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Study of Antimicrobial Activity & Structural Elucidation of Purified Compound from Potential Solvent Extract of *Bruguiera cylindrica [L]*

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Article History

Abstract

B.Cylindrica is used as a mangrove plant in folk medicine to cure various diseases and disorders which include tumors, blood clotting, infections, fevers, pains etc., The Present study was to isolation, purification and characterization of antibacterial compound from the leaves along with phytochemical analysis. Healthy leaves of *B. cylindrica* were collected from natural habitat, washed, air dried and ground into powder. The solvents, such as chloroform, ethyl acetate, ethanol, methanol, and butanol were used to extract the powdered leaves. Crude extracts were tested for phytochemical constituents like carbohydrates, glycosides, proteins, steroids, triterpenoids flavonoids, alkaloids, tannins, saponins and phenols. Antimicrobial activities for crude extracts were tested against bacteria and fungi using standard well diffusion agar plate method. The highest antibacterial activity was identified in ethyl acetate extract, which was further purified using column chromatography. Activity-guided ethyl acetate fraction contains potential compound was used for structure elucidation using LC-MS, FTIR and NMR studies. Mass spectrometry of purified compound showed major peak at 6.901 minutes with a molecular weight of 256.32 g/mol, suggesting that the compound belongs to carboxylic acid group with a molecular formula CHN_2O_3 when compared to LC-MS standard from the NIST library. The Pure compound from *Bruguiera cylindrica* showed the stretch at 2561 indicated the presence of (O-H) hydroxyl group, 3341 N- H group, a stretch at 1709 indicated the presence of C=O group. The data obtained showed the presence of nitrogen containing amine, amide and carboxylic acid functional groups 7-(dimethylamino)-3ethyl-8-oxo-5-thia-1-azabicyclo(4.2.0)-oct-2-ene-2-carboxylic acid with a molecular formula CHN_2O_3. Based on ^{1H} NMR spectrum and database of NIST, purified fraction was identified as 7-(dimethylamino)-3ethyl-8-oxo-5-thia-1-azabicyclo(4.2.0)-oct-2-ene-2-carboxylic acid. The results show that *B cylindrica* contains significant phytochemical and antibacterial activity against tested bacteria and fungi that helps to scientifically understand potentiality of tribal medicine.

Keywords: Phytochemical, Anti-fungal, antibacterial, NMR, *Bruguiera cylindrica*

Introduction:

The mangrove plants grow along the coastal areas with brackish water. Although the environment is unfavorable for rich plant growth, Mangroves are extremely adaptable to their environment in terms of anatomy, physiology, and morphology. Many researchers have referred to the usefulness of mangrove plants in traditional medicine because mangrove plant extracts have been used for centuries by the local people as folk medicine for curing many health disorders like HIV, rheumatism, cancer etc., (Kokpal *et al.,* 1990; Premanathan *et al.,* 1996). They contain medicinally important bioactive compounds. Common phytochemicals such as flavonoids, alkaloids, terpenoids, tannins, Saponins etc., are produced by plants and are enlisted as secondary metabolites having pharmaceutical importance such as antimicrobial agents and antioxidant agents (Santhi and Sengottuve, 2016; Shelar *et al.*, 20). Plant medications have several advantages over synthetic ones, including being comparatively safer, providing significant therapeutic effects, and being more reasonably priced. Previous reports have stated the presence of medicinally important phytochemicals in barks, stems, roots, leaves, flowers, and fruits of the genus *Bruguiera*. (Han, L., *et al*., 2004; Homhual, Bunyapraphatsara, *et al*., 2006; Nguyen *et al*., 2020 , Cai *et al*., 2011; Huang *et al*., 2009; Karalai & Laphookhieo, 2005; Ponglimanont & Chantrapromma, 2004). The present study was undertaken to investigate the presence or absence of phytochemicals of *Bruguiera cylindrica*, as this plant has been reported to have many medicinal properties, but very few reports are available on chemical profiling (Ali *et al*., 2012; Ravikumar *et al*., 2011; Shamsuddin *et al*., 2013; Krishnamoorthy *et al*., 2011; Vadlapudi and Naidu 2009; Agoramoorthy *et al*., 2008; Chantrapromma *et al*., 2003; Chantrapromma *et al*., 2007)

Materials and Methods:

Collection of plant material and Preparation of extracts:

Healthy *Bruguiera cylindrica* leaves were obtained from the Mada forest near Nizam Patnam, in Andhra Pradesh, India. Prof. K. Madhava Chetty of Sri Venkateswara University Department of Botany in Tirupati, provided the plant species authentication. In order to cease the enzymatic degradation of secondary metabolites, alcohol was sprayed on the plant samples and shade dried for 7-10days. After the leaves were dried. We used a sterilized mortar and pestle to crush them into a fine powder. The powder was kept in an airtight container to prepare for future usage. The standard Soxhlet Extraction procedure was used to prepare plant extracts using various solvents, followed by the rotary flash evaporation method to remove excess solvents in extraction**.** (Aiyelaagbe *et al*., 2009).

Qualitative analysis of Phytochemicals:

To evaluate different phytochemical constituents like flavonoids, amino acids, carbohydrates, glycosides, tannins, phenolic compounds, saponins, steroids, terpenoides, phytosterols and triterpenoids, various solvent extracts such as chloroform, ethyl acetate, methanol and butanol, were used. Carbohydrates by Fehling's method, Glycosides test by Legal's test, Amino acid by Ninhydrin test, Steroids and Triterpenoids test by Salkowski'sTest, Flavonoid by Lead acetate test, Alkaloids by Mayer's test, Saponins test by foam method, Phenols and Tannins by Ferric chloride test were carried out in this study. (Tukiran, 2013; Bhatt and Dhyani, 2012; Mouafi *et al*., 2014; Vittaya and Chalad, 2016)

Quantitative estimation of Phytochemicals: (Malik *et al.*, 2018, Ushie *et al*., 2018; Garg and Garg 2019)

Quantitative analysis of steroids:

1 ml of plant extract was transferred into 10 ml volumetric flasks. 2 ml 4N H_2SO_4 and 2 ml of 0.5% iron (III) chloride were added, followed by 0.5 ml of 0.5% potassium hexacyanoferrate (III) solution . The mixture was heated for thirty minutes at a temperature of 70±20 °C in a water bath. The steroid was calibrated, and its absorbance was measured at 780nm compared to the reagent blank.

Quantification of flavonoid total content:

To 4 ml of distilled water, 1 ml of plant extract was added, along with 0.3 ml of 5% sodium nitrite, and it was allowed to sit for 5 minutes. Then, 0.3 ml of 10% aluminum chloride was added and left to sit for another 5 minutes. Following incubation, 10 ml of distilled water was used to treat and dilute 2 ml of 1M NaOH. The absorbance was then measured using a reagent blank at 510nm. The total flavonoid concentration was expressed in mg of QE/g of extract.

Alkaloids' total content:
1 ml of plant extract diluted in dimethyl sulfoxide (DMSO) was mixed with 1 ml of 2N HCl and filtered. Following continuous shaking, 5 ml of Bromocresol green solution, 5 ml of phosphate buffer, and 4 ml of chloroform were added to the filtrate. The mixture was then collected in a 10 ml volumetric flask, and the absorbance was measured at 470 nm against the reagent blank. The total content of alkaloids was expressed as mg of AE/g of plant extract.

Estimation of Total Saponins:
20 g of the plant powdered material was mixed with 100 ml of 20% aqueous ethanol. The samples were heated at 55°C for 4 hours in a hot water bath using continuous stirring. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over a hot water bath maintained at 90°C. The concentrate was transferred into a 250 ml separation funnel and to it 20 ml of diethyl ether was added and wobble vigorously. The aqueous layer was recovered, while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath for evaporation and was further dried in the oven to a constant weight; the saponins' content was calculated.

Evaluation of Antibacterial activity of Plant extracts: (Okla and Garg 2019; Haq *et al*., 2011)
Pseudomonas aeruginosa, E.coli, Salmonella typhi, Klebsiella pneumoni, and Staphylococcus aureus were used in the present study to determine the antimicrobial activity of various plant extracts. The agar well diffusion method investigated the antibacterial activity of different plant extracts. Test bacterial strains were inoculated on Muller Hinton agar using the spread plate method. Further wells were punctured using a sterile well borer, and test plant extracts of 10 µl (10 mg/ml concentration) were loaded into the wells. After loading, the plates were incubated at 37oC for 20-48 hrs. Observed for Zone of inhibition around well indicating the antimicrobial activity of the plant extract. DMSO was used as the negative control, and streptomycin (Hi-media) was used as the positive control.

Antifungal activity of Plant Extract: (Abeysinghe, 2010)
The fungal culture *Aspergillus niger, Fusarium oxysporum, Candida albicans* and *Penicillium chrysogenum* were evenly spread on the PDA medium by using sterile cotton swabs and wells were made in the medium using sterile cork borer. 200µl of plant extracts were added into separate wells, and plates were incubated at 27ºC for 72 hours. After the incubation period, the results were measured in the zone of inhibition around each well.

Purification of bioactive metabolite:
Purification of compounds was performed by Column chromatography for the separation of pure compounds. The sample was loaded into a column pre-packed with silica gel (Hi-media 100-200 mesh) and purified fractions were collected and stored for further research. (Lin *et al*., 2020).

Structural elucidation of active compound:
FTIR and NMR analysis were used to clarify the active compound's structure. Fourier Transform Infrared Spectroscopy (FTIR) can identify compounds with distinct functional groups. FTIR spectroscopy analysis of pure compound was scanned within the frequency range between 4000cm-1 and 500cm-1 using an Alpha-T Bruker Spectrometer. Liquid chromatography and mass spectra were carried out in an Apex mass spectrum analyzer in the negative ion measurement mode with the detection voltage of 1.6kv, an APCI temperature of 400ºC, a curved dissolution line of 250ºC and the block temperature of 200ºC. ^{1}H NMR spectra of compounds were recorded by Bruker DRX 500.13 MHz spectrometer and acquired on a Bruker Avance-2 model spectrophotometer (Barik *et al*., 2016; Li *et al.*, 2010).

Results:

Collection of plant material and phytochemical analysis of extracts:
The phytochemical components of many solvent extracts from healthy *Bruguiera cylindrica* leaves such as methanol, butanol, ethanol, ethyl acetate, and aqueous extract, were investigated qualitatively and quantitatively. Based on the primary quantitative phytochemical screening Carbohydrates, Flavonoids, Alkaloids, and Saponins were present in all the extracts, Glycosides, Protein, Triterpenoids, Tannins, and

Phenols were not detected in all extracts, whereas Steroid was reported in all samples except butanol as indicated in **(Table:1a).** Solvent choice and polarity matter in extracting various phytochemicals. According to the qualitative analysis, medicinally important bioactive compounds such as flavonoids, alkaloids like compounds, have been extracted (Singh *et al*., 2007; Hong *et al*., 2011; Vijayavel *et al*., 2006; Loo *et al*., 2007; Loo *et al*., 2008; Rahim *et al*., 2008; Gao and Xiao, 2012; Premanathan *et al*., 1999; Benbott *et al*., 2012; Sulaiman *et al*., 2011). According to Saad *et al*., 2011, Saponins have antimicrobial activity. Based on the quantitative analysis of phytochemical constituents, in compared with other solvent extractions Steroids, Flavonoids, Alkaloids were more in ethyl acetate extract as indicated in **(Table:1b).** Previous reports have stated that phenolic compounds, Saponins and Alkaloids exhibit antimicrobial, antifungal and antioxidant properties (Aboaba *et al.*, 2006).

Evaluation of antimicrobial and antifungal activity of plant extracts:

The antimicrobial activity of plant extract was evaluated against five human pathogens, including *Pseudomonas aeruginosa, E.coli, Salmonella typhi, Klebsiella pneumonia and Staphylococcus aureus. Based* on its antimicrobial potential, ethyl acetate has shown broad and potential activity compared to other extracts and the standard drug tetracycline. Moreover, it is given as in **(Table: 3).** In case of *Pseudomonas aeruginosa* except methanol, butanol remaining extracts have inhibited the growth in *E.coli and Staphylococcus aureus* no activity was seen in aqueous. Methanol, butanol, ethanol and remaining extracts have inhibited the growth in *Salmonella typhi* and *Klebsiella pneumonia,* all extracts have inhibited growth except in butanol as shown in **(Table:2).** Antifungal activity of solvent extracts was checked against 4 different fungal species (*Aspergillus niger, Fusarium oxysporum, Candida albicans, Penicillium chrysogenum).* Based on the results, ethyl acetate extract and ethanol extract have inhibited all four fungal species. In comparison with other extracts and standard antifungal agent, ethyl acetate extract showed more potential activity. *Aspergillus niger* and *Fusarium oxysporum* were inhibited with all extracts except methanol and butanol. *Penicillium chrysogenum* was inhibited in ethyl acetate, ethanol but not in other extracts. *Candida albicans* were inhibited by all extracts except butanol extracts as shown in **(Table: 3).**

Purification of active compound:

Four fractions were obtained by column chromatography. Biological activity has revealed fraction 3 had good biological properties **(Table: 4).** Hence, fraction 3 was further used for structural elucidation studies by LCMS, FTIR and NMR. The preliminary compound from *Bruguiera cylindrica* leaves fraction 3, revealed a strong peak at 6.901 minutes based on mass spectral analysis with a 256.32 g/mol molecular weight. Mass spectral analysis suggested that the compound belongs to a carboxylic acid group with molecular formula CHN_2O_3 compared to the LC-MS standard from the NIST library, as shown in **Fig. 1**. The Pure compound from *Bruguiera cylindrica* showed the stretch at 2561, indicated the presence of (O-H) hydroxyl group, 3341 N- H group, a stretch at 1709, indicated the C=O group. The result obtained showed the presence of nitrogen containing amine, amide and carboxylic acid functional groups 7-(dimethylamino)-3ethyl-8-oxo-5-thia-1-azabicyclo(4.2.0)-oct-2-ene-2-carboxylic acid with a molecular formula as shown in **Fig. 2**. ^{1}H NMR spectrum of a purified compound from fraction 3 *Bruguiera cylindrica* shows the triplet at d 1.091 is due to a terminal methyl group, and the strong singlet at d 1.127 is due to long chain methylene groups. The strong signals at d 2.384, 3.430 and 2.469 are due to methylene protons attached to unsaturated systems, and the signal at d 3046 is due to two bis allylic protons. Based on spectral characteristics of the ^{1}H spectrum and the database of NIST, the purified fraction was identified as 7-(dimethylamino)-3ethyl-8-oxo-5-thia-1-azabicyclo(4.2.0)-oct-2-ene-2-carboxylic acid **Fig. 3.**

Table: 1a Qualitative analysis of Phytochemicals

Sr no.	Phytochemical Analysis	Aqueous extract	Methanol extract	Ethyl acetate extract	Butanol Extract	Ethanol Extract
1	Carbohydrate	-	-	-	-	-
2	Glycosides	-	-	-	-	-
3	Protein	-	-	-	-	-
4	**Steroids**	+	+	+	-	+
5	Triterpenoids	-	-	-	-	-
6	**Flavonoids**	+	+	+	+	+
7	**Alkaloids**	+	+	+	+	+
8	Tannins	-	-	-	-	-
9	**Saponins**	+	+	+	+	+
10	Phenols	-	-	-	-	-

Table: 1b Quantitative analysis of Phytochemicals

Sr no.	**Phytochemical Analysis**	**Aqueous extract**	**Methanol extract**	**Ethyl acetate extract**	**Butanol Extract**	**Ethanol Extract**
1	Steroids	5.64+0.04^{d}	6.64+0.03^{c}	10.23+0.08^{a}	0.00	8.36+0.04^{b}
2	Flavonoids QE/g of extract	9.72+003^{b}	5.76+0.02^{d}	11.56+0.04^{a}	7.64+0.02^{c}	5+0.02^{d}
3	Alkaloids AE/g of plant extract	9.28+0.09^{d}	12.03+0.03^{c}	20.39+0.43^{a}	15+0.05^{b}	12+0.04^{c}
4	Saponins	6.03+0.02^{b}	3.09+0.04^{d}	8.06+0.03^{a}	5.36+0.06^{c}	2.03+0.04^{e}

(Duncan's Multiple Range Test: DMRT) is significant at 0.05 level zone of inhibition diameter from higher to lowest values

Table: 2 Antibacterial activity of Plant Extract (Zone of inhibition in mm):

Extract	***Pseudomonas aeruginosa***	***E.coli***	***S.typhi***	***Klebsiella pneumonia***	***S.aureus***
Aqueous	0.40+0.10^{c}	-	1+0.10^{d}	0.65+0.10^{a}	-
Methanol	-	-	0.56+0.10^{d}	0.22+0.10^{d}	-
Ethyl acetate	5.12+0.10^{a}	8.56+0.10^{a}	6.28+0.10^{a}	0.56+0.10^{b}	2.56+0.10^{a}
Butanol	-	-	-	-	-
Ethanol	0.13+0.30^{d}	-	0.1+0.10$_{d}$	0.22+0.10^{d}	-
Amoxicillin	3.13+0.20^{b}	0.45+0.30^{b}	2.32+0.10^{c}	0.45+0.10^{c}	0.49+0.10^{b}
Tetracycline	3.32+0.20^{b}	0.32+0.30^{c}	3.26+0.10 ^{b}	0.40+0.10^{c}	0.36+0.10^{c}

(Duncan's Multiple Range Test: DMRT) is significant at 0.05 level zone of inhibition diameter from higher to lowest values

Table: 3 Antifungal activity of Plant Extract (Zone of inhibition in mm):

Extract	***Aspergillus niger***	***Fusarium oxysporum***	***Candida albicans***	***Penicillium chrysogenum***
Aqueous	1.14+0.10^{c}	0.54+0.10^{d}	1.54+0.10^{c}	-
Methanol	-	-	1.13+0.10^{c}	-
Ethyl acetate	**5.31**+0.10^{a}	**7.31**+0.10^{a}	**4.52**+0.10^{a}	**3.14**+0.10^{a}
Butanol	-	-	-	-
Ethanol	2.50+0.10^{d}	3.28+0.10^{c}	0.50+0.10^{d}	0.28+0.10^{c}
Fluconazole	3.56+0.10^{b}	4.63+0.10^{b}	3.56+0.10^{b}	2.56+0.10^{b}

(Duncan's Multiple Range Test: DMRT) is significant at 0.05 level zone of inhibition diameter from higher to lowest values

Table: 4 Biological property of Purified ethyl acetate extract fractions (Zone of inhibition in mm):

Activity against	Fraction 1	Fraction 2	**Fraction 3**	Fraction 4
Ps.aeruginosa	5.12	0	**7.03**	2.31
E.coli	6.56	0	**8**	0
S.typhi	6	0	**8.62**	0
Klebsiella pneumonia	0.82	0	**2.23**	0
S.aureus	2.87	0	**3.89**	0
Aspergillus niger	5.87	0	**7.34**	0
Fusarium oxysporum	7.31	0	***9.56***	0
Candida albicans	3.52	0	***6.54***	1.23
Penicillium chrysogenum	4.14	0	**5.21**	0

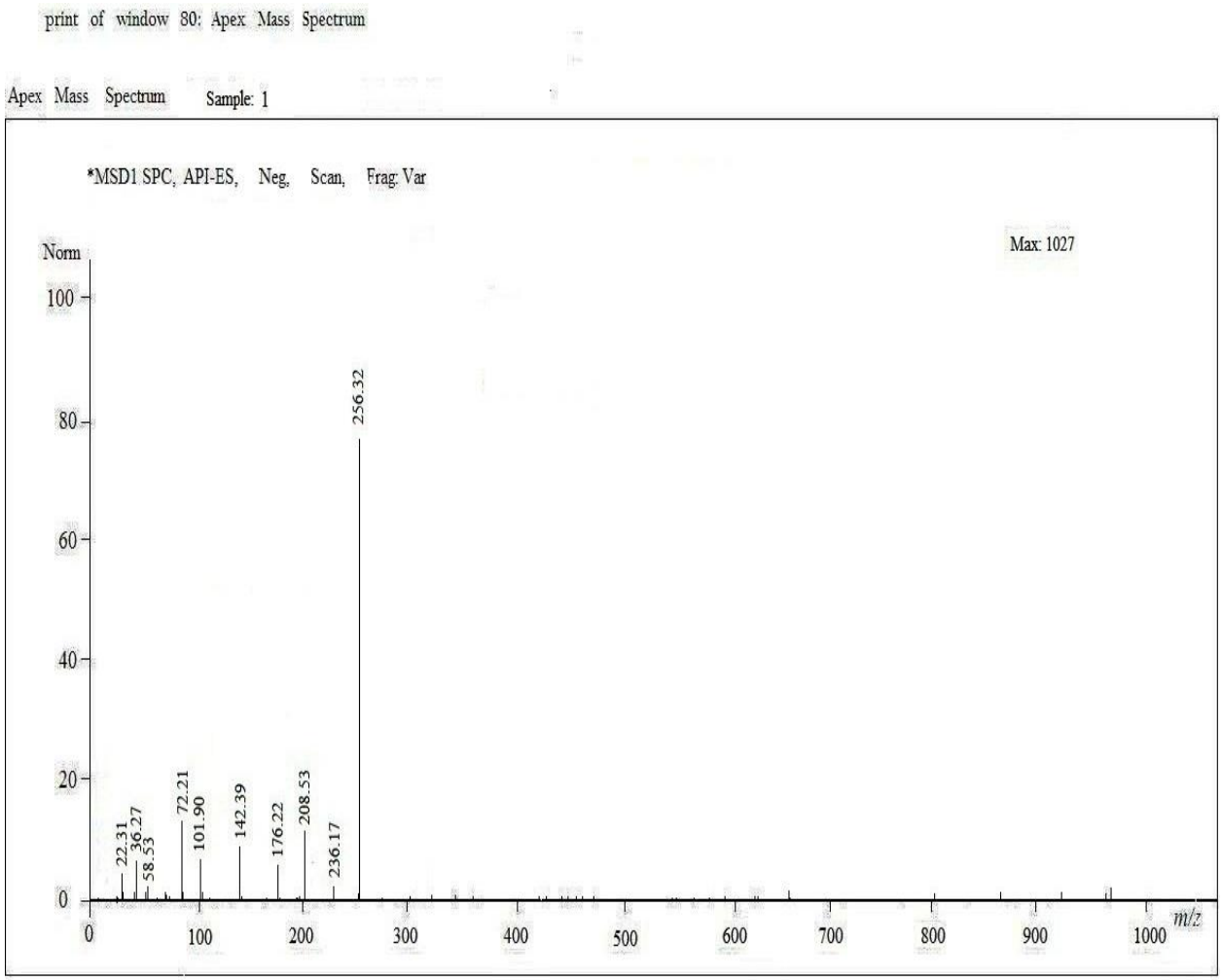

Fig 1: LC-MS chromatogram of purified fraction from *Bruguiera cylindrica* shows the isolated major peak at 256.32m/z.

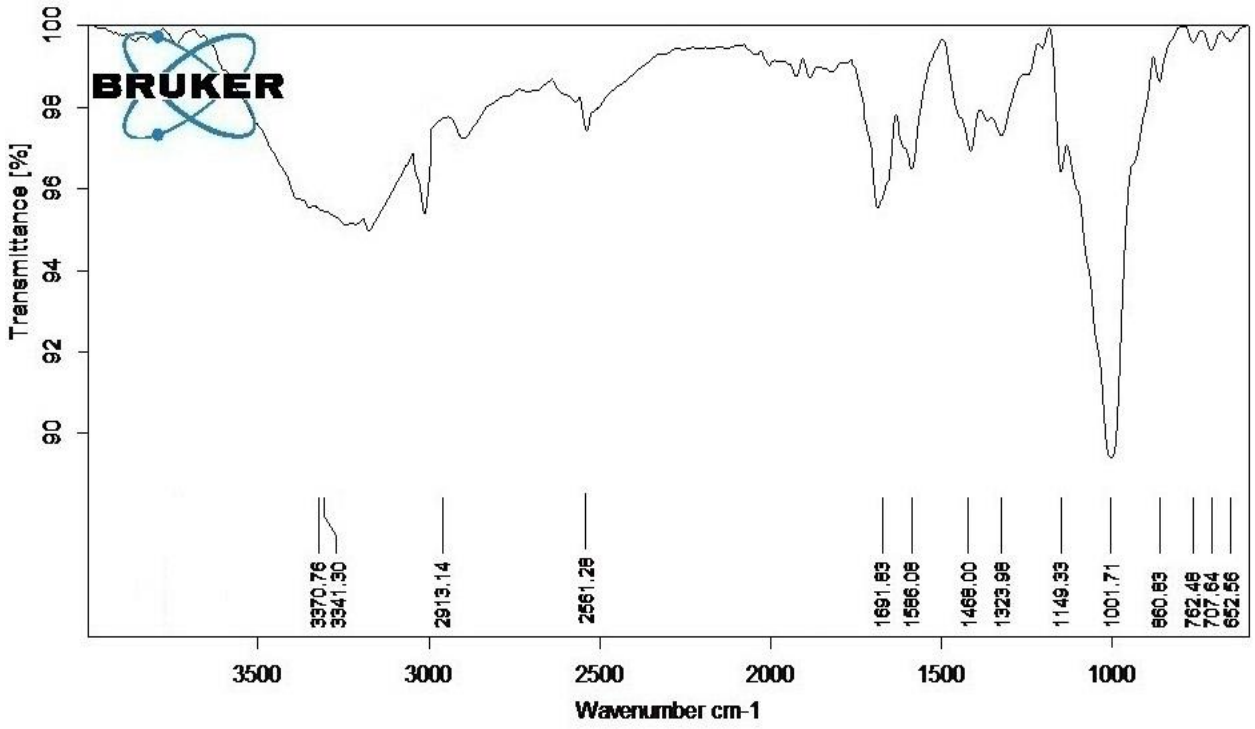

Fig 2: FT-IR Spectrum showing functional groups of purified compound from fraction from *3*

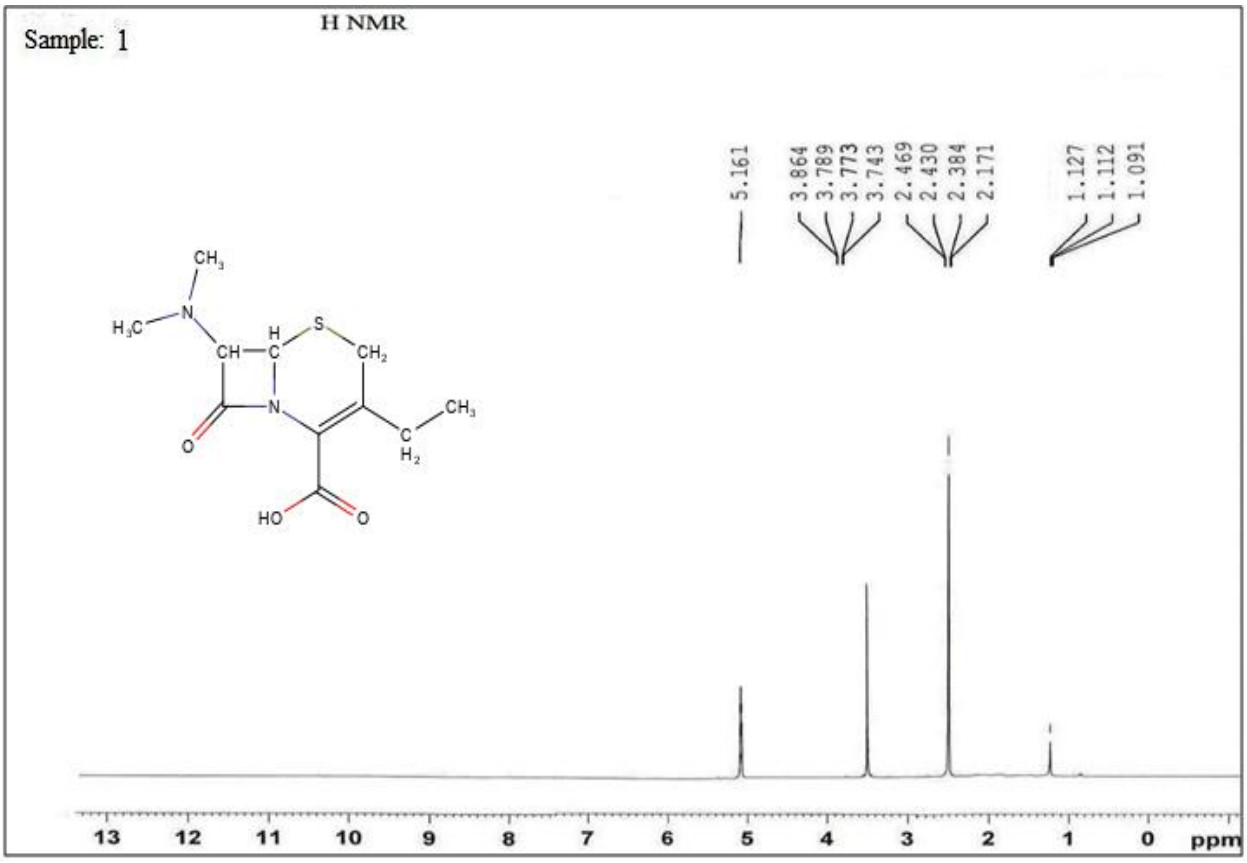

Fig 3: NMR Spectra of purified compound isolated from fraction from *Bruguiera cylindrica*

Conclusion:

Based on the studies conducted the leaf extracts can be categorized as bacteriostatic due to the capability of bacterial growth inhibition. Isolation and characterization of the antibacterial compounds from ethyl acetate extracts seemed may pave a way for developing antimicrobial agents which may lead to development of cost effective, less allergic phytomedicine against pathogenic microbes.

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