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Lovly MS
Department of Botany, St. Teresa's College, Ernakulam, Kerala, India

Merlee Teresa MV
Department of Botany, St. Teresa's College, Ernakulam, Kerala, India

Correspondence
Lovly MS
Department of Botany, St. Teresa's College, Ernakulam, Kerala, India

Phytochemical analysis and antimicrobial properties of *Nypa fruticans* Wurmb. from Kerala

Lovly MS and Merlee Teresa MV

Abstract
Nypa fruticans, Wurmb.is a mangrove palm and its plant parts are traditionally used by the local people curing herpes, toothe ache, head ache etc. Preliminary phytochemical screening of plant parts revealed the presence of variety of phytochemicals such as phenols, flavonoids, alkaloids, tannins, glycosides, terpenoids, coumarins and saponins. In the present study, hot extracts of leaves and stem of *N. fruticans* in different solvents such as water, methanol, ethyl acetate, chloroform and hexane were used to test the antimicrobial properties against *B. subtilis*, *S. aureus*, *K. pneumoniae*, *E.coli*, *P. aeruginosa*, *A. flavus* and *A. niger*. Antimicrobial study was conducted in different extracts by disc diffusion method. Different fractions of methanolic extracts in leaves were used to get the MIC value. Water extract obtained highest inhibition against both gram positive strains and gram negative strains. Maximum inhibition obtained in stem extract in water against *B. subtilis* (22.16±0.76mm). Lowest MIC of 5 µl or 0.125mg/100µl observed in *S. aureus* (18.00±1.21mm) and *E. coli* (15.00±1.29mm). Inhibition of leaf and stem extracts in different solvents against fungal strains were less compared to bacterial strains. High MIC recorded in both *A. flavus* and *A. niger*. Highest percentage of inhibition (56%) comparable with positive control (60%) obtained in 20 µl or 0.5 mg/100 µl concentration.

Keywords: phytochemicals, antimicrobial, disc diffusion, well diffusion, MIC

Introduction
People throughout the world largely depend on antibiotics for fighting against diseases. Antibiotics are considered as one of the wonders ever created by humans. Many pathogenic organisms are developing resistance in plasmid genes against the existing drugs. Ayurvedic therapy uses natural compounds from plants in the treatment of human diseases. Many plants and plant products we use in medicine and food are having antioxidant properties. Plants are the chief source of chemotheraputants and provide valuable natural products in the control of several bacterial diseases. Studies have shown that plants contain bioactive compounds such as peptides, glycosides, alkaloids, terpenoids, flavonoids, saponins etc with antimicrobial activity against fungal, bacterial and viral infections [1, 2, 3]. *Nypa fruticans*, Wurmb. is a mangrove palm with antioxidant[4] and anti-diabetic potential [5]. Nypa palm plant parts are rich in phenol and flavonoids, thus rich source of antioxidants [6]. Antimicrobial property of ethanolic and aqueous leaves extracts against bacterial strains showed its maximum antimicrobial potential[7]. In the present study, Nypa palm plant parts such as leaf and stem in different extracts (water, methanol, ethyl acetate, chloroform and hexane) were tested for screening of primary and secondary metabolites. Extracts in various solvents were used for antibacterial and antifungal studies by disc diffusion method and well diffusion method.

Materials and Methods
Sample processing
Leaves and stem of *N. fruticans*, Wurmb. were collected from Muziris Pattanam and cleaned with autoclaved double distilled water and shade dried. Dried leaves and stem were powdered and stored in air tight containers.

Qualitative phytochemical analysis
Powdered samples of leaves and stem extracted with five solvents viz. water, methanol, ethyl acetate, chloroform and water using soxhlet apparatus. The extract obtained used for qualitative analysis by the methods described by Brindha. (1981) [8]; Harbone, (1984) [9]; Trease and Evans, (1985) [10] and Kokate, (2001) [11].

Antimicrobial study
Methanol, water, ethyl acetate, chloroform and hexane extracts of leaves and stem were prepared by Soxhlet extraction method and evaporated to dryness. Powdered extract dissolved

in Dimethyl sulfoxide (DMSO) was used for the antimicrobial studies. Five bacterial strains and two fungal strains were used for the study and are given along with their code in the Table: 1

Table 1: List of Pathogen and the stain code

Name of Pathogen	Strain code
Gram positive bacteria	
Bacillus subtilis	ATCC 11778
Staphylococcus aureus	ATCC 6538
Gram negative bacteria	
Klebsiella pneumoniae	ATCC 9621
Pseudomonas aeruginosa	ATCC 9027
Escheritia coli	ATCC 8739
Fungal strains	
Aspergillus flavus	ATCC 9643
Aspergillus niger	ATCC 16888

Antibacterial study by disc diffusion method

Antibacterial activity of leaf and stem of five selected mangrove plants were studied using disc diffusion method (Kirby-Bauer method) of Bauer *et al.* (1966) [12]. Agar and nutrient broth were used to prepare the medium. Sterile discs of 6mm loaded with different solvent extracts (Methanol, water, ethyl acetate, chloroform and hexane) of leaf and stem were used in the study and all the solvent extracts were evaporated to dryness and dissolved in DMSO for the ease of work. Vancomycin, gentamycin and chloramphenicol discs were used as positive control. Zone of inhibition was measured in millimetre (mm) ± SD.

Antifungal study by Disc diffusion method

Antifungal study of leaf and stem extracts (Methanol, water, ethyl acetate, chloroform and hexane) of five selected mangroves was conducted using disc diffusion method (Kirby-Bauer method) of Bauer *et al.* (1966) [12].

Antibacterial study by Well diffusion method

Well diffusion method of antibacterial study was carried out by the method described by Perez *et al.* (1990) [13]. Leaf extract in methanol was prepared in different dilutions in Dimethyl sulfoxide (DMSO) and used for well diffusion method to find the MIC value. Five wells were placed in the agar plate and three dilutions in DMSO of methanolic extracts of selected mangrove leaves, one DMSO solvent and one control were poured and incubated for 12 hours.

Anti-fungal study by Well diffusion method

Antifungal study by well diffusion method was conducted based on the method described by Morton and Stroube, (1955) [14]. Methanolic leaf extract and test fungi were placed on the wells in the opposite halves of the PDA plate at equal distance from the periphery. Clotrimazole used as positive control was placed in one plate with opposite test fungi and another plate with only test fungi served as negative control. Inoculated plates were incubated at 27 ± 3° C for 5 days. Percent inhibition was calculated by the following equation suggested by Fokkema and Shearer (1973) [15].

Percent inhibition = $(R_1 – R_2/ R_1) \times 100$ (R_1- radius away from the antagonist, R_2 - radius in direction of the antagonist)

Results

Qualitative phytochemical analysis

Screening of primary and secondary metabolites obtained positive results more in the leaves extracts than in the stem extracts. Carbohydrates observed in all the extracts. Amino acids and proteins were observed in water, methanol and ethyl acetate extracts. Alkaloids, flavonoids and phenolic compounds recorded excellent results in water, methanol, ethyl acetate, chloroform and hexane extracts more appreciable in leaf extracts. Saponins appeared in most of the extracts except in chloroform and hexane stem extracts. Results are depicted in the table: 2.

Table 2: Phyto chemical screening of primary and secondary metabolites

Name of Test	Metabolites	Water		Methanol		Ethyl acetate		Chloroform		Hexane	
		L	S	L	S	L	S	L	S	L	S
Molish's Test	Carobohydrates	+	+	+	+	+	+	+	+	+	+
Fehling's Test		+	+	+	+	+	+	+	+	+	+
Biuret Test	Proteins	-	+	+	+	+	+	-	-	-	-
Ninhydrin Test	Amino acids	-	-	+	-	-	--	-	-	-	-
Dragendroff's test	Alkaloids	+	+	+	+	+	-	+	-	-	-
Mayer's Test		+	+	+	-	+	-	+	-	+	-
Wagner's Test		+	-	+	-	+	-	+	-	-	-
Hager's Test		+	-	+	-	+	+	+	-	-	-
Shinoda Test	Flavonoids	+	+	+	+	+	-	+	-	+	-
Lead acetate Test		+	-	+	-	+	-	+	-	+	-
Ferric chloride Test	Tannins	+	-	+	+	+	-	-	-	-	-
Gelatin Test		-	-	+	-	+	-	-	-	-	-
Iodine Test		+	-	+	-	+	-	+	-	+	-
Nitric acid Test		+	-	+	+	-	-	+	-	+	-
Liberman Burchard Test	Sterols and Triterpenoids	+	-	+	+	+	+	+	-	+	-
Salkowski's Test		+	-	+	-	+	-	+	-	+	-
Keller Kiliani Test	Glycosides	+	-	+	-	+	-	+	-	+	-
Foam Test	Saponins	+	+	+	+	+	+	+	-	+	-

Anti-bacterial activity by Disc diffusion method

Water extract of leaves produced significant activity to both the gram positive strains and gram positive strains. Water extract inhibited *B. subtilis* in 13.00±0.55mm and *S. aureus* in16.36±3.36 mm. Similarly gram negative strains were highly susceptible to water extract with zone of inhibition 18.70±0.82mm for *E. coli*, 20.03±1.08 mm for *K. pneumoniae* and 20.33±0.95mm for *P. aeruginosa*. Gram positive strains of *B. subtilis* and *S. aureus* were highly susceptible to the stem extract, particularly in the water and methanol extracts. Water extract of stem showed high inhibition of 22.16±0.76 mm in *B. subtilis* comparable to the antibiotic positive control. Gram negative bacteria, *E.coli* exhibited maximum inhibition of 11.97±0.45 mm in chloroform extract. *K.*

pneumoniae had high susceptibility of 13.13±0.81 mm in water extract, whereas *P. aeruginosa* was resistant to most of the extract except in water extract which inhibited the growth in 10.20±0.72 mm. Activity of various solvent extracts are recorded in Table: 3.and Plate: 1.

Table 3: Antibacterial activity of *N. fruticans* leaf and stem extracts (zone of inhibition in mm ± SD)

Extract	Pathogens	Water	Methanol	Ethyl Acetate	Chloroform	Hexane
Leaf extract	*B. subtilis*	13.03±0.55	12.66±0.76	7.00±0.60	11.23±1.66	08.00±0.50
	S.aureus	16.36±3.36	11.23±0.87	11.20±0.72	12.36±1.00	08.17±1.25
	K. pneumaoniae	20.03±1.08	16.26±0.93	14.26±0.93	14.33±1.04	08.16±0.76
	P.aeruginosa	20.33±0.95	15.43±0.93	14.17±0.76	12.23±0.87	11.03±0.55
	E.coli	18.70±0.82	15.43±1.11	09.41±1.03	12.16±0.76	10.00±0.40
Stem extract	*B. subtilis*	22.16±0.76	21.06±1.10	21.20±1.31	15.16±0.76	10.06±0.90
	S.aureus	20.33±1.04	21.00±0.50	15.36±1.00	09.20±0.72	10.26±0.64
	K. pneumaoniae	13.13±0.81	10.20±0.72	11.26±0.93	08.17±0.76	10.33±1.04
	P.aeruginosa	10.20±0.72	07.03±0.55	00.00±0.00	00.00±0.00	00.00±0.00
	E.coli	10.00±0.30	11.00±1.00	11.03±0.25	11.97±0.45	10.86±0.47

Anti-fungal activity by disc – diffusion method

Leaf extracts showed moderate inhibition against both fungal strains and stem extracts recorded negligible or less inhibition. Water extract of leaves was more active against both the fungal strains of *A. flavus* (17.00±1.00 mm) and *A. niger* (15.3±1.52 mm), but no activity was shown by hexane extract against *A. flavus*. All the stem extracts obtained low inhibition with both the fungal strains (Table: 4, Plate: 2).

Table 4: Antifungal activity of *N. fruticans* leaf and stem extracts. (Zone of inhibition in mm ± SD)

Extract	Fungal strains	Water	Methanol	Ethyl Acetate	Chloroform	Hexane
Leaf extract	*A. flavus*	17.00±1.00	15.00±0.57	10.00±1.00	13.30±1.52	00.00±0.00
	A. niger	15.3±1.52	12.00±0.00	11.00±0.57	09.00±0.57	09.00±1.00
Stem extract	*A. flavus*	00.00±0.00	00.00±0.00	07.00±0.00	08.00±2.00	00.00±0.00
	A. niger	07.00±1.52	00.00±0.00	07.00±1.00	07.00±2.00	00.00±0.00

Antibacterial activity by well diffusion method

Among the leaf and stem extracts studied by disc diffusion method, more results obtained for the leaf extracts than the stem extracts. So MIC values determined for the methanolic leaf extracts in various concentrations. All the plant extracts had given a dose dependent sensitivity against bacterial strains. Methanolic extracts of mangrove leaves recorded activity against both the gram positive strains and gram negative strains. Highest activity was obtained against *S. aureus* (18.00±1.21mm) in 5 µl concentration of 0.125mg/100 µl extract. Activity against *E. coli* showed moderate results (15.00±1.29 mm) in 5 µl concentration of 0.125mg/100 µl extract. Activity against other bacteria such as *B. subtilis, K. pneumonia, P. aeruginosa* was moderate. (Table: 5, Plate: 3)

Table 5: Antibacterial activity of *N. fruticans*, Wurmb. leaf extract in methanol by well diffusion method (zone of inhibition in mm ± SD)

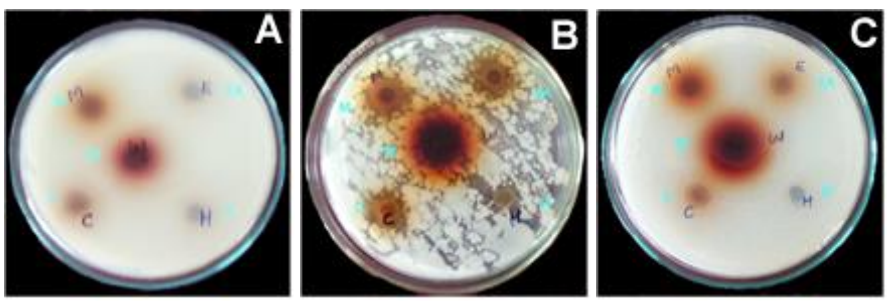
Bacterial strains	Methanolic leaf extract fractions of *N. fruticans* / well			
	5µl	15µl	20µl	+ve control
B.subtilis	10.00±1.32	15.00±1.40	15.00±0.95	15.00±1.32
S.aureus	18.00±1.21	18.00±1.89	18.00±0.72	12.00±1.25
K.pneumoniae	10.00±1.32	14.00±1.52	15.00±1.04	30.00±1.80
P.aeruginosa	10.00±1.53	12.00±1.32	15.00±2.08	30.00±2.52
E.coli	15.00±1.29	14.00±1.36	15.00±2.00	25.00±1.75

Antifungal activity by well diffusion method

Percentage of inhibition observed for methanolic leaf extract against *A. niger* was comparable with the positive control. MIC obtained for 56% of inhibition was 20 µl (0.5mg/100 µl). The percentage of inhibition against *A. flavus* was less (28%) in 20 µl concentration. Results are recorded in the table: 6. and Plate: 4.

Table 6: Antifungal activities of *N. fruticans* leaf extract in methanol by well diffusion method

Fungal strains	Methanolic leaf extract fractions of *N. fruticans*, Wurmb./well			
	10µl	20 µl	Negative control	Positive control
A. flavus	24	28	NIL	56
A. niger	44	56	NIL	60



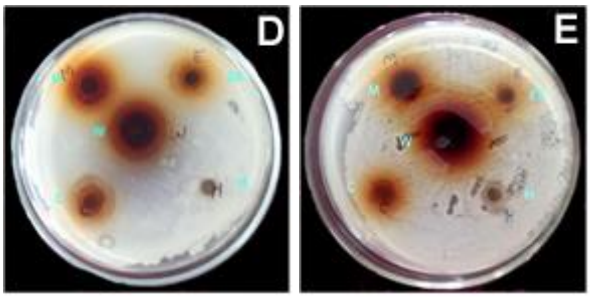


Plate 1: Antibacterial activity of *N. fruticans* leaf extracts. (A) - *B. subtilis*, (B)- *S. aureus*, (C)- *K. pneumoniae*, (D)- *P. aeruginosa*,(E)- *E. coli*, (W-Water, M-Methanol, E-Ethyl acetate, C-Chloroform, H-Hexane)

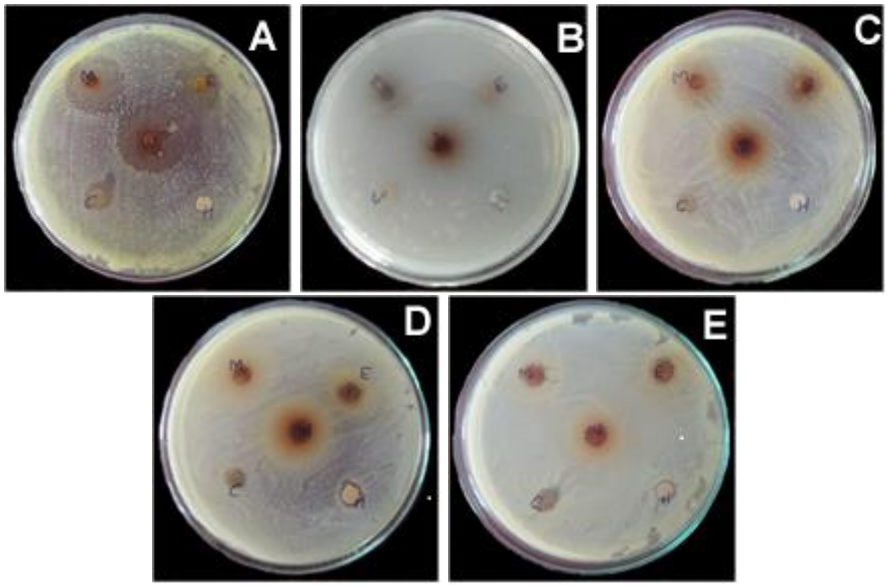


Plate 2: Antibacterial activity of *N. fruticans* stem extracts. (A) - *B. subtilis*, (B)- *S. aureus*, (C)- *K. pneumoniae*, (D)- *P. aeruginosa*,(E)- *E. coli*, (W-Water, M-Methanol, E-Ethyl acetate, C-Chloroform, H-Hexane

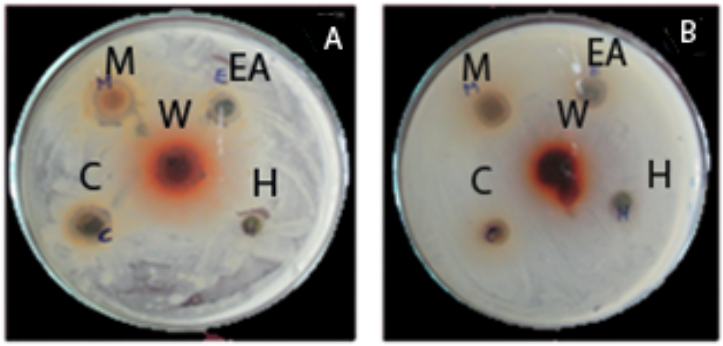
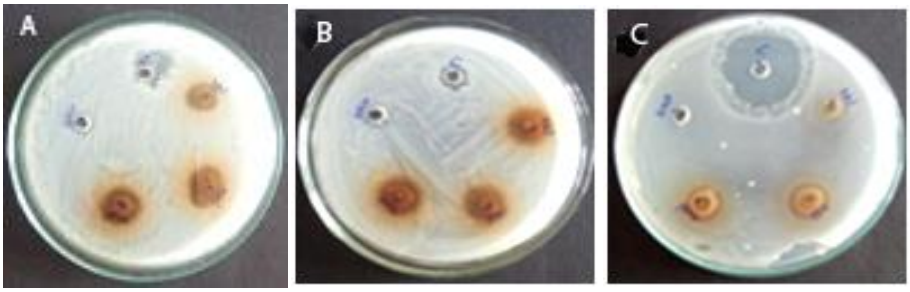


Plate 3: Antifungal activity of *N. fruticans*, Wurmb. by disc diffusion method A- *N. fruticans* leaf extracts against *A. flavus* ; B- *N. fruticans* leaf extracts against *A. niger*



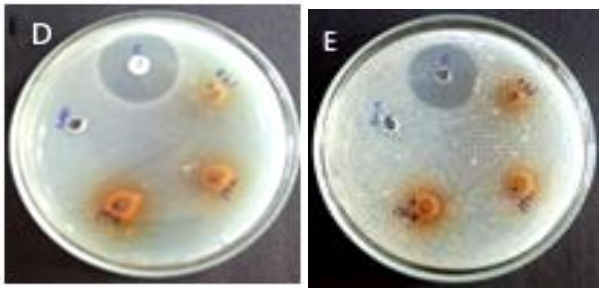


Plate 4: Antibacterial activity of methanolic leaf extract fractions of *N. fruticans*, Wurmb. (A)-*Bacillus subtilis*, (B)-*Pseudomonas aeruginosa*, (C)- *Staphylococccus aureus*, (D)- *Klebsiella pneumaoniae* (E)-*E.coli*.

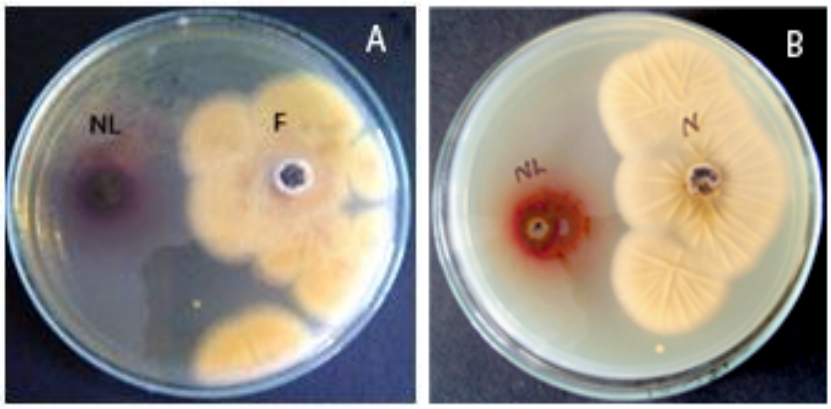


Plate 5: Antifungal activity of *N. fruticans* by dual culture method; A- *A. niger*, B - *A. flavus*, NL - *N. fruticans* leaf extract

Discussion

Kerala, a state of India, is well known for its rich biodiversity and heritage. *N. fruticans*, a mangrove palm is not identified naturally in any part of the Kerala, eventhough the plant could flourish in the mangrove belts and coastal areas. Recently, the plant is proved to have anti-diabetic [5], antioxidant [4] and anti-inflammatory potential [6]. Antimicrobial potential of the plant parts such as leaves, husks and mid vein tissues was reported by Ebana *et al.*(2015) [7]. Detailed analysis of antimicrobial potential was not conducted so far in leaf and stem parts in different extracts. Phytochemical screening done in different extracts revealed the therapeutic potential of extracts in distinct solvents and the antimicrobial activity observed, further supported the presence of phytochemicals. Hence the isolation and characterization of therapeutically important compounds will be significant.

Conclusion

Although *N. fruticans* can be used in variety of applications, the plant has not given much consideration in India. This fast growing plant used in different purposes such as in the fixation of carbon, production of biofuels [16], sap [17, 18, 19], aromatic tea [19], vineager [17, 19], paper manufacturing [1, 20], animal feed [19], and in thatching houses [19]. Phytochemical and pharmacological studies have proved its potential in the treatment of diseases. Continuous productivity, adsorbance of heavy metal ions ($Pb^{2+}$and Cu^{2+}) [21, 22] from aqueous solution and checking of coastal erosion suggests the need for the cultivation of this plant in the coastal areas of Kerala.

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