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Seasonal and Geographical Variations in Antimicrobial Activity of Selected Mangroves from Krishna Estuary

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ABSTRACT

Objective: To determine the seasonal and geographical impact on plant secondary metabolite production and antimicrobial activity.

Methods: Three mangrove plants viz., *Ceriops tagal*, *Bruguiera cylindrica* and *Lumnitzera racemosa* commonly found in Gilakaladindi and Malakayalanka regions of Krishna Estuary, Andhra Pradesh, India were selected for the present study. The study was done during 2014-15 summer, rainy and winter in both the stations. Leaf samples were extracted with methanol and subjected to preliminary phytochemical screening. The methanolic leaf extracts were tested against bacterial pathogens *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli* and a fungal pathogen *Candida albicans*.

Results: The methanolic extracts of leaves collected during rainy season showed maximum zone of inhibition at both the stations Gilakaladindi and Malakayalanka. Among the studied plants leaf methanolic extract of *C.tagal* was recorded maximum antibacterial activity against *B. subtilis* and *B. cereus* (18±0.05 mm), followed by *Bruguiera cylindrica* both at Gilakaladindi and Malakayalanka respectively. But the extracts of *L.racemosa* showed less inhibition activity (7±0.00 mm) in all the seasons at both the stations.

Conclusion: The study demonstrated that mangroves produce bio-active compounds optimally in rainy season that are effective against microbial proliferation which may further help in synthesis of natural antibiotics in place of commercial antibiotics to heal infectious diseases.

Key Words: Gilakaladindi, Malakayalanka, Phytochemicals, Rainy

INTRODUCTION

Mangroves are a specific group of plant communities, habituated to adverse environments includes high salt content, relative humidity with fluctuated water currents.^[1] These contrary conditions consequently results in reduced photosynthetic rate and thereby reduced growth.^[2,3] Of all the stressors soil and water salinity inflict more on plant development especially in semiarid and arid regions.^[4] In order to cope up with these unfavorable conditions, mangrove switch on to different modifications at morphological, physiological, biochemical and molecular levels.^[5] Production of secondary metabolites is one of such physiological change which tends the plant to protect themselves against harmful microorganisms along with grazing threat.^[6] Bryant et

al.^[7] have hypothesized that when plants are stressed, an exchange occurs between carbon to biomass production or formation of defensive secondary compounds.

The local geo-climate, seasonal changes, external factors such as light, temperature and humidity may affect composition of secondary metabolites. The synthesis of secondary metabolites in response to environmental factors is nothing but an adoptable strategy leading to tolerance to abiotic stress.^[8,9] On the other side the mangrove plant extracts have proven activity against human, animal and plant pathogens and have been used in folklore medicine since ages.^[10,11,12] Till now more than 200 bio-active compounds have been isolated from true mangroves of tropical and subtropical population.^[13] As said, seasonal changes influence much on production

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of secondary metabolites which is less explored and badly overlooked. On the other side right season for the collection of leaf material to extract maximum secondary metabolites is inevitable in order to get much benefit. Krishna estuary is one such neglected area with less exploration specifically on seasonal changes. The Krishna deltaic region is in subtropical humid climate with hot summers and moderate winters, which get annual rainfall due to south west monsoon.^[14,15] Gilakaladindi and Malakayalanka are the prominent biodiverse mangrove reserves in Krishna estuary on East Coast of India located at latitudes 16° 8' 56.1624'', 15° 58' 34.5354 and longitudes 81° 9' 46.029'', 81° 5' 53.3616'' respectively. Coupled with their richness and diversity mangroves of Gilakaladindi and Malakayalanka have also physiological peculiarities as a result of environmental conditions to which they belong. Each species of mangroves has a particular range of tolerance to environmental factors.^[16, 17]

The present study aims at identify the seasonal and geo-climatic changes on secondary metabolite production in terms of antimicrobial activity on selected mangrove species *Lumnitzera racemosa*, *Ceriops tagal* and *Bruguiera cylindrica*.

MATERIALS AND METHODS

Collection of Plant Material

Leaf samples were collected during summer (March to May) rainy (July to September) coinciding with South west monsoons and in winter season (October to February). Two different sites were chosen and the distance between the sites was about 25 kms. Healthy and fresh leaves were collected from selected three mangroves i.e. *Lumnitzera racemosa*, *Ceriops tagal* and *Bruguiera cylindrica* of Krishna estuary. The collected leaves were washed with tap water and double distilled water until dust is removed from surface of the leaves. The leaves are shade dried at room temperature. Dry leaf material was powdered with the help of mechanical grinder and sieved.

Extraction

The leaf powders were extracted with 80% methanol using soxhlet apparatus for about 18 hours. The crude extracts obtained were evaporated and concentrated by using vacuum rotary evaporator (Buchi Labortechnik Ag, model I, R-215) under reduced pressure. The dried extracts were preserved at 4°C until further use.

Antimicrobial screening

The antibacterial activity of the obtained leaf extracts was carried out by agar well diffusion method. Nutrient agar (NA) was used for culturing the test bacteria. Nutrient agar medium was sterilized at 15 lbs pressure (121 °C) for 15 min,

later cooled and inoculated with 0.1 ml of test bacterial suspension. The inoculated medium was poured into petri plates under aseptic conditions. After solidification, wells of about 5 mm diameter were made with sterilized cork borer^[18]. Solvent extract (50 µl, 100 µl and 150 µl) was added to each well and the addition of solvent alone served as control. The inoculated plates were incubated at 30 °C and the diameter of the inhibition zone was measured after 24 h on bacterial pathogens such as *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (MTCC 430) *Escherichia coli* (MTCC 43) and fungal pathogen *Candida albicans* (ATCC 10231).

Phytochemical screening

Shade dried plant material was extracted with 80% methanol and were tested for qualitative phytochemical screening.^[19,20]

Alkaloids

The methanolic extract was evaporated to dryness and the residue is dissolved in 1% HCl. To this solution Mayer's reagent was added. Appearance of any precipitate or turbidity indicates the presence of alkaloids.

Saponins

The plant extract is evaporated to dryness. Tap-water was added and shaken vigorously. Formation of persistent foam of about 2 cm is taken as a positive reaction.

Terpenoids and steroids

A 50% H₂SO₄ is added along the sides of the test tube containing a mixture of methanolic HCl and acetic anhydride. If there is any change in color, from green to blue-green (sometimes via red or blue) indicates the presence of terpenoids and steroids.

Tannin

The methanolic extract is evaporated to dryness and the residue was dissolved in water and tested with 1% gelatin solution and 1% gelatin salt solution (1 g) gelatin dissolved in 10 g of NaCl (w/w) to separate volumes. The appearance of white precipitate will be regarded as a positive reaction.

Anthocyanin

To the plant extract was added equal volume of methanolic HCl. Appearance of red or purple color indicates the presence of anthocyanidins.

Phenolic compounds

The formation of intense color in the extract, on adding 1-2 drops of 1% ferric chloride to the extract is considered as a positive reaction test.

Flavonoids

Few ml of methanolic extract is added with conc. HCl and Mg powder. The presence of flavonoids can be identified by the development of pink or magenta or red colored foam.

Coumarin

To the methanolic extract, a few drops of alcoholic sodium hydroxide were added. Formation of yellow color indicated the presence of coumarins.

Quinones

To the 1 ml of methanolic extract, 1 ml of conc. H₂SO₄ was added. Formation of red color shows the presence of Quinine's.

Resins

Plant extracts were treated with acetone. To this, small amount of water was added and shaken. The appearance of turbidity indicates the presence of resins.

Test for glycosides

To the methanolic extract a little amount of anthrone was added. To this a few drops of conc. H₂SO₄ was added and warmed gently over water bath. The presence of glycosides was identified by dark green color formation.

Soil and Water analysis

Field data like temperature, salinity and pH were recorded during morning to noon. Water salinity was estimated with the help of hand refractometer and pH was measured using Elico pH meter (model L.C-120). Data was collected on five

plants of each species growing in different locations in both the sites. Soils are air-dried, crushed with a wooden mallet, passed through a 2 mm sieve and were analyzed for inorganic ions using the standard procedure.^[21] Saturation extracts of soils are analyzed for electrical conductivity using an electrical conductivity meter. Soil pH was determined on air-dried samples using a 1: 2.5 soil to 1 M KCl ratio. Exchangeable Na⁺, Ca²⁺, Mg²⁺, K⁺, P, Zn²⁺, Cu²⁺ and Mg²⁺ present soil and water samples were analyzed.^[22]

RESULTS

The methanolic leaf extracts were screened for various phytochemical constituents and represented in Table 1. The Phytochemical analysis of the methanol extracts revealed the presence of alkaloids, flavanoids, phenols, resins, saponins and tannins in all the studied plants of both Gilakaladindi and Malakayalanka estuaries in all the seasons (Table 1). But the existence of anthocyanins, quinines, glycosides and terpenoids were not reported in *L. racemosa* in all the seasons at both the stations. Along with the *L. racemosa* the presence of anthocyanins and quinines not observed in *B. cylindrica* in all the seasons. The existence of flavonoids also not recorded in *B. cylindrica* in all the seasons at both the stations. On the other side the presence of coumarins and glycosides not observed in *C. tagal* in both the stations during all the seasons. These variations in presence or absence of phytochemicals among three selected mangroves may correspond to different bioactive constituents and may be responsible for variations in antimicrobial potency among the species.

Table 1: Preliminary phytochemical analysis

Compound	Gilakaladindi			Malakayalanka		
	L. racemosa	C. tagal	B. cylindrica	L. racemosa	C. tagal	B. cylindrica
Anthocyanin	-	+	-	-	+	-
Phenolics	+	+	+	+	+	+
coumarin	+	-	+	+	-	+
Quinine	-	+	-	-	+	-
Glycosides	-	-	+	-	-	+
Saponin	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Flavonoids	+	+	-	+	+	-
Terpenoids & Steroids	-	+	+	-	+	+
Resins	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+

* '+' indicates the presence of particular compound

* '-' indicates the absence of particular compound

Antimicrobial activity

Antibacterial and antifungal activities of selected mangrove plants were studied and analyzed. The present study revealed the significant influence of seasons on potential antimicrobial activity of plant extracts. All the test organisms responded varied to different plant extracts at different concentrations of crude extracts i.e 50 μ l, 100 μ l, 150 μ l and maximum inhibitory action was obtained at 150 μ l.

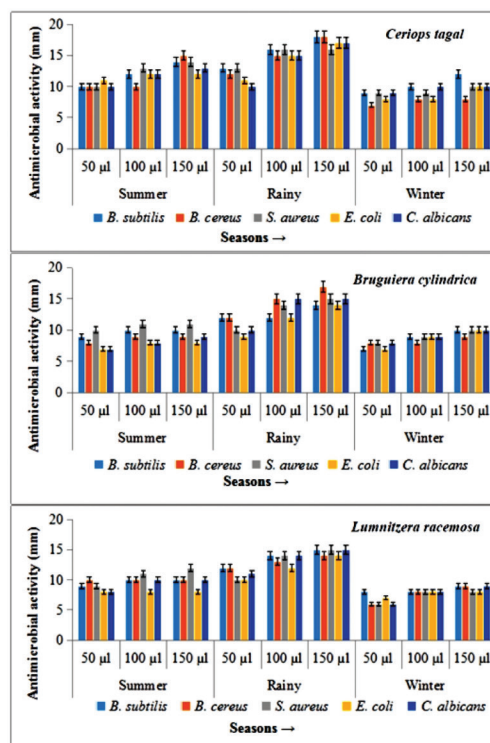
Summer season

The leaf methanolic extracts of Gilakaladindi showed significant inhibitory activity. The plant extracts of mid-summer reported antimicrobial activity ranged from 7-11 mm with a mean average of 9.06 mm at 50 μ l concentration. At 100 μ l plant extract concentration the sensitivity of test organisms ranged from 8-13 mm with an average mean of 10.26 mm. On the other side at 150 μ l the maximum inhibition was ranged from 8-15 mm with a mean average 11.00 mm. The extracts of *C. tagal* showed potential antimicrobial activity against *E. coli* (11.00 \pm 1.25 mm), *S. aureus* (13.00 \pm 1.00 mm) and *B. cereus* (15.00 \pm 0.25 mm) at 50 μ l, 100 μ l and 150 μ l concentrations respectively (Figure 1). In case of Malakayalanka antimicrobial activity ranged between 7-10 mm with a mean average of 8.26 mm at 50 μ l concentration. At 100 μ l plant extract concentration microbial activity ranged from 7-12 mm with an average mean of 9.00 mm. At 150 μ l the maximum zone of inhibition was ranged from 8-14 mm with a mean average 9.66 mm. At 50 μ l concentration, leaf extract of *C. tagal* recorded maximum antimicrobial activity with *S. aureus*, *E. coli* and *C. albican* (10.00 \pm 1.102). Test organisms *B. subtilis* and *E. coli* showed highest sensitivity (12.00 \pm 0.958) when medium diffused with 100 μ l leaf methanolic extracts of *C. tagal*. When all the test organism treated with 150 μ l methanolic leaf extracts *C. tagal* extracts found to be more potential against *B. subtilis* with a zone of inhibition of 14.00 \pm 0.085 (Figure 2). The results of the present study indicated that the methanolic leaf extracts of summer season of Gilakaladindi showed more inhibitory activity when compared to Malakayalanka.

Rainy season

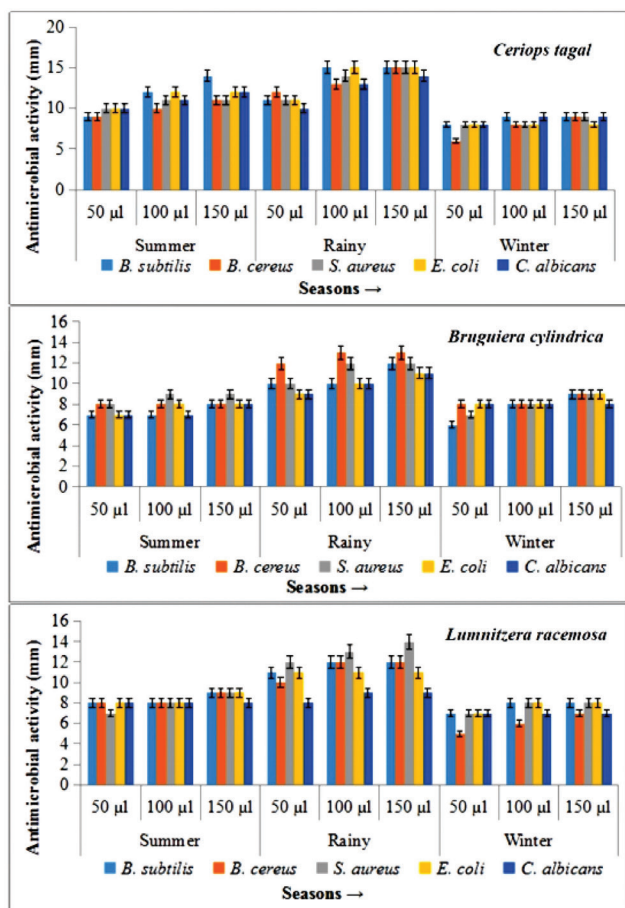
Rainy season showed highest positive antimicrobial activity and antifungal activity in all the plant species during all the seasons in both Gilkaladindi and Malakayalanka stations. The methanolic extracts of peak rainy season of Gilakaladindi, reported a varied antimicrobial activity 9-13 mm with a average mean of 11.13 mm at 50 μ l concentration whereas at 100 μ l concentration the inhibitory activity differed between 12-16 mm with an average mean of 14.13 mm. At 150 μ l concentration of plant extract the maximum sensitivity is occurred between 14-18 mm with a mean average 15.6 mm. Samples collected during rainy season showed maximum inhibitory activity against all the test organisms. At 50

μ l and 100 μ l concentrations, leaf methanolic extracts of *C. tagal* showed high microbial activity 13 \pm 0.00 and 16 \pm 0.45 against *B. subtilis* and *S. aureus* respectively. Increased concentration of methanolic leaf extracts i.e 150 μ l especially *C. tagal* extracts potentially increased the inhibitory activity (18.00 \pm 0.00) in *B. subtilis* and *B. cereus* (Figure 1) (Plate 1 A, B). Samples collected from Malakayalanka are not potentially bioactive when compared with Gilakaladindi samples in combating harmful microorganisms (Figure 2). In case of Malakayalanka antimicrobial activity varied from 8-12 mm with an average mean of 10.46 mm at 50 μ l concentration. At 100 μ l concentration inhibitory activity ranged from 9-15 mm with an average mean of 12.13 mm. The potential inhibitory activity at 150 μ l concentration ranged from 9-15 mm with a mean average 12.8 mm. *B. Cereus* (12.00 \pm 0.12 mm) strains found to be more sensitive against *C. tagal* and *B. cylindrica* at 50 μ l concentration. Methanolic leaf extract of *C. tagal* recorded maximum antimicrobial activity with *B. subtilis* and *E. coli* (15.00 \pm 0.04) at 100 μ l concentration. In case of 150 μ l concentration leaf extracts of *C. tagal* recorded maximum antibacterial activity (15.00 \pm 0.00) on *S. aureus* (Plate 1C), and against fungal organism *C. albican* (14.00 \pm 0.02) (Figure 2) (Plate 1D). Results revealed that the leaf methanolic extracts of rainy season are more sensitive against *Bacillus subtilis* and *Bacillus cereus* at Gilakaladindi and *S. aureus* and *C. albican* at Malakayalanka stations.



*significant at 5% level

Figure 1: Antimicrobial activity of methanolic leaf extracts of Gilakaladindi.



*significant at 5% level

Figure 2: Antimicrobial activity of methanolic leaf extracts of Malakayalanka.

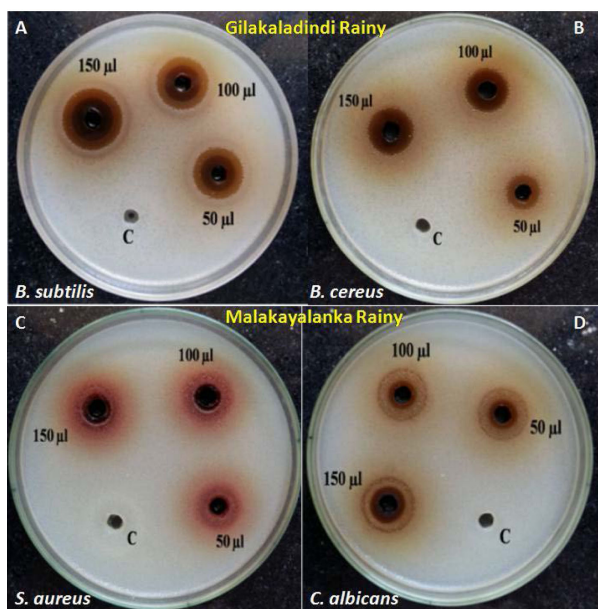


Plate 1: Antimicrobial activity of *C. tagal* methanolic leaf extracts.

Winter season

In winter season all the extracts recorded lowest antibacterial and antifungal activity when compared to summer and rainy seasons. In case of Gilakaladindi antimicrobial activity differed between 6-9 mm with a mean average of 7.53 mm at 50 µl concentration. Methanolic leaf extracts 100 µl concentration recorded antimicrobial activity ranged from 8-10 mm with an average mean of 8.6 mm. At 150 µl concentration of leaf extract antimicrobial sensitivity was ranged from 8-12 mm with a mean average of 9.46 mm. Of all the plants highest microbial activity was reported with *C. tagal* leaf methanolic extracts in all three concentrations. At 50 µl maximum inhibitory activity (9.00 ± 1.24 mm) was observed against *B. subtilis*, *S. aureus*, *C. albicans* whereas at 100 µl concentration strains *B. subtilis*, *C. albicans* recorded more inhibition (10.00 ± 0.110). In case of 150 µl concentration, leaf extract of *C. tagal* recorded maximum antimicrobial activity with *B. subtilis* (12.00 ± 0.00) (figure 1). Plant extracts collected from Malakayalanka showed relatively less activity when compared to Gilakaladindi. At 50 µl concentration the mean antimicrobial activity was ranged from 6-8 mm, with a mean of 7.20 mm. At 100 µl, 150 µl concentrations antimicrobial activity varied between 6-9 mm, 7-9 mm and with a mean average of 7.93 mm and 8.40 mm respectively. Except *E. coli* all the strains showed maximum sensitivity (9.00 ± 0.11) towards *C. tagal* extracts when compared to other two plant extracts.

Nutrient analysis

Both in Gilakaladindi and in Malakayalanka field stations the available soil and water nutrients were measured and tabulated. As compared with Malakayalanka the soil samples of Gilakaladindi contained less nitrogen (S:81 kg/a; R:81 kg/a and W:80 kg/a), more phosphorus (S:1.99 kg/a; R:1.99 kg/a and W:1.98 kg/a), zinc (S:1.015 ppm; R:1.019 ppm and W:1.015 ppm), iron (S:13.79 ppm; R:13.77 ppm and W:13.78 ppm) and copper (S:6.70 ppm; R:6.54 ppm and W:6.65 ppm). The availability of manganese (S:24.0 ppm; R:24.48 ppm and W:23.50 ppm) and sulphur (S:67 ppm; R:68 ppm and W:67 ppm) were found to be more in Gilakaladindi in all the seasons. The availability of all these components were increased during rainy season when compared to summer and winter seasons (Table 2 and Table 3). Electrical conductivity is an important parameter to evaluate the water quality. Any change in the concentration of chlorides and sulphates may influence the changes in the conductivity. In present study significant variation in electrical conductivity was observed in all the seasons (Table 2 and Table 3). The electrical conductivity values were found to be recorded more during summer in both Gilakaladindi (5.75 m.mmhos/cm) and Malakayalanka (7.58 m.mmhos/cm) stations. Among all the components the concentration of CO_3 was increased slightly (5.7 m.e.q/Lts) during winter season

in Malakayalanka region. The availability of chlorides, sulphates, sodium, potassium were slightly decreased during rainy seasons both in Gilakaladindi and Malakayalanka regions (Table 2 and Table 3).

Table 2: Soil seasonal analysis at two field stations

S. no	Content	Gilakaladindi			Malakayalanka		
		Summer	Rainy	Winter	Summer	Rainy	Winter
1	pH	8.0	8.5	8.1	8.0	8.2	8.1
2	EC	5.75	2.27	4.01	7.58	5.01	6.56
3	N (Kg/a)	81	81	80	84	84	82
4	P (Kg/a)	1.99	2.29	1.98	1.87	2.16	1.88
5	K (kg/a)	247	248	248	244	246	246
6	Zn (ppm)	0.619	0.806	4.140	1.056	1.289	1.176
7	Fe (ppm)	13.79	13.71	13.78	6.807	9.594	8.646
8	Mn (ppm)	21.66	24.48	23.55	24.0	24.0	23.50
9	Cu (ppm)	6.70	6.54	6.65	2.288	3.808	2.028
10	S (ppm)	67	71	65	67	68	67

Table 3: Seasonal Water analysis at two field stations

S.no	Content	Gilakaladindi			Malakayalanka		
		Summer	Rainy	Winter	Summer	Rainy	Winter
1	pH	8.0	8.0	8.0	7.9	8.0	8.2
2	EC (m.mhos/cm)	12.00	7.18	10.10	14.15	10.80	12.17
3	CO ₃ (m.e.q/Lts)	5.7	5.5	5.5	5.6	5.6	5.7
4	HCO ₃ (m.e.q/Lts)	-1.61	-1.61	-1.60	-1.52	-1.50	-1.49
5	Cl (m.e.q./Lts)	462	456	459	468	460	462
6	Na+K (m.e.q/Lts)	65	60	62	68	65	66
7	Ca+Mg (m.e.q/Lts)	135	137	134	136	136	135

DISCUSSION

In mangrove ecosystem nutrients are considered as the significant components that influence growth and development of the plants.^[23] and their availability is majorly based upon season, tidal forces and influx of fresh water. In present study also the availability of nutrients found to be increased during rainy season (Table 2). This increased nutrient availability during rainy season is may be due to influx of not only fresh water but also due to organic minerals carried by the rain water. Moreover the availability of nutrients observed to be more at Gilakaladindi as compared with Malakayalanka is because of high tidal currents at Gialakaladindi when compared to Malakayalanka. Frequent changes in water sources from ocean to fresh water or vice-versa may have

significant effect on growth and production of bioactive compounds and result in seasonal variations in microbial activity.^[24,25] Seasonal variations in protein, polyphenol and tannin concentration in leaves of Rhizophoraceae was reported and the organic constituents proteins, polyphenol and tannin concentration were generally highest in rainy seasons, intermediate in summer and lowest in winter of mangrove leaves.^[26] Earlier studies indicated that anti bacterial activity of the leaves of *Ceriops tagal* showed 9.78±0.32 mm zone of inhibition against *Bacillus cereus* and 9.25 ±0.84 mm against *Staphylococcus aureus*^[27,28]. *Bruguiera cylindrica* showed zone of inhibition of 6.8 ±0.84mm against *Pseudomonas aeruginosa*.^[29,30] Highest antibacterial activity of aqueous and methanolic extracts of leaves and shoots of *Lumnitzera racemosa* and *Ceriopsis decandra* was previously reported.^[31,32]

In the present study also rainy season samples showed highest overall mean zone of inhibition of 13.62 mm and 11.79 mm. The high concentration of organic constituents in leaf extracts could be the reason for pronounced microbial activity in rainy season. *Excoecaria agallocha* leaf extracts of rainy season collected from Mumbai coast reported highest antimicrobial and anti fungal activity in *Klebsiella pneumonia*, *Aspergillus flavus* and *Phytophthora infestans*.^[33] In present study also irrespective of location, samples collected during rainy season showed more activity compared to summer and winter samples. In fact samples collected from Gilakaladindi station showed comparatively more antimicrobial activity over samples of Malakayalanka.

CONCLUSION

The present study concluded that the seasonal and geographical variations were found to be significant in production secondary metabolites. Of all the seasons rainy season is relatively more preferable for sample collection followed by summer. As the methanolic leaf extracts of rainy season showed highest inhibitory activity on bacterial and fungal growth at both sampling sites. Among the three mangrove species investigated leaf extracts of *Ceriops tagal* showed more inhibitory activity followed by *Lumnitzera racemosa* and *Bruguiera cylindrica*. Further research in isolation and quantification of secondary metabolites from the mangrove leaf samples of rainy season would provide information in developing natural antibiotics with therapeutic potential and pharmacological efficacy.

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