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Research Paper

Antibacterial Steroids from Roots of *Bersama Abyssinica*

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Abstract

Bersama abyssinica is one of the medicinal plants used traditionally to treat various diseases such as leprosy, wound, diarrhea, fever, eye disease, rabies and tumor/cancer. Phytochemical screening test of dichloromethane/methanol (1:1) and methanol extracts revealed the presence of glycosides, alkaloids, tannins, flavanoids, saponins, terpenoids, steroids and phytosterols. Silica gel column chromatography separation of dichloromethane/methanol (1:1) root extracts afforded *β*-sitosterol (**1**), 7-hydroxysitosterol (**2**) and 2-methylamino-butyric acid (**3**) of which the latter is isolated for the first time from natural source. The crude extracts and isolated compounds were screened for *in vitro* antibacterial activity against strains of *Salmonella thphimurium*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. Dichloromethane/methanol (1:1) extract, methanol extract and *β*-sitosterol (**1**) showed moderate activity against *E. coli* and *S. aureus* (zone of inhibition 13±0, 13±2 and 12.6±0.48, respectively) and (zone of inhibition 13.6±0.55, 12±2, and 12.5±0.5 mm, respectively) compared to ciprofloxacin (28.6±1.25 and 26±5.1 mm) at 0.5 mg/mL. The structures of compounds were determined by spectroscopic techniques (IR and NMR) and comparison with literature report.

1. Introduction

The use of traditional medicine for treating human diseases still remains widespread in low income countries with a wide range of biological and pharmacological activities (Ajayi et al., 2011; Ejele 2010). *Bersama abyssinica* (Melianthaceae) is an ever green shrub to small tree up to 18 m tall and its bark, leaf and root decoctions are widely taken as a purgative to treat a range of stomach disorders, such as abdominal pain, colic, diarrhea, cholera, intestinal worms, dysentery, and also for the treatment of rabies, tumour, syphilis, gonorrhea, malaria, rheumatism, aphrodisiac and snake bites (Djemgou et al., 2010; Lather *et al*., 2010; Kuete et al., 2008; Teklehaymanot et al., 2007). *B. abyssinica* is known in Ethiopia as *Azamer* (Amharic) and *Lolchissa* (Afan Oromo) (Verdcourt, 1989). It is also distributed in Democratic Republic of Congo, Tanzania, Mozambique, Zambia, Zimbabwe, Angola, Nigeria, Ethiopia, Kenya, Sudan and Uganda (Mikkelsen and Seberg, 2001). Previous phytochemical works reported compounds including 3,11,15-tetramethyl-2-hexadecen-ol, 7,8-epoxyanostan-11-ol,3-acetoxy, pyrogallol, capric ether, 2,3-dimethylfumaricacid, 5-methyl-2-furancarboxyaldhyde, *β*-sitigmasterol and ethyl iso-allocholate (Zekeya et al., 2014; Kuete et al., 2008). This study report the isolation, spectroscopic identification, and antibacterial analysis of the roots of *B. abyssinica*.

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2. Experimental section

2.1. General

TLC was performed using precoated aluminum backed supported silica gel 60 F254 (0.2 mm thickness) and glass supported silica gel 60 F254 (1.0 mm thickness), respectively. Phytosterols were detected on TLC stained with the Salkowiski reagent. Column chromatography was carried out using silica gel 60-120 mesh. ^{1}H and ^{13}C NMR data were obtained in $CDCl_3$ on a Bruker Avance 400 MHz.

2.2. Plant material collection and identification

The roots of *B. abyssinica* were collected in December, 2018 from Oromia Region Arsi zone, Asela town, Tiyo woreda which is 175 km far from Addis Ababa, the capital of Ethiopia. The plant was identified with the help of botanist and voucher specimen was deposited (FL 001) at National Herbarium of Ethiopia, Addis Ababa University. The roots were cut into small pieces, air-dried and ground into a fine powder.

2.3. Extraction and isolation

Air-dried root powder (300 g) was extracted exhaustively with dichloromethane/methanol (1:1) (2 L) for 72 h at room temperature. The marc left was further extracted with methanol (2 L) soaked for 72 h at room temperature. The extracts were evaporated under reduced pressure at 40ºC using Rotary evaporator to afford 23.68 g (7.89%) and 30.08 g (10.03%) crude extracts, respectively. The crude dichloromethane/methanol (1:1) crude extract (15 g) was adsorbed on 15 g silica gel and subjected to silica gel (160 g) column chromatography separation. Elution was carried out with increasing gradient of ethyl acetate in *n*-hexane followed by increasing gradient of methanol in dichloromethane. A total of 145 fractions were collected each concentrated under reduced pressure to dryness. Fractions that showed similar R_f values and the same characteristic color on TLC were combined. Fraction 33-38 afforded single spot (*β*-sitosterol (**1**, 10.8 mg)) derivative of *β*-stigmasterol (EtOAc/*n*-hexane 1:1 as eluent on TLC with R_f value of 0.64). Fraction 22-26 afforded single spot (7-hydroxy-*β*-sitosterol (**2**, 9.3 mg, EtOAc/*n*-hexane 3:7 as eluent on TLC with R_f value 0.85). Fractions 42-79 afforded 2-methylbutyric acid (**3**, 26.5 mg, EtOAc/*n*-hexane 8:2 as eluent on TLC with R_f value of 0.6).

2.4. Phytochemical screening test

2.4.1. Test for flavonoids**:** To DCM/MeOH (1:1) and MeOH crude extracts (0.5 g), 10 mL of ethyl acetate was added and heated for 3 min using steam bath. The mixture was filtered, and mixed with 1 mL of dilute ammonia solution. Formation of intense yellow color ratifies the presence of flavonoids (Sofowora and Debiyi, 1978).

2.4.2. Test for saponins: To DCM/MeOH (1:1) and MeOH crude extracts (0.5 g), 5 mL of distilled water was added and shaken while heating to boil. Frothing showed the presence of saponins (Evans and Trease, 1989).

2.4.3. Test for phenols: To DCM/MeOH (1:1) and MeOH crude extracts (0.5 g), 5 drops of 2 % of $FeCl_3$ were added and formation of bluish green to black color indicates the presence of phenols (Roopashree et al., 2008).

2.4.4. Test for tannins: The crude extracts (DCM: MeOH (1:1) and MeOH extract) (0.5 g each) was boiled in 10 mL of water in a test tube and filtered. To the filtrate, 5 drops of 0.1 % $FeCl_3$ were added to give a brownish green or a blue-black color which confirms the presence of tannins (Ayoola et al., 2008).

2.4.5. Test for terpenoids (Salkowski test): DCM/MeOH (1:1) and MeOH crude extracts (0.5 g) were mixed with 2 mL of chloroform and 3 mL concentrated H_2SO_4 carefully to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids (Ugochukwu et al., 2013).

2.4.6. Test for steroids: To DCM/MeOH (1:1) and MeOH crude extracts (0.5 g), 10 mL of chloroform and 10 mL of concentrated H_2SO_4 were added by sides of the test tube. The upper layer turns red and H_2SO_4 layer showed yellow with green fluorescence indicating the presence of steroids (Alhadi et al., 2015).

2.4.7. Alkaloids (Wagner's test): DCM/MeOH (1:1) and MeOH crude extracts (0.5 g each) were dissolved individually in wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate was examined (Sadoon et al., 2014).

2.4.8. Detection of phytosterols (Salkowski's test): To DCM/MeOH (1:1) and MeOH crude extracts (0.5 g each), 10 mL of chloroform was added and filtered. To the filtrate, 5 drops of concentrated H_2SO_4 was added, shaken and examined for the appearance of the golden yellow color (Roopashree et al., 2008).

2.4.9. Test for anthraquinones (Borntrager's test): DCM/MeOH (1:1) and MeOH crude extracts (0.5 g each) were boiled with concentrated hydrochloric acid for few minutes in water bath and filtered. The filtrate was allowed to cool and equal volume of $CHCl_3$ was added to it. Few drops of ammonia were added to the mixture and heated in water bath. Formation of rose-pink color was inspected (Roopashree et al., 2008).

2.5. Antibacterial testing

2.5.1. Preparation of discs containing extracts

The same concentrations of 0.5 mg/mL were prepared from the extract, isolated pure compounds and the standard. The concentration was incorporated into sterile agar-disc diffusion and dried at 37°C. The agar disc was weighed carefully to confirm the exact amount of the extract and isolated pure compounds being incorporated (compared to preweighed blank discs).

2.5.2. Bacterial culture

Escherichia coli was isolated from stool specimens collected from clinic and identified according to routine cultural properties and biochemical tests. Four strains of each were included in the study. A few colonies from the overnight culture of Eosin Methylene Blue (EMB) agar was transferred into approximately 4-5 mL Tripticase soy broth (TSB) medium. The broth was incubated at 37 °C for 3-4 h, and the turbidity of suspension was adjusted to that of 0.5 McFarland barium sulfate standards. The standard suspension was used for both qualitative and quantitative antibacterial assays.

2.5.3. Bacterial susceptibility testing

Standardized inoculums (0.5 mg/mL) were introduced on to the surface of sterile agar plates, and a sterile glass spreader was used for even distribution of the inoculums. Sterile agar-disc diffusion previously soaked in a known concentration of extract or pure compound (0.5 mg/mL per disc) was carefully placed at the center of the labeled seeded plate. The same procedure was used for all the MRSA strains used. The plates were incubated aerobically at 37°C and examined for zones of inhibition after 24 hr. The inhibition zones were measured with a ruler and compared with the control disc (disc containing only physiological saline). Strains of human pathogen microorganisms used in this study were as follows: two Gram-negative bacteria, *Escherichia coli*, *Salmonella thphimurium* and two Gram-positive bacteria *Staphylococcus aureus and Bacillus subtilis*. The bacterial stock cultures were incubated for 24 h at 37°C on nutrient agar medium (Adama Science and Technology University, Department of Applied Biology, Adama). The bacterial strains were grown in the Mueller–Hinton agar (MHA) plates at 37°C. The agar was melted (50°C), and the microorganism cultures were then added aseptically to the agar medium at 45°C in plates and poured into sterile petri dishes to give a solid plate. All these experiments were performed in triplicate. The plates were incubated for 24-48 h at 37°C for bacteria. The inhibition zones produced by the plant extracts were compared with the inhibition zones produced by commercial standard antibiotics (ciprofloxacin). One dilution (0.5 mg/mL) of *B. abyssinica* extract, pure compound, and standard drugs was prepared in DMSO using nutrient agar tubes. Mueller–Hinton sterile agar plates were seeded with indicator bacterial strains (1.3×10^8 cfu/mL) and allowed to stay at 37°C for 3 h. Control experiments were carried out under similar conditions by using ciprofloxacin for antibacterial activity as a standard drug. The zones of growth inhibition around the disks were measured after 24 h of incubation at 37°C for bacteria. The sensitivities of the microorganism species to the plant extract and isolated pure compounds were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks, and values < 6 mm were considered as not active against microorganisms. DMSO used as negative control during the whole test on bacteria. The results were expressed as mean value ± standard deviation (SD) (Murai et al., 1995). The results are calculated as averages of triplicate tests. The zone of inhibitions in all cases were includes the diameter of the wells.

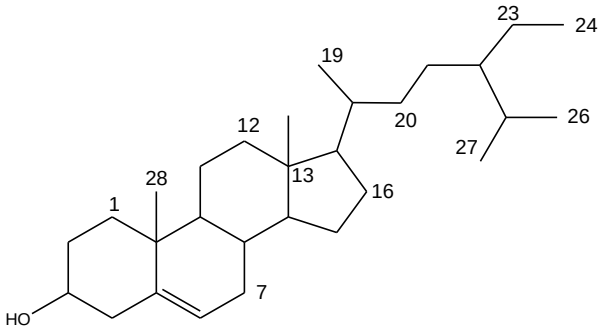
3. Result and Discussion

3.1. Phytochemical screening

Phytochemical screening test of dichloromethane/ methanol (1:1) and methanol roots extracts revealed the presence of alkaloids, flavonoids, phytosterols, phenols, steroids, tannins, terpenoids, coumarins, anthraquinones, terpenes and saponins in both extracts, whereas, saponins were found to be absent in methanol extract (Table 1). The presence of these secondary metabolites may be attributed to the traditional use of the plant to treat various diseases.

3.2. Characterization of compounds

Compound **1** was isolated as white solid with melting point 134-136°C and R_f value of 0.64 (50% EtOAc in *n*-hexane as eluent). The ^{1}H NMR spectrum (Table 2) showed a series of proton signal at δ 1.0-1.8 due to overlapping of methylenes and methines, a characteristic frame work of steroid. Oxygenated sp^3 methine proton was observed at δ 3.68 (m, 1H, H-3) which is a characteristic of steroids with hydroxyl group at C-3 position. Olefinic proton was observed at δ 5.3 suggesting that the proton is next to methylene. The presence of six methyl groups at δ 0.68, 0.93, 0.83, 0.81, 0.84 and 1.01 is also in agreement with the steroidal nucleus. The ^{13}C NMR spectrum (Table 2) revealed the presence of twenty nine carbon signals which is a characteristic feature of triterpenes. The ^{13}C NMR and DEPT-135 spectra displayed the presence of six methyl carbon signals which resonated at δ_C 11.9, 14.0 18.8, 19.2, 19.4 and 19.8. Eleven methylene carbon signals were observed at δ_C 21.1, 23.1, 25.9, 27.9, 28.3, 31.9, 32.0, 35.5, 37.3, 39.8 and 42.3 in (Table 2). Presence of five methine carbons (δ_C 48.0, 48.30, 50.5, 55.3 and 38.1), two olefinic carbons (δ_C 143.8 and 121.2), of which the former suggests sp^2 quaternary carbon, and two sp^3 quaternary carbon signals (at δ_C 36.2 and 42.8) were also confirmed. Oxygenated sp^3 methine was observed at δ_C 76.8 (C-3), in agreement with oxygenation pattern of steroids at C-3. Thus, based on the above spectral data and comparison with literature, the structure of the compound was identified as *β*-sitosterol (**1**) (Chaturvedula and Prakash, 2012; Anjoo et al., 2011; Pateh et al., 2008).



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Compound **2** was isolated as white solid (mp: 135-137°C) with R_f value of 0.6 (50% EtOAc in *n*-hexane as eluent). The ^{1}H NMR and ^{13}C NMR spectra displayed comparable spectral feature to that of *β*-sitosterol except additional peak observed at δ_C 79.1 suggesting the presence of additional sp^3 oxygenated methine. Comparison with literature reports and with NMR features of *β*-sitosterol, compound 2 was identified as 7-hydroxy-*β*-sitosterol (**2**).

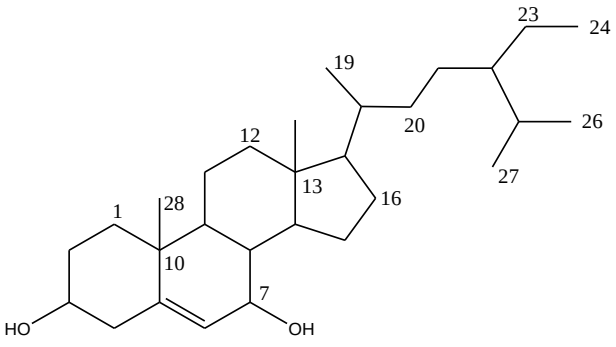
Table 1: Phytochemical screening tests of crude extracts of DCM: MeOH (1:1) and MeOH (100%)

Phytochemical screening	Test	DCM:MeOH(1:1) extract	MeOH extract
Alkaloids	Wagner's test	+	+
Flavonoids	Ammonia test	+	+
Phytosterols	Salkowski's test	+	+
Steroids	Salkowski's test	+	+
Phenols	Ferric Chloride test	+	+
Tannins	Gelatin Test	+	+
Terpenoids	Salkowski's test	+	+
Anthraquinones	Borntrager's test	+	+
Terpenes	Salkowski test	+	+
Saponins	Froth test	+	-

Key: DCM=Dichoromethane and MeOH=Methanol

Table 2: ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data of *β*-sitosterol (**1**)

Position	δ_H (δ in ppm)	δ_C	Chaturvedula and Prakash, 2012	
1		37.3		37.2
2		31.9		31.6
3	3.18 (1H, t)	71.4	3.53 (dd, 1H, *J* = 4.5, 4.2, 3.8 Hz)	71.7
4		42.3		42.2
5		143.8		140.9
6	5.38 (1H, t)	121.2	5.36 (t, 1H)	121.9
7		32.0		32.1
8		33.1		32.1
9		50.4		50.3
10		36.2		36.7
11		21.1		21.3
12		39.8		39.9
13		42.8		42.6
14		56.8		56.9
15		25.9		26.3
16		28.3		28.5
17		56.1		56.3
18		36.2		36.3
19	0.91 (d, 3H, *J* = 6 Hz)	19.4	0.93 (d, 3H, *J* = 6.5 Hz)	19.2
20		35.5		34.2
21		27.9		28.4
22		45.9		46.1
23		23.1		23.3
24	0.85 (t, 3H)	14.0	0.84 (t, 3H)	12.2
25	2.06 (m, 1H)	29.2		29.4
26	0.96 (d, 3H, *J* = 6 Hz)	19.8	0.83 (d, 3H, *J* = 6.4 Hz)	20.1
27	0.97 (d, 3H, *J* = 6 Hz)	19.2	0.81 (d, 3H, *J* = 6.4 Hz)	19.6
28	0.65 (s, 3H)	18.8	0.68 (s, 3H)	19.0
29	0.99 (s, 3H)	11.9	1.01 (s, 3H)	12.0



2

Compound **3** was isolated as a white solid with R_f value of 0.7 (*n*-hexane/EtOAc (8/2) as eluent. The ^{1}H NMR spectrum showed (Table 3) the presence of one terminal methyl protons at δ 1.27 (3H, t) suggesting it is adjacent to methylene. The spectrum also displayed a multiplet methine signal at δ_H 3.5 (q, 1H), methylene at δ_H 2.36 (2H, t) and methyl at δ_H 2.8 (3H, m) where the former suggests a methine protons next to a carbonyl of carboxylic acid and also connected to hetroatom whereas the later suggests methyl attached to hetroatom. The ^{13}C NMR spectrum with the help of DEPT-135 (Table 3) revealed the presence of five well resolved carbon signals of which one carbonyl carbon (δ_c 179.2), methyl (δ_c 17.6), methine (δ_c 48.7), one methylene (δ_c 29.0) and methyl (δ_c 30.7). Its DEPT-135 spectrum displayed that signal at δ_c 29.0 pointing down attributed to methylene signal (C-3). Thus, based on the above spectral data the compound was found to be 2-methylamino-butyric acid (**3**) isolated for the first time from a natural source.

Table 3: ^{1}H and ^{13}C NMR spectral data of compound **3**

Position	δ_H (multiplicity)	^{13}C NMR (δ_C in ppm)	DEPT-135 (δ_C in ppm)
1		175.1	-
2	3.5 (1H, m)	49.4	49.4
3	2.3 (2H, m).	29.0	29.0
4	1.27 (3H, t).	17.6	17.6
5	2.8 (3H, s)	30.7	30.7

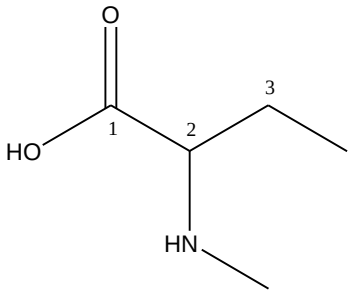


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Table 4: Zone of bacterial growth inhibition diameter (mm)

Sample name	Zone of inhibition (mm) Mean ± standard deviation			
	E. coli	*S. thyphimerium*	*S. aureus*	*B. subtlis*
DCM/MeOH (1:1) extract	13±0.00	11.6±0.48	13±2.00	12.3±1.25
MeOH extract	13.6±0.55	12±0.00	12±2.00	11.6±0.48
β-sitosterol (**1**)	11.6±0.55	11.6±0.48	12.5±0.50	11±0.00
7-Hydroxy-*β*-sitosterol (**2**)	12.5±0.48	10.6±0.48	12.5±0.50	11±0.82
2-methylamino-butyric acid (**3**)	11.3±0.48	11± 0.82	11.5±0.50	11±0.00
Ciprofloxacin	28.6±1.25	28.6±0.94	26±5.1.00	34.3±0.94

3.3. Antibacterial activity

The antibacterial activity of the crude extracts of DCM:MeOH(1:1), MeOH and isolated compound were examined at a concentration of 0.5mg/mL against four pathogenic bacterial strains. Promising antibacterial activity was observed for DCM:MeOH (1:1) and methanol extracts against *E. coli*, *S. thyphimerium*, *S. aureus* and *B. subtlis* with zone of inhibition of 13±0, 11.6±0.48, 13±2, and 12.3±1.25, respectively, for DCM: MeOH extract and 13.6±0.55, 12±0, 12±2 and 11.6±0.48, respectively, for methanol extract. 7-hydroxy-β-sitosterol (**2**) showed promising antibacterial activity against *E. coli* and *S.aureus* with zone of inhibition of 12.6±0.48 and 12.5±0.5, respectively, compared to ciprofloxacin 28.6±1.25 and 26±5.1 (Table 4).

4. Conclusion

For decades traditional medicines have been used and continued to be an alternative approach on treatment for various diseases caused by protozoan, bacteria, fungi, viruses and helminthes. Currently, the growing interest of consumers in substances of natural origin in association with the increasing concern of potentially harmful infectious disease has directed to a rising interest in the use of plant extracts as functional ingredients in many pharmaceutical products. *B. abyssinica* is one of these medicinal plants used traditionally to heal various infectious diseases. The phytochemical screening tests showed that crude extracts of root barks *B. abyssinica* plants are rich in alkaloids, flavonoids, saponins, phenols, tannins, terpenoids, steroids, phytosterols and glycosides. Silica

gel column chromatography separation of the DCM:MeOH (1:1) crude extract furnished two triterpenoids named *β*-sitosterol (**1**), 7-hydroxy-*β*-sitosterol (**2**) and 2-methylamino-butyric acid (**3**). The extracts and isolated compounds were evaluated *in vitro* for antibacterial activity using the disc diffusion method against *E coli, S. aureus, S. thphimurium* and *B. subtilis.* Moderate antibacterial activity was observed for DCM/MeOH (1:1) and methanol extracts against *E. coli, S. thyphimerium, S. aureus* and *B. subtlis* with zone of inhibition of 13±0, 11.6±0.48, 13±2, and 12.3±1.25, respectively, for DCM/MeOH (1:1) extract and 13.6±0.55, 12±0, 12±2 and 11.6±0.48, respectively, for methanol extract. 7-Hydroxy-*β*-sitosterol (**2**) showed moderate antibacterial activity against *E. coli* and *S. aureus* with zone of inhibition of 12.6±0.48 and 12.5±0.5, respectively, compared to ciprofloxacin 28.6±1.25 and 26±5.1.

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