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Research Article

EXPLORING THE CHEMICAL COMPOSITION AND THERAPEUTIC POTENTIAL OF *AVICENNIA MARINA*: A MULTIMODAL ANALYTICAL AND BIOASSAY APPROACH

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ABSTRACT

Objectives: Mangrove ecosystems are rich sources of bioactive natural products with significant pharmacological potential. This study aimed to comprehensively evaluate the hydroalcoholic extract of *Avicennia marina* leaves collected from the Shirgaon mangroves, Ratnagiri District, India, through phytochemical, biological, and spectroscopic analyses.

Methods: Preliminary phytochemical screening was conducted to detect secondary metabolites. Bioactive constituents were isolated, characterized, and quantified for total phenolic and flavonoid content. Antioxidant activity was assessed in correlation with phytochemical levels. Biological evaluations included antimicrobial, antifungal, cytotoxicity, and anthelmintic assays.

Results: The extract contained alkaloids, flavonoids, phenolics, and saponins, with high phenolic and flavonoid content supporting strong antioxidant activity. Isolated fractions demonstrated broad-spectrum antimicrobial and antifungal activity, which showed inhibitory effects against bacteria and *Candida albicans*. Cytotoxicity assays indicated markable preliminary screening for general toxicity. In addition, the extract showed significant anthelmintic activity, inducing rapid paralysis and mortality in worms in a dose-dependent manner.

Conclusion: The integrated phytochemical and biological analyses confirm the multifaceted bioactivity of *A. marina*. Its strong antioxidant, antimicrobial, general toxicity assay, and antiparasitic properties highlight its potential as a promising natural source for novel therapeutic agents. These findings support further development of mangrove-based natural products in drug discovery.

Keywords: *Avicennia marina*, Mangroves, Phytochemical screening, Antibacterial, Antifungal.

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INTRODUCTION

Mangroves are unique intertidal ecosystems which are located at the interface of sea and land in subtropical and tropical regions. These are characterized by saline, waterlogged soils, and fluctuating tidal conditions, mangrove forests host a diverse array of plant and animal species that have developed specialized adaptations to survive in extreme environmental conditions. These adaptations include aerial roots for gas exchange, viviparous seeds, salt-excreting leaves, and unique biochemical pathways that enable them to produce specialized secondary metabolites. These metabolites not only contribute to the survival of mangrove species but also hold significant pharmaceutical, ecological, and biotechnological importance [1]. Globally, mangrove ecosystems are recognized for their high productivity and ecological services. They act as buffers against coastal erosion, serve as nursery and breeding grounds for various marine organisms, and also contribute to carbon sequestration and nutrient cycling. It is estimated that more than 80% of the world's marine catch depends either directly or indirectly on mangroves and other coastal wetlands for part of their life cycles [2]. However, beyond their ecological role, mangroves have also emerged as important reservoirs of novel natural products with therapeutic potential [3]. Numerous studies have documented the presence of bioactive secondary metabolites in mangrove species, including flavonoids, saponins, terpenoids, alkaloids, tannins, phenolic acids, and steroids. These compounds exhibit a wide range of biological activities such as antimicrobial, antioxidant, anti-inflammatory, anticancer, antiviral, and antidiabetic properties [4]. The harsh conditions of mangrove environments exert strong cancer conditions encouraging the biosynthesis of structurally diverse and potent compounds. As a result, mangrove plants are considered valuable candidates in the search for new pharmacologically active molecules, particularly in the era of rising antibiotic resistance and emerging viral threats [5]. One of the notable mangrove genera with immense pharmacological significance is *Avicennia*. Among its species, *Avicennia marina* (commonly known as grey mangrove) is widely distributed along the Indian coastline, including the west coast regions such as the Ratnagiri district of Maharashtra. This species exhibits remarkable ecological plasticity, enabling it to thrive in a range of salinity gradients and sediment types. Conventionally, *A. marina* is reported to be used in folk medicine in various conditions such as respiratory disorders, ulcers, rheumatism, and skin diseases [6]. Despite its wide distribution and ethnopharmacological importance, the phytochemical and pharmacological attributes of *A. marina* remain underexplored, particularly in the context of regional biodiversity such as the Shirgaon mangroves.

The phytochemical profile of *A. marina* is characterized by an array of secondary metabolites, which are synthesized as part of the plant's adaptive responses to its environment. Nitrogen-containing compounds such as alkaloids and amines play crucial roles in plant defense, while compounds containing phenols such as tannins and flavonoids contribute to antimicrobial and antioxidant activity. Terpenoids another major class of metabolites found in *A. marina* contribute to their cytotoxic and anti-inflammatory properties [7]. These compounds are not only responsible for plant's survival but also hold promise for applications in human health. Recent studies have marked the antimicrobial efficacy of mangrove-derived compounds against various pathogens, including multidrug-resistant strains of *Staphylococcus aureus*, *Proteus mirabilis*, and *Escherichia coli* [8]. Such findings underscore the therapeutic potential of mangrove plants in

addressing global health challenges, especially the growing threat of antibiotic resistance.

Moreover, *A. marina* has shown promise in antiviral research, particularly in the wake of the severe acute respiratory syndrome coronavirus 2 pandemic. While antiviral activity has been documented in crude extracts, systematic fractionation and identification of specific antiviral compounds have been largely lacking [9]. Given the global distribution and ecological resilience of *A. marina*, it represents a sustainable and scalable resource for natural product drug discovery. Employing advanced analytical techniques such as high-resolution liquid chromatography mass spectrometry quadrupole time-of-flight (HRLCMS-QTOF), nuclear magnetic resonance (NMR), and gas chromatography-mass spectrometry (MS) allows for comprehensive metabolite profiling [10,11].

Despite the rich ethnobotanical heritage and chemical diversity of *A. marina*, several research gaps persist. There is a lack of comprehensive phyto-geographical studies comparing the chemical profiles of *A. marina* populations across different coastal regions. Furthermore, the mapping of traditional medicinal knowledge to scientific validation remains inadequate, with limited efforts to connect ethnomedicinal uses to specific phytochemicals and their pharmacological actions. In addition, there is a need for more integrative studies that combine phytochemical analysis with bioassays to provide a holistic understanding of bioactivity [12,13].

The present study seeks to address some of these gaps by investigating the phytochemical and antimicrobial potential of leaves of mangrove *A. marina* collected from the Shirgaon mangroves of Ratnagiri District, Maharashtra biodiversity rich coastal region of India. This study involves a multi-faceted approach of phytochemical screening, isolation of key constituents from isopropyl alcohol and water (hydroalcoholic) extracts of leaves, characterization of isolated compounds using HRLCMS-QTOF, and *in vitro* evaluation of antibacterial and antifungal activities against clinically relevant pathogens as well as general toxicity and anthelmintic activity. By integrating marine ethnobotany and metabolomics, this research is aiming to uncover novel bioactive compounds from *A. marina* and contributes to the growing repository of marine and estuary derived therapeutic agents.

This study also aligns with the broader goals of sustainable bioprospecting, emphasizing the use of regionally abundant and ecologically important plant species to develop pharmaceuticals with minimal environmental impact. As antimicrobial resistance continues to pose a global public health crisis, the discovery of new, effective compounds from natural sources like mangroves becomes increasingly urgent. The findings of this study not only highlight the biomedical value of *A. marina* but also underscore the importance of conserving mangrove ecosystems as repositories of chemical biodiversity and future drug leads.

Therefore, this study aims to provide the first comprehensive phytochemical and pharmacological profile of an isopropanol-water extract of *A. marina* leaves from the under-explored Shirgaon mangroves of Ratnagiri District, Maharashtra, India.

METHODS

Study area and sample collection

Leaf samples of *A. marina* were collected from the Shirgaon mangrove ecosystem located along the Konkan coast of Maharashtra, India (latitude: 17.0393093° N, longitude: 73.2962153° E). The site is characterized by tidal influence, saline soil, and high biodiversity, making it an ideal location for the study of marine medicinal flora. Fresh, healthy leaves were harvested and washed with distilled water to remove debris and salt, and shade-dried before being powdered for further analysis. Plant authentication was confirmed by a taxonomist in the Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhajinagar. Herbarium was deposited and received authentication letter with accession number 00869.

Plant extract preparation

Using isopropyl alcohol and water as a solvent (7:3), secondary metabolites from *A. marina* leaves were extracted using a conventional extraction method [14-17]. 500 g of powdered leaves were used initially for pet ether extraction followed by hydroalcoholic extraction with 7:3 ratio using maceration method for 48–72 h. After a complete extraction process, the extract was concentrated under 40°C in a rotary vacuum evaporator of ROTAVAP SUPERFIT model. The resultant extract was further use for isolation of phytoconstituents using column chromatography. The glass column was packed with silica gel 100–120 using slurry method. To separate a phytoconstituents efficiently, gradient elution technique was used starting with a non-polar solvent hexane and gradually increasing the polarity of solvent to ethyl acetate and finally methanol. All fractions were analyzed using thin-layer chromatography (TLC) and tests for phytochemicals identification focusing flavonoids and phenolic compounds. Two fractions were pooled on basis of TLC profile for flavonoids and phenolic compounds and collected. The mobile phase chloroform: Ethyl acetate: Acetic acid (4:4:2, v/v/v) was used for TLC screening, and spots were visualized under ultraviolet (UV) (254/366 nm) and by spraying with vanillin-sulfuric acid reagent. The prepared extracts and isolated two fractions (named here as AMI and AMII) were kept at 4°C in sealed containers until they were used.

Phytochemical screening

The preliminary phytochemical screening of extract was conducted to detect the presence of bioactive compounds such as saponins, phenols, flavonoids, alkaloids, tannins, and terpenoids using standard qualitative methods. Quantitative estimation of total phenolic and flavonoid content was performed using Folin-Ciocalteu and aluminum chloride colorimetric assays, respectively [18].

Qualitative phytochemical screening

Preliminary qualitative phytochemical screening was performed using chemical tests for extract of leaves of *A. marina* for the presence of different chemical groups of compounds. Extract was screened for the presence of glycosides, tannins, anthraquinones, coumarin, saponins, alkaloids, flavonoids, triterpenoids, carbohydrates, reducing sugars, starch, steroids, gum, mucilage, protein, as well as amino acids.

Determination of total phenolic content (TPC)

For calculation of the leaf extract's TPC, "The Folin-Ciocalteu" method was used [14]. In 10 mL of hydroalcoholic solvent, 200 mg of the dry extract were reconstituted. Test tubes were filled with 0.5 mL of the extract in triplicate, 2.5 mL of 0.2 N Folin-Ciocalteu reagent, and 2 mL of Na_2CO_3 (7.5% weight by volume [w/v]). After complete conversion of yellow color to blue color, the test tubes were incubated at room temperature for 60 min. Absorbance was measured at 765 nm using a Shimadzu UV-Visible Spectrophotometer 1900i. Gallic acid equivalent (GAE) that is TPC was expressed as mg/L of material obtained from the gallic acid standard curve (5–500 µg/mL) using the calibration equation y = ×0.0005–0.0079 (r^2 = 0.999), where x is the absorbance and y is the gallic acid solution concentration in µg mL.

$$\text{TPC} = \frac{C \times V}{m}$$

Here, TPC = Total phenolic content, C = concentration of gallic acid obtained from calibration curve in µg mL, V = volume of extract in mL, m = weight of dry extract in g.

The above formula was used to quantify the total phenolics as GAE in milligrams of GAE g-11 dried extract after they were determined using spectrophotometry at 765 nm.

Determination of total flavonoid content (TFC)

The $AlCl_3$ colorimetric method was used to determine the leaf extracts' TFC [14]. In triplicate, 0.1 mL of the reconstituted extract in hydroalcoholic solvent, 0.2 mL 2.5% $AlCl_3$, 1 mL 2% NaOH,

and 0.3 mL 2.5% $NaNO_2$ were added to test tubes. After 30 min at ambient temperature, the solution progressively became yellow in the test tubes. Using Shimadzu UV-Visible Spectrophotometer 1900i absorbance was measured at 450 nm. TFC was determined using the catechin equivalent (CE) mg/mL of extract from the catechin standard curve (5–500 µg/mL) and the calibration equation y = ×0.0005–0.0017 (R^2 = 0.9986).

Total flavonoids content was measured using spectrophotometry at 415 nm scanning and is calculated as CE in milligrams of CE/g dry extract using the following formula:

$$TFC = = \frac{C \times V}{m}$$

Here, TFC = Total flavonoid content, m = weight of dry extract in g, V = volume of extract in mL, and C = concentration of catechin obtained from calibration curve in µg/mL.

(1,1-diphenyl-2-picrylhydrazyl) (DPPH) radical scavenging potential

A. marina leaf hydroalcoholic extract's antioxidant capability was assessed using the DPPH free-radical scavenging assay which checks for the capacity to scavenge free radicals like DPPH. 50, 100, and 150 µg/mL were the concentrations at which extracts dilutions were prepared. Experiment was performed in triplicate. The experiment involved adding 1 mL of extract solution to 2 mL of 0.1 mM DPPH produced in methanol. After 40 min for the reaction to complete, absorbance was measured at 517 nm using a UV spectrophotometer against an appropriate blank. The conventional reference used was catechin. As a control, 1 mL of methanol in 2 mL of 1,1-diphenyl-2-picrylhydrazyl solution was used. After calculating the percentage of scavenging, the inhibitory concentration 50% (IC_{50}) was established [19,20].

Fourier transform infrared (FTIR) spectroscopy of the crude extract

200 mg of FTIR-grade KBr (overnight-dried) and 2 mg of each fraction of *A. marina* leaf extract were combined and the mixture was uniformly mashed in a mortar and pestle. A mechanical pelletizer was used to turn the resulting mixture into a thin pellet with a diameter of around 13 mm while exerting 150 kg/cm of pressure. FTIR spectroscopy was performed using the pellets using a Perkin-Elmer FTIR spectrophotometer. Spectrophotometer was equipped with optical system with KBr beam splitter at the best resolution of 0.4/cm, at mid-infrared with a spectral range of 8,300–350 cm^{-1} [21].

NMR spectroscopy

Carbon-13 NMR (^{13}C NMR) and proton NMR (^{1}H NMR) spectroscopy were carried out using NMR instrument of Bruker BioSpin GmbH for two fractions of leaf extracts of *A. marina* AMI and AMII. The analyses were performed using a Bruker instrument with the following acquisition parameters: Spectrometer program PULPROG "zgpg," time domain of 32768, and solvent dimethyl sulfoxide. The probe head used was Z125331_0022. These spectra aided in the identification and structural elucidation of the phytoconstituents present in the respective fractions.

HRLC-MS-QTOF metabolic profiling

HRLCMS-QTOF was used to analyze two fractions of extract of *A. marina* leaves AMI and AMII that were obtained following column chromatography to capture the various bioactive ingredients. To help with structural elucidation, important metrics were recorded, including molecular weight, chemical structure, base peak intensity, and ion fragmentation patterns. The high-resolution liquid chromatography MS (HR-LCMS)-QTOF data were obtained using an Agilent Q-TOF mass spectrometer (model G6550A) fitted with a Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI ion) source in ionization mode. The HR-LCMS-QTOF method employed a Binary Pump, HiP Sampler, and diode array detector with MS/MS thresholds set at 0.010% for both metabolite positive and negative results. MS data were collected during a 30-min runtime, and the system was calibrated using the default autotune file. Identification was done using a number of databases.

Cytotoxicity assay

Brine shrimp (*Artemia salina*) nauplii were used for *in vitro* cytotoxicity assay using Meyer *et al*. methods [22]. The cytotoxicity test is a universal assay that may detect hazardous concentrations of crude extracts of medicinal herbs. Assay was conducted using the brine shrimp lethality bioassay. In a tank, brine shrimp were hatched at ambient temperature. 50, 100, and 150 µg/mL extract concentrations were made. In different glass vials, above stated test fluids were combined with ten live nauplii and ten milliliters of simulated saltwater. After a 24 h, vials were examined using magnifying glass and the number of survived nauplii in each vial was recorded. The percentage of nauplii lethality was calculated for each concentration and control as well. Finally, hydroalcoholic leaf extracts' Lethal Concentration 50% (LC_{50}) values were determined and noted.

Anthelmintic activity

Indian adult earthworms (Eisenia foetida), which resemble intestinal parasites in humans, were used to investigate the anthelmintic activity [22-24]. The earthworms were purchased from local market of Karad, India, and were kept under standard conditions of living. Only healthy, active earthworms of uniform size (6–8 cm in length and 0.3–0.4 cm in diameter) were selected. Weak, injured, or sluggish worms were discarded.

For the experiment, nine groups were created, each with five worms. 25, 50, 75, and 100 µg/mL concentrations of extract of *A. marina* leaves and common standard albendazole were prepared, roughly 0.9% (w/v) of regular saline solution was taken as control. The paralysis and death time of earthworms was observed and measured in minutes.

Antibacterial and antifungal activity

In high-performance liquid chromatography grade isopropyl alcohol (Sigma-Aldrich), stock solutions of the two separated fractions of the hydroalcoholic extract of *A. marina* AMI and AMII were made at a concentration of 1 mg/mL. The antibacterial and antifungal activity of several samples was assessed at doses ranging from 25 to 100 µg/mL [25]. Clinical and laboratory standards institutes' standard was used for performing modified well diffusion technique. The antimicrobial qualities of the two fractions AMI and AMII were evaluated against both Gram-positive and Gram-negative bacteria as well as fungal pathogens. *Bacillus cereus* National Collection of Industrial Microorganisms (NCIM 2703) and *Bacillus subtilis* (NCIM 5433) are the microorganisms used in the aforementioned investigations. The well diffusion method was used for *S. aureus* (NCIM 2654), *Proteus vulgaris* (NCIM 5266), *E. coli* (NCIM 2832), and *Pseudomonas aeruginosa* (NCIM 5031) [26-28]. For additional research, the corresponding test pathogen suspension was made in sterile saline. Pathogens were dispersed on plates using a sterile spreader after being inoculated on the surface of sterile Muller and Hinton agar for the antimicrobial activity test. Following the spreading of agar, 100 µL of various test solutions (25–100 µg/mL) were placed into wells made on the agar plates using a sterile cork borer with a 0.7 cm diameter. After 10 min of sample diffusion in culture media at 4°C, plates were moved to an incubator set at 37°C for 24 h.

In addition, the findings were recorded and compared with streptomycin as a positive control and the appropriate solvent as a negative control. The diameter of the inhibitory zone was measured in millimeters (mm), and the results were compared with streptomycin (Table 1).

Similarly, three test pathogens were utilized for antifungal activity, *A. niger* (NCIM 2683), *Candida albicans* (NCIM 3471), and Microsporumcanis (ATCC 36299). Sterile MGYP (Hi-media) plates were used to transmit these infections. After 10 min of sample diffusion in a culture medium at 4°C, the plates were moved to an incubator set at 27°C for 24 to 48 h.

Table 1: FTIR stretching frequencies observed in hydroalcoholic extracts fraction AM1

Peak No.	Wavenumber (cm^{-1})	Group/stretching type	Possible functional group/class
1	767.8	C–H bending (out of plane)	Aromatic or substituted alkene
2	842.4	=C–H bending (out of plane)	Aromatic ring (para/ortho-substituted)
3	954.2	=C–H bending or C–O stretch	Alkene/ether/phenol
4	1051.1	C–O stretching	Alcohols, esters, ethers
5	1099.6	C–O stretching	Alcohols, phenols, esters
6	1267.3	C–O or C–N stretching	Esters, ethers, amines
7	1416.4	CH_2/CH_3 bending, C–C stretching	Alkanes, aromatic ring
8	1468.6	CH_2 scissoring	Alkanes, methyl groups
9	1572.9	C=C stretching (aromatic ring)	Aromatic compounds
10	2228.9	C≡C or C≡N stretching (sharp, medium-strong)	Alkyne or nitrile group
11	2698.6	O–H stretch (acidic –COOH dimer), weak C–H	Carboxylic acid, aldehyde (Fermi resonance)
12	2791.8	Aldehyde C–H stretch (Fermi doublet)	Aldehyde
13	2967	C–H stretching (sp^3 CH_3/CH_2 groups)	Alkanes, possibly methyl or methylene groups

FTIR: Fourier transform infrared

Table 2: FTIR stretching frequencies observed in hydroalcoholic extracts fraction AM2

Peak No.	Wavenumber (cm^{-1})	Functional group/type of stretching	Possible functional class
1	767.8	C–H bending (out of plane)	Aromatic ring substitution (mono/para)
2	849.8	=C–H bending (aromatic ring)	Aromatic compounds
3	1051.1	C–O stretch	Alcohols, ethers, esters
4	1151.7	C–O–C or C–N stretch	Ether, ester, amine
5	1200.2	C–O stretch	Alcohol, phenol, ester
6	1256.1	C–O/Ar–O–C stretch	Phenols/aromatic ethers
7	1334.4	CH bending/C–N stretch	Aliphatic amines
8	1449.9	CH_2/CH_3 scissoring/aromatic ring	Alkanes, aromatics
9	1476	CH_2/CH_3 deformation	Alkanes
10	1513.3	C=C stretch (aromatic)	Aromatic ring
11	2348.2	C≡C/C≡N stretch (uncommon doublet region)	Alkyne/nitrile
12	2374.3	C≡C/C≡N continuation (weak, often CO_2 artifact)	Same as above (may also be background noise)
13	2825.3	Aldehyde C–H (Fermi doublet)	Aldehyde
14	2937.1	C–H stretch (sp^3 CH_2/CH_3)	Alkane
15	3060.1	=C–H stretch (aromatic or alkene)	Aromatics, alkenes
16	3108.6	=C–H (aromatic C–H stretch)	Aromatics

FTIR: Fourier transform infrared

The diameter of the inhibitory zone was measured in millimeters, and the results were recorded and compared with fluconazole as a positive control and the corresponding solvent as a negative control. In addition, the acquired results were compared with the antifungal agent fluconazole [25,26] (Table 2).

RESULTS AND DISCUSSION

Qualitative phytochemical screening

The hydroalcoholic extract of *A. marina* leaves was shown to include flavonoids, tannins, saponins, phenolic compounds, triterpenoids, glycosides, steroids, anthraquinones, reducing sugars, and carbohydrates.

Total phenolic and TFC

The TPC value for the *A. marina* leaf extract in this investigation was found to be 102 mg GAE/g dry extract, and the TFC value for the hydroalcoholic extract was 207.4 mg CE/g dry extract. In the present study, the *A. marina* extract displayed significant TPC and TFC, suggesting a rich profile of bioactive secondary metabolites. Strong antioxidant qualities are frequently associated with high TPC and TFC. Mangrove species may have elevated phenolics as a result of the particular environmental stressors that they face [29]. By scavenging reactive oxygen species (ROS), these phenolic compounds help to lessen oxidative damage in biological systems.

DPPH radical scavenging potential

Comparing with catechin, the hydroalcoholic extract of *A. marina* leaves showed higher antioxidant activity (Fig. 1). As the extract concentration rose, the % inhibition of the DPPH free radical rose monotonically. The hydroalcoholic extract's maximum DPPH

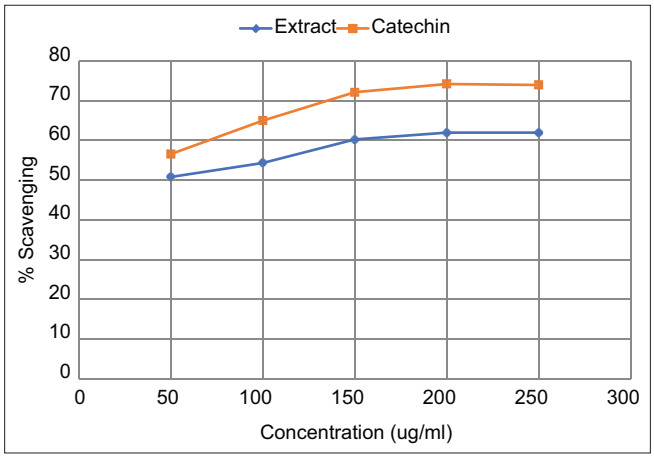

Fig. 1: (1,1-diphenyl-2-picrylhydrazyl) scavenging activity of leaf extract from *Avicennia marina*

scavenging percentage at 250 µg/mL was found as 61.96%. Compared to the hydroalcoholic extract (44.20 µg/mL), the catechin's IC_{50} value (49.22 µg/mL) was noticeably greater. The extracts' antioxidant activity was shown to be marginally higher than that of the standard catechin, as indicated by the DPPH assay's IC_{50} value. IC_{50} value is the quantity of an antioxidant agent needed to scavenge 50% of the DPPH free radicals. Such antioxidant effect is may be due to flavonoids and phenolics present in extract as they are having capacity to donate hydrogen [30].

FTIR spectroscopy of the crude extract

For two fractions of *A. marina* AMI and AMII, FTIR spectra revealed a broad range of stretching frequencies, suggesting the existence of many functional groups. Alkaloids, flavonoids, phenolic compounds, glycosides, terpenoids, tannins, saponins, fatty acids, and steroids, among other substances, have been found in *A. marina*, according to earlier investigations [4,21]. The comprehensive FTIR analysis of the *A. marina* fractions AMI and AMII, as well as the corresponding class of compounds, are shown in Tables 1 and 2; Figs. 2 and 3.

The FTIR spectra revealed broad bands at ~3,400 cm^{-1} (–OH groups of phenolics), 2,900 cm^{-1} (C–H stretches), 1,620 cm^{-1} (aromatic C=C), and 1,050 cm^{-1} (C–O–C stretching), indicative of flavonoids, phenolic acids, and terpenoids in the extract [31].

Table 3: Antibacterial activities of Fractions of extracts of leaves of *Avicennia marina* against bacterial test organism (Values are mean±SD of three parallel measurements, 00=No zone of inhibition)

Test pathogens	Zone of inhibition (mm), mean±SD (n=3)									
	AM I				AM II				Solvent	Streptomycin
	25	50	75	100	25	50	75	100		100
Bacillus cereus	0.00±0.00^{e}	19.00±1.00^{d}	21.00±1.00^{c}	25.00±1.00^{b}	0.00±0.00^{e}	22.00±1.00^{c}	23.00±1.00^{c}	26.00±0.00^{b}	0.00±0.00^{e}	29.00±1.00^{a}
Bacillus subtilis	0.00±0.00^{d}	22.00±1.00^{c}	25.00±1.00^{b}	26.00±1.00^{b}	0.00±0.00^{d}	20.00±1.00^{c}	21.00±0.00^{c}	24.00±1.00^{b}	0.00±0.00^{d}	30.00±0.00^{a}
Staphylococcus aureus	0.00±0.00^{d}	20.33±0.58^{c}	21.00±1.00^{c}	23.00±1.00^{b}	0.00±0.00^{d}	22.00±1.00^{c}	24.00±1.00^{b}	25.00±1.00^{b}	0.00±0.00^{d}	28.00±1.00^{a}
Proteus vulgaris	0.00±0.00^{d}	22.00±1.00^{b}	24.00±1.00^{a}	25.00±1.00^{a}	0.00±0.00^{d}	19.00±1.00^{c}	21.00±1.00^{b}	24.00±0.00^{a}	0.00±0.00^{d}	25.00±1.00^{a}
Escherichia coli	0.00±0.00^{d}	21.00±1.00^{b}	22.00±1.00^{a}	24.00±1.00^{a}	0.00±0.00^{d}	15.00±0.00^{c}	17.00±1.00^{c}	21.00±1.00^{b}	0.00±0.00^{d}	24.00±0.00^{a}
Pseudomonas aeruginosa	0.00±0.00^{d}	21.00±1.00^{b}	23.00±1.00^{b}	25.00±0.00^{a}	0.00±0.00^{d}	19.00±1.00^{c}	21.00±1.00^{b}	23.00±1.00^{b}	0.00±0.00^{d}	27.00±1.00^{a}

Values are mean±SD (n=3). Different superscript letters within a column indicate significant differences at P<0.05. SD: Standard deviation

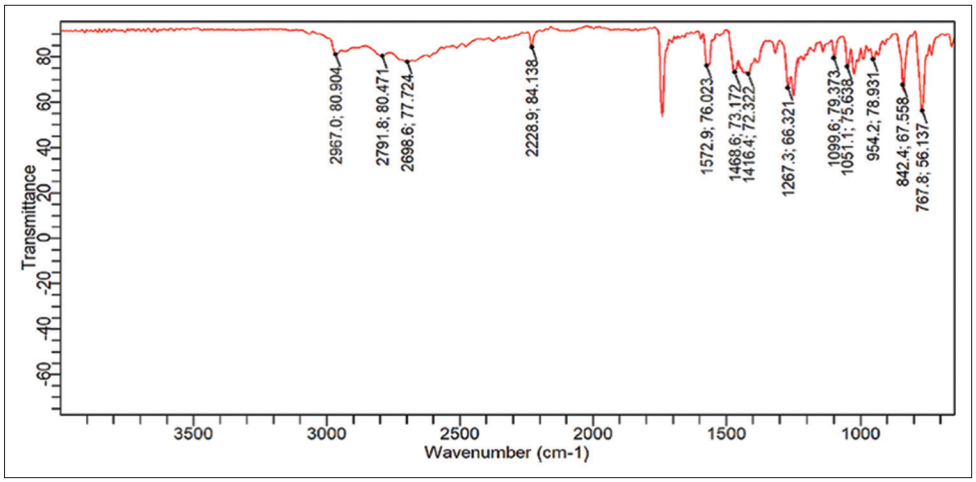

Fig. 2: Fourier-transform infrared spectrum of fraction 1 (AM1)

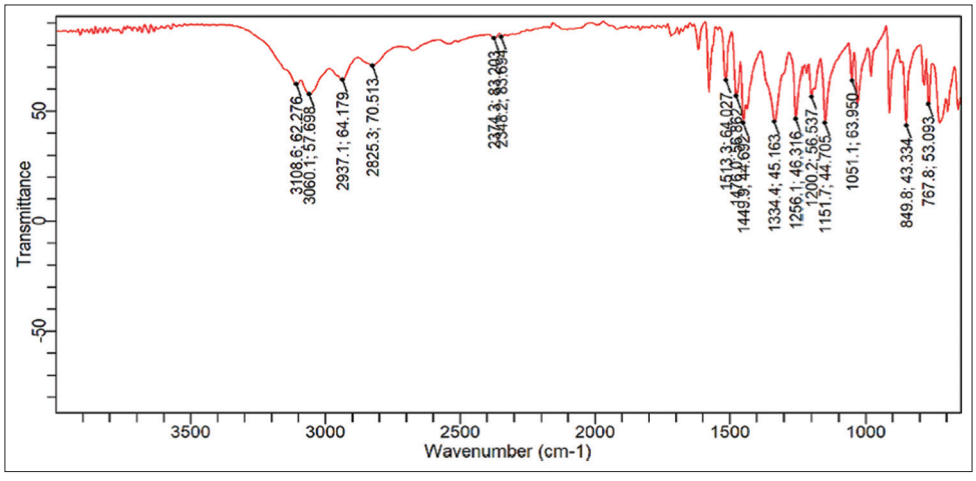

Fig. 3: Fourier-transform infrared spectrum of fraction 2 (AM2)

NMR spectroscopy

From chemical shift values of both C13 and H NMR analysis for fraction AMI, likely structural features are found to be aromatic ring possibly phenyl or substituted phenyl, aliphatic side chains with CH_2 groups, no peaks above 160 part per million (ppm), which means carbonyl carbons (C=O) such as ketones, esters, and acids are likely absent. Hence, possible compound classes are phenolic compounds, aromatic amines, aromatic ethers or glycosides, and flavonoid or polyphenol-type molecules. Figs. 4 and 5 provide the detailed H NMR and C13 NMR analysis of the fraction AMI of *A. marina.* The 1H NMR spectrum displayed characteristic aromatic proton signals between δ 6.0 and 7.8 ppm and aliphatic signals between δ 0.8 and 2.5 ppm, suggesting the presence of aromatic compounds and fatty acid residues. The 13C NMR spectrum further confirmed the presence of aromatic carbons (~110–160 ppm) and aliphatic carbons (~10–50 ppm), supporting the identification of flavones, flavonols, and long-chain aliphatic moieties.

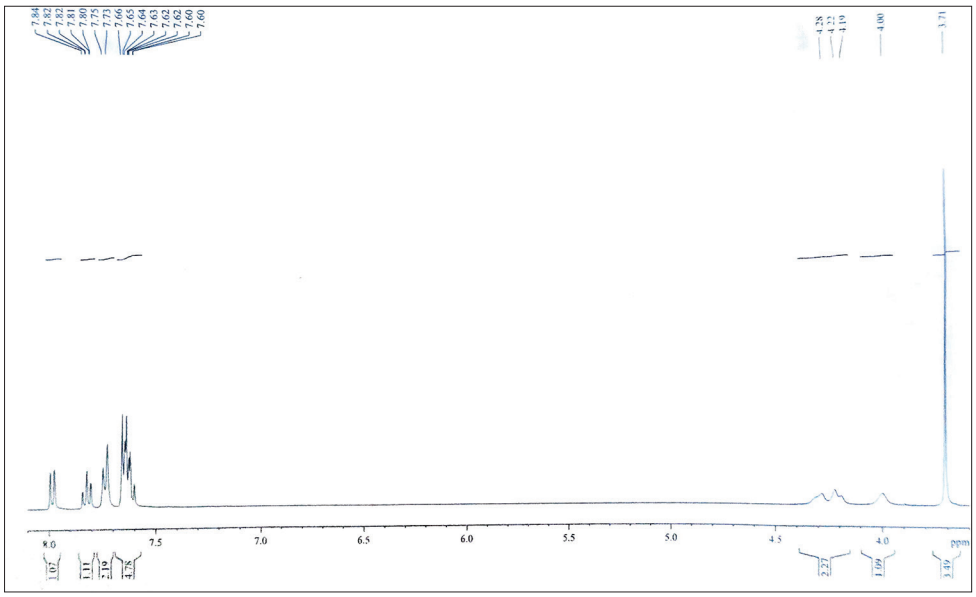

Fig. 4: H nuclear magnetic resonance of the fraction AM1 of *Avicennia marina*

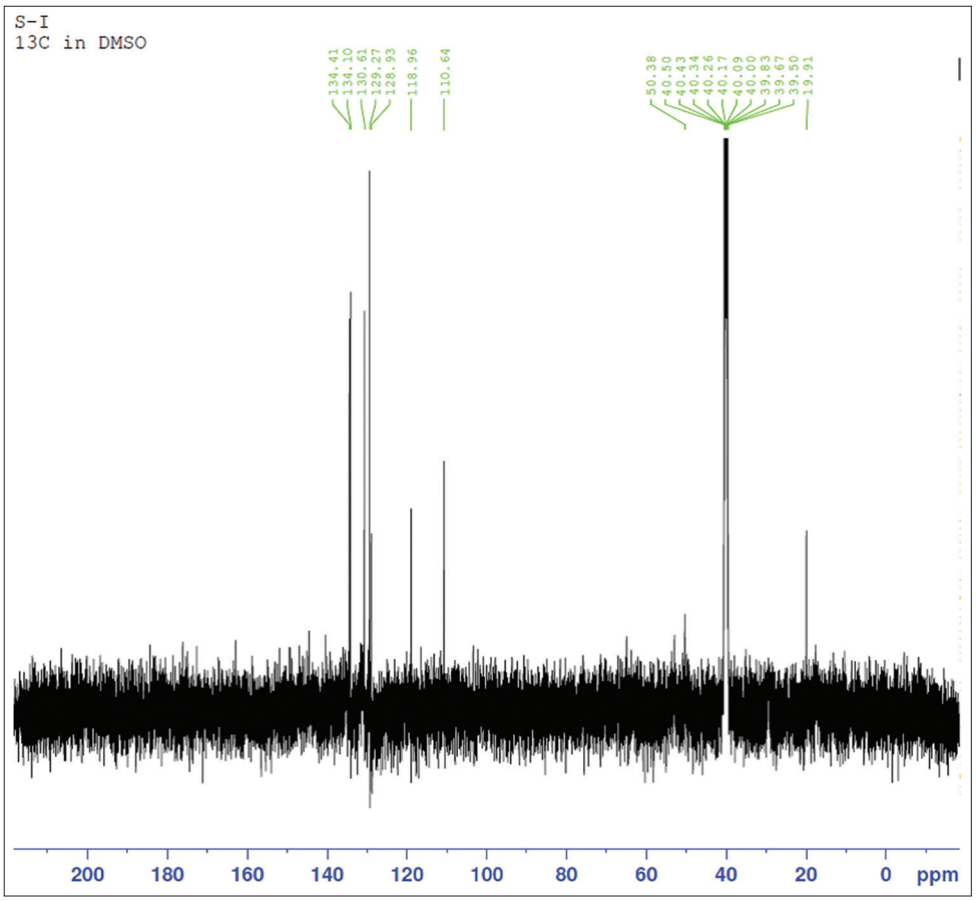

Fig. 5: C13 nuclear magnetic resonance of the fraction AM1 of *Avicennia marina*

The overall interpretation from H NMR and C13 NMR for second fraction AMII of *A. marina* (Figs. 6 and 7) indicates a phenolic acid or substituted cinnamic acid derivative, possibly flavonoid-type or aromatic aldehyde system, which is most likely. Given the presence of downfield aromatic protons and both deshielded and shielded carbons, the compound could resemble the vanillic acid, ferulic acid, a hydroxybenzoic acid derivative, and hence possibly a natural product plant-derived polyphenol, flavone, or coumarin analogue.

HRLC-MS-QTOF metabolic profiling

High-resolution liquid chromatography mass spectrometry coupled with QTOF detection (HRLC-MS-QTOF) was employed for comprehensive metabolic profiling of the extract. This advanced analytical technique enabled the high-sensitivity detection and precise mass measurement of a wide array of metabolites under both positive and negative ionization modes. The secondary metabolic profile of the two isolated fractions AMI and AMII from *A. marina* was identified and

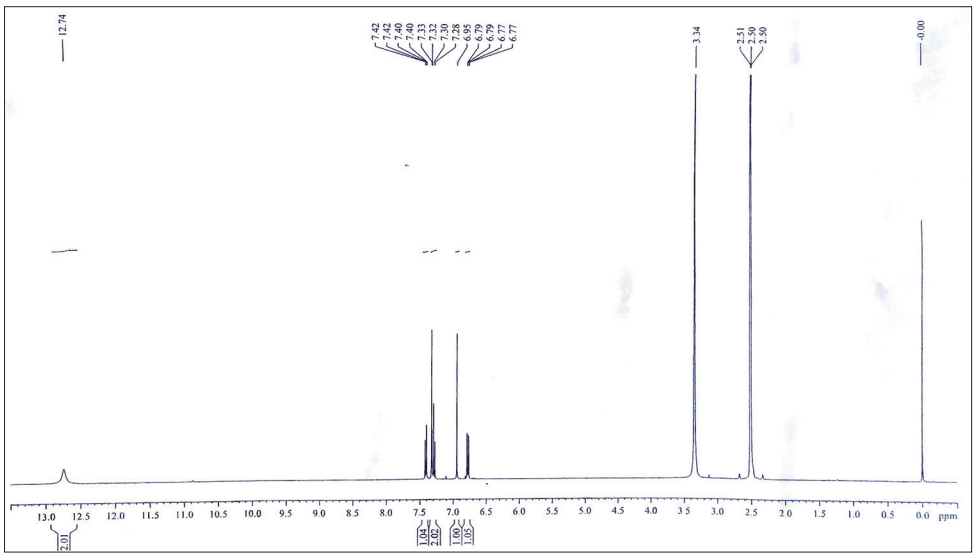

Fig. 6: H nuclear magnetic resonance of the fraction AMII of *Avicennia marina*

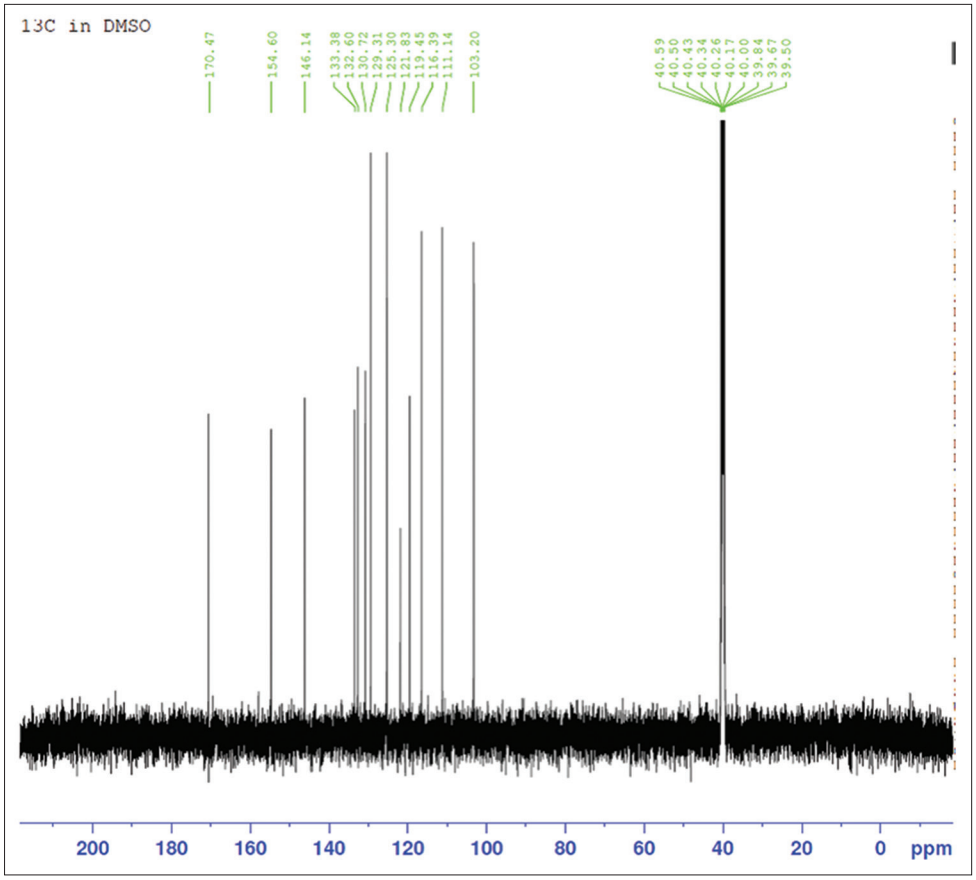

Fig. 7: C13 nuclear magnetic resonance of the fraction AMII of *Avicennia marina*

characterized by comparing the mass spectrum with Mass Bank library. A total of twelve compounds from the library could be identified and matched with the AMI fraction while total 25 compounds were analyzed from AMII fraction. Figs. 8 and 9 are the HRLC-MS-QTOF chromatogram for fraction AMI at positive and negative ionization modes, respectively.

For fraction AMI, a few compounds demonstrated significant mass spectral matching scores and potential pharmacological relevance. The most prominent compound matched and identified from library was Paromamine ($C_{12}H_{25}N_3O_7$) with a high spectral score of 91.12 and a molecular ion peak at *m/z* 323.1707. Paromamine is a known aminocyclitol derivative. Its presence suggests potential antibacterial activity.

Another major compound consistently detected and matched with available library across multiple peaks was Gardenal ($C_{20}H_{22}N_2O_2$), registering the highest score of 91.83 among its isomers. Gardenal is a known quinazoline alkaloid reported to exhibit antifungal, cytotoxic, and antineoplastic activities. Its repeated identification with high accuracy underlines its abundance and possible contribution to the extract's overall bioactivity profile.

In addition, diphenylamine ($C_{12}H_{11}N$), with a score of 80.53, was identified as a potential antioxidant molecule due to its aromatic amine structure. Diphenylamine derivatives have previously been reported for their neuroprotective and radical-scavenging properties.

Another significant compound was 4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside] ($C_{32}H_{32}O_{13}$), which yielded a score of 76.74. This polyphenolic glycoside is structurally rich in functional groups associated with anti-inflammatory, antioxidant, and antidiabetic effects, attributed to the presence of galloyl and coumaroyl moieties.

Flavonoid derivatives such as Catechin 3',5-diglucoside ($C_{27}H_{34}O_{16}$) were also identified, with a matching score of 61.33. This compound is

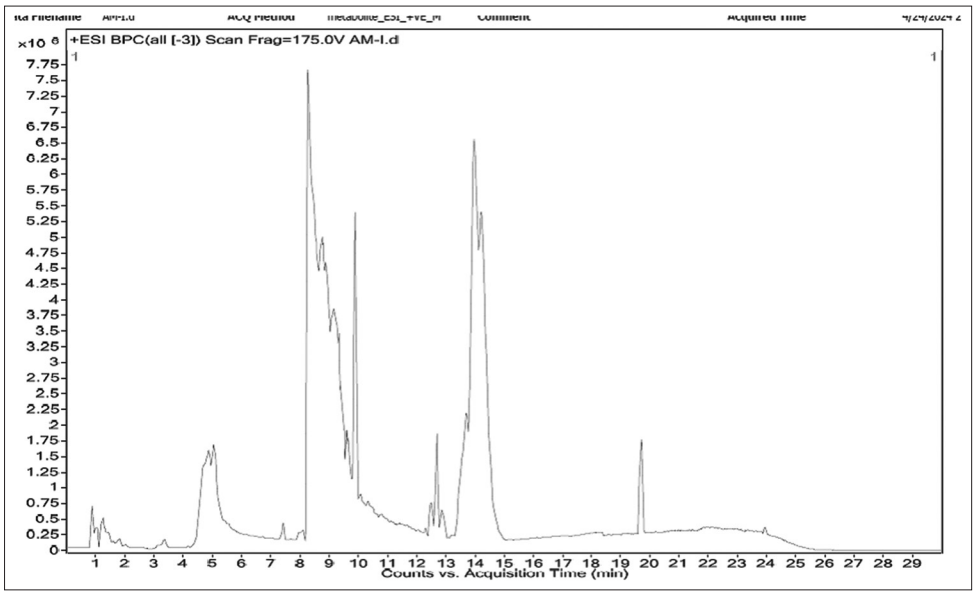

Fig. 8: High-resolution liquid chromatography mass spectrometry-quadrupole time-of-flight chromatogram for fraction AMI at positive ionization mode

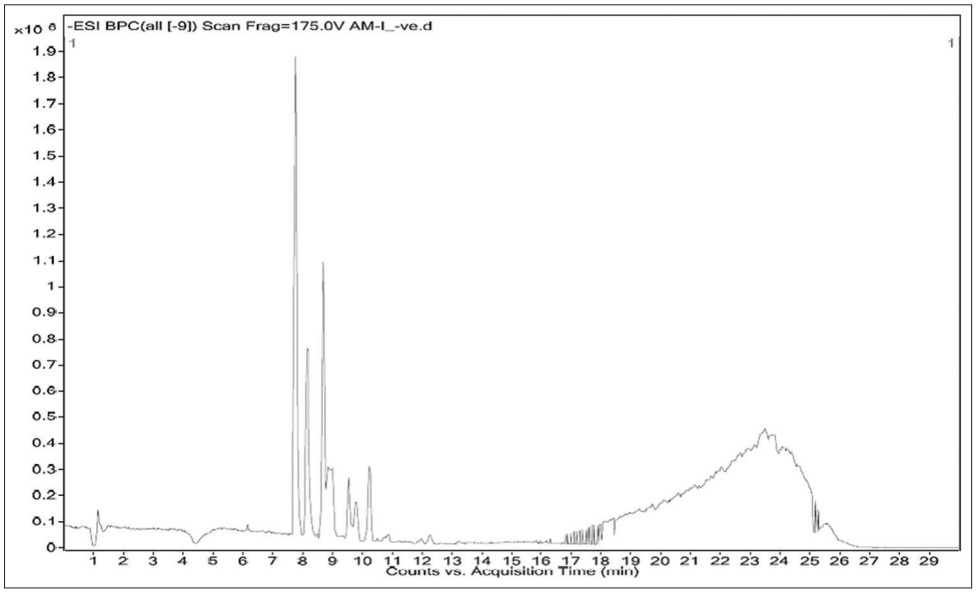

Fig. 9: High-resolution liquid chromatography mass spectrometry quadrupole time-of-flight chromatogram for fraction AMI at negative ionization mode

widely studied for its antioxidant, cardioprotective, and antimicrobial properties, particularly due to its ability to scavenge reactive oxygen species and modulate signaling pathways.

The detection of these key phytoconstituents suggests a diverse metabolic profile of the extract, rich in phenolics, alkaloids, and glycosides, which may synergistically contribute to its biological potential.

For sample, AMII HR-LCMS profiling of the test extract revealed a diverse array of compounds in both positive and negative ionization modes, highlighting the chemical complexity and bioactive potential of the sample. Figs. 10 and 11 are the HRLC-MS-QTOF chromatogram for fraction AMII at positive and negative ionization modes, respectively.

In the positive mode, several notable compounds were identified. Quinidine ($C_{20}H_{22}N_2O_2$) exhibited the highest spectral match score of 91.74 with a corresponding *m/z* of 323.174, confirming its molecular ion mass at 322.1666 Da. As a quinoline alkaloid, quinidine is reported to have antiplasmodial, antiarrhythmic, and anti-inflammatory effects, indicating strong therapeutic relevance. Paromamine ($C_{12}H_{25}N_3O_7$), with a mass of 323.1706 Da and a score of 78.2, is a well-known aminoglycoside precursor found as match with available library data. Its identification points to possible antibacterial activity. Diphenylamine ($C_{12}H_{11}N$), identified at 169.0903 Da, scored 80.53 and is recognized for its antioxidant and neuroprotective properties due to its aromatic amine scaffold. 5-Methyl-2-pentylthiazole ($C_9H_{15}NS$), though with a lower score of 56.17, was also detected at 169.0934 Da. Thiazole derivatives are pharmacologically active, with applications ranging from antibacterial to anticancer agents.

Several unknown compounds with characteristic base peaks in the range of 342–398 Da showed consistent ion signatures and may represent novel or less-studied phytoconstituents. Their prevalence in both modes suggests that they contribute significantly to the extract's bioactivity and warrant further structural elucidation.

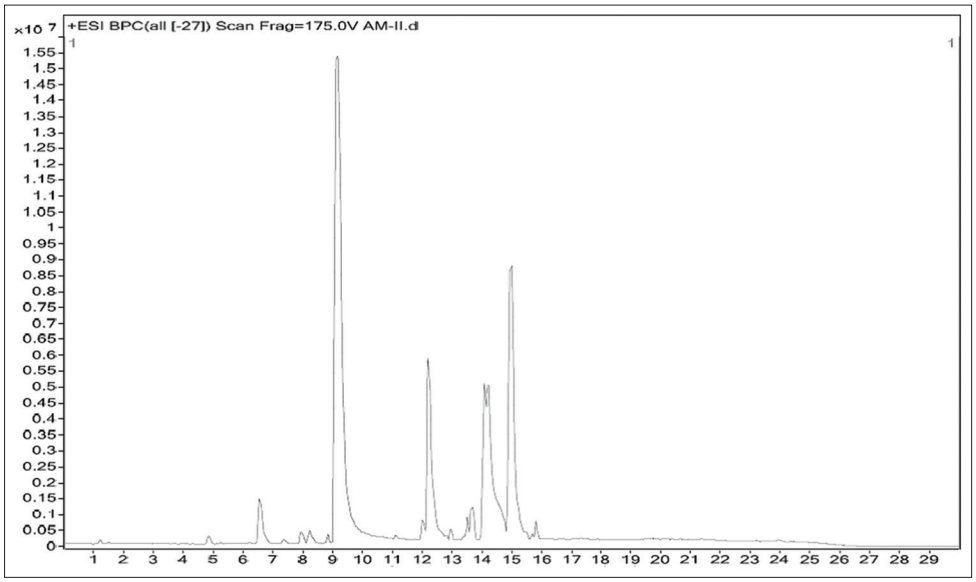

Fig. 10: The high-resolution liquid chromatography mass spectrometry quadrupole time-of-flight chromatogram for fraction AMII at positive ionization mode

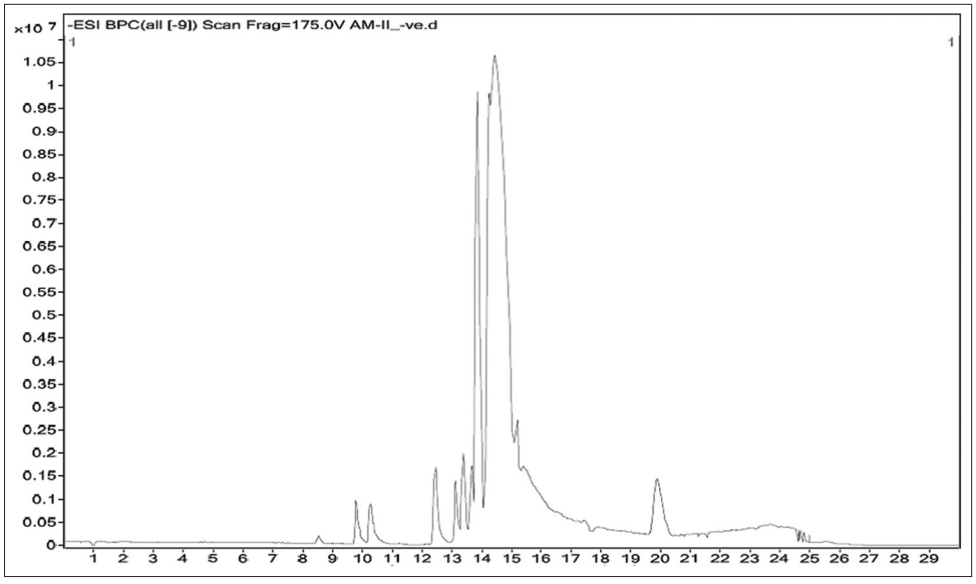

Fig. 11: The high-resolution liquid chromatography mass spectrometry-quadrupole time-of-flight chromatogram for fraction AMII at negative ionization mode

Cytotoxicity assay

The LC50 value of *A. marina* leaf extract was 45.38 µg/mL, which means at a concentration of 45.38 µg/mL, the leaf extract was able to kill 50% of the brine shrimp. At 3 concentrations (10 µg, 50 µg, and 100 µg/mL), the death rate was found to be 30%, 40%, and 70%, respectively (Fig. 4). These findings suggest that the extract may contain cytotoxic metabolites which further need to be explored.

Anthelmintic activity

Using varying concentrations of A. marina leaf extract 10, 50, and 100 µg/mL, the brine shrimp lethality bioassay was performed. Mortality was recorded after 24 h, and data were analyzed. LC50 value of A. marina leaves hydroalcoholic extract was found to be 45.38 µg/mL representing a highly significant anthelmintic effect by causing paralysis and death of the earthworm compared to the standard drug (Figs. 13 and 14), hence suggesting that extract contains potent bioactive compounds warranting further pharmacological evaluation. Therefore, A. marina extract was further studied for the identification of the responsible phytoconstituents. This study supports the traditional medicinal use of A. marina as a vermifuge, with tannins and flavonoids playing a key role in disrupting neuromuscular coordination.

Antibacterial activity

The antibacterial activity of AM-I and AM-II extracts was evaluated against *B. cereus*, *B. subtilis*, *S. aureus*, *P. vulgaris*, *E. coli*, and *P. aeruginosa* using agar well diffusion. The dataset was analyzed by one-way ANOVA followed by Tukey's HSD test (α=0.05). Solvent controls showed no inhibition and served as a negative reference, while streptomycin served as the positive standard. One-way ANOVA revealed a highly significant treatment effect (p<0.001). Streptomycin produced the largest inhibition zone and formed the highest statistical group (*a*). Higher concentrations of AM-I (100 µg/mL) and AM-II (100 µg/mL) showed moderate but significant activity (*b*), while AM-I and AM-II at 50–75 µg/mL showed lower activity (*c and d*). All 25 µg/ml treatments were inactive and grouped with the solvent (*e*). These findings demonstrate a clear dose-dependent increase in antibacterial activity for both extracts. ANOVA revealed a highly significant difference among treatments (p<0.001). Streptomycin remained the most active (*a*). AM-I and AM-II at 75–100 µg/mL formed a statistically similar second tier (*b*), followed by moderate activity at 50 µg/mL (*c*). All 25 µg/mL groups produced no inhibition (*d*). The results show that both extracts express meaningful antibacterial activity at higher doses, with AM-I generally showing slightly stronger potency than AM-II. A significant treatment effect was detected (p<0.001). Streptomycin again produced the highest inhibition (*a*), while AMI-100, AMII-100, and AMII-75 formed the next highest group (*b*). AMI-75, AMI-50, and AMII-50 formed intermediate groups (*c*), while zero-response treatments clustered as a separate inactive group (*d*). These results indicate substantial susceptibility of *S. aureus* to both extracts, particularly at concentrations ≥75 µg/mL. ANOVA indicated significant differences across treatments (p<0.001).

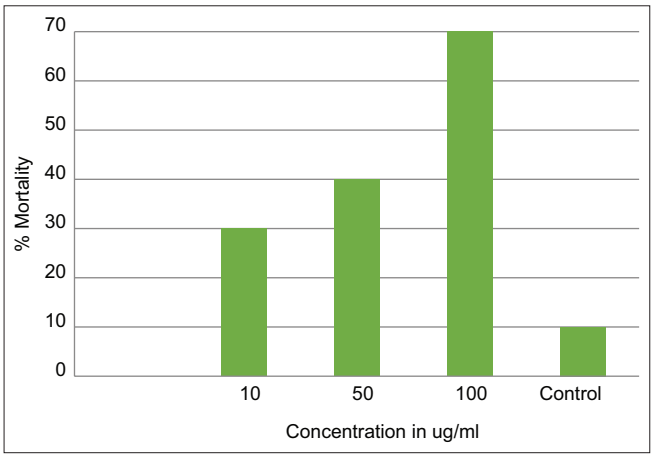

Fig. 12: Cytotoxic activity of *Avicennia marina*

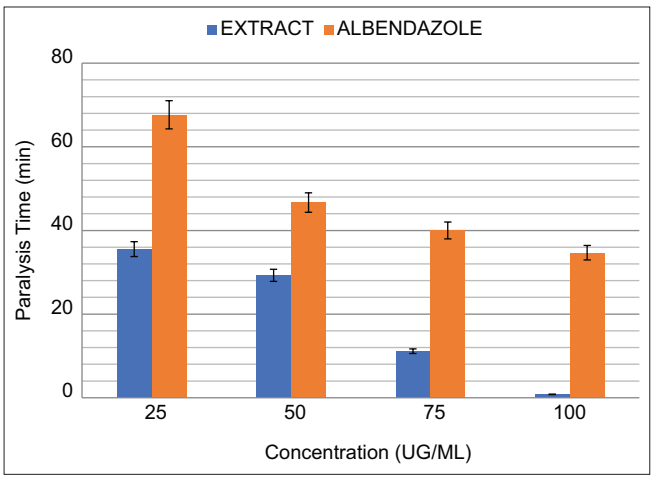

Fig. 13: Anthelmintic activity (Paralysis time) for *Avicennia marina* (Data shown as mean±standard deviation D, n=3)

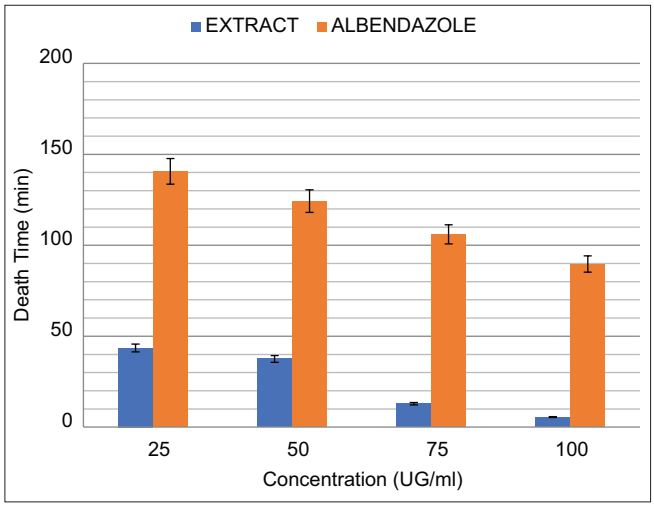

Fig. 14: Anthelmintic activity (Death time) for *Avicennia marina* (Data shown as mean±standard deviation, n=3)

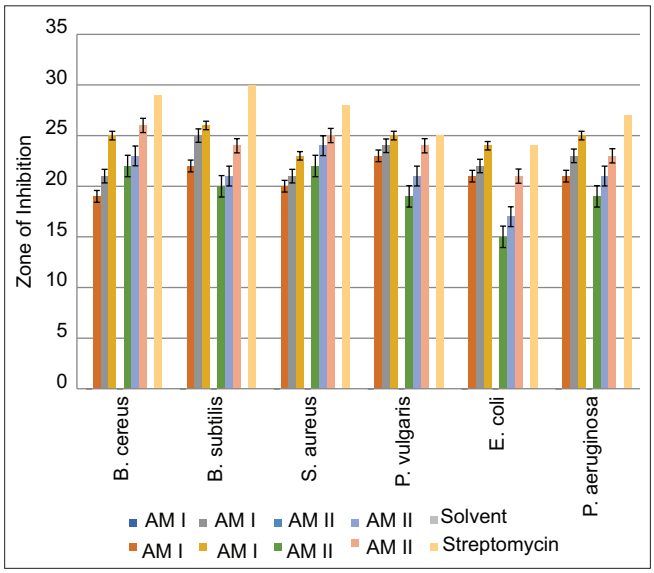

Fig. 15: Antibacterial activity for fractions AMI and AMII (Data shown as mean±standard deviation, n=3)

AM-I at 75 and 100 µg/mL, AM-II at 100 µg/mL, and streptomycin showed comparable high activity (*a*). AM-I 50 µg/mL and AM-II 75 µg/mL formed an intermediate group (*b*), while AM-II 50 µg/mL showed lower inhibition (*c*). All low-dose (25 µg/mL) treatments and the solvent remained inactive (*d*). These findings suggest that *P. vulgaris* is moderately sensitive, with efficacy improving sharply at higher extract concentrations. ANOVA revealed significant treatment differences ($p<0.001$). Streptomycin, AM-I 100 µg/mL, and AM-II 100 µg/mL displayed the strongest inhibition (*a*). AM-I 75 µg/mL and AM-II 100 µg/mL formed the next tier (*b*), while AM-II 50 µg/mL was markedly less active (*c*). All 25 µg/mL treatments were inactive (*d*). These results highlight a strong dose response, with AM-I demonstrating slightly higher potency than AM-II.

A significant treatment effect was observed ($p<0.001$). Streptomycin and AMI-100 produced the most potent activity (*a*). AMI-75, AMII-75, and AMII-100 formed a statistically similar intermediate group (*b*), while AMI-50 and AMII-50 demonstrated lower inhibition (*c*). All 25 µg/mL treatments were inactive (*d*). Considering the notorious resistance profile of *P. aeruginosa*, the activity of AM-I and AM-II at higher doses is noteworthy.

Collectively, across all six bacterial species, both AM-I and AM-II showed significant and dose-dependent antibacterial activity. High concentrations (75–100 µg/mL) consistently formed high-activity statistical groups, often approaching the efficacy of the positive control streptomycin. Low concentrations (25 µg/mL) were uniformly inactive. AM-I generally exhibited slightly higher activity than AM-II but both extracts demonstrated meaningful, broad-spectrum antibacterial potential.

These results collectively suggest that the extracts – especially at higher concentrations – possess promising antibacterial properties, with the most pronounced effects observed against Gram-positive bacteria (*Bacillus* spp. and *Staphylococcus aureus*), and moderate activity against Gram-negative strains.

Antifungal activity

The antifungal activity of AM-I, AM-II, and the standard drug fluconazole was evaluated against *Aspergillus niger*, *C. albicans* and *Microsporum canis* using the agar well diffusion method, and the results were subjected to one-way ANOVA followed by Tukey's HSD test ($\alpha=0.05$) after exclusion of treatments that produced no measurable inhibition (0 mm). As shown in Fig. 16 and Table 4, all concentrations of AM-I and AM-II, as well as the solvent control, failed to produce any inhibition zone (0 mm) against *A. niger*. Therefore, these groups were excluded from statistical comparison. Fluconazole remained the only active treatment, producing a zone of inhibition of 27.0±1.0 mm. As only one non-zero group was present, ANOVA and *post hoc* analyses were not applicable. These results indicate that *A. niger* was highly resistant to both AM-I and AM-II extracts, while fluconazole retained full antifungal potency. In contrast, *C. albicans* exhibited measurable sensitivity to several concentrations of both extracts. After excluding the zero-response treatments, one-way ANOVA revealed a significant difference among treatments ($F_{6,14}=24.71$, $p<0.001$). Tukey HSD grouping demonstrated that AMI-100, AMII-100, and fluconazole formed a statistically equivalent high-activity cluster (letter *a*), indicating comparable antifungal effectiveness among these treatments. Intermediate inhibition was observed with AMI-50, AMI-75, and AMII-75 (letter *b*), while AMII-50 produced significantly lower activity (letter *c*). Overall, the data suggest that the antifungal effect of higher concentrations of AM-I and AM-II approaches that of fluconazole against *C. albicans*, indicating strong dose-dependent activity. Only three treatments – AMI-100, AMII-100, and fluconazole – produced inhibition zones against *M. canis*. ANOVA revealed no significant difference among these treatments ($F_{2,6}=1.00$, $p=0.422$). Consistent with this, Tukey HSD assigned all three treatments to the same statistical group (*a*), indicating that the extracts at their highest test concentrations exhibited antifungal efficacy equivalent to fluconazole. All lower concentrations failed to inhibit fungal growth

Table 4: Antifungal activities of fractions of extracts of leaves of *Avicennia marina* against fungal test organism (Values are mean±SD of three parallel measurements 00=No zone of inhibition)

Test pathogens	Zone of inhibition in mm								Solvent	Fluconazole	p-value
	AM I				AM II						
	25	50	75	100	25	50	75	100			
Aspergillus niger	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	27.00±1.00	Zero-response treatments were excluded from statistical comparison
Candida albicans	0.00±0.00	23.00±1.00^{b}	25.00±1.00^{b}	28.00±1.00^{a}	0.00±0.00	21.00±1.00^{c}	24.00±1.00^{b}	27.00±1.00^{a}	0.00±0.00	29.00±1.00^{a}	Significant differences among the non-zero treatments ($p<0.001$).
Microsporum canis	0.00±0.00	0.00±0.00	0.00±0.00	25.00±1.00^{a}	0.00±0.00	0.00±0.00	0.00±0.00	26.00±1.00^{a}	0.00±0.00	26.00±1.00^{a}	Not significant ($p=0.422$)

(25=25 µg/mL, 50=50 µg/mL, 75=75 µg/mL, 100=100 µg/mL concentration of respective compound. Streptomycin=100 µg/mL)

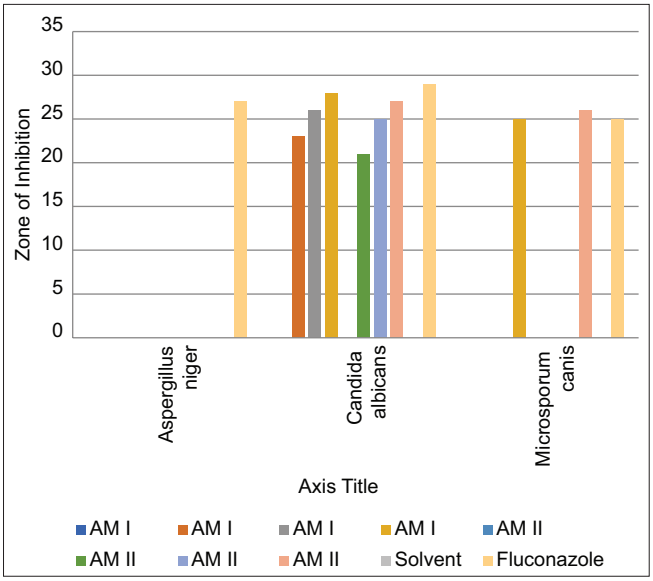

Fig. 16: Antifungal activity for fractions AMI and AMII (Data shown as mean±standard deviation, n=3) (25=25 μg/mL, 50=50 μg/mL, 75=75 μg/mL, 100=100 μg/mL concentration of respective compound. Fluconazole=100 μg/mL)

and were, therefore, excluded from analysis. These findings suggest that *M. canis* is moderately susceptible to higher doses of both extracts.

Values are mean±SD (mm), n=3. Zero-response treatments (0 mm) were excluded from the statistical analysis. One-way ANOVA was performed on the remaining non-zero groups per pathogen; *post hoc* pairwise comparisons were conducted using Tukey's HSD (α=0.05). Different letters indicate statistically significant differences (Tukey HSD).

Collectively, the fungal strains demonstrated varied susceptibility to AM-I and AM-II. *A. niger* was completely resistant to all extract concentrations, whereas *C. albicans* and *M. canis* showed dose-dependent inhibition. The highest concentrations of AM-I and AM-II yielded antifungal activity comparable to the standard fluconazole in *C. albicans* and *M. canis*. This outcome highlights the potential of the extracts – particularly at elevated concentrations – as promising antifungal agents against susceptible fungal pathogens.

CONCLUSION

Avicenniaceae family has become the interest of researcher nowadays due to its abundance in mangrove world even in Indian mangrove system. The study validated the biochemical wealth of mangrove *A. marina* from the Ratnagiri Coast, presenting a comprehensive evaluation of *A. marina*, integrating phytochemical quantification, *in vitro* biological assays, and advanced spectroscopic and spectrometric analyses. Numerous studies have been conducted on *A. marina* species from mangroves around the world, and numerous publications indicates that the extracts mostly consist of tannins, flavonoids, fatty acids, glycosides, sterols, alkaloids, terpenoids, esters, carotenoids, etc. However, there are not many reports on the mangroves of Ratnagiri as well as on isopropyl alcohol and water (hydroalcoholic) extract of *A. marina* leaves. Secondary metabolites with a high polyphenolic content are confirmed by the findings of the flavonoid and total phenolic assays.

The isopropyl hydroalcoholic extract of *A. marina* leaves collected from Shirgaon mangroves in Ratnagiri districts is also rich in natural antioxidants and can be used as a radical scavenger to stop damage from ROS, according to the antioxidant test. The phytochemical profile of *A. marina* leaf extract was shown to be highly diverse when viewed from the standpoint of the class of chemicals that were detected. Several analytical techniques, including as FTIR spectroscopy, H and C13 NMR spectroscopy, and HRLCMS QTOF analysis, are used to confirm the existence of diverse substances.

A comprehensive molecular fingerprint of the two fractions AMI and AMII, isolated from hydroalcoholic extract of *A. marina* leaves extract were obtained by the use of FTIR, NMR, and HR-LCMS QTOF studies. This fingerprint revealed a wide variety of bioactive components, such as phenolic acids, terpenoids, and flavonoids. The untapped potential of *A. marina* as a rich source of novel natural compounds with medicinal value is highlighted by this integrative study. Our results demonstrate the species' potential with high phenolic content, strong antioxidant activity, and broad-spectrum antimicrobial and anthelmintic effects. Furthermore, the discovered phytochemicals will act as a model for novel chemical compounds derived from nature that can be used to create environmentally friendly and sustainable chemical processes. Therefore, more research is needed, focusing on the isolation and more targeted testing of the specific compounds such as Gardenal, 4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside], and catechin identified in this study.

ETHICAL STATEMENTS (AND/OR INFORMED CONSENT IN CASE OF HUMAN STUDY)

Not applicable.

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AUTHORS' CONTRIBUTIONS

Shital S Chavan: designed and performed study, collected and analyzed data, and wrote paper. Prashant B Shamkuwar: Analyzed data and overall supervision. Sachin S Bhusari: Analyzed data and overall supervision.

CONFLICTS OF INTEREST

No any conflicts of interest.

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