Research Article
Volume 8:3, 2022

Journal of Pharmacognosy & Natural Products

ISSN: 2472-0992 **Open Access**

Chemical Constituents of the Stem Barks of *Podocarpus falcatus* and Evaluation for Antibacterial Activity

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Abstract

Chromatographic separation of equal ratio of CH_2Cl_2-MeOH extract of the stem barks of *Podocarpus falcatus* led to the isolation of three compounds namely: 4β-carboxy-19-nor-totarol (1), β-sitosterol (2), 4-hydroxybenzoic acid (3) and (E)-methyl-3, 4, 5-trimethylhex-2-enoate (4). The structures of the compounds were established based on the analysis of 1D and 2D NMR spectroscopic data. These compounds were reported from this plant for the first time. The crude extract and isolated compounds were evaluated for their antibacterial activity using disk diffusion assay method. The crude extract showed a strong activity against *S. aureus*. Compounds (1) and (2) showed a relatively moderate activity against *S. flexineri* and S. typhimurium respectively, whereas, compound (3) and (4) demonstrated a strong activity against *S. aureus*. The crude extract and the isolated compounds showed antibacterial activity as compared to the reference gentamycin indicating that this plant has potentially antibacterial properties.

Keywords: Antibacterial activity • *Podocarpus falcatus* • 4β-carboxy-19-nor-totarol • β-sitosterol • p-hydroxybenzoic acid; Methyl-3, 4, 5-trimethylhex-2-enoate

Introduction

The Podocarpus (family, Podocarpaceae) is one of the largest genera of all conifers of the family containing about 94 species distributed from south temperate zones through the tropical highlands, West India and Japan [1]. Species from this genus have been reported to produce cytotoxic nor- and bisnor-diterpenoid dilactones generally known as nagilactones or podolactones [2]. In addition, totarane-type diterpenes such as totarol and their dimers (macrophyllic acid) have also been reported from many species of Podocarpus, These compounds were considered to be chemical markers of the genus [3]. *Podocarpus falcatus* (Thunb.) is belongs to with the vernacular name zigiba. It is an evergreen, dioecious, medium to large-sized tree up to 60m tall widely distributed in Ethiopia, Kenya, Tanzania, Mozambique, South Africa, and Madagascar [1,2]. Besides its commercial and ecological importance, it has also known for its ethnobotanical values in Ethiopia. The roots of this plant were used as anticancer remedies [3,4]. Crushed and juice of the leaves were taken for vomiting, whereas the root dried powdered, mixed with water were taken for febrile illness. Decoction of the fruit serves as a tonic for cleaning the kidneys, lungs and stomach [5]. Despite of the wider use of this plant by the communities for medicinal purposes, the phytochemical and bioactivity information pertaining to the stem bark of this plant is limited. Therefore, as part of the search for new bioactive molecules from Ethiopian communities for medicinal purposes, the phytochemical and bioactivity information pertaining to the stem bark of this plant medicinal plants, the isolation of five compounds and antibacterial activities of the extract and the compounds were reported here.

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Received 16 February, 2022, Manuscript No. jpnp-22-54608; Editor Assigned: 18 February, 2022, PreQC No. P-54608; Reviewed: 12 March, 2022, QC No. Q-54608; Revised: 18 March, 2022, Manuscript No. R-54608; Published: 28 March, 2022, DOI: 10.37421/2472-0992.22. 8.175

Materials and Methods

General method

Solvents and reagents used for extraction and purification of the compounds are of analytical and HPLC grade. Analytical TLC pre-coated sheets ALUGRAM®Xtra SIL G/UV_{254} (layer: 0.20 mm silica gel 60 with fluorescent indicator $UV_{F254l/365}$) was used for purity analysis. For column chromatography, silica gel 100-200 mesh was used. Chromatograms were visualized on TLC by spraying with 10% H_2SO_4 and heating on hot plate. NMR spectra data were recorded on an Avance 600 MHz spectrometer (Bruker, Billerica, MA, USA, at 600 MHz (^{1}H) and 150 MHz (^{13}C). Chemical shifts were expressed in parts per million (ppm) downfield of Trimethylsilane (TMS) as internal reference for ^{1}H resonances, and referenced to the central peak of the appropriate duetrated solvent's resonances (residual $CDCl_3$, $(CD_3)_2CO$, MeOD and $(CD_3)_2SO$ at δ_H7.26, 2.20, 3.35, 2.52 for protons and δc79.16, 205.87, 49.77, 40.76 for carbons respectively). Whatman filter paper No.3, DMSO, Petri dishes and gentamycin were used in antibacterial analysis.

Plant materials

The stem bark of *P. falcatus* was collected from Horro Buluk, Horro Guduru Wollega zone, Oromia regional state, Ethiopia in September, 2020. The plant material was identified by botanist Dr. Fekadu Gurmessa and the voucher specimen (DAD002Pf) has been deposited in Wollega University Herbarium. The collected plant part was washed thoroughly with tap water and cut into smaller pieces and dried under shade.

Extraction and isolation

The powdered stem bark of *P. falcatus* (850 g) was extracted with equal ratio of CH_2Cl_2-MeOH (3 × 3L) at room temperature for 48 hr each with occasional shaking. The crude extract was filtered from marc using Whatman filter paper. The solvent was evaporated under reduced pressure using rotary evaporator at 40°C to yield (20 g, 2.7%) of dark brown crude extract. About 18 g of the extract was adsorbed on 25 g of silica gel and subjected to column chromatography, packed with silica gel (320 g). The column was eluted with hexane with increasing gradient of ethyl acetate to afford 30 major fractions ca. 100 ml each. Fractions with similar TLC profiles were combined together for further purification. Fractions 6–10 (2% EtOAc in hexane) were combined together 20 mg and refined by Sephadex LH-20 (eluting with CH_2Cl_2/MeOH; 1:1) afforded (1, 15 mg) while fractions 15-20 (3% EtOAc in hexane) showed

similar TLC profiles were combined together gave 18 mg and purified by washing with excess petroleum ether gave compound (2, 12 mg). Fractions 26-30 (6% EtOAc in hexane) showed similar spots combined together afforded 15 mg and purified further by Sephadex LH-20 (eluting with CH_2Cl_2/MeOH; 1:1) afforded compound (3, 10 mg) and compound (4, 8 mg).

Pathogenic bacterial strains

Five pathogenic bacterial strains, one gram-positive (*Staphylococcus aureus* (ATCC25923)) and four gram-negative (*Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella typhimurium* (ATCC13311), and *Shigella flexneri* (ATCC29903)) were obtained from the Department of Biology, Wollega University and used for evaluation of antibacterial activities.

Antibacterial activity assay

Antibacterial activities of the methanol extract and isolated compounds were tested against five bacterial strains using the disc diffusion method as described in [6] with slight modification. The test solutions were prepared with known weight of crude extract (0.0015 g/mL) and isolated compounds were dissolved in 1 ml of DMSO. A 0.6 mm diameter sterile Whatman test disks were placed on the surface of the inoculated Mueller Hinton Agar in a 90 mm petridishes and then 0.0015g/mL of the crude extract and the isolated compound (1, 2, 3 and 4) were applied onto the disks. The plates were incubated at 370C for 24 hr. The antibacterial activity was determined by measuring the zone of growth inhibition surrounding the disks. Gentamycin (10 µg) and DMSO were used as the positive and negative controls, respectively. The test samples were allowed to diffuse for 30 minutes and the plates were then kept in an incubator at 37°C for 48 hr [7]. The experiments were carried out in triplicate and the mean of the diameter of the inhibition zones were calculated. Antibacterial inhibition activities were measured against the standard.

Results and Discussion

The stem bark of *P. falcatus* was exhaustively extracted with equal ratio of CH_2Cl_2-MeOH solvent combination. The extract was subjected to column chromatography on silica gel followed by purification on Sephadex LH-20 and afforded four compounds 1-4 (Figure 1).

Compound 1 was isolated as white powder with melting points of 175-177°C. The 1HNMR spectrum showed 12 signals, with a highly downfield shifted proton signal at 12.07 assigned to carboxylic acidic proton whereas, proton signals at 6.96 (1H, d, 8.6 Hz) and 6.41 (1H,d, 8.6 Hz) assigned to ortho-coupled aromatic protons (H-11, H-12), two overlapped doublets at 1.33 (6H, d, 7.1 Hz) were an isopropyl moiety and the rest proton signals correspond to non-aromatic protons. ^{13}C NMR spectrum showed six aromatic carbon signals resonating at δC 149.5, 142.1, 134.6, 133.4, 126.6 and 113.4 assigned to C-13, C-9, C-14, C-8, C-11 and C-12 carbons, respectively. The remaining protons and carbons were assigned on the basis of 2D-NMR data, notably, HSQC and HMBC. The COSY spectrum showed coupling between H-2/H-3, H-5/H-6. The HSQC spectrum showed the presence of twelve signals suggested that compound 1 possesses four saturated methylene groups, although their respective proton signals could not be fully determined due to their significant overlapping. The HMBC correlations between methyl protons H-16 and H-17 with C-14 indicated that the isopropyl group is attached to the aromatic ring at C-14. Moreover, a cross peak in the HMBC spectrum between H-18 and δC at 178.9 ppm, confirmed the presence of a carboxylic acid group attached at C-4. Similarly, C-4 at δC 43.4 was assigned on the basis of a HMBC cross peak (Figure 2) to H-18. The above evidence was in agreement with a totarane-type diterpenes skeleton and corresponded to the known compound 4β-carboxy-19-nortotarol, which matched with the reported data for this compound [8] (Table 1).

Compound 2 was isolated as a white powder with melting points of 134-136˚C. The structure of this compound was identified to be β-sitosterol using ^{1}H and ^{13}C spectra data. The ^{1}H NMR spectrum showed an olefinic proton at δH 3.54 (1H, tdd, 11.2, 6.5, 4.6 Hz) corresponds H-6 and oxymethine proton at δH 3.54 (1H, tdd, 11.2, 6.5, 4.6 Hz) for H-3. It also showed proton signals at δH 0.69 (3H, s), 1.02 (3H, s), 0.94 (3H,d, 6.5 Hz), 0.84 (3H, d, 6.8 Hz), 0.81 (3H, d, 6.8 Hz), 0.85 (3H, t, 7.2 Hz) for six methyl groups and were assigned to H-18, H-19, H-21, H-26, H-27 and H-29, respectively.

The ^{13}C NMR spectrum showed signals for 29 carbon atoms including signals for six methyl (19.8, 19.4, 19.1, 18.8, 11.9 and 11.8), eleven methylene (δc 42.2, 39.8, 37.3, 33.9, 31.9, 31.6, 28.3, 26.1, 24.3, 23.1 and 21.1), nine methine (δc 121.7, 71.8, 56.8, 56.1, 50.1, 45.8, 36.2, 31.9 and 29.2) and three quaternary (δc 140.7, 42.3 and 36.5) carbon atoms. The recognizable signals at 140.9 (C-5) and 121.9 (C-6) are typical alkenes double bonds. The signals at δ 19.2 and 12.1 correspond to angular methyl carbon atoms (C-19) and (C-18) respectively. Signal at 71.9 is assignable to the β-hydroxyl group attached to the carbon at (C-3). Therefore, based on spectral data which is in agreement with existing literature reported for β-sitosterol [9,10] (Table 2).

Compound 3 was isolated as white amorphous with melting points of 214-2150C. The 1H-NMRspectrum showed four proton signals. Signals at δ_H 7.78 (2H, d, 8.5Hz) assigned to two overlapping aromatic protons (H-2, H-6) and signal at 6.82 (2H, d, 8.6Hz) allocated to two overlapping protons (H-3, H-5). Whereas, proton signals at 12.41 (1H; s) corresponds to carboxylic acid proton and signal at 10.22 (1H, s) assigned to OH proton.

The ^{13}C NMR spectrum showed seven carbons signals corresponding to four aromatic methine at δc 131.9 assigned to two overlapping carbons (C-2, C-6) and at 115.6 assigned to two overlapping carbons (C-3, C-5), three quaternary, one for carboxylic acid at 167.6 (C-7),121.8 (C-1) and 162.1 (C-4). The 2D experiment COSY and HSQC spectra of compound 3 allowed, respectively, the detection of the scalar couplings of the protons and connectivity of each proton to directly linked carbon atom. The COSY spectrum shows coupling between H-2/ H-3 and H-5/H-6. The HSQC shows

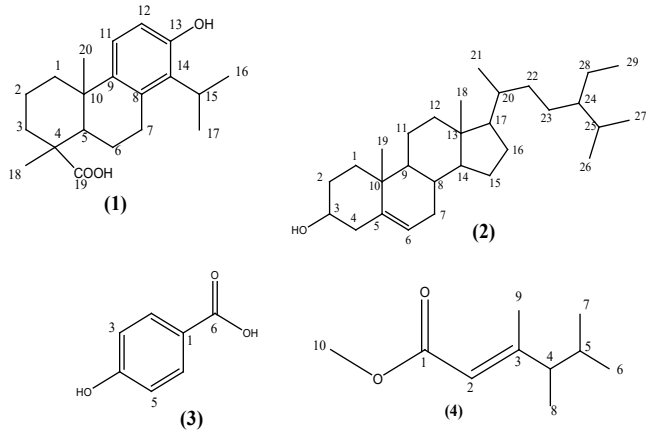

Figure 1. Structure of isolated compounds from stem bark of *P. falcatus*.

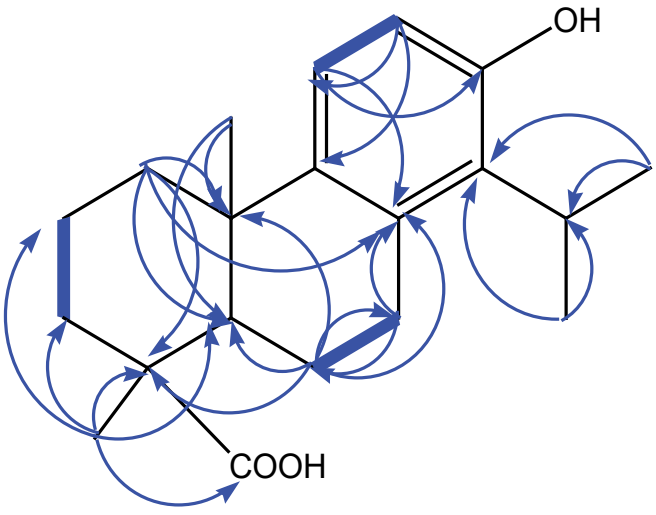

Figure 2. COSY and HMBC correlations of compound 1.

Table 1.H and 13C NMR spectra data of compound (1).

Carbon No.	Appearance	^{13}C NMR	δH (int., mult., J in Hz)	HMBC
1	CH_2	40.5	2.53 (1H,m),2.17 (1H,m)	C-5, C-7, C-8, C-10
2	CH_2	20.4	1.51 (1H,overlap),1.38 (1H,m)	-
3	CH_2	37.5	2.09 (1H,overlap), 1.02 (1H,m)	-
4	C	43.4	-	-
5	CH	51.7	1.41 (1H,dd, 12.3,1.5 Hz)	-
6	CH_2	21.5	2.18 (1H,br dd, 12.3, 5.1 Hz) 2.16 (1H,ddd, 12.3, 6.7,5.1,1.6 Hz)	C-5, C-7,C-8, C 10
7	CH_2	29.9	2.91 (1H,dd, 16.7, 4.8 Hz) 2.60 (1H,ddd,16.7, 12.4, 6.5 Hz)	C-5,C-6, C-8,C-9 C-5, C-8, C-9, C-10
8	C	133.4	-	-
9	C	142.1	-	-
10	C	38.5	-	-
11	CH	126.6	6.96 (1H,d, 8.6 Hz)	C-8, C-13
12	CH	113.4	6.41 (1H,d, 8.6 Hz)	C-9, C-11
13	C	149.5	-	-
14	C	134.6	-	-
15	CH	28.8	3.17 (1H,m)	-
16	CH_3	20.6	1.33 (3H,d, 7.1 Hz)	C-14, C-15,C-17
17	CH_3	21.1	1.33 (3H,d, 7.1 Hz)	C-14, C-15,C-16
18	CH_3	21.2	1.23 (3H,s,)	C-2, C-3, C-4, C-5, C19
19	C	178.9	-	-
20	CH_3	23.7	1.06 (3H,s)	C-1,C-5, C-9, C-10

Table 2. ^{1}H and ^{13}C NMR spectra data of compound (2) and β-sitosterol.

Carbon No.	Experimental ^{13}C NMR	^{1}H NMR	Literature ^{13}C NMR	^{1}H NMR	Appearance
1	37.4	-	37.28	-	CH_2
2	32.1	-	31.69	-	CH_2
3	71.9	3.54 (tt, 1H)	71.82	3.53 (m,1H)	CH
4	42.5	-	42.33	-	CH_2
5	140.9	-	140.70	-	C
6	121.9	5.37 (dd,1H)	121.72	5.36 (dd,1H)	CH
7	31.8	-	31.69	-	CH_2
8	32.1	-	31.93	-	CH
9	50.3	-	50.17	-	CH
10	36.7	-	36.52	-	C
11	21.2	-	21.10	-	CH_2
12	39.9	-	39.80	-	CH_2
13	42.5	-	42.33	-	C
14	56.2	-	56.79	-	CH
15	24.5	-	24.57	-	CH_2
16	28.4	-	28.25	-	CH_2
17	56.9	-	56.09	-	CH
18	12.1	0.70 (s, 3H)	11.86	0.63 (s, 3H)	CH_3
19	19.2	1.03 (s, 3H)	19.40	1.01 (s, 3H)	CH_3
20	36.3		32.52	-	CH
21	18.9	0.94 (d, 3H)	18.79	0.93 (s, 3H)	CH_3
22	34.1	-	33.98	-	CH_2
23	26.2	-	26.14	-	CH_2
24	45.9	-	45.88	-	CH
25	29.3	-	28.91	-	CH
26	19.9	0.84 (3H , d, 6.4Hz)	19.80	0.84 (s, 3H)	CH_3
27	19.6	0.88 (3H, d, 6.4Hz)	18.79	0.83 (s, 3H)	CH_3
28	23.2	-	23.10	-	CH_2
29	12.0	0.84 (s, 3H)	11.99	0.81 (s, 3H)	CH_3

two protonated carbons at 7.79 (H-2 and H-6) linked with 131.9 (C-2 and C-6) and 6.82 (H-3 and H-5) linked with 115.6 (C-3 and C-5). The HMBC spectrum reveals the correlation between the cross peak δH 7.79 (H-2and H-6) correlated with 115.6 (C-3 and C-5), 131.9 (C-4) and 167.6 (C-7), proton at 6.82 (H-3 and H-5) correlated with 121.8 (C-1), 115.6 (C-3), 162.1 (C-4) and proton at 10.22 (OH proton) correlated with 115.6 (C-3 and C-5) and 162.1 (C-4) that reveals the position of OH at C-4. Based on the basis of spectral analysis of 1D and 2D NMR compound 3 was identified as 4-hydroxybenzoic acid as shown in Figure 3.

Compound 4 was isolated as white powder compound. The 1 HNMR spectrum of the compound displayed one olefinic protons at δH 8.11 (1H, s)

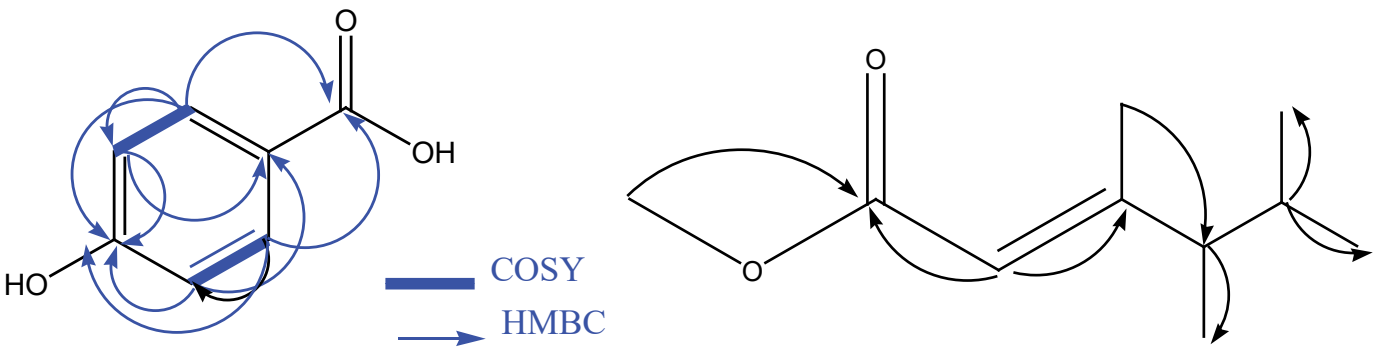

Figure 3. COSY and HMBC correlations of compound 3 and 4.

Table 3. ^{1}H and ^{13}C NMR spectra data for compound (3) and (4).

Compound 3			Compound 4				
Carbon No.	**^{13}C NMR**	**δ_H (m, J in Hz)**	**Carbon No.**	**^{13}C NMR**	**δ_H (m, J in Hz)**	**Appearance**	**HMBC**
1	121.8	-	1	165.3	-	C	-
2 & 6	131.9	7.79 (2H, d, 8.5 Hz)	2	129.7	8.11 (1H, s)	CH	C-1, C-3
3 & 5	115.6	6.82 (2H, d ,8.6 Hz)	3	133.8	-	C	
4	162.1	-	4	33.3	2.38 (1H, m)	CH	C-8
7	167.6	-	5	31.6	1.28 (1H, m)	CH	C-6, C-7
			6	29.7	1.26 (3H, d, 6.4 Hz)	CH_3	-
			7	29.7	1.26 (3H, d, 6.4 Hz)	CH_3	-
			8	22.7	1.63 (3H, d, 6.2 Hz)	CH_3	-
			9	14.1	0.90 (3H, s)	CH_3	C-4
			10	62.8	4.71 (3H,s)	CH_3	C-1

Table 4. Antibacterial activity test for crude and isolated compounds from *P. falcatus*. ±

Crude Extract/Isolated Compound	Bacteria Inhibition Zone (mm)				
	E.coli	*S. aureus*	*S. flexineri*	*S. typhimurium*	*P. aeruginosa*
PfME	10.03 ± 0.03	23.03 ± 0.05	19.07 ± 0.02	15.23 ± 0.21	13.13 ± 0.04
1	7.1 ± 0.24	7.33 ± 0.47	10.16 ± 0.24	7.33 ± 0.47	7.66 ± 0.24
2	8.00 ± 0.41	7.16 ± 0.24	8.16 ± 0.24	8.67 ± 0.47	8.33 ± 0.24
3	8.06 ± 0.09	22.13 ± 0.12	11.23 ± 0.33	9.22 ± 0.47	8.10 ± 0.08
4	8.36 ± 0.02	9.60 ± 0.22	7.10 ± 0.01	9.15 ± 0.20	7.25 ± 0.20
Gentamycin	22.13 ± 0.05	19.5 ± 0.04	20.03 ± 0.05	20.10 ± 0.03	14.06 ± 0.06
DMSO	-	-	-	-	-

assigned to (H-2), two saturated methines at 2.38 (1H, m, H-4) and 1.28 (1H, m, H-5). The spectrum also showed three methyl protons at 1.32 (3H, d, H-8), 1.26 (3H, d, H-6/7), 0.90 (3H, s, H-9) and one methoxy proton at 4.71 (3H, s, H-10). The ^{13}C NMR showed a carbonyl resonance at δc 165.3 (C-1), olefinic carbons at 133.8 (C-3), 129.7 (C-2) and as well as five signals assignable at 62.8 (C-10), 33 (C-4), 31 (C-5), 29.7 (C-6/7), 22.7 (C-8) and 14.1 (C-9). The DEPT spectrum showed three methine carbons at 129.7 (C-2), 33 (C-4), 31 (C-5), four methyl carbons at 62.8 (C-10), 29.7 (C-6/7), 22.7 (C-8) and 14.1 (C-9). The COSY spectrum displayed three coupling protons H-4/H-8, H-4/H-5 and H-5/H-6/7. The HMBC spectrum showed the correlation between carbons and protons were shown in Figure 3. Based on the spectroscopic data analysis, compound 4 was identified as (E)-methyl 3, 4, 5-trimethylhex-2-enoate (Table 3).

The antibacterial activity of the crude extract and the isolated compounds were determined by the disk diffusion method against different bacteria. The bacterial strains were *E. coli, S. aureus, S. flexineri, S. typhimurium* and *P. aeruginosa*. The results of the diameters of inhibition zones are shown in Table 4.

The antibacterial activity test result showed varying degree of inhibition of the growth of bacterial strains. The crude extract showed considerable activity on both Gram-positive and Gram-negative bacterial strains with zone of inhibition ranging from 10.03 ± 0.03-23.03 ± 0.05 mm with the highest activity (23.03 ± 0.05 mm) was observed against *S. aureus*, which is even greater than that of the reference drug (gentamycin, 19.5 ± 0.04 mm) against the same strain Whereas, the isolated compounds showed moderate activities against all the test strains. This variation in inhibition of the bacterial growth by the crude extract and isolated compounds could be related to the synergetic effects of the various kinds of compounds present in the crude extracts or the minor compounds in the extract that could showed this activity have not been isolated. In general, the remarkable activities of the crude extract from this medicinal plant (*P. falcatus*) support the traditional use of the plant and could be used as a potential candidate in the development of novel antibacterial agents.

Conclusion

Phytochemical investigation of DCM-MeOH extract of stem bark *P. falcatus* led to the isolation of four compounds 4β-carboxy-19-nor-totarol (1), β-sitosterol (2),4-hydroxybenzoic acid (3) and (E)-methyl 3, 4, 5-trimethylhex-2-enoate (4)and their structures were established on the basis of their ^{1}H and ^{13}C NMR spectral data and comparing with existing literature. Compound 3 is reported for the first time from the genus *Podocarpus* and compound 4 was reported for the first time. The crude extract showed strong activity against *S. aureus*. Whereas, the isolated compounds showed moderate activity against all test strains. The antibacterial activity displayed by the extract support the traditional use of this plant against various ailments caused by bacteria. Further comprehensive evaluations including *in vivo* activity and cytotoxicity tests could be done for conclusive decision on potential candidacy of the plant for formulation and medicinal uses.

Conflict of Interests

The authors declare that they have no conflict of interest.

Acknowledgment

Mr. Desalegn. A is thankful to Wollega University, Ethiopia for material and financial support for his PhD study.

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How to cite this article: Deresa, Desalegn Abebe, Zelalem Abdissa, Getahun Tadesse Gurmessa and Negera Abdissa. "Chemical Constituents of the Stem Barks of Podocarpus falcatus and Evaluation for Antibacterial Activity" J Pharmacogn Nat Prod 8 (2022): 175.