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Research Article

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

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ABSTRACT

Objective: The aim of the study is to isolate and characterize the phytochemicals from the leaves of a rare and unexplored mangrove *Aegialitis rotundifolia* and evaluate the antimicrobial properties of the crude extract.

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 phytocompounds. The isolated compounds were characterized using infrared (IR), carbon-13 nuclear magnetic resonance (^{13}C NMR), proton nuclear magnetic resonance (^{1}H NMR), mass spectroscopy, 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.

Results: Three pure compounds were isolated from the leaves of *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 *Staphylococcus aureus* and *Pseudomonas aeruginosa*, whereas *Escherichia coli* and *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, *Candida albicans* and *Aspergillus niger*.

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 *E. coli* and *B. cereus* which showed average and nil activity, respectively.

Keywords: *Aegialitis rotundifolia*, Mangrove, Phytochemical isolation, Flavonoids, Antibacterial**,** Antifungal.

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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].

Aegialitis is a genus consisting of only two shrubby mangrove species, namely, *Aegialitis rotundifolia* which is native to Southeast Asia and *Aegialitis annulata* which is native to Australia and Papua New Guinea [2]. *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 and is endemic to the coastal parts of South Asia. In Orissa, it is locally known as Banrua [3]. This mangrove species is reported to produce one of the best quality honey [4]. Conventionally, the leaf is used in the treatment of sundry cutaneous injuries accompanied by pain and inflammation and is locally utilized as an anti-ache agent [5]. Further, the leaf of the plant is pounded with oil to make a paste which acts as an antidote for insect bites [6]. According to the present literature available for this plant, there have been very few scientific reports of pharmacological screening conducted such as *in vitro* antioxidant [7], antimicrobial [8,9], antibacterial [10], anticancer [11], analgesic, [6], anti-inflammatory [6,10], and *in vitro* thrombolytic activities [10]. The phytochemical content of this mangrove species has not been scientifically explored much. There is also no report of any isolated pure phytocompounds. 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, (-)-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].

For 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 Specialities 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 after which 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 ethanol extract obtained was stored at 4°C before analysis.

Isolation of phytoconstituents

The crude ethanol 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 the n-hexane and ethyl acetate fractions. The ethyl acetate fraction obtained was subjected to silica gel column chromatography (CC) (60–120 mesh) using $CHCl_3$-MeOH (30:1 → 15:1 → 10:1 → 5:1 → 1:1) gradient system to give eleven sub-fractions. TLC was performed on all the resulting fractions which were then combined based on their TLC profiles in A-D junctions. Fraction A was further subjected to silica gel CC (60–120 mesh) with CH_2Cl_2-MeOH (9:1 → 1:1) gradient system to yield three sub-fractions (Fr. A1-A3). Fr. A1 was then recrystallized from hot H_2O to yield pure compound 1 (115 mg). Fraction B was also subjected to silica gel CC (60–120 mesh) with $CHCl_3$-MeOH gradient (9:1 → 1:1) system to yield two sub-fractions (Fr. B1 and B2). Fr. B1 was then recrystallized from MeOH to yield pure compound 2 (86 mg). Fraction D was then subjected to silica gel CC (60–120 mesh) with $CHCl_3$-MeOH gradient (9:0.5 → 1:0.5) system to yield three sub-fractions (Fr. D1, D2, and D3). Sub-fraction D3 was recrystallized from acetone to yield pure compound 3 (102 mg).

Characterization of the isolated phytoconstituents

General instrumentation

Melting points of the isolated compounds were determined by melting point apparatus (Analab, India) using a one end open capillary tube. The infrared (IR) spectra of the pure compounds were recorded on IR Affinity 1 Spectrophotometer (Shimadzu, Japan) using KBr pellets. The NMR data of the isolated pure compounds were recorded using AMX-400 spectrometer (Bruker, Rheinstetten, Germany). The ^{13}C NMR spectra were recorded at 100 MHz, and ^1H NMR spectra were obtained at 400 MHz. Deuterated methanol (CD_3OD) and deuterated chloroform ($CDCl_3$) were used as the solvents and tetramethylsilane (TMS) was used as the internal standard. Mass spectral data were recorded using Synapt Mass Spectrometer (Waters, UK).

3,4-dimethyl benzoic acid (1)

Colorless needles (115 mg); m.p.: 189–193°C; IR (KBr) v cm^{-1}: 3525.9, 3408.3, 3311.8–3010.9, 2916.5, 2895.2, 1753.4, 1670.4, 1591.3, 1444.7 to 1388.8, 1274.9 to 1197.8, 1138.0, 1074.4 to 1030.0, 989.5, 869.9 to 821.7, 759.9, 673.2 to 574.8;^1H NMR (400 MHz, CD_3OD): δ_H 4.893 (H-1, s), 4.818 (H-2, s), 3.941 (H-5, s), 3.918 (H-6, s), 3.704 (OCH_3-3), 3.325 (OCH_3-4);^{13}C NMR (100 MHz, CD_3OD): δ_C 1–173.47, 2-76.87, 3-70.55, 4-154.73, 5-119.80; EIMS m/z: 182 (100) $[M^+]$, $C_9H_{10}O_4$.

3'-methoxy-4'-hydroxy-flavan-3-ol (2)

Pale yellow powder (86 mg); m.p. 272–277°C; IR (KBr) v cm^{-1}: 3524.1 to 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, $CDCl_3$): δ_H 7.276 (OH-3, s), 5.557 (OH- 3', s), 5.138 (H- 5',s), 4.881 to 3.430 (H- 5, 6, 7 & 8, s), 3.269 (OMe-3', s), 2.175 (H- 4, d), 1.67 to 1.33 (H- 5', 6' and 2', s);^{13}C NMR (100 MHz, $CDCl_3$): δ_C 2-77.41, 3-130.29, 4-50.41, 5-76.99, 6-73.42, 7-76.57, 8-70.62, 3'-139.41, 4'-131.99, 1'-170.39, 2'-121.69, 5'-123.09, 6'-123.51; EIMS m/z: 272 (100) $[M^+]$, $C_{16}H_{16}O_4$.

3',7-dimethoxy-dimethyl-4',3,5-trihydroxy flavone (3)

Colorless powder (102 mg); m.p. 260–263°C; IR (KBr) v cm^{-1}: 3508.6, 3371.7, 2939.6, 1691.6, 1450.5, 1288.4, 1211.3, 1070.5, 983.7, 918.2, 833.3, 765.8-736.8, 663.5;^1H NMR (400 MHz, $CDCl_3$): δ_H 5.625 (OH-3, s), 4.889 (OH-4', s), 4.725 (OH-5, s), 4.255 (H-3', d), 3.838 (H-7, d), 3.411 (H-8, s), 3.482 (H-6), 3.091 (H-2'), 3.062 (H-5', d), 3.379 (H-6', d);^{13}C NMR (100 MHz, $CDCl_3$): δ_C 2–75.09, 3-130.26, 4-106.91, 5-133.68, 6-90.13, 7-104.20, 8-90.54, 3'-130.26, 4'-181.52, 1'-178.94, 2'-73.93, 5'-72.93, 6'-71.44; EIMS m/z: 358 (100) $[M^+]$, $C_{19}H_{18}O_7$.

Antimicrobial activity of the crude extract

Culture media preparation

The culture media for conducting both 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 at 15 psi for 15–30 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 after 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

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 airflow. Then, wells were made in each plate with the help of borer of 8 mm diameter. In these well, 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 airflow. Then, wells were made in each plate with the help of borer of 8 mm diameter. In these well, 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 – nystatin (20 µg/mL). All the procedures have been done under aseptic conditions. Petri plates were incubated at 25°C ± 0.5°C for 72 h 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 of 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, ^{13}C NMR, ^{1}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 H_2O. Its mass spectroscopic analysis showed molecular ion peak at m/z: 182 which corresponds to molecular formula $C_9H_{10}O_4$. The IR spectrum of compound 1 showed strong absorption bands which indicated the presence of benzoic acid group (3525.9 cm^{-1}), O-H, free hydroxyl group (3408.3 cm^{-1}), O-H, str (3311.8 to 3010.9 cm^{-1}), cyclic C-H, str (2916.5 cm^{-1}), Ali- C-H, str (2895.2 cm^{-1}), COOH stretch (1753.4 cm^{-1}), ring C=C stretch (1670.4 cm^{-1}), asymmetric carboxylate anion (1591.3 cm^{-1}), C-C ring stretch (1444.7 to 1388.8 cm^{-1}), C-C stretching (1274.9 to 1197.8 cm^{-1}), -CO, stretch (1138.0, 1074.4 to 1030.0 cm^{-1}), O-H, out of plane bend (989.5, 869.9 to 821.7 cm^{-1}), monosubstituted in aromatic ring (759.9 cm^{-1}), and out of plane C=C (673.2 to 574.8 cm^{-1}).

The ^{1}H NMR spectrum of the compound 1 revealed four singlets at δ_H 4.893 (H-1, s), 4.818 (H-2, s), 3.941 (H-5, s), and 3.918 (H-6, s) which are attributable to H-1, H-2, H-5, and H-6, respectively. Peak signal at δ_H 3.704 and 3.325 indicated the presence of OCH_3-3 and OCH_3-4, respectively.

The ^{13}C 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 δ_C 1-173.47, 2-76.87, 3-70.55, 4-154.73, and 5-119.80. By performing TLC, the R_f value of the compound was found to be 0.53 (chloroform:acetone, 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 $C_{16}H_{16}O_4$. The IR spectrum of compound 2 indicated the presence of O-H, free hydroxyl group (3524.1 to 3294.53 cm^{-1}), cyclic C-H, str (2966.6 cm^{-1}), Ali- C-H, str (2864.4 cm^{-1}), C=O stretch (1708.9 cm^{-1}), C-C ring stretch (1554.7 cm^{-1}), CH_3 of CH_3CO (1454.4 cm^{-1}), C-C stretching (1238.3 to 1192.0 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 ^{1}H NMR spectrum of the compound 2 revealed characteristic signals at δ_H 7.276 (OH-3, s), 5.557 (OH-3', s), 5.138 (H-5', s), 4.881 to

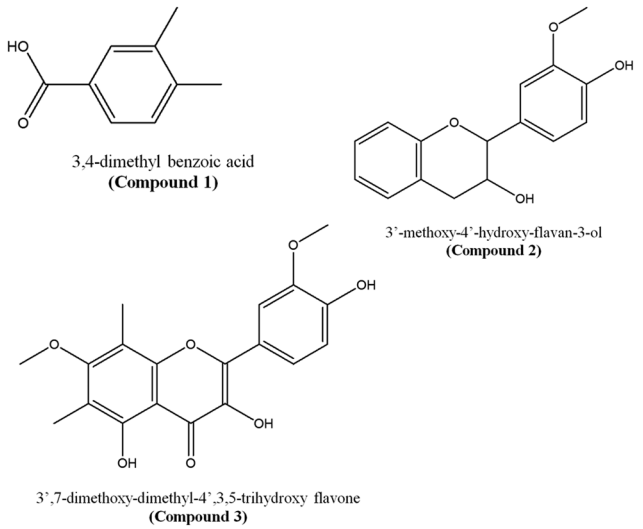

Fig. 1: Chemical structures of the isolated compounds

3.430 (H-5, 6, 7, & 8, s), 3.269 (OMe-3', s), 2.175 (H-4, d), 1.67 to 1.33 (H-5', 6', and 2', s). In the ^{1}H NMR spectrum, the double doublets were recorded for methine proton at 5, 6, 7, 8, 2', 5', and 6' position of the ring. The singlet for the proton of hydroxyl group was found at 3 and 4' while for methoxy at 3' position of carbon, and these signals are typical of a flavonoid nucleus with an unsubstituted ring. The double doublets protons at 2', 3', 5', and 6' of carbon suggested that third ring was saturated. This splitting pattern was due to the coupling between the H-2 axial proton and the H-3 geminal protons. The above data suggested a flavan nature for compound 2.

The ^{13}C NMR spectrum of compound 2 portrayed the characteristic signals at δ_C 2-77.41, 3-130.29, 4-50.41, 5-76.99, 6-73.42, 7-76.57, 8-70.62, 3'-139.41, 4'-131.99, 1'-170.39, 2'-121.69, 5'-123.09, 6'-123.50. The result of the ^{13}C NMR supported the presence of two hydroxyl group, methoxyl group and other groups such as CH and CH_2 groups. By performing TLC, the R_f value of the compound was found to be 0.63 (ethyl acetate:methanol, 1:0.25).

Compound 3, namely, 3',7-dimethoxy-dimethyl-4',3,5-trihydroxy flavone, was obtained as colorless powder after recrystallization of fraction D3 with acetone. The IR spectrum of compound **3** indicated the presence of O-H, free hydroxyl group (3508.6 cm^{-1}), O-H, str (3371.7 cm^{-1}), cyclic C-H, str (2939.6 cm^{-1}), C=O stretch (1691.6 cm^{-1}), C-C ring stretch (1450.5 cm^{-1}), C-C stretching (1288.4 cm^{-1}), C-C stretching (1211.3 cm^{-1}), -CO, stretch (1070.5 cm^{-1}), O-H, out of plane bend (983.7, 918.2, 833.3 cm^{-1}), monosubstituted in aromatic ring (765.8 to 736.8 cm^{-1}), and out of plane C=C (663.5 cm^{-1}). The ^{1}H NMR spectrum of the compound **3** revealed characteristic signals at δ_H 5.625 (OH-3, s), 4.889 (OH-4', s), 4.725 (OH-5, s), 4.255 (H-3', d), 3.838 (H-7, d), 3.411 (H-8, s), 3.482 (H-6), 3.091 (H-2'), 3.062 (H-5', d), 3.379 (H-6', d). The ^{13}C NMR spectrum of compound **3** showed the characteristic signals at δ_C 2-75.09, 3-130.26, 4-106.91, 5-133.68, 6-90.13, 7-104.20, 8-90.54, 3'-130.26, 4'-181.52, 1'-178.94, 2'-73.93, 5'-72.93, 6'-71.44. Its mass spectroscopic analysis showed molecular ion peak at m/z: 358 which corresponds to molecular formula $C_{19}H_{18}O_7$.

The occurrence of a flavone skeleton in the molecule could be easily deduced from the ^{1}H-NMR and ^{13}C-NMR spectrums. From the above-mentioned data of ^{13}C NMR signal indicated the presence of three hydroxyl group at C-3, C-5, and C-4' and unsaturated keto function. The ^{13}C NMR signal also reported the presence of two methoxy group at C-7 and C-3' of flavones skeleton. Presences of two methyl group were identified at C-6 and C-8. The ^{1}H-NMR further showed the presence of three hydroxyl, two methoxyl group two methyl groups, and three methine group. By performing TLC, the R_f value of the compound was found to be 0.74 (Chloroform:ethyl acetate, 6:4).

Effect of ARELE on antimicrobial activity

The development of effective drugs to treat microbial infections has taken a huge leap since the 1940s. Development of effective antimicrobial drugs has led to a significant reduction of diseases caused by microbes, but currently, drug-resistant microbes have emerged which has given rise to serious complications in the treatment of microbial diseases [16]. Natural products of plant origin are a source of chemical diversity and can be used to combat microbial diseases and even drug-resistant microbes [17].

The antibacterial activity of ARELE was tested against two Gram-positive (*S. aureus* and *B. cereus*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria, and the results are shown in Fig. 2. The results revealed that the activity was dose-dependent for all bacteria. The negative control (5% DMSO) showed no zone of inhibition at all against all the tested bacteria. The test extracts at different concentrations showed strong antibacterial activity against *S. aureus* and *P. aeruginosa,* whereas average activity was noticed for *E. coli.* The test extract was found to be inactive against *B. cereus* at all concentrations. ARELE at a concentration of 100 µg/mL showed maximum activity against *P. aeruginosa* (9.16±1.25 mm) closely followed by *S. aureus* (9.11±1.16 mm) and

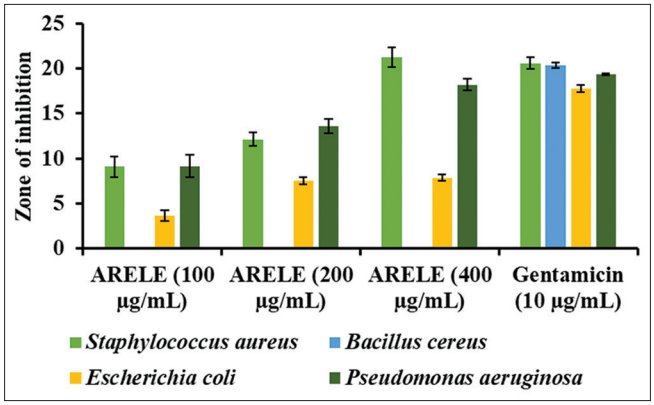

Fig. 2: Antibacterial activity of *Aegialitis rotundifolia* ethanolic leaves extract. All experiments were carried out in triplicate (n=3), and the results are expressed mean±standard deviation

then *E. coli* (3.66±0.57 mm). At a concentration of 200 µg/mL, ARELE also showed maximum activity against *P. aeruginosa* (13.61±0.78 mm) followed by *S. aureus* (12.16±0.76 mm) and then *E. coli* (7.55±0.38 mm). At 400 µg/mL dose of ARELE, the highest activity was shown against *S. aureus* (21.27±1.11 mm) followed by *P. aeruginosa* (18.22±0.69 mm) and then *E. coli* (7.88.0.34 mm). The mean inhibition zone calculated for reference standard gentamicin was found to be 20.60±0.63, 20.33±0.28, 17.77±0.38, and 19.38±0.09 mm for *S. aureus, B. cereus, E. coli,* and *P. aeruginosa,* respectively. The above results show that ARELE was able to inhibit the growth of *S. aureus* highly successfully followed by *P. aeruginosa* and *E. coli,* whereas it was not able to produce any inhibition against *B. cereus.* 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. E. coli* is a rod-shaped, Gram-negative coliform bacterium mainly found in the lower intestine of warm-blooded organisms. They are usually harmless, but virulent strains are reported to cause gastroenteritis, hemorrhagic colitis, urinary tract infections, etc. [20]. 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),

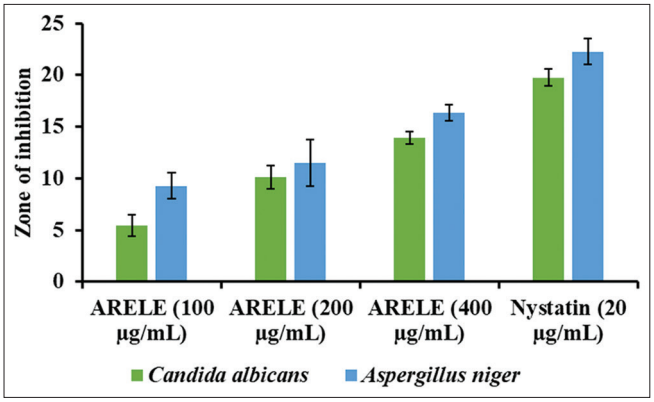

Fig. 3: Antifungal activity of *Aegialitis rotundifolia* ethanolic leaves extract. All experiments were carried out in triplicate (n=3) and the results are expressed mean±standard deviation

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, *Mycovellociella* 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 Cushnie 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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