ORIGINAL ARTICLE

Chemical constituents of *Securidaca longipedunculata* root bark and evaluation of their antibacterial activities

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ABSTRACT

The Bioassay-guided sequential extraction and column chromatographic separation of the root bark of *Securidaca longipedunculata* led to the isolation of three phenolic compounds; two benzoic acid derivatives (1 and 2) and a xanthone (3). The structures of these compounds were established based on the spectroscopic analyses including NMR (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC, and HMBC), MS, IR, UV-Vis and comparison with reported literature. In an in vitro antibacterial assay of the crude extracts and the pure compounds against four bacterial strains (*S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853), almost all crude extracts showed significant antibacterial activity with acetone extract exhibited highest activity against *B. subtilis* and *P. aeruginosa*. Whereas, only compound 3 showed activity against the tested bacterial strains with the highest activity observed against *B. subtilis* with inhibition zone diameter 15 mm, which is comparable to that of the reference drug (gentamycin).

Keywords: Antibacterial activity; Extract; Medicinal plants; *Securidaca longipedunculata*

INTRODUCTION

Securidaca (family Polygalaceae) is a widespread genus comprising of about 80 species widely distributed in woodlands and arid of tropical and subtropical Africa (Wallnofer, 1998; Yang *et al.,* 2001). It is known to contain a large number of chemically complex bioactive compounds including xanthones, flavonoids, terpenes, coumarins and steroids (Simeon *et al.,* 2017). *Securidaca longipedunculata* is known with its vernacular name "*king of medicines*" as it is used for almost every conceivable ailment. It is commonly visited by traditional healers for the treatment of sexually transmitted disease, fungal infection, diabetes, and wounds in Ethiopia (Semenya and Potgieter, 2013). However, the phytochemical investigation and its antimicrobial activities have not been exhaustively carried out. Thus, in our continuing efforts at identifying medicinal plants with antimicrobial activity and establishing scientific evidence for activity,

the claimed potency of the traditional use of this plant stimulated our interest to establish the microbial activity of the phytochemicals from the roots barks of *S. longipedunculata.*

MATERIALS AND METHODS

General information

Analytical grade solvents (*n*-hexane, petroleum ether, chloroform, acetone, ethyl acetate and methanol) were used for successive extraction and column elution. Column chromatography was performed on oxalic acid impregnated silica gel, the silica gel was deactivated by mixing 1 kg silica gel 60-120 mesh with 3% of oxalic acid (30 g in 1L of distilled water) and allowed to stand for 30 min, filtered and dried in an oven at 100 ^{O}C for 45 min. Standard antibiotic drug (gentamycin), Mueller Hinton agar, nutrient agar and saline solution were used as a culture medium during antibacterial test. Thin layer chromatography (TLC) was performed on pre-coated silica gel on aluminium foil and viewed under UV chamber 254 and 365 nm (LF-260.LS, EEC) for detection of spot. Infrared (IR) spectra were measured on Perkin-Elmer IR spectrophotometer. The 1D (^{1}H-NMR, ^{13}C-NMR and DEPT-135) and 2D (COSY, HSQC and HMBC) spectra were recorded on Bruker Avance NMR in deuterated solvent.

Collection of plant materials

The root bark of *s. longipedunculata* was collected from western Ethiopia, Oromia regional state, Kelem Wollega Zone, Sayyo district (located at 8°27'15.1"n 34°39'16.6"e), which is about 636 km away from Addis Ababa in march 2017. It was collected from none protected area since it is a wild plant. The plant material was identified at Wollega University, Biology Department and the voucher specimen (tt1/2017) has been deposited. The plant material was air-dried and powdered so as to allow the penetration of the solvents during the extraction.

Extraction and isolation

The air-dried roots bark (1 kg) of *S. longipedunculata* was milled into powder and then sequentially extracted with equal volume (2.5 L) of hexane, chloroform, acetone and methanol four times for 24 h each at room temperature. The crude extracts of each solvent were filtered with Whatmann No.1 filter paper and then concentrated under reduced pressure using rotary evaporator at 40 °C to yield 5 g (0.53%), 12.5 g (1.32%), 10 g (1.05%), 40 g (4.23%) of n-hexane, chloroform, acetone, and methanol crude extracts, respectively. The acetone extract (8 g) was subjected to column chromatography on oxalic acid impregnated silica gel (200 g) eluting with petroleum ether containing increasing amount of ethyl acetate to afford 210 fractions *ca*. 20 mL each.

Fractions 42-56 (4% ethyl acetate in n-hexane) showed similar TLC profiles (*Rf* value) and were combined and further purified using column chromatography (column size: 40 cm length and 2 cm diameter) to give 3, 4-dimethoxy-7-hydroxyxanthone (3, 20 mg). Fractions 99-101 (12% ethyl acetate in n-hexane) were combined and further purified in the same way to give 3-hydroxy benzoic acid (1, 12 mg). Fractions 147-162 (30% ethyl acetate in n-hexane) were also combined and allowed to precipitate in petroleum ether, which was washed with chloroform to give 3-hydroxy- 4-methoxy benzoic acid (2, 13 mg).

Antibacterial assay (agar diffusion test)

The crude extracts and isolated compound were subjected to antibacterial activity test. The antibacterial activity test was done using disc diffusion method following

standard procedures Singh *et al.,* 2002). The test solution of the extracts were prepared by dissolving 200 mg of crude extract in 1 mL of dimethyl sulfoxide (DMSO) to get the final stock concentration of 200 mg mL^{-1} solution of the test sample. The same concentration of both DMSO (negative) and gentamycin (positive) control were used. The *in vitro* antibacterial activity of the crude extracts and isolated compounds were determined against four bacterial strains (*S. aureus*, *E. coli*, *P. aeruginosa* and *B. subtilis*). The strains were first activated at 37 ˚C for 24 h prior to inoculation on to the nutrient agar. Some colonies of the cultured bacteria were transferred into a nutrient broth and incubated until adequate growth obtained. The cell density was adjusted to turbidity equivalence of 0.5 on McFarland scale through dilution of the activated culture using sterile nutrient broth. The bacterial culture was then marked onto Muller Hinton agar plate with a sterile cotton swab to obtain a uniform thick lawn of growth. Sterile paper discs (6 mm diameter, Whatmann No.3) were separately soaked in a pre-prepared crude extracts (whose stock solutions were prepared at concentration of 200 mg mL^{-1} by dissolving 200 mg of the crude extracts separately in DMSO) and pure compounds before aseptically placed on an already inoculated Muller Hinton agar plate. It was allowed to diffuse for five minutes at ambient temperature and then incubated at 37 ˚C for 24 h. Finally, the antibacterial activity was evaluated by measuring diameter of zone of growth inhibitions (mm) using transparent ruler after 24 h of incubation.

RESULTS AND DISCUSSION

The root bark of *S. longipedunculata* was sequentially extracted with hexane, chloroform, acetone and methanol. The acetone extract was subjected to column chromatography on oxalic acid impregnated silica gel for further purification following its superior antibacterial activity in comparison to the other extracts, and has resulted three compounds 1-3 (Fig. 1).

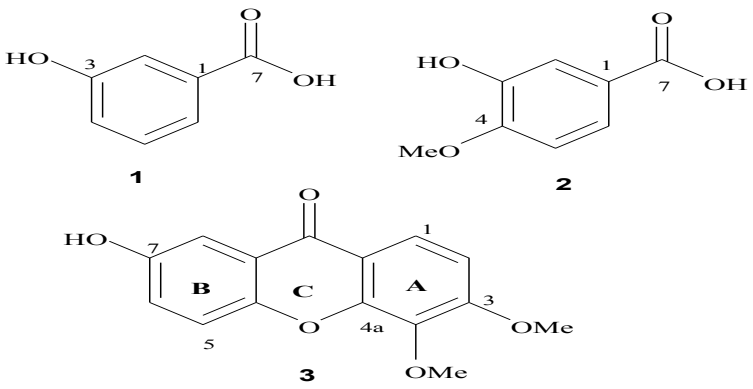

Fig. 1. Structures of the Isolated Compounds

Compound 1 was isolated as a white solid. The positive mode ESI-MS spectrum showed molecular ion peak of *m/z* 161 $[M+Na]^+$, corresponding to the molecular formula of $C_7H_6O_3$, which indicated five degrees of unsaturation. Its IR spectral analysis showed a strong absorption band in the region of 3238 cm^{-1} and 1659 cm^{-1} for the hydroxyl (OH) and carbonyl (C=O) stretching respectively.

The ^1H NMR spectrum (400 MHz) showed signals for six protons (Table 1) including the downfield shifted exchangeable signal at δ_H 11.05 for carboxylic acid group. The presence of a carbonyl carbon signal at δ_C 172.7 in ^{13}C NMR spectrum confirmed the existence of carboxylic acid group. The presence of a *meta* coupled aromatic proton at δ_H 6.96 (d, J = 2.4 Hz) for H-2 and three mutually coupled aromatic protons at δ_H 6.94 (dd, J = 8.2, 2.4 Hz), 7.62 (dd, J = 8.2, 7.6 Hz), 7.55 (dd, J = 7.6, 2.4 Hz) for proton H-4, H-5 and H-6, respectively, confirmed by HMBC and COSY analyses has indicated the presence of disubstituted aromatic ring. The ^{13}C NMR spectrum (Table 1) showed signals for seven carbon atoms, accounted for four methine, three quaternary (one is for acid carbonyl) carbon atoms. The position of the carboxylic acid (δ_H 11.05; δ_C 172.7) and hydroxyl groups were established at C-1 (δ_C 136.1 and C-3 (δ_C 163.0), respectively based on the HMBC analysis. Therefore, based on these spectroscopic data, compound 1 was identified as 3-hydroxy benzoic acid, previously reported from grape fruit (*Citrus paradisi*), olive oil (*Olea europaea*) and medlar fruit (*Mespilus germanica*) (Shahriar, 2010).

Compound **2** was isolated as a white crystalline solid. The EI-MS showed *quasi*-molecular ion $[M+Na]^+$ at *m/z* 191, consistent with the molecular formula of $C_8H_8O_4$.The benzoic acid nature of the compound was also deduced from the ^{13}C NMR spectrum (Table 1) which displayed eight non-equivalent carbon atoms, of which one was down-field shifted (δ_C 166.5) for carboxylic acid carbon. The ^1H and ^{13}C NMR spectra were similar to that of compound **1** except for the presence of extra methoxyl group (δ_H 3.94; δ_C 55.4). The molecular mass of compound 2 (168) was found to be higher by 16 amu than that of compound **1** (138) supporting that compound **2** is a methoxyl derivative of 1. This was further confirmed by the presence of two oxygenated quaternary carbon signals at δ_C 147.1 and 151.2 in the ^{13}C NMR spectrum. Interestingly, the upfield shifted chemical shift of these two carbon signals for the oxygenated aromatic carbon indicating that the methoxyl and hydroxyl groups are *ortho* to one other. Furthermore, the presence of two *ortho* coupled aromatic protons at δ_H 7.58 and 6.93 (d, J = 8.2 Hz) and a *pseudo* singlet aromatic proton at δ_H 7.62 established the position of the methoxyl group (δ_H 3.94; δ_C 55.4) at C-4 (δ_C 151.2)

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectral data for compounds 1, 2 and 3 in acetone-d_6

Position	1		2		3	
	δ_H (m, *J* in Hz)	**δ_C**	**δ_H (m, *J* in Hz)**	**δ_C**	**δ_H (m, *J* in Hz)**	**δ_C**
1	-	136.7	-	122.0	7.59 (d, 7.6)	123.1
1a	-	-	-	-	-	115.8
2	6.96 (d, 2.4)	113.2	7.62 (s)	112.5	7.36 (d, 7.6)	113.7
3	-	163.0	-	147.1	-	150.5
4	6.94 (dd, 8.2, 2.4)	118.1	-	151.2	-	146.4
4a	-	-	-	-	-	145.0
5	7.62 (dd, 8.2, 7.6)	131.2	6.93 (d, 8.2)	114.6	7.45 (d, 8.2)	119.0
5a	-	-	-	-	-	149.9
6	7.55 (dd, 7.6, 2.4)	119.9	7.58 (d, 8.2)	124.0	7.24 (d, 8.2, 2.4)	123.9
7	11.05 (brs)	172.7	-	166.5	-	155.9
8	-	-	-	-	7.40 (d, 2.4)	105.8
8a	-	-	-	-	-	122.3
C=O	-	-	-	-	-	175.0
3-OMe	-	-	-	-	3.92 (s)	55.2
4-OMe	-	-	3.94 (s)	55.4	3.91(s)	61.3

Thus, based on the above spectroscopic evidence the compound was characterized as 3-hydroxy- 4-methoxy benzoic acid (2) which had previously been reported from rhizomes of *Curcuma singularis* species (Nguyen, 2016).

Compound 3 was isolated as a yellow amorphous solid from 4% ethyl acetate in n-hexane. The IR spectrum showed absorption bands at 3391, 1659 and 1476 cm^{-1} corresponding to the stretching vibration peak of the hydroxyl (OH), conjugated carbonyl (C=O) groups, and aromatic moieties, respectively. The UV-Vis spectrum (in MeOH showing absorption bands λ_{max} at 244, 263, 319, 374 nm and the NMR spectra (Table 1) suggested a xanthone derivative. ^{1}H NMR spectrum displayed proton resonances for two sets of aromatic spin system; a three-proton *ortho-meta* spin system resonating at δ_H 7.45 (d, J = 8.2 Hz), 7.24 (dd, J = 8.2, 2.4 Hz) and 7.40 (d, J = 2.4 Hz) for ring B and an AB spin system at δ_H 7.59 (d, J = 7.6 Hz) and 7.36 (d, J = 7.6 Hz) for ring A. It also displayed two singlet protons at δ_H 3.91 and 3.92 for two methoxyl groups. The ^{13}C NMR displayed signals for sixteen carbon atoms corresponding to the highly downfield shifted carbon signals at δ_C 175.0 for carbonyl group, five oxygenated quaternary aromatic carbons at δ_C 150.5, 146.4, 145.0, 149.9, 155.9. Five methine signal at δ_C 123.1, 113.7, 119.0, 123.9 and 105.8, and two signal (δ_C 55.2 and 61.3) for the two methoxy groups. Hence, based on the spectroscopic data and comparison of this data with the related literature (Yang *et al*., 2003), the compound was identified to be 3, 4-dimethoxy-7-hydroxyxanthone (Fig. 1), which is a common metabolite in the genus.

The crude extracts (n-hexane, chloroform, acetone and methanol) and the isolated compounds (1 - 3) were *in vitro* assayed against four bacterial strains (Table 2). The activities of the tested samples were comparatively assessed by the diameter of zone of inhibition in millimetres and zones of inhibition more than 6 mm were taken into consideration.

The crude extracts showed superior inhibitory activities against the four tested bacterial strains, with the highest activity observed for acetone and methanol extract. The growth inhibitory potential of these extract were even greater than the reference drug (gentamycin). Acetone displayed potent activity against *p. aeruginosa, B. subtilus, E. coli, and S. aureus* bacterial strain with the inhibition zone of 32, 32, 30 and 26 mm, respectively, whereas, methanol extract showed greater inhibition zone against *E. coli* and *P. aeruginosa.* However, n-hexane extract showed the lowest activity against all the test strains as compared to positive control (gentamycin). The low activity of hexane extract could be related to the absence of polar bioactive secondary metabolites, since hexane interact only with non-polar organic molecules.

Table 2. *In vitro* antibacterial activities of the extracts and isolated

Samples	Growth Inhibition (mm)			
	Bacterial Strains			
	B. subtilis	S. aureus	E. coli	p. aeruginosa
HE	14	NA	12	10
CE	20	12	30	11
AE	32	26	30	32
ME	30	26	34	28
1	NA	NA	NA	NA
2	NA	NA	NA	NA
3	15	11	15	11
G	15	20	26	20
DMSO	NA	NA	NA	NA

***Key*:** These results are average results of three replicates. NA= Not active, HE = Hexane extract, CE = Chloroform extracts, AE = Acetone extracts, ME = Methanol extracts.

On the other hand, among the isolated compounds, only compound 3 displayed marginal activity against the tested bacterial strains, whereas the two benzoic acid derivatives (1 and 2) showed limited or no inhibitory activity against these bacterial strains. In generally, the activity observed for crude extracts could be aroused from the synergistic interactions of several compounds present in the extracts and support the traditional use of medicinal plants by local community for the treatment of different bacterial ailments. It is worth mentioning that the antimicrobial activities of *S. longipedunculata* roots extracts were attributed to the presence of flavonoids and saponins (Arnold and Gulumian, 1984; Ajali and Chukwurah, 2004).

CONCLUSION

The bioassay guided extraction of the root bark of *S. longipedunculata* demonstrated significant antibacterial activity whose activity for some of the extract is greater than the reference drugs against some of the tested bacterial strains. The chromatographic purification of bioactive compounds from acetone extract resulted in the isolation of three compounds (1, 2 and 3). Compound 3 displayed moderate antibacterial activity; whereas compounds 1 and 2 showed no inhibitory activity. The observed activities of the crude extracts could give perception about the promising potentials of the minor compounds present in the extract and suggested to search for the minor compounds that can have potential application in the development of antibacterial drugs.

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