitions may be due to inhibitory compounds present in the extracts of different parts. In this study two concentrations, T1 (10%) and T2 (5%) were tested for antibacterial response. The results revealed that T1 extracts of leaves of *A. marina* and *R. mucronata* showed better zones of inhibitions against *E.coli* followed by *S. aureus* and *K. pneumoniae* as compared to T2 (Table 2). We recorded 13.5±0.4 and 8.0±0.3 mm zone of inhibition in T1 extracts of A. marina against *E.* coli and S. *aureus* while Al Maqtari and Nagi (2014) reported 12, 6, and 7 mm zones of inhibitions in *A. marina* extracts against *E. coli, S. aureus,* and *B. subtilis* respectively. Ravikumar *et al*., (2010) reported that hypocotyls extracts of *R. apiculata, R. mucronata, and A. marina* showed the highest antibacterial activity against the urinary tract infectious bacterial pathogens and it was concluded that this activity may be due to the presence of active compounds such as alkaloids, anthroquinone, flavonoids, phenolic group and triterpenoids.

In present study, phytoconstituents and antibacterial response of *A. marina* and *R. mucronata* collected from Keti Bander were analyzed; however, the defense mechanism of plants against microorganisms is not fully understood and debatable. The mechanisms through which differrent microorganisms survive against antimicrobial agents are poorly understood (Woolfrey and Enright, 1990). The presence of phytochemicals and antibacterial activity may have interactions and are correlated. Terras *et al*., (1995) noted that small proteins play a significant role in defense system of plants which protects them from microbial invaders. Furthermore, plants also accumulate

phytoalexins that protect them from microbes (Maher, *et al*., 1994).

Conclusion: *A. marina* and *R. mucronata* are part of mangrove forests which not only provide unique ecosystems of the world but also rich source of secondary compounds. The qualitative analysis confirmed that alkaloids, flavonoids, tannins, terpenoids, saponins and sterols are present in various parts of all these mangrove species. The quantitative analysis confirmed that higher amount of total phenol contents, flavonoids and antioxidants are present in leaves extracts of *A. marina* and *R. mucronata* which showed better response of bioactivity against *E. coli*, *S. aureus*, and *K. pneumoniae*.

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