SPECTROSCOPIC CHARACTERIZATION OF PHYTOCONSTITUENTS ISOLATED FROM A RARE MANGROVE \textit{AEGIALITIS ROTUNDIFOLIA} ROXB., LEAVES AND EVALUATION OF ANTIMICROBIAL ACTIVITY OF THE CRUDE EXTRACT

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\textbf{ABSTRACT}

\textbf{Objective:} The aim of the study is to isolate and characterize the phytochemicals from the leaves of a rare and unexplored mangrove \textit{Aegialitis rotundifolia} and evaluate the antimicrobial activity of the crude extract.

\textbf{Methods:} The dried powdered plant material was extracted with ethanol and the ethanol extract obtained was dissolved in distilled water and partitioned using n-hexane first and then ethyl acetate. The ethyl acetate fraction was subjected to column chromatography for isolation of phytoconstituents. The isolated compounds were characterized using infrared (IR), carbon-13 nuclear magnetic resonance (\textsuperscript{13}C-NMR), proton nuclear magnetic resonance (\textsuperscript{1}H NMR), mass spectrometry, and thin-layer chromatography (TLC). Antimicrobial activity of the crude extracts was performed using the well diffusion method against four bacterial strains and two fungal strains.

\textbf{Results:} Three pure compounds were isolated from the leaves of \textit{Aegialitis rotundifolia}, namely, 3,4-dimethyl benzoic acid, 3'-methoxy-4'-hydroxy-flavan-3-ol, and 3,7-dimethoxy-dimethyl-4',3,5-trihydroxy flavone which were confirmed by spectroscopic studies. Strong antibacterial activity was shown by the test extract against \textit{Staphylococcus aureus} and \textit{Pseudomonas aeruginosa}, whereas \textit{Escherichia coli} and \textit{Bacillus cereus} showed average and nil activity, respectively. The antifungal activity of the test extract was found to be strong for both the fungal strains, namely, \textit{Candida albicans} and \textit{Aspergillus niger}.

\textbf{Conclusion:} The results of the present study show that the isolated compounds were confirmed to be 3,4-dimethyl benzoic acid, 3'-methoxy-4'-hydroxy-flavan-3-ol, and 3,7-dimethoxy-dimethyl-4',3,5-trihydroxy flavone and the test extracts showed potent antimicrobial activity for all the bacterial and fungal strains except \textit{E. coli} and \textit{B. cereus} which showed average and nil activity, respectively.

\textbf{Keywords:} \textit{Aegialitis rotundifolia}, Mangrove, Phytochemical isolation, Flavonoids, Antibacterial, Antifungal.

INTRODUCTION

Mangrove plants are a rich source of alkaloids, flavonoids, triterpenes, steroids, saponins, and tannins. Many metabolites belonging to diverse chemical classes and possessing novel chemical structures have been identified from mangroves plants, which are of considerable interest to modern industries and medicine [1].

\textit{Aegialitis} is a genus consisting of only two shrubby mangrove species, namely, \textit{Aegialitis rotundifolia} which is native to Southeast Asia and \textit{Aegialitis annulata} which is native to Australia and Papua New Guinea [2]. \textit{Aegialitis rotundifolia} Roxb. is a small mangrove tree or shrub belonging to family Plumbaginaceae. It usually grows up to a height of 2-3 m and is available in shorelines of the Andaman Sea and the Bay of Bengal from Orissa to Mergui and on the Andaman Islands which is native to Australia and Papua New Guinea [2]. The phytochemical content of this mangrove species has not been scientifically explored much. There is also no report of any isolated pure phyto compounds. Recently, we have reported that the presence of gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, rutin, coumarin, and quercetin by performing quantitative high-performance liquid chromatography (HPLC) analysis and an organosilicon compound, (\textsuperscript{-})-\textsuperscript{spiro\{1-[(tert-Butyldimethylsiloxy)methyl]-3,5,8-trimethyl-bicyclo[4,3,0]non-2-en-5,7-diol-4,1'-cyclopropane\} was detected in gas chromatography-mass spectrometry analysis as the most abundantly found compound [3].

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The treatment of various diseases caused by microorganisms, several medicinal compounds are employed to destroy microorganisms or to prevent their growth are called antimicrobial agents. Today, most of the antimicrobial agents are losing their efficacy due to an increase in microbial resistance. Presently, the impact of multidrug-resistant bacteria on public health is a major concern which resulted in failures to treat several microbial infected diseases. For this reason, there is an urgent need to develop new antibiotics. Plants and other natural sources can provide an array of new and a wide range of chemical compounds which could be beneficial for the treatment of various diseases caused by microbes [12,13].

According to these findings and hypothesis, the present work deals in isolation of phytoconstituents from the leaves of this rare and unexplored mangrove plant and to investigate the potential of the crude leaves extract as an antimicrobial agent.
METHODS

Chemicals and reagents
Ethanol 99.9% was procured from Changshu Hongsheng Fine Chemicals Co. Ltd., China. Muller-Hinton Agar (MHA), Nutrient broth (NB), Sabouraud dextrose agar (SDA), and Sabouraud dextrose broth (SDB) were procured from HI-MEDIA Laboratories (Mumbai, India). All major chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck Specialties Pvt. Ltd., (Mumbai, India). The solvents used were of high purity and HPLC grade. All other chemicals and reagents used in the whole study were of analytical grade.

Collection and authentication of plant materials
The fresh leaves of *Aegialitis rotundifolia* Roxb. were collected from healthy and fully-grown plants from Bichitrapur mangrove located in Kharibil, Orissa, India (21°34′54.0″N - 87°25′25.4″E). The plant materials were then authenticated from Botanical Survey of India (BSI), Central National Herbarium, Botanic Garden, Howrah, West Bengal, India, and were assigned with a Voucher no. CNH/Tech/II/2016/11a and specimen no. DG-01.

Preparation of extracts
The collected plant materials were gently washed in tap water to remove dirt, and then they were shade dried in the laboratory under room temperature (24 ± 2°C) for 3–4 weeks. After complete drying, the dried plant materials were pulverized using a mechanical grinder followed by sieving to obtain a coarse powder. The powdered plant material was then extracted with ethanol (99.9%) using reflux technique. The crude extract solution obtained was filtered using Whatman No. 42 filter paper and the excess solvents were evaporated by rotary vacuum evaporator (Evator, Media Instrument Mfg. Co., Mumbai, India) and concentrated on a water bath to obtain *Aegialitis rotundifolia* Roxb., ethanolic leaves extract (ARELE). The crude ethanolic extract obtained was stored at 4°C before analysis.

Isolation of phytoconstituents
The crude ethanolic extract was suspended in distilled water and partitioned first with n-hexane to remove the fatty materials and then partitioned with ethyl acetate using a separating funnel. Both n-hexane and ethyl acetate were used several times until convinced that most of the n-hexane and ethyl acetate soluble constituents have been extracted. The mixture was then filtered using Whatman No. 42 filter paper, and the excess solvents were removed using rotary vacuum evaporator and concentrated on a water bath to obtain *Aegialitis rotundifolia* Roxb., ethanolic leaves extract (ARELE). The crude ethanolic extract obtained was stored at 4°C before analysis.

Characterization of the isolated phytoconstituents

General instrumentation
Melting points of the isolated compounds were determined by melting point apparatus (Analytik Jena, India) using a one end open capillary tube. The infrared (IR) spectra of the pure compounds were recorded on a Perkin Elmer 1720X FT-IR spectrophotometer (Shimadzu, Japan) using KBr pellets. The NMR data of the isolated pure compounds were recorded using a Bruker Avance III HD 400 spectrometer. The 1H NMR spectra were recorded at 400 MHz, 13C NMR spectra were recorded at 100 MHz, and 1H NMR spectra were obtained at 400 MHz. Deuterated methanol (CD3OD) and deuterated chloroform (CDCl3) were used as the solvents and tetramethylsilane (TMS) was used as the internal standard. Mass spectral data were recorded using a Finnigan MAT95 (Thermo Scientiﬁc Inc., USA).

3,4-dimethyl benzoic acid (1)
Colorless needles (115 mg; m.p.: 189–193°C); IR (KBr) v cm−1: 3294.53, 2966.6, 2864.4, 1708.9, 1554.7, 1454.4, 1238.3 to 1192.0, 1028.1 to 962.5, 709.8 to 667.4; 1H NMR (400 MHz, CDCl3): δ 6.276 (OH-3, s), 5.557 (OH-3′, s), 5.138 (H-5, s), 4.881 to 3.430 (H-5′, 6, 6.7, 8, s), 3.269 (Me-3′, s), 2.175 (H-4, s), 1.67 to 1.33 (H-5′, 6′, and 2′, s); 13C NMR (100 MHz, CDCl3): δ 170.4 (C-6), 135.3 (C-5, 5′), 133.6 (C-4′), 123.0 (C-3′), 110.9 (C-2′), 102.9 (C-2), 77.4 (C-4′), 76.6 (C-5), 76.5 (C-3′), 71.1 (C-7), 51.7 (C-8), 38.8 (C-2′); 3508.6, 3436.4, 3294.5, 2966.6, 2864.4, 1708.9, 1554.7, 1454.4, 1238.3 to 1192.0, 1028.1 to 962.5, 709.8 to 667.4; MS (EI): m/z: 164.1 [M]+, 150.1 [M−H]+, 127.0, 115.9, 103.9, 91.8, 80.0, 67.9, 55.8, 43.8 [M−H,O]+, 32.8.

3,4,5-trihydroxy-flavan-3-ol (2)
Pale yellow powder (86 mg); mp: 272–276°C; IR (KBr) v cm−1: 3524.1 to 3294.5, 2966.6, 2864.4, 1708.9, 1554.7, 1454.4, 1238.3 to 1192.0, 1028.1 to 962.5, 709.8 to 667.4; 1H NMR (400 MHz, CDCl3): δ 6.276 (OH-3, s), 5.557 (OH-3′, s), 5.138 (H-5, s), 4.881 to 3.430 (H-5′, 6, 6.7, 8, s), 3.269 (Me-3′, s), 2.175 (H-4, s), 1.67 to 1.33 (H-5′, 6′, and 2′, s); 13C NMR (100 MHz, CDCl3): δ 170.4 (C-6), 135.3 (C-5, 5′), 133.6 (C-4′), 123.0 (C-3′), 110.9 (C-2′), 102.9 (C-2), 77.4 (C-4′), 76.6 (C-5), 76.5 (C-3′), 71.1 (C-7), 51.7 (C-8), 38.8 (C-2′); 3508.6, 3436.4, 3294.5, 2966.6, 2864.4, 1708.9, 1554.7, 1454.4, 1238.3 to 1192.0, 1028.1 to 962.5, 709.8 to 667.4; MS (EI): m/z: 272 (100 [M]+, C13H10O6).

Antimicrobial activity of the crude extract

Culture media preparation
The culture media for testing both the antibacterial and antifungal activities were prepared according to the standard instruction provided by the HiMedia Laboratories (Mumbai, India). The media used for antibacterial activity were MHA and NB, whereas, for antifungal activity, SDA and SDB were used. They were prepared and sterilized at 121°C for 15 min in an autoclave.

Plate preparations
In an aseptic environment, 25 mL of pre-autoclaved MHA for antibacterial activity and 25 mL of pre-autoclaved SDA for antifungal activity were poured separately into 90 mm diameter pre-sterilized Petri plates. The culture media in the Petri plates were then allowed to solidify at room temperature under ultraviolet light (265 nm wavelength) for 15–20 min.

Evaluation of antibacterial activity using well diffusion method
The antibacterial activity of ARELE was conducted according to standard procedures [14,15] with a few minor modifications. The activity was performed against four bacterial strains, namely, Gram-positive bacteria including *Staphylococcus aureus* and *Bacillus cereus* and Gram-negative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa*. The bacterial strains were maintained at the Department of Biotechnology, GITAM Institute of Pharmacy, GITAM (Deemed to be University). The microbial cultures were checked for purity by conventional biochemical methods. These bacterial cultures were maintained on nutrient agar slants at first being incubated at 37°C for about 18–24 h and then stored at 4°C as stock for antibacterial activity. Fresh cultures were obtained by transferring a loop full of culture into NB and then incubated at 37°C overnight. The well diffusion method was followed to test the antibacterial activity of the test extract, and reference standard (Gentamicin) was used for activity comparison. This method depends on the diffusion of leaves extracts from the hole through the solidified

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agar layer of Petri dish to such an extent that the growth of the added microorganism is prevented entirely in a circular area or zone around the hole containing the test extracts.

After the culture media in Petri plates have been solidified, the freshly prepared microbial growth culture suspension (about 100 µL) was poured over the MHA media using micropipette and spread uniformly over the media using L shaped sterilized glass spreader separately under the aseptic condition using laminar air flow. Then, wells were made in each plate with the help of borer of 8 mm diameter. In these wells, about 40 µL of positive control, negative control, and ARELE of different concentrations (100 µg/mL, 200 µg/mL, and 400 µg/mL) were individually loaded. The following control agents were used for the study: Negative control agent – 5% DMSO and positive control agent – gentamicin (10 µg/mL). All the procedures have been done under aseptic conditions. Petri plates were incubated for overnight at 37°C ± 0.5°C in the incubator. After incubation, the diameter of the clear zone of incubation produced around the well or holes was measured in mm by digital caliper and compared with the standard drug. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was calculated.

**Evaluation of antifungal activity using well diffusion method**

The antifungal activity of ARELE was conducted according to standard methods [14,15]. The activity was performed against two fungal strains, namely, Candida albicans and Aspergillus niger. The fungal strains were maintained at the Department of Biotechnology, GITAM Institute of Pharmacy, GITAM (Deemed to be University). The microbial cultures were checked for purity by conventional biochemical methods. These cultures were maintained on SDA at first after being incubated at 30°C for about 32-48 h and then stored at 4°C as stock for antifungal activity. Fresh cultures were obtained by transferring a loop full of culture into SDB and then incubated at 25°C for 72 h. The well diffusion method was followed to test the antifungal activity of the test extract, and reference standard (Nystatin) was used for activity comparison.

After the culture media in Petri plates have been solidified, the freshly prepared microbial growth culture suspension (about 100 µL) was poured over the SDA media using micropipette and spread uniformly over the media using L shaped sterilized glass spreader separately under the aseptic condition using laminar air flow. Then, wells were made in each plate with the help of borer of 8 mm diameter. In these wells, about 40 µL of positive control, negative control, and ARELE of different concentrations (100 µg/mL, 200 µg/mL, and 400 µg/mL) were individually loaded. The following control agents were used for the study: Negative control agent – 5% DMSO and positive control agent – gentamicin (10 µg/mL). All the procedures have been done under aseptic conditions. Petri plates were incubated for overnight at 37°C ± 0.5°C in the incubator. After incubation, the diameter of the clear zone of incubation produced around the well or holes was measured in mm by digital caliper and compared with the standard drug. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was calculated.

**Statistical analysis**

The results were calculated and expressed as mean ± Standard deviation. The data obtained in the studies were subjected to one-way analysis of variance (ANOVA) for determining the significant difference. The intergroup significance was analyzed using Dunnett’s t-test. p<0.01 was considered to be significant. All the statistical analysis and data presentation were done using GraphPad InStat Version 3.06 (GraphPad Software, Inc. La Jolla, CA, USA) and Microsoft Excel 2013 standard (Microsoft Corp., Redmond, WA, USA).

**RESULTS AND DISCUSSION**

Isolation and characterization of phytochemicals

Leaves of A. rotundifolia were extracted with ethanol to give crude ethanol extract. The crude extract was then subjected to liquid-liquid fractionation to yield n-hexane and ethyl acetate fractions. The ethyl acetate fraction obtained was subjected to CC on silica gel (60–120 mesh). Three pure compounds (1, 2, and 3) were isolated after series of chromatographic separation which was characterized using IR, 1H NMR, 13CNMR, H NMR, mass spectroscopy, and TLC. The chemical structures of the isolated phytocompounds are given in Fig. 1.

Compound 1, namely, 3,4-dimethyl benzoic acid, was obtained as colorless needles after recrystallization of fraction A1 with hot H2O. Its mass spectroscopic analysis showed molecular ion peak at m/z: 182 which corresponds to molecular formula C7H6O2. The IR spectrum of compound 1 showed strong absorption bands which indicated the presence of benzoic acid group (1708.9 cm⁻¹), –COOH stretch (1591.3 cm⁻¹), aromatic ring (1670.4 cm⁻¹), asymmetric carboxylate anion (1444.7 to 1388.8 cm⁻¹), C-C stretching (1274.9 to 1197.8 cm⁻¹), O-C stretch (1138.0, 1074.4 to 1030.0 cm⁻¹), O-H, out of plane bend (985.9, 869.9 to 821.7 cm⁻¹), and monosubstituted in aromatic ring (759.9 cm⁻¹) and out of plane C=C (673.2 to 574.8 cm⁻¹).

The 1H NMR spectrum of the compound 1 revealed four singlets at δH 4.893 (H-1, s), 4.181 (H-2, s), 3.941 (H-5, s), and 3.198 (H-6, s) which are attributable to H-1, H-2, H-5, and H-6, respectively. Peak signal at δH 3.704 and 3.325 indicated the presence of OCH3-3 and OCH3-4, respectively.

The 13C NMR spectrum of compound 1 showed 9 carbon signals. Three of the nine signals belong to methine carbons at position 2, 3, and 6. One carbon of the benzene ring was substituted by a carboxylic acid group and two carbon of ring was substituted by methoxy group. The characteristic signals were displayed at δC 173.47, 176.87, 135.85, 145.44 cm⁻¹, and 119.20 cm⁻¹. O-H, out of plane bend (1028.1 to 962.5 cm⁻¹), and monosubstituted in aromatic ring (709.8 to 667.4 cm⁻¹).

The 1H NMR spectrum of the compound 2 revealed characteristic signals at δH 7.276 (OH-3, s), 5.557 (OH-3', s), 5.138 (H-5', s), 4.881 to 3.705, 4.5-14.73, and 5-119.80. By performing TLC, the R value of the compound was found to be 0.53 (chloroform:methanol, 1:1).

Compound 2, namely, 3-methoxy-4′-hydroxy-flavan-3-ol, was obtained as pale-yellow powder after recrystallization of fraction B1 with MeOH. Its mass spectroscopic analysis showed molecular ion peak at m/z: 272 which corresponds to molecular formula C14H12O4. The IR spectrum of compound 2 indicated the presence of O-H, free hydroxyl group (3522.41 to 3294.53 cm⁻¹), cyclic C-H, str (2966.6 cm⁻¹), anti-C-H, str (2895.2 cm⁻¹), ring C-C stretch (1554.7 cm⁻¹), monosubstituted in aromatic ring (1454.4 cm⁻¹), asymmetric carboxylate anion (1444.7 to 1388.8 cm⁻¹), C-C stretching (1274.9 to 1197.8 cm⁻¹), O-C stretch (1138.0, 1074.4 to 1030.0 cm⁻¹), O-H, out of plane bend (985.9, 869.9 to 821.7 cm⁻¹), and monosubstituted in aromatic ring (759.9 cm⁻¹) and out of plane C=C (673.2 to 574.8 cm⁻¹).

The 1H NMR spectrum of compound 2 showed 9 carbon signals. Three of the nine signals belong to methine carbons at position 2, 3, and 6. One carbon of the benzene ring was substituted by a carboxylic acid group and two carbon of ring was substituted by methoxy group. The characteristic signals were displayed at δC 173.47, 176.87, 135.85, 145.44 cm⁻¹, and 119.20 cm⁻¹. O-H, out of plane bend (1028.1 to 962.5 cm⁻¹), and monosubstituted in aromatic ring (709.8 to 667.4 cm⁻¹).

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**Fig. 1: Chemical structures of the isolated compounds**
deduced from the mass spectroscopic analysis showed molecular ion peak at m/z: 358, 8-90.54, 3’-130.26, 4’-181.52, 1’-178.94, 2’-73.93, 5’-72.93, 6’-71.44. Its signals at δ (OH-3, s), 4.889 (OH-4’, s), 4.725 (OH-5, s), 4.255 (H-3’, d), 3.838 (H-7, d), (9.16±1.25 mm) closely followed by of 100 µg/mL showed maximum activity against inactive against average activity antibacterial activity against bacteria. The test extracts at different concentrations showed strong (5% DMSO) showed no zone of inhibition at all against all the tested bacteria, and the results are shown in Antibacterial activity of ARELE was tested against two Gram-positive bacteria, and no activity against E. coli. The above results were further supported to a major extent by a previous antibacterial study conducted on this plant by Sett et al. [9]. The antibacterial activity of A. rotundifolia leaves was also studied previously by Hasan et al. [10] and they have concluded that the test extract was mostly inactive against all the bacterial strains, which was inconsistent with our findings. S. aureus is a round-shaped Gram-positive bacterium which is common in the normal flora of the body and is frequently found in the nose, respiratory tract, and on the skin. It is also known to cause several diseases mainly skin and soft tissue infections, endocarditis, etc. [18]. Our study showed good activity against S. aureus which may indicate that the test extracts might be effective against any kind of skin infections. B. cereus is a rod-shaped, Gram-positive bacterium which is responsible for foodborne diseases such as heavy nausea, vomiting, and diarrhea [19] and out test extracts were found to be ineffective against B. cereus. The above results were further supported by the negative control (5 % DMSO) showed no zone of inhibition at all against both Candida albicans and Aspergillus niger. The average activity was noted against E. coli. Moderate activity against E. coli and no activity against B. cereus might indicate that the test extract might be ineffective in the treatment of microbial intestinal and urinary tract diseases. P. aeruginosa is a rod-shaped, Gram-negative bacterium which is mainly known as an opportunistic bacteria as they cause infections when an individual’s immune system is weakened [21]. The test extract showed high activity against P. aeruginosa which may be used to cure infections caused by the bacterium. Fungal infections are usually very difficult to cure and may take a prolonged duration of time [22]. The antifungal activity of ARELE was tested against two fungal strains, namely, Candida albicans and Aspergillus niger, and the results are shown in Fig. 3. The results showed that the activity was dose-dependent for both the fungal strains. The negative control (5 % DMSO) showed no zone of inhibition at all against both Candida albicans and Aspergillus niger. The test extracts at different concentrations showed strong antifungal activity against both Candida albicans and Aspergillus niger. Maximum zone of inhibition at all the doses of ARELE was observed against Aspergillus niger which was 9.27±1.25 mm (100 µg/mL).
11.5±2.29 (200 µg/mL), and 16.38±0.78 mm (400 µg/mL). The zone of inhibition against *Candida albicans* was 5.44±1.07, 10.11±1.16, and 13.94±0.58 mm for 100, 200, and 400 µg/mL, respectively, which was slightly lower compared to that of *Aspergillus niger*. The reference standard nystatin (20 µg/mL) showed slightly higher zone of inhibition than ARELE showing the value of 19.77±0.83 mm for *Candida albicans* and 22.27±1.27 mm for *Aspergillus niger*.

This study will give the first report of the antifungal activity of *A. rotundifolia* against two important fungal strains, namely, *Candida albicans* and *Aspergillus niger*. However, one previous study on this plant was conducted by Sett et al. [8] against an isolated leaf fungus, namely, *Myccovellosiella* sp., where the test extract showed strong activity. Our study also showed strong antifungal activity, which may indicate that *A. rotundifolia* leaves extract can be potentially used to cure fungal infections.

The antimicrobial activity found in the test extracts could be due to the presence of various phytochemicals and also the isolated compounds [23]. In a previous report by Cusnife and Lamb, 2005 [24] flavonoids and their derivatives are reported to possess strong antimicrobial properties.

**CONCLUSIONS**

This study provides the first information report of isolation of phytochemicals from the leaves of *A. rotundifolia*. Three pure compounds were isolated, namely, 3,4-dimethyl benzoic acid (1), 3'-methoxy-4'-hydroxy-flavan-3-ol (2), and 3,7-dimethoxy-dimethyl-4',3,5-trihydroxy flavone (3) which were confirmed by spectroscopic studies. Antibacterial activity of the extracts was performed against two Gram-positive (*S. aureus* and *B. cereus*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria and the results revealed strong activity against *S. aureus* and *P. aeruginosa*, whereas average activity was noted for *E. coli* and no activity for *B. cereus*. Antifungal activity was performed against *Candida albicans* and *Aspergillus niger*, and the results showed strong antifungal activity against both the fungal strains. However, further study is required for revealing the mechanism of antifungal activity and the phytochemicals responsible for the activity.

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**AUTHORS’ CONTRIBUTION**

This research work has been performed in collaboration between all authors. All authors revised and approved the final manuscript.

**CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest.

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