ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 5, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

BIOACTIVE FRACTION OF *TROPIDIA CURCULIOIDES,* A RARE ORCHID OF ARUNACHAL PRADESH, INDIA: PHYTOCHEMICAL PROFILE AND MARKER COMPOUNDS

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Received: 05 December 2017, Revised and Accepted: 24 January 2018

ABSTRACT

Objective: Chemical profiling of the most active fraction of *Tropidia curculioides*, isolation and characterization of marker compounds along with the evaluation of biological activity.

Methods: The most active Et_2O fraction of roots of *T. curculioides* (TC) was analyzed by gas chromatography coupled with a mass selective detector. Repetitive chromatography followed by high-performance liquid chromatography of the active fraction afforded three major compounds. The structure of the compounds was established by nuclear magnetic resonance and mass spectral analysis. Antibacterial activity of the compounds was determined by agar well diffusion method against five multidrug-resistant (MDR) clinical isolates while minimum inhibitory concentration (MIC) and minimum bactericidal concentration were determined by microdilution method. Scanning electron microscopy was performed with compound 2 against *Escherichia coli* cells at MIC (15 µg/ml).

Results: Analysis of the gas chromatography–mass spectrometry (GC–MS) spectra revealed that the isolated compounds constituted 27.16% of the total constituents. Two other chemical classes, namely, saturated fatty acids and sterol constituted 38.04% and 12.49%, respectively. The structure of the compounds was characterized as 4-hydroxy benzaldehyde (1), bisphenol F (2), and 3,5-dihydroxy-4-methoxybenzoic acid (3). The most significant bacteriostatic and bactericidal activity against *E. coli* comparable to that of tetracycline and gentamicin was observed with compound 2. Microscopic study confirmed that compound 2 affects integrity of cell membrane severely, causing death of the bacterium. Compound 1 and 3 showed moderate to good antibacterial activity against *E. coli* and *Enterococcus* sp. Cell cytotoxicity of the compounds was well within permissible limit**.** This is the first report of compounds in TC.

Conclusion: The study identified three marker compounds from the less polar fraction of the roots of TC with significant antibacterial activity. The GC–MS spectra with marker compounds could be treated as the chemical fingerprinting of the bioactive fraction. The outcome justifies the use of the plant in traditional medicine.

Keywords: Orchidaceae, *Tropidia curculioides*, Antibacterial activity, 4-hydroxy benzaldehyde, Bisphenol F, 3,5-dihydroxy-4-methoxybenzoic acid.

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INTRODUCTION

Bioprospecting of natural resources is of immense importance in search of potential drug candidates. Although large number of plants has already been exploited in traditional medicine, still a substantial number remained unexplored. The North-Eastern state of India, Arunachal Pradesh (AP) is a natural habitat of 550 species of orchids including 37 species of medicinal importance [1]. Local people of this region use folk medicine substantially, but very little systematic investigation has been carried out with these plants. Few orchids, namely, *Orchis latifolia*, *Dendrobium nobile*, and *Vanda roxburghii* are reported to possess alkaloids, terpenoids, bibenzyls, flavonoids, and stilbenoids [2,3] as secondary metabolites. Preclinical studies showed that it possesses antimicrobial, antioxidant, hepatoprotective, anti-inflammatory, antiarthritic, and wound healing properties [4]. In our previous study, we investigated three medicinal orchids of AP, namely, *Rhynchostylis retusa, Tropidia curculioides,* and *Satyrium nepalense* by bioassay-guided fractionation for antimycobacterial, leishmanicidal, and antibacterial activity [5]. This resulted in the identification of the diethyl ether (Et_2O) fraction of the roots of *T. curculioides* (TC) as most active fraction with maximum antibacterial potency of 125 µg/ml minimum inhibitory concentration (MIC). The bioactivity was comparable to that of *Curcuma longa*, well-known for antibacterial property [6]. Hence, we conducted the present investigation to identify the chemical constituents and total chemical profile of the bioactive fraction.

TC Lindl. (Orchidaceae) is an endangered plant of tropical and subtropical region of Arunachal Pradesh, India [7]. The genus *Tropidia* is reported to possess more than 30 species worldwide [8]. TC in particular, is a rare orchid, native of AP in Indian subcontinent. The plant is traditionally used in the form of decoction for cold stage of malaria and diarrhea [9]. As any systematic phytochemical and pharmacological investigation has not been conducted so far, we thought of investigating the plant for the same. Chemical profiling of the most active fraction was conducted by gas chromatography–mass spectrometry (GC–MS), while, isolation and characterization of compounds was conducted to identify marker compounds. Further, all isolates were evaluated for antibacterial activity against five MDR clinical isolates.

METHODS

General

Ultraviolet (UV) spectra were recorded on UV-240 spectrophotometer (Spinco tech) GC–MS was performed on GCMS-Q2010 Ultra Shimadzu, coupled with mass selective detector. The SPB-5 fused-silica capillary column (30 m × 0.25 mm × 0.25 µm), Supelco was used for the separation of analytes. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker spectrometer (500 MHz) using TMS as internal standard. HR-ESI-MS spectra were recorded on Bruker APEX II mass spectrometer. Silica gel 60 GF_{254} precoated on aluminum sheets (200 mm, 20 × 20 cm) (Merck, India) were used for analytical thin-

layer chromatography (TLC). The spots on TLC plates were visualized under UV light (254 and 366 nm) as well as by staining with iodine. The high-performance liquid chromatography (HPLC) experiments were performed on Shimadzu LC system equipped with manual injector and PDA detector SPD-M20A. HPLC grade water and acetonitrile was used as mobile phase. An aliquot of 500 µl at 5 mg/ml concentration was injected on Phenomenex Luna RP-18 semi-prep - ODS column (10 µm, 10 × 250 mm). All solvents used were of analytical grade (Merck, India).

Plant material

The whole plant was collected from Tipi district of AP (N 2700′14.9″ &E 092036′44.2″, Altitude: 339 m) in the month of April 2014 and was authenticated by Dr. Ona Apang, Scientist, State Forest Research Institute, Itanagar. A voucher specimen (AUUP/AIB/2014/02) has been maintained in the herbarium of Amity Institute of Biotechnology, Amity University, Noida.

Extraction and fractionation

The dried plant material (1.6 kg) of TC was divided into root (0.922 g), stem (0.302 g), and leaves (0.310 g). Each part was macerated separately and was extracted with methanol:water (9:1, 1 l × 24 h) for 3 consecutive days. To prepare the aqueous extract, the residue was extracted with H_2O (0.5 l) at room temperature. The concentrated MeOH extracts of TC were suspended in water and were partitioned with Et_2O and n-BuOH successively. Thereafter, the organic solvents were removed under vacuum, below 40°C in a rotary evaporator to produce the following fractions which have been presented in Table 1. Subsequently, the aqueous (Aq) extracts were lyophilized and refrigerated at 4°C. All fractions were evaluated for antibacterial activity by agar well diffusion method and the most active fraction was further analyses by GC–MS.

GC–MS analysis

Sample preparation for GC–MS analysis

The sample for GC–MS was prepared by dissolving 5 mg of TCR-Et_2O in methanol. The solution was centrifuged at 3000 rpm for 15 min; the supernatant was collected and evaporated to dryness. The dried material was reconstituted with methanol and an aliquot of 1 µl was injected for GC–MS experiment.

GC–MS analysis

The Et_2O fraction of roots of TC was analyzed by GCMS-Q2010 (ultra operated in EI mode, electron energy = 70eV, scan range = 40–650 amu, and scan rate = 1.98 scans/s) and GC–MS solution workstation software system. The GC column used was SPB-5 fused-silica capillary column (30 m × 0.25 mm × 0.25 µm), Supelco, Sigma-Aldrich. The carrier gas used was helium with a column head pressure of 45.6 kPa and a flow rate of 1.0 ml/min. The injector and detector were operated at 260°C and 270°C, respectively. The GC oven temperature program was used as follows: 70°C initial temperature, held for 2 min; increased at 3°C/min to 250°C, held for 5 min; and increased at 2°C/min to 280°C, held for 23 min. The sample solution (1 µl) in methanol (1:100) was injected in pulsed split mode (first 4 min at 1.5 ml/min and the rest time period at 1.0 ml/min; split ratio1:10) [10]. Retention indices (RIs) were determined in relation to a homologous series of *n*-alkanes (C7-C33) under the same conditions. The percentage area of each component has been reported with retention time. Peak identification was accomplished by comparison of their mass spectra with those stored on GC–MS databases (NIST 11 and Wiley 8).

Isolation and characterization

Column chromatography of 5.18 g of the sample on silica gel (100–200 mesh, 0.8 kg) with *n*-hexane and increasing concentration of EtOAc (0–100%) as eluent afforded nine fractions (F-1 to F-9). Repeated chromatography of F-4 (540 mg) with *n*-hexane and EtOAc (0–30%) afforded eight subfractions (SF4.1 to SF4.8). As the subfraction SF4.3 showed a single spot on TLC, further, the separation was conducted by HPLC on C-18 column (10 × 250 mm) using MeCN-H_2O (45:55, v/v, 20 min, flow rate of 5 ml/min) as mobile phase. This afforded compound 1 (40 mg, 0.0044%) at 12.5 min of the run. Similarly, repeated column chromatography followed by HPLC afforded compound 2 (35 mg, 0.0038%) and 3 (20 mg, 0.0022%) from F-5 and F-6, respectively.

- Compound 1: Light brown amorphous powder, UV/visible λmax (MeOH): 284 nm (log ε) 3.68; ^{1}H NMR (500 MHz, CD_3COCD_3): δ 9.86 (H, s, -CHO), δ 7.81 (2H, d, J = 9Hz, H-2, H-6), δ 7.03 (2H, d, J = 9Hz, H-3, H-5); ^{13}C NMR (500 MHz, CD_3COCD_3): δc 190.1 (-CHO), δc 163.1(C-4), δc 131.9 (C-2, C-6), δc 129.4 (C-1), δc 115.8 (C-3, C-5); HRESIMS (+ve): $[M + Na]^+$ m/z 145.0429 (calculated for $C_7H_6O_2Na$, 145.1232).
- Compound 2: Brownish amorphous powder, UV/visible λmax (MeOH): 254 nm(log ε) 3.69; ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.00 (4H, d, J = 8.5 Hz, H-2, H-6, H-2' and H-6'), δ 6.73 (4H, d, J = 8.5Hz, H-3, H-5, H-3' and H-5'), δ 3.76 (2H, s, CH_2); ^{13}C NMR (500 MHz, CD_3COCD_3): δc 155.5 (C-4, C-4'), δc 132.8 (C-1, C-1'), δc 129.6 (C-2, C-6, C-2' and C-6'), δc 115.0 (C-3, C-5, C-3', C-5'), δc 39.8 (CH_2); HRESIMS (+ve): $[M + Na]^+$ m/z 223.0829 (calculated for $C_{13}H_{12}O_2Na$, 223.2332).
- Compound 3: Amorphous solid, UV/visible λmax (MeOH): 284 nm (log ε) 3.39; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39 (2H, s), δ 3.96 (6H, s, $-OCH_3$); ^{13}C NMR (500 MHz, CD_3COCD_3): δ 171.0 (-COOH), δc 146.7 (C-3, C-5), δc 139.9 (C-4), δc 120.0 (C-1), δc 107.1 (C-2, C-6), δc 56.5 ($-OCH_3$); HR-ESI-MS (+ve): $[M + Na]^+$ m/z 207.0429 (calculated for $C_8H_8O_5Na$, 207.1532).

Biological assay

In vitro antibacterial assay

The compounds were evaluated against MDR clinical isolates of five bacterial strains.

Microorganisms and culture media

Five MDR bacterial isolates including *Staphylococcus aureus* (2413), *Enterococcus* sp. (2449), *Serratia* sp. (2442), *Acinetobacter* sp. (2457), and *Escherichia coli* (2461) were obtained from Dr. Kumardeep Dutta Choudhary, Department of Medical Oncology, Rajiv Gandhi Cancer Research Institute, Delhi, India, with respective antibiotic resistance profiles as shown in Table 2. The bacterial strains were grown on 5% nutrient broth (NB) and after initial incubation, organisms were suspended in 15 ml of NB and optical density was measured. The readings were compared with 0.5 McFarland standard [11]. For the

Table 1: Details of extraction and fractionation of *T. curculioides*

Name	Part used for fractionation	Fraction/extract	Yield (g)
T. curculioides (1600 g)	Root (0.922 g)	Diethyl ether fraction (TcR Et2O)	5.18
		n-butanol fraction (TcR *n*-BuOH)	16.54
		Aqueous extract (TcR Aq ext)	38.99
	Stem (0.302 g)	Diethyl ether fraction (TcS Et2O)	2.6
		n-Butanol fraction (TcS*n*-BuOH)	15.6
		Aqueous extract (TcS Aq ext)	4.19
	Leaves (0.310 g)	Diethyl ether fraction (TcL Et2O)	16
		n-butanol fraction (TcL*n*-BuOH)	11.96
		Aqueous extract (TcL Aq ext)	1.65

T. curculioides: Tropidia curculioides

determination of MIC, bacterial suspension at 5×10^6 colony-forming units (CFU)/ml was employed [12].

Agar well diffusion assay

Antibacterial activity of the isolates was determined by agar well diffusion method as described previously [13]. Briefly, nutrient agar plates were inoculated with 0.1 ml of the organisms (1×10^8 CFU/ml). Subsequently, wells of 6 mm size were prepared in nutrient agar plates containing bacterial culture and 40 µL of sample. The sample solutions were prepared from a stock solution at 500 µg/ml in 2% dimethyl sulfoxide (DMSO). The plates were incubated at 37°C for 24 h. Standard antibiotic disc of tetracycline and gentamycin (30 µg) was considered as positive control, whereas 2% DMSO solution was taken as negative control. Assays were carried out in triplicate. The antimicrobial activity was expressed by zone of inhibition (mm) with standard deviation.

Determination of MIC and minimum bactericidal concentration (MBC)

MIC was determined by microdilution method described by Wiegand *et al.* [14]. Briefly, the cultures were diluted in Mueller-Hinton broth at a density of 0.5 McFarland turbidity. Thereafter, 0.5 ml of the bacterial suspension (5×10^6 CFU/ml) was added to 4.5 ml of susceptibility test broth, which contained diluted compound solution, prepared by serial two-fold dilution (250–15.62 µg/ml) of the stock solution (500 µg/ml). A number of wells were reserved in each plate for various controls including control of sterility (no inoculum was added); inoculum viability (no sample solution was added); and DMSO inhibitory effect. The plates were then incubated for 24 h at 37°C. After 24 h of incubation, the absorbance was recorded at 570 nm. The experiments were conducted in triplicate and the lowest concentration of sample that prevented visible growth was considered as MIC.

The MBC assays were performed as described by Holla *et al*. [15]. Briefly, the wells showing complete absence of bacterial growth were identified and aliquots of 10 µl were transferred to each well on Muller-Hinton agar plates. Subsequently, the plates were incubated at 37°C for 24 h. Experiments were conducted in triplicate and complete absence of growth was considered as the MBC.

Sample preparation for scanning electron microscopy (SEM)

By following the method described by Tang *et al.,* [16] *E. coli* cells were treated at MIC of compound 2 and were observed under SEM. Both control and treated cells were considered for morphological study. After centrifugation, the cells were washed with 0.1 M phosphate buffer, pH 7.3 and were fixed in 2.5% glutaraldehyde in the same buffer and postfixed in 1% osmium tetroxide for 1 h at room temperature. Thereafter, the cells were washed thrice and were dehydrated by a series of ethanol at 20, 50, 70, 90, and 95% concentrations for 15 min each. The cells were finally treated with 100% ethanol and CO_2 to achieve the critical point. An aliquot of 20 µL of bacterial pellets was applied on poly-L-lysine slide and subjected to gold coating. Finally, it was observed under Zeiss Evo 40 (Germany) SEM at magnifications of 8–30 KX. The bacterial cell suspension in saline without any treatment of compound was considered as control.

Cell cytotoxicity assay

The cell cytotoxicity was assessed against J774G8 mammalian macrophage cells (1×10^6 cells/ml) with different concentrations of compounds by MTT-based colorimetric assay as described by Koday *et al.* [17]. The experiments were performed in duplicate with three independent experiments.

Statistical analysis

All experiments were carried out in triplicates and the results were expressed as mean ± SD values wherever applicable.

RESULTS

The chemical profile of TCR-Et_2O fraction was analyzed by GC–MS, which showed the presence of 27 compounds, each constituting more than 1% of the total constituents (Table 3).

Analyses of GC–MS data exhibited five major chemical classes, namely, fatty acid and esters (40.5%), aromatic aldehydes (18.8%), sterols (12.5%), aromatic acids (11.3%), and phenolic compounds (10.1%). Rest of the compounds constituting 6.8% of the total constituents were placed as miscellaneous group (Fig. 1).

Characterization of the compounds

The GC–MS analyses furnished a broad idea about the nature of chemical constituents present in the active fraction. This was further confirmed by isolation and characterization of compounds from the same. Column chromatography of TCR-Et_2O afforded nine fractions (F-1 to F-9). Repeated chromatography of F-4 followed by HPLC on C-18 column using MeCN-H_2O as mobile phase afforded compound 1 (40 mg, 0.0044%). Similarly, repeated column chromatography followed by HPLC of F-5 and F-6 furnished compound 2 (35 mg, 0.0038%) and 3 (20 mg, 0.0022%), respectively. The structure of the compounds (Fig. 2) was determined by ^1H, ^{13}C, and mass spectral data analysis.

The HR-ESI-MS (+ve) spectra of compound **1** exhibited molecular ion peak at m/z 145.1232 for $[M+Na]^+$ corresponding to the molecular formula $C_7H_6O_2$. ^1H NMR spectrum of the compound showed the presence of two pair of protons at δ_H 7.81 and δ_H 7.03 (each 2H, d, J = 9Hz) along with an aldehyde proton at δ_H 9.86 (1H, s, -CHO). In addition, ^{13}C NMR spectra demonstrated signals for an aldehyde group (δ_C 190.1), two quaternary carbons at δ_C 163.1 and δ_C 129.4, and two olefin carbons at δ_C 131.9 and δ_C 115.7. The NMR data suggested the presence of a 1,4

Table 2: Antibiotic resistance profiles of MDR clinical isolates

Antibiotics	*S. aureus* (2413)	*Enterococcus* sp. (2449)	*E. coli* (2461)	*Acinetobacter* sp. (2457)	*Serratia* sp. (2442)
Amikacin	S	R	S	R	S
Ampicillin	-	-	R	-	R
Ciprofloxacin	S	R	R	R	R
Ceftriaxone	S	R	R	-	R
Chloramphenicol	-	-	R	-	R
Gentamicin	S	R	S	R	R
Imipenem	S	R	S	R	S
Levofloxacin	S	R	R	-	R
Meropenem	S	R	S	R	S
Nalidixic acid	-	-	R	-	-
Nitrofurantoin	-	-	S	-	-
Norfloxacin	-	-	R	-	-
Ofloxacin	S	R	R	-	R
Piperacillin	S	R	R	R	S
Vancomycin	S	R	-	-	-
Tobramycin	-	-	R	R	R

R: Resistant, S: Sensitive. *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli*, MDR: Multidrug resistant

Table 3: The list of chemical constituents from Et2O fraction of the roots of *T. curculioides* by GC–MS

Number of peak	Rt[a] (min)	Chemical constituents[b]	RI[c]	Composition (area%)	Formulas	MW
1	8.21	3-cyano-3-methyl-4-oxo-pentanoic acid amide	0	1.97	$C_7H_9NO_3$	55.15
2	10.25	3,5-dihydroxy-4-methoxybenzoic acid	1780	5.89	$C_8H_8O_5$	184.15
3	11.02	Phenol, 4-(methoxymethyl)	1190	1.58	$C_8H_{10}O_2$	138.16
4	12.26	4-hydroxybenzaldehyde	0	14.04	$C_7H_6O_2$	122.04
5	12.48	4-hydroxy-3-methoxybenzaldehyde	0	4.72	$C_8H_8O_3$	152.15
6	14.99	3-hydroxy-benzoic acid	0	1.42	$C_7H_6O_3$	138.12
7	15.39	3-hydroxy-4-methoxybenzoic acid	1560	3.96	$C_8H_8O_4$	168.15
8	19.14	Pentadecanoic acid	1869	3.44	$C_{15}H_{30}O_2$	242.40
9	19.85	Hexadecanoic acid, methyl ester	0	4.23	$C_{17}H_{34}O_2$	270.45
10	20.47	*n*-hexadecanoic acid	1968	14.17	$C_{16}H_{32}O_2$	256.42
11	21.08	Heptadecanoic acid, methyl ester	1968	2.01	$C_{18}H_{36}O_2$	284.48
12	21.60	Heptadecanoic acid	0	4.64	$C_{17}H_{34}O_2$	270.45
13	21.89	Phenol, 4,4'-methylene-bis	0	7.23	$C_{13}H_{12}O_2$	200.23
14	21.96	9-octadecenoic acid, methyl ester, (E)	2085	1.92	$C_{19}H_{36}O_2$	296.49
15	22.25	Methyl stearate	2077	2.58	$C_{19}H_{38}O_2$	298.50
16	22.49	9,12-octadecadienoic acid (Z)	2183	2.93	$C_{18}H_{32}O_2$	280.45
17	22.54	Cis-vaccenic acid	2175	1.18	$C_{18}H_{34}O_2$	282.46
18	22.79	Octadecanoic acid	2167	3.37	$C_{18}H_{36}O_2$	284.48
19	22.96	2-methoxy-6-[(pyridinylamino) methyl] phenol	0	1.33	$C_{16}H_{19}N_3O_3$	301.34
20	38.07	Dotriacontane	3202	1.10	$C_{32}H_{66}$	450.87
21	38.44	3-benzofuranmethanol, 2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxy-1-propenyl)-7-methoxy	3136	1.24	$C_{20}H_{22}O_6$	358.04
22	38.81	(R)-14-methyl-8-hexadecyn-1-ol	1907	1.07	$C_{17}H_{32}O$	252.1
23	39.16	Stigmasterol	2739	6.72	$C_{29}H_{48}O$	412.1
24	40.62	γ-sitosterol	2731	1.68	$C_{29}H_{50}O$	414.1
25	42.06	Z-verongulasterol	0	1.17	$C_{30}H_{50}O$	426.1
26	42.93	Stigmast-5-en-3-ol, (beta)	0	2.92	$C_{29}H_{50}O$	414.1
27	45.74	24-norursa-3,12-diene	0	1.50	$C_{29}H_{46}$	394.1

[a]Retention time, [b]compounds listed in order of elution, chemical constituents chromatographed on capillary column (SPB-5 fused-silica) and GC–MS detection, [c]retention indices measured relative to *n*-alkanes (C7-C33), under same experimental condition, compounds identified through NIST11/Wiley8 mass spec library. *T. curculioides: Tropidia curculioides,* GC–MS: Gas chromatography–mass spectrometry

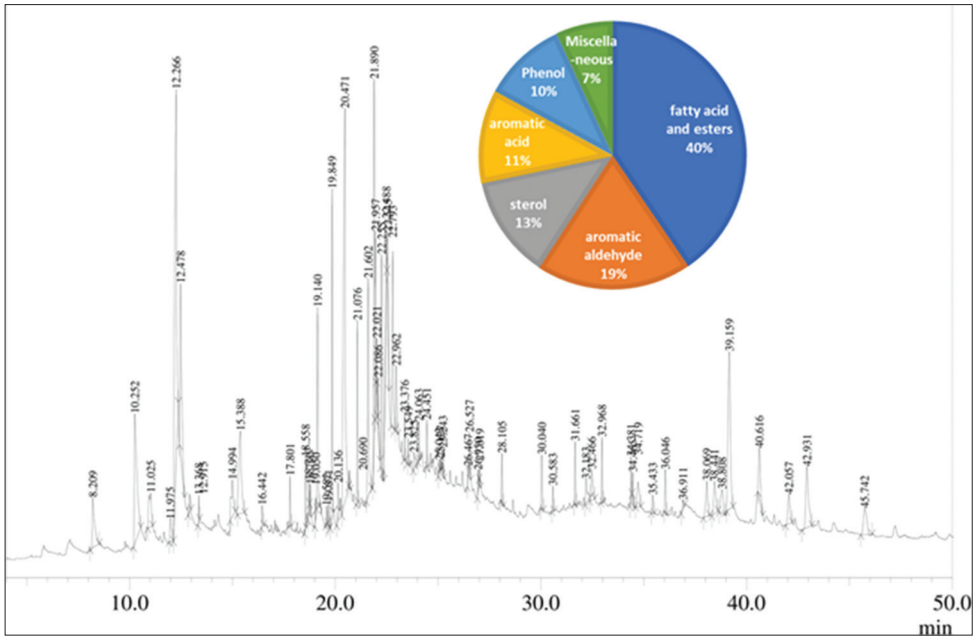

Fig. 1: Gas chromatography–mass spectrometry of Et_2O fraction of roots of *Tropidia curculioides* (compounds constituting more than 1% of the total constituents were classified on the basis of chemotypes and represented in pie chart)

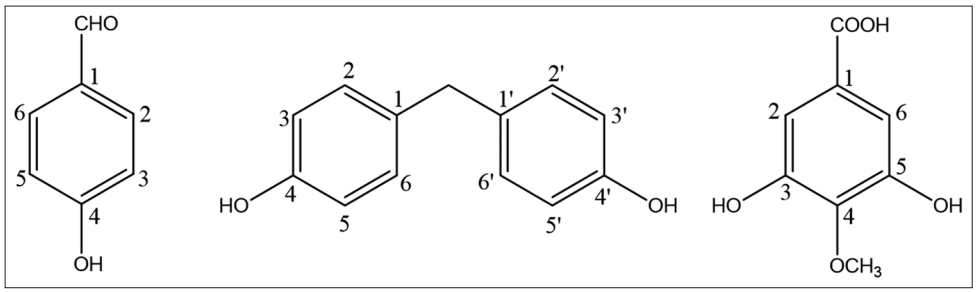

Fig. 2: Chemical structure of isolated compounds 1-3

substituted benzenoid moiety, with an aldehyde group at position 1 and a hydroxyl group at position 4. Finally, the structure was confirmed as p-hydroxybenzaldehyde by comparing the experimental data with that reported in literature [18].

The molecular formula of $C_{13}H_{12}O_2$ for compound 2 was suggested on the basis of molecular ion peak at m/z 223.0829 $[M + Na]^+$ on HR-ESI-MS (+ve) spectra. The ^1H NMR spectrum revealed two pair of aromatic protons at δ_H 7.00 and δ_H 6.73 (each 4H, d, J = 8.5 Hz) along with a methylene group appearing as a singlet at δ_H 3.76 (2H, s). Preliminary analyses of ^1H NMR spectra suggested the presence of two 1,4 disubstituted benzenoid moieties connected together by a methylene group. The partial structure analyses were further substantiated by ^{13}C NMR spectra which showed two quaternary carbons at δ_C 155.5 and δ_C 132.8, two aromatic –CH- at δ_C 129.6 and δ_C 115.0, and a methylene carbon at 39.8. The intensities of ^{13}C signals suggested the presence of two identical 1,4 disubstituted benzenoid moieties. Hence, on the basis of molecular ion peak and NMR data, the structure of the compound was established as 4,4'-methylenediphenol, also known as bisphenol-F. The structure was further confirmed by comparing NMR data with that reported in literature [19]. This is the first report of occurrence of the compound in TC.

The molecular formula of $C_8H_8O_5$ for compound 3 was indicated by the molecular ion peak at m/z 207.0429 $[M+Na]^+$ in HR-ESI-MS (+ve) spectra. The ^1H NMR spectra showed signals for a methoxyl group at δ_H 3.96 (3H, s) and two aromatic protons at δ_H 7.39 (2H, s), suggesting the presence of a 1,3,4,5 tetrasubstituted benzenoid moiety. Here, two meta coupled protons appeared as singlet instead of doublet as they were identical in nature. In commensurate to this observation, the ^{13}C NMR spectra showed signals for a carboxyl group (δ_C 171.0); four quaternary carbons [δ_C 146.67 (2) 139.9 and 120.1]; two aromatic –CH– (δ_C 107.06); and a methoxyl group (δ_C 56.45). Accordingly, the structure of the compound was suggested as 3,5-dihydroxy-4-methoxybenzoic acid which was confirmed by comparing the experimental data with that reported in literature [20]. Here, we report the occurrence of the compound for the 1st time in TC.

Antibacterial activity

The antibacterial activity of the compounds was evaluated by agar well diffusion method against two Gram-positive, namely, *S. aureus* (2413) and *Enterococcus* sp. (2449). Furthermore, the compounds were evaluated against three Gram-negative strains including *E. coli* (2461), *Acinetobacter* sp. (2457), and *Serratia* sp. (2442) at a concentration of 500 µg/ml of sample in 2% DMSO solution. The result of this study has been summarized in Table 4. A close look to the data identified compound 2 as most active against *E. coli* (Gram-negative) and *Enterococcus* sp. (Gram-positive) showing inhibition zones of 15.66 ± 0.47 and 14.66 ± 0.47 mm, respectively. The bactericidal activity of the compounds was expressed by MIC and MBC values (Table 5) which confirmed the potency of compound 2.

To understand the effect of the compounds on cell morphology, the *E. coli* cells were treated with MIC of compound 2. Treated cells were compared with untreated ones under SEM as presented in Fig. 3a-d.

Table 4: *In vitro* antibacterial activity expressed as diameter of zone of inhibition in mm of the compounds 1-3 against MDR clinical isolates

Compounds	MDR resistance clinical isolates of bacterial strains				
	Gram-positive strains		Gram-negative strains		
	S. aureus (2413)	*Enterococcus* sp. (2449)	*E. coli* (2461)	*Acinetobacter* sp. (2457)	*Serratia* sp. (2442)
1	7.33±0.57	11.66±0.47	12.33±0.47	7.33±0.57	7.33±0.57
2	7.33±0.57	14.66±0.47	15.66±0.47	7.00±0.00	7.33±0.57
3	7.33±0.57	8.66±0.57	9.33±0.47	7.33±0.57	7.33±0.57
Tetracycline	16.56±0.58	15.71±0.58	18.85±0.58	15.76±0.58	17.42±0.58
Gentamicin	15.76±0.58	15.71±0.58	15.79±0.58	14.56±0.58	11.56±0.58

Antimicrobial activity expressed as diameter of zone of inhibition in mm including 6 mm as diameter of the well. Value (mm)±S. D of three replicates. All compounds were tested at 500 µg/ml concentration. 30 µg tetracycline and gentamycin discs were used as positive control. *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli*, MDR: Multidrug resistant

Table 5: MICs and MBCs expressed in µg/ml of the most active compounds 1–3 against MDR resistance clinical isolates

Compounds	*Enterococcus* sp. (2449)		*E. coli* (2461)	
	MIC	MBC	MIC	MBC
1	125	125	62.5	125
2	31.25	62.5	15.62	31.25
3	125	125	62.5	125
Tetracycline	1.87	3.75	4	16
Gentamicin	0.93	1.87	1.56	3.12

E. coli: Escherichia coli, MDR: Multidrug resistant, MDR: Multidrug resistant, MIC: Minimum inhibitory concentration

DISCUSSION

In continuation, to our previous work, we conducted chemical profiling of the most active fraction by gas chromatography. The RIs of the compounds were determined in relation to the homologous series of *n*-alkanes (C_7-C_{33}). The constituents were identified by comparing their RIs with those available on GC–MS database. Analyses of GC–MS data exhibited five major chemical classes, namely, fatty acid and esters (40.5%), aromatic aldehydes (18.8%), sterols (12.5%), aromatic acids (11.3%), and phenolic compounds (10.1%). The GC–MS data provided an insight about the number and nature of phytoconstituents in the active fraction. In addition, the chemical classes presented less polar character. It is worthy to mention that a number of less polar fractions of high altitude and terrestrial plants also showed significant antimicrobial property [21]. Further, chromatography was conducted with the active fraction to identify marker/major compounds which afforded three compounds, namely, 4-hydroxy benzaldehyde (1), bisphenol F (2), and 3,5-dihydroxy-4-methoxybenzoic acid (3). The presence of the compounds in GC–MS spectra was confirmed by comparing the molecular mass and formula of the isolated compounds with the corresponding database representing each peak. The analysis suggested, peak 4 in Table 3 as compound 1, while peak 2 and 13 represented compound 3 and 2, respectively. The occurrence of the compounds in GC–MS spectra validated the efficacy of the analysis and they were established as marker compounds. Such validation by marker compounds is accepted as chemical fingerprinting and nurtures immense importance in commercialization of any plant extract.

The compounds were evaluated for antibacterial activity against two Gram-positive and three Gram-negative bacterial strains. However, the compounds showed activity against two strains only, namely, *E. coli* (Gram-negative) and *Enterococcus* sp. (Gram-positive). Compound 2 exhibited most significant activity followed by compound 1 and 3. The bacteriostatic and bactericidal activity observed by compound 2 against *E. coli* cells was comparable to that of standard drug tetracycline and gentamicin. The SEM performed with compound 2 against *E. coli* cells suggested that being a less polar compound it could penetrate through the cell membrane causing death of the bacterium. Overall, the study

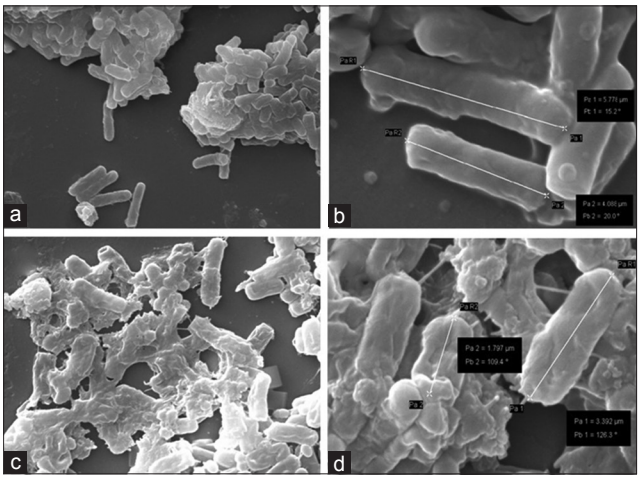

Fig.3: Scanning electron micrograph of *Escherichia coli* cells treated with compound 2. (a and b) the untreated cells appeared smooth, intact, and rod-shaped, (c and d) compound 2 treated cells are swollen up and have distorted cell morphology at magnification 8–30 KX

showed that each compound played a positive role in bactericidal activity of the active fraction and could be considered as marker compounds.

CONCLUSION

The present study demonstrated identification of three marker compounds along with chemical profiling of the most active Et_2O fraction of the roots of TC. The compounds exhibited significant antibacterial activity against *E. coli* and *Enterococcus* sp. SEM analysis also supported the role of the compound in causing disintegration of cell membrane. The outcome could be used as the chemical fingerprint of the active fraction with marker compounds. The result could further be verified through *in vivo* experiments to establish the efficacy of the compounds as drug candidates.

ACKNOWLEDGMENT

Authors acknowledge the Department of Biotechnology, Government of India, for financial support. Dr. Ajay Kumar, JNU, New Delhi, for NMR and GC–MS and Dr. A.K. Chauhan, Founder President, Amity University, for his continuous motivation and encouragement.

CONFLICTS OF INTERESTS

None of the authors of the above manuscript have declared any conflict of interest being named as an author on the manuscript.

AUTHOR'S CONTRIBUTION

NS performed extraction, isolation, purification, GC–MS analysis, and part of evaluation of antibacterial activity. ASA contributed in agar well

diffusion assay. SG planned the study, harmonized the investigation, and corrected the manuscript. All authors have read and approved the final manuscript.

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