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Research Article

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# Identification of Bioactive Principles of *Avicennia officinalis* Fruit Extract in Methanol and Screening for Antibacterial Activity

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**ABSTRACT**

*Avicennia officinalis* (AO) belongs to family *Avicenniaceae*. It finds a prominent place in folk medicine. Hence the present work was carried out for the identification of bioactive principles in the fruits of *Avicennia officinalis* and screened for the antibacterial activity against selected test cultures. The methanolic extracts has high efficacy against the test cultures. The methanolic extracts were further purified by column chromatography. With the aid of $^{1}H$ NMR, $^{13}C$ NMR and GC-MS analysis. The structure of the compounds were detected as Phenols namely 1, 2, 3 Benzene triol and 4, 4'-(1-methylethyldiene)bis2-methyl and both the compounds exhibited antibacterial activity. This report of ours is the first one regarding the 1, 2, 3 Benzene triol and 4, 4'-(1-methylethyldiene) bis 2-methyl in the plant *Avicennia officinalis.*

**Keywords:** *Avicennia officinalis*, Fruit, Methanol, 1, 2, 3 Benzene triol, Phenol, 4, 4'-(1-methylethyldiene) bis 2-methyl, Antibacterial activity.

## INTRODUCTION

India is the birth place of indigenous medicine such as Ayurveda, Siddha and Unani. Most of the pharmacopeias are obtained from plant sources. Since ancient times plants have been used in traditional medicine to overcome many ailments. Modern medicine has rooted from folk medicine only after thorough phytochemical and pharmaceutical screening. [1] Nature possesses a rich resource of potential compounds that are structurally novel and biologically active principles. Secondary metabolites such as alkaloids, flavonoids, terpenoids, glycosides, tannins etc which are called as phytoalexins are produced by plants to serve as defense mechanism agents against microorganisms. [2] Mangroves are perennial plants that grow in coastal regions of tropical regions. Mangrove plant products have been used for centuries in natural remedies in the treatment of several health disorders. [3] India's traditional healers used *Avicennia officinalis* to treat smallpox infection in the past. [4] Antibacterial activity of *Avicennia officinalis* fruit has still not been studied extensively as compared to other plants parts of *Avicennia officinalis*. With this background, the present study was undertaken with an aim of evaluating the antibacterial activity of the fruits of *Avicennia officinalis* (AO) in different organic solvents.

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## MATERIALS AND METHODS

**Collection of Fruit Samples:** The fruits of *Avicennia officinalis* have a slight oval shape with a beak of about

2.5 cm long. They are either green or brown in colour. Fresh fruits were collected in the month of January from Corangi Reserved Forest, Kakinada, East Godavari, District, Andhra Pradesh, India. Geographic location- between 16° 39′ N longitude - 17° N longitude and 82° 14′ E latitude - 82° 23′E latitude. The fruits were transported to the laboratory in new polythene bags. All the fruits were surface sterilized with 1% mercuric chloride solution and thoroughly washed with filter sterilized distilled water. The washed fruits were then chopped to small pieces and shade dried until they become suitable for extraction in the selected solvents.

**Extraction**

Fruit extracts in ethyl acetate, acetone, ethanol and methanol were prepared according to the standard protocols. [5] The chopped fruit material (500 g) was initially soaked in 2000 ml of the respective solvent in round bottom flask at room temperature for 24 h. Subsequently, the soaked material was refluxed for 6 h below the boiling point of the respective solvent. Infusions were filtered through Whatman No.1 filter paper and the residual material was re-extracted with fresh solvent. After 24 h the process was repeated. Pooled extracts were individually concentrated by removing the solvent under reduced temperatures using vacuum rotator evaporator. These extracts were further concentrated by solvent evaporation using thin film method. Dried fruit extract of 100 mg each was dissolved in 10ml of 1:10 diluted DMSO in sterile distilled water so as to obtain the final concentration of 10 mg/ml. [6-7] All the extracts thus prepared were stored in a refrigerator at 4°C.

**Determination of antibacterial activity**

Antibacterial activity of the fruit extracts of *Avicennia officinalis* prepared in different solvents was determined using standard agar well diffusion method. [8] Bacterial suspensions of the test cultures were prepared by using 24 h old bacterial culture. The amount of bacteria needed to undertake the study was determined using UV/Vis spectrophotometer (ELICO) at 625 nm so that the absorbance of the suspension was held at 0.1 which was assumed to contain 1-2×$10^8$CFU/ml. About 20 ml of melted Mueller Hinton agar was mixed with 1 ml of bacterial suspension homogeneously and allowed to solidify in petri dishes (143 mm diameter). Wells (8 mm diameter) were made using a sterile cork borer on the solidified medium and are filled with 100µl of the original crude extract (10 mg/100µl) or purified compound (1 mg/100µl). The bacterial strains used in our study were *Enterobacter cloacae, Proteus vulgaris, Bacillus cereus* and *Enterococcus faecalis.* The diameters of the inhibition zones were measured and their means were calculated. DMSO in water was taken as control. The zones were compared with that of Gentamicin (0.1 mg/100µl).

**Determination of MIC**

Minimum Inhibitory Concentration (MIC) was determined by broth dilution assay method. [9] Fruit extracts were serially diluted in Mueller Hinton broth to get the concentrations of 1.25, 2.5, 5.0 and 10 mg/100µl. Each experiment was repeated thrice and the mean values were tabulated.

**Fractionation**

The fruit extract in methanol was separated by column chromatography with silica gel (100-200 mesh) and eluted with chloroform and methanol (9:1 to 1:9) followed by acetone and methanol (9:1 to 1:9). Altogether 24 fractions were collected and every fraction was screened for the antibacterial activity. [10] The 6th fraction (compound A) and 15th fraction (compound B) were subjected to further studies

**$^1$H NMR, $^{13}$C NMR**

The $^1$H NMR and $^{13}$C NMR techniques were carried at Laila Impex R & D centre Vijayawada, Andhra Pradesh. These techniques were used to identify the bioactive principles present in the extracts responsible for the antibacterial activity. Spectra were run on Bruker spectrometers operating at 400 MHz for $^1$H NMR and 100MHz for $^{13}$C NMR. The chemical shifts were given in ppm (δ) and coupling constant was expressed in Hertz.

**GC-MS analysis**

The GC-MS technique was carried at Lucid Laboratory, Hyderabad, Andhra Pradesh. GC-MS analysis of the samples was carried out using Shimadzu Make QP-2010 with non polar 60 M RTX 5MS Column. Helium was used as the carrier gas and the temperature programming was set with initial oven temperature at 40°C and the final temperature of the oven was 480°C. A 2µL sample was injected with split less mode. Mass spectra was recorded over 35-650 amu range with electron impact ionization energy 70 eV. The total running time for a sample is 45 min. The chemical components from the methanolic extracts of plant were identified by comparing the retention times of chromatographic peaks using Quadra pole detector with NIST Library.

**Statistics:** Results were expressed as mean ± SD and the data was analyzed using one-way analysis of variance (ANOVA) to discover the significant difference at the 5% ($P$<0.05) level.

**RESULTS**

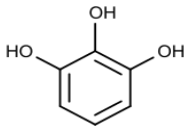
**Antibacterial Activity of crude extract (10mg/100µl):** *In vitro* antibacterial activity of all the extracts of AO fruit was determined by agar well diffusion method. This experimental data is presented in Figure 1. All the extracts were found to possess various degrees of antibacterial activity against both Gram positive and Gram negative bacteria. Among the tested extracts, methanol and ethanol soluble of AO fruits exhibited different degree of antibacterial activity (range between 10.66 mm to 19.33 mm). However, components of AO infused into ethyl acetate were found to be active only against gram positive cultures. The efficacy of acetone soluble is varied among the test cultures used. It inhibited both the Gram positive test cultures but only one of the gram negative culture i.e., *Enterobacter cloacae*. Further, these extracts were analyzed to

determine the minimum inhibitory concentration against the test cultures used. The MIC values ranged from 1.25-10 mg/100µl and varied from one extract to the other extract (Table 1).

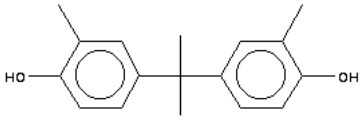
**Antibacterial activity of pure extracts (1mg/100µl)**

The results are given in Fig. 2. From this, it is very clear that both compound A and B are responsible for the antibacterial activity. The efficacy of inhibitory effect differs among them.

Compound A was obtained as white crystals and its molecular formula was determined to be $C_6$ $H_6$ $O_3$ and molecular weight as 126. The $^{13}C$ NMR spectrum led to the confirmation that this compound has 6 carbon atoms with the following chemical shifts. $C_1$ and $C_3$ (142 s), $C_2$ (129 s), $C_4$ and $C_6$ (109 d), $C_5$ (123 d). $^1H$-NMR proton A δ (4.5 ppm), proton B δ (6.07 ppm) and proton C δ (6.47 ppm). This compound is pyrogallol which is seen in many plants (e.g. *Embellica officinalis*) and holds biological activities like antioxidant and antibacterial activity.




Compound B was obtained as light yellow crystals and its molecular formula was determined to be $C_{17}$ $H_{20}$ $O_2$ and molecular weight as 256. The $^{13}C$ NMR spectrum led to the authentication that this compound has 17 carbon atoms with the following chemical shifts. $C_1$ and $C_1'$ (153 s), $C_2$ and $C_2'$ (124 s), $C_3$ and $C_3'$ (129 d), $C_4$ and $C_4'$ (136 s), C5and $C_5'$ (127 d), $C_6$ and $C_6'$ (115 d), $C_7$ (21 s), $C_8$ and $C_8'$ (11q), $C_9$ and $C_9'$ (21q). $^1H$-NMR proton A δ (4.5 ppm ), proton B δ (2.3 ppm) and proton C δ (6.57 ppm). proton D δ (6.62 ppm ), proton E δ (6.67 ppm) and proton F δ (1.25 ppm).




This compound is 4 4'-(1-methylethylidene)bis(2-methyl) found in many plants and has biological activities like antioxidant and antibacterial activity.

AO fruit extract in methanol was purified on column chromatography with chloroform-methanol and acetone-methanol as developing solvent system. By the GC-MS two bioactive compounds were identified. The compounds were recognized based on the mass spectrum, peak areas, molecular weight and molecular formula using the GC-MS database of National Institute Standard and Technology (NIST). The two compounds were identified as 1, 2, 3 benzenetriol also known as pyrogallol or 1, 2, 3 tri hydroxy benzene and 4 4'-(1-methylethylidene)bis(2-methyl) also be called as Bisphenol C.

**DISCUSSION**

The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes such as alkaloids, flavonoids, glycosides, terpenoids, tannins, polyphenols and saponins. [11] The solubility of these compounds differ, some of them are soluble in inorganic solvents but major proportions are soluble in organic solvents. Among them some compounds are soluble in less polar solvents and other in highly polar solvents. An attempt was made with ethyl acetate, acetone, methanol and ethanol as solvent for the fruit extracts of AO. The antibacterial activity of AO fruit extracts could be due to presence of flavonoids and phenols. [12] In India and other tropical countries, the infusion of AO is being used in the treatment of several ailments. So far all the plant parts of AO were extensively studied for antioxidant and antimicrobial activities except the fruit. [13-14] In the present study, we have established the antibacterial activity of the fruit extracts in ethyl acetate, acetone, methanol and ethanol solvents with *Enterobacter cloacae, Proteus vulgaris, Bacillus cereus* and *Enterococcus faecalis,* by agar well diffusion method. Of all the solvents used, methanol extract showed higher degree of inhibitory action against the test cultures, similar results were earlier reported by Sharief *et al*. [15] Hence, we concentrated our study on the methanol infusions to know the key bioactive principles responsible for the antibacterial activity. From the $^1H$ and $^{13}C$ NMR, as well as the GC-MS analysis, it was concluded that the active principles in the extracts are Pyrogallol and Bisphenol C. This report of identification of 1, 2, 3 Benzene triol (or) Pyrogallol and 4, 4'-(1-methylethyldiene)bis 2-methyl (or) Bisphenol C in *Avicennia officinalis* by us is of its first kind.

**Table 1: MIC of fruit extracts of AO in mg/100µl**

| Organism | Ethyl acetate | Acetone | Methanol | Ethanol |
|---|---|---|---|---|
| *Bacillus cereus* | 10 | 5 | 1.25 | 2.5 |
| *Enterococcus faecalis* | 10 | 5 | 1.25 | 2.5 |
| *Enterobacter cloacae* | - | 10 | 5 | 5 |
| *Proteus vulgaris* | - | - | 5 | 5 |

Pyrogallol is the compound formed in plants during the catabolism of tannic acid via gallic acid. [16] Tannin is metabolized into tannic acid which in turn gets converted to gallic acid and finally into pyrogallol. Tannins are reported in many plants including the mangrove especially the AO. [17] Both Pyrogallol and Bisphenol C have been reported early in *Eugena caryophyllus* plant. [18] Pyrogallol hold antioxidant activity [19] and compounds like polyphenol having pyrogallol group incur antibacterial activity. [20] Our reports of antibacterial activity are in concurrence with them. AO fruit infusion extracted in ethyl acetate is exclusively active only against Gram positive test cultures. This could be due to variation in the cell wall composition of Gram negative and Gram positive bacteria. The Gram negative bacteria restrict the influx of many antibiotics. [21] Multidrug efflux pumps at the transmembrane are also responsible for a higher intrinsic resistance in Gram negative bacteria. [22] The MIC data revealed that the gram negative bacteria

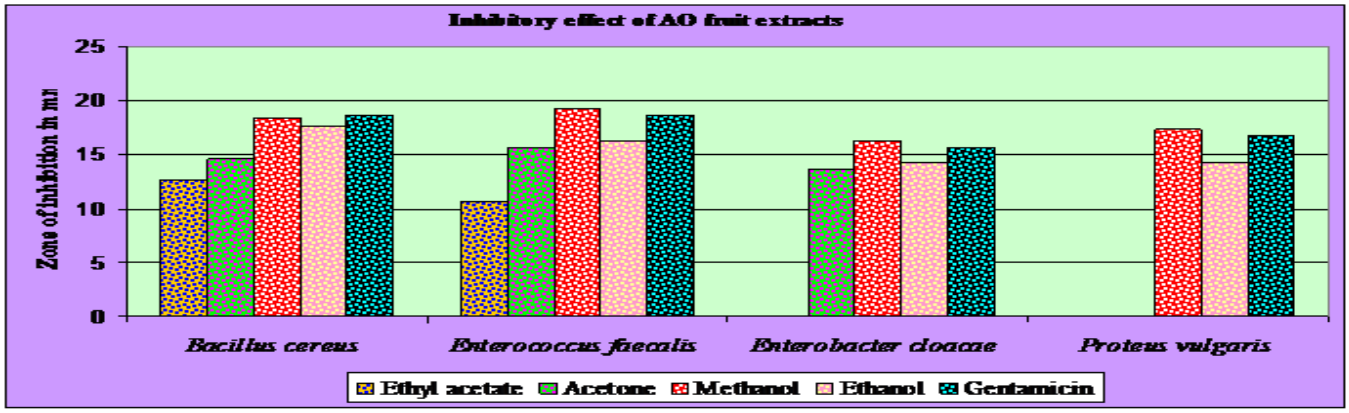



**Fig. 1: Antibacterial activity of AO fruits extracts with test cultures in comparison with gentamicin.**

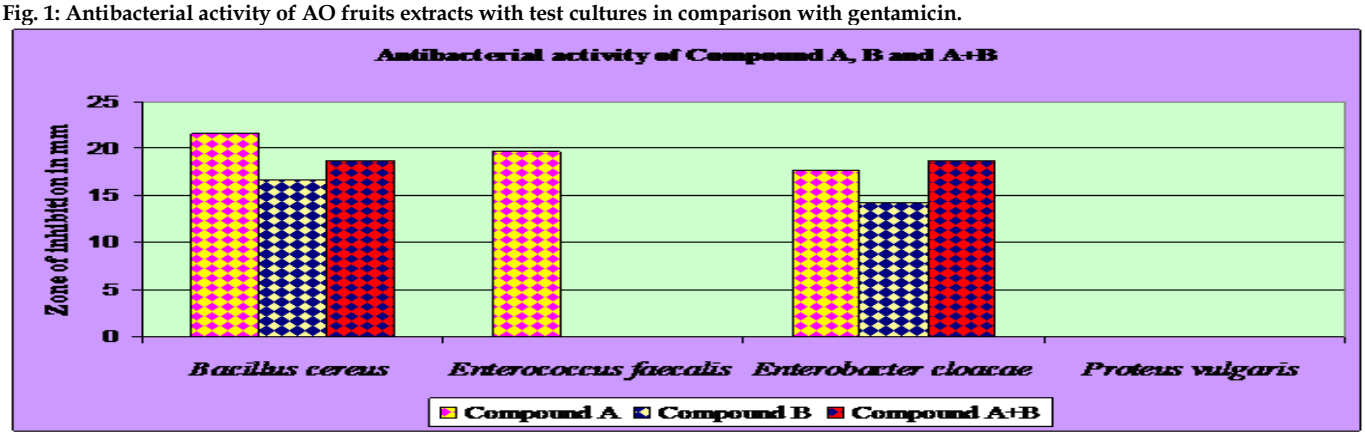



**Fig. 2: Antibacterial Activity of pure extracts against the test cultures**

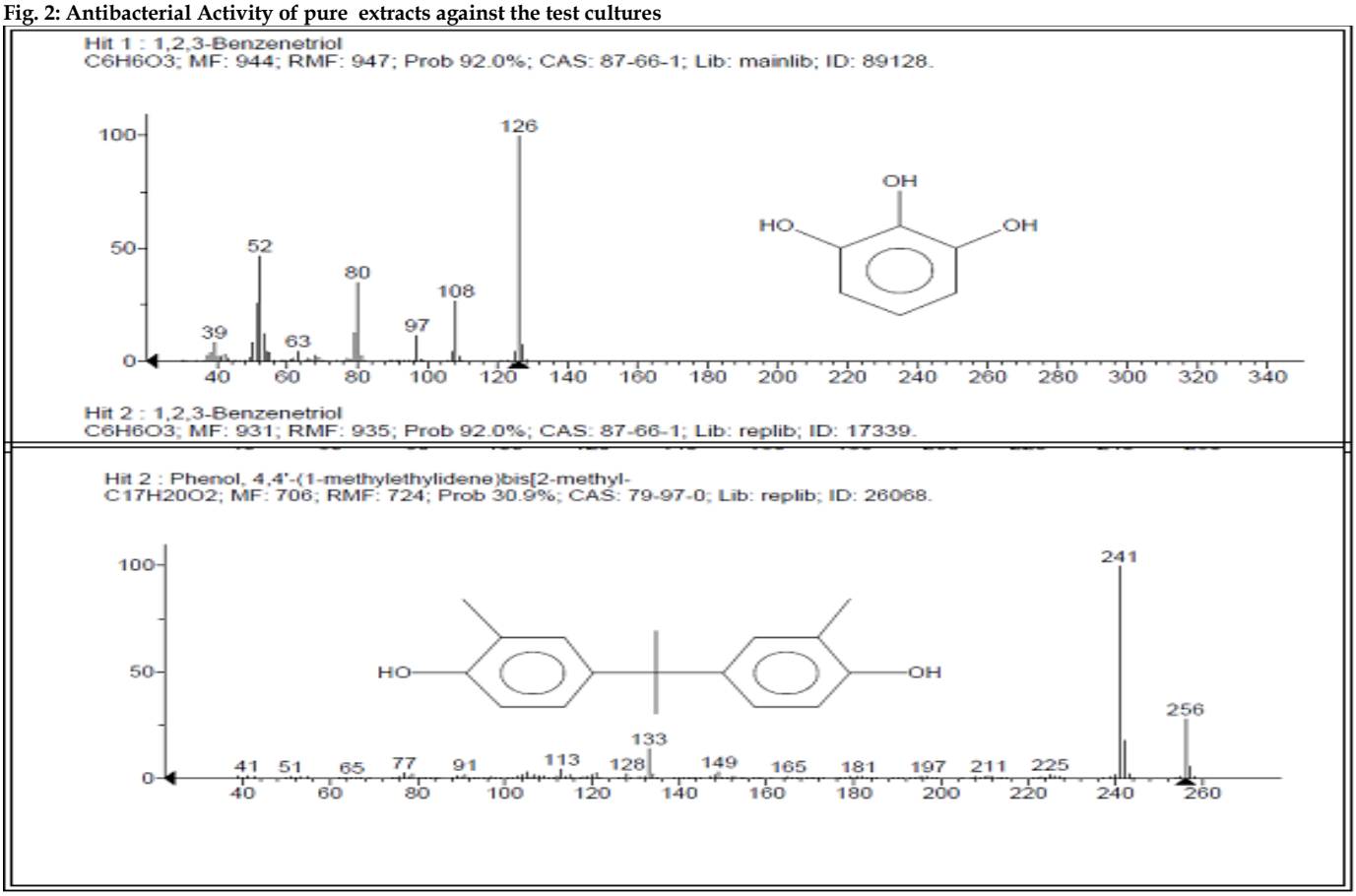



**Fig. 3: GC-MS Chromatogram of purified fraction of AO fruit extract in Methanol.**

had higher values than that of the gram positive bacteria for acetone, ethanol and methanol extracts. Our result is in agreement with the earlier studies. [23] However, the infusion in methanol and ethanol had same values for gram negative bacteria. This may be due to the active principle present in the extracts, as well as the permeability property of the metabolite across the cell wall.

Sangeetha and Vijayalakshmi reported that highest % of pyrogallol is seen in *Punica granatum* rind extract and they confirmed that the pyrogallol has various biological activities like antidermatitic, antioxidant, antisoriac from Dr. Duke's phytochemical and Ethanobotanical databse. [24] The antioxidant activity of AO fruit extracts in methanol has been reported by Sharief *et al.* [25] Bisphenol C also possesses antimicrobial activity as reported by Panagal Mani *et al.* [17] They screened on *Candida albicans* and suggested that it has antifungal activity.

The crude methanol extract was active against all the test cultures used. The purified compound A does not have inhibitory effect on *Proteus vulgaris*. Whereas, the zone of inhibition exhibited against the other test cultures were higher than the crude extract. However, the compound B was effective only with *Bacillus cereus* and *Enterobacter cloacae*. We even studied the effect of compound A and B against the same test cultures. Interestingly we observed synergistic effect of these compounds against *Enterobacter cloacae* and antagonistic effect against *Enterococcus faecalis*. So, we robustly recommend the AO fruit for consideration as a valuable source for the isolation and identification of bioactive principles with different solvents and also to screen for action against other pathogenic microorganisms. Finally there is a need to explore mangrove plants towards the development of novel medicines in order to combat the diseases caused by the microorganisms.

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