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Phenolic Glycoside and Steroid from the Roots of Comberetum Paniculatum

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Abstract
Combretum paniculatum has been used widely in ethnomedicine where its root is traditionally used around Kaffa Zone and other central highlands of the country for the treatment of various illnesses, including conjunctivitis, eye ailments, leprosy, chronic diarrhea, dysentery, flatulence, vomiting and liver. Phytochemical screening tests of the methanol root extract revealed the presence of steroids, alkaloids, terpenoids, phenols, flavonoids, saponins, tannins, glycosides and the absence of anthraquinones. Silica gel chromatographic separation of the methanol extract gave cholest-5-en-3-ol (**1**) and mutiniside (**2**) for the first time from the roots. Complete characterizations of the isolated compounds were done with the help of spectroscopic techniques (UV-Vis, IR, 1D NMR).
Keywords: Combretum paniculatum, phytochemical screening,cholest-5-en-3-ol, mutiniside

Introduction
Medicinal plants represent a rich source of antimicrobial agents (Khaing, 2011).Majority of rural population in Ethiopia still uses traditional medicine for their healthcare need (Kassaye*et al*, 2006). In Ethiopia, *Combretum paniculatum*i grow in the warm, moist areas of Kaffa, Jimma, Wollega and Shewa Ethiopia, and flowers in January and February. The local name of this plant is "baggo" (KafiNoono), "baggii" (Afaan Oromo) and "baye" (Amharic)(Figure 1) (Getahun, 1976). The sap expressed from flowers is used to treat conjunctivitis and eye ailments. It is also externally applied to treat leprosy (Schmelzer, 2012).

Fig 1.*Combretum paniculatum* (*Baggo*) [Photo taken by Birhanu Bekele, Nov, 2017]

Materials and methods
Instrumentation
Column chromatographic separation was carried out on silica gel (230-400 mesh size, Merck). Thin layer chromatography was done on silica gel 60 F-254, 0.2 mm thick layer on aluminum sheets for detection of spots. The UV-Vis spectrum was recorded on UNICAM UV-300 double beam spectrophotometer using CHCl3 as internal standard. The IR absorption spectrum was determined by Shimadzu 440 instrument using KBr disk in the range of 500-4000cm-1. The 1H NMR, 13C NMR, DEPT-135, spectra were recorded using Bruker Avance 400MHz spectrometer using TMS as internal standard. Chemical shift values for all NMR data are reported in parts per million (ppm) relative to internal standard. All the chemicals used were analytical grade.
Plant material collection and authentication
The roots of *Combretum paniculatum* were collected in November, 2017 from natural forest of Bonga town around Barta River 730 km from Hawassa. The plant species was identified by botanist Mr. Seyoum Robo at Mizan-Tepi University.
Preparation of plant extract
Powdered roots (500g) were soaked with CH_3OH for 72hr with occasional shaking. The extract was filtered and concentrated using rotary evaporator at 40^oC to give brown crude (45.6g, 9.12% yield).

Isolation and purification of compounds

Crude extract (30g) was subjected to silica gel column chromatographic separation (150g silica gel) and eluted with increasing gradient of ethyl acetate in petroleum ether. A total of 31 fractions (each 50 mL) were collected. Out of the 31 fractions (petroleum ether/ethyl acetate) collected. Fractions 14-18 revealed single spot showing yellow spot under UV light having the R_f value of 0.42 in (90:10) petroleum ether/ethyl acetate solvent system. After concentrating, the solid material left was repeatedly washed with *n*-hexane to yield compound **1** (20mg). The remaining sample in column chromatography was further washed with increasing gradient of methanol in chloroform and a total of 29 fractions were collected. Fractions 12-16 revealed single spotwith R_f value of 0.35 in (90:10) chloroform/methanol solvent system. After concentrating, the solid was washed repeatedly with *n*--hexane to yield compound **2** (12mg).

Preliminary phytochemical screening

Tests for steroids (Liebermann-Burchard test)

Each extract (100 mg) was shaken with chloroform in a test tube; few drops of acetic anhydride was added to the test tube and boiled in a water bath and rapidly cooled in iced water. Concentrated H_2SO_4 (2 mL) was added alongside of the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids (Joshi et al., 2013).

Test for alkaloids (Dragendroff's Test)

Crude extract (300mg) was mixed with 2mL of concentrated hydrochloric acid. The mixture was then filtered and mixed with small amount of amyl alcohol at room temperature. Few drops of dragendroff's reagent (Solution of Potassium Bismuth Iodide) was added to the acid layer and a reddish brown precipitate was observed (Ganjewala et al., 2009).

Test for tannins (Gelatin Test)

Small quantity of the extract was mixed with water and heated on water bath. To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins (Saklani *et al*., 2012).

Detection of phenols (Lead acetate test)

Crude extract (5mg) was dissolved in 1 milliliter of distilled water and 3 mL of 5% lead acetate solution was added. A bulky white precipitates indicated the presence of phenols (Harborne *et al* 1998).

Test for flavonoids (Alkaline Reagent Test)

Few drops of sodium hydroxide solution was added to the extract and formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids (Saklani et al., 2012).

Test for saponins (Froth Test)

Crude extract (0.1g) was dissolved in 20mL of water shaken in a graduated cylinder for 15 minutes. Formation of 1cm layer of foam indicates the presence of saponins (Roopashree et al., 2008).

Detection of glycosides

Glacial acetic acid (2mL) and 3 drops of 5% ferric chloride were added to 0.5 mL of an aqueous solutionof plan extract. Then, 1 mL of concentrated sulphuric acid was added. Formation of brown ring at the interface indicated presence of glycosides.

Test of terpenes

Methanol extract (0.25g) was mixed with 2mL of $CHCl_3$ and 30mL of concentrated H_2SO_4 was added carefully to form a layer. Reddish-brown coloration of the interface was inspected (Debjyoti, 1995).

Test for anthraquinones

Methanol extract (0.5g) was 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 (Sofowora, 1982).

Results and Discussion

Phytochemical screening test results

The results from the phytochemical screening of the methanol extract revealed the presence of steroids, phytosterols, alkaloids, terpenoids, phenols, flavonoids, saponins, tannins, cardiac glycosides and the absence of anthraquinones.

Structural elucidation of isolated compounds

Compound **1** was obtained as a white crystalline solid (20mg) from the methanol extract with R_f value of 0.42 in petroleum ether/ethyl acetate (90:10) solvent system. The UV spectrum (Appendix 1) indicated maximum absorbance (λ_{max}) (chloroform) at 298 and 260 nm which indicated the presence σ-π and π-π^* transitions, respectively. IR spectrum revealed (Appendix 2) broad absorption at 3425 cm^{-1} attributed to hydroxyl moiety. The bands at 1632 cm^{-1} and 1050 cm^{-1} can be attributed to olefinic C=C stretch and carbon-oxygen (C-O) stretch, respectively. The bands at 1464 cm^{-1} and 1387cm^{-1} also represent in-plane bending or scissoring of methyelenes

stretching vibrations and in-plane O-H bend, respectively. Moreover, intense peaks at 2850 $cm^{-1}$and 2930 cm^{-1}indicate methyelenes (sp^2) and methyls (sp^3) C-H stretching vibrations, respectively.

The ^{1}H-NMR spectrum ($CDCl_3$, 400MHz) (Appendix 3) revealed peaks at δ5.35 (t, 1H,H-6) and δ3.53 (m, 1H, H-3) attributed to olefinic proton adjacent to methylene and oxygenated methine (C-3), respectively. Moreover, five methyl groups at δ 0.68 (*s,* 3H, H-18), δ0.93 (*s,* 3H, H-19), δ0.92 (dd, 3H, H-21), δ0.83 (d, 3H, H-26) and δ 0.84 (*dd*, 3H, H-27) were observed. Peak observed at δ2.27 is attributed to methyelene proton (H-4) adjacent to olefinic carbon.

The ^{13}C-NMR spectrum ($CDCl_3$, 150 MHz) (Appendix 4) showed twenty seven carbon signals assigned to five methyl, eleven methylene, eight methine groups and three quaternary carbon signals. The peak at δ71.8 (C-3) indicates sp^3 oxygenated methine whereas peaks at δ140.7 (C-5) and δ121.7(C-6) are attributed to olefinic carbons (C-5,6).

The DEPT-135 spectrum (Appendix 5) supported the presence of five methyl groups at δ19.0(C-18), δ11.9(C-19), δ18.8 (C-21), δ19.8 (C-26) and δ11.9 (C-27). Eleven methylene groups at δ37.2 (C-1), δ31.5 (C-2), δ45.8 (C-4), δ29.7 (C-7), δ21.1 (C-11), δ39.8(C-12), δ24.3(C-15), δ26.0 (C-16), δ33.9 (C-22), δ28.3 (C-23) and δ19.4 (C-25). Eight methine groups at δ71.8 (C-3), δ121.7 (C-6), δ31.9 (C-8), δ50.1 (C-9), δ56.8 (C-14), δ56.0 (C-17), δ36.2 (C-20) and δ23.1 (C-24). The absence of peak at δ140.7(C-5), δ36.5 (C-10) and δ42.3 (C-13) in the DEPT-135 spectrum which were observed in the ^{13}C-NMR spectrum also confirmed the presence of three quaternary carbon atoms in compound **1**.

Thus, based on the above spectral data and comparison with literature (74-76), the structure of compound **1** is similar to cholest-5-en-3-ol (Tesemma et al., 2013; Mekoya, 2007) (Figure 2, Table 1).

Table 1.^{1}H-NMR ($CDCl_3$, 400 MHz), ^{13}C-NMR and DEPT-135 (150MHz) spectral data compound **1**

Position	**^{1}H-NMR**	**^{13}C-NMR**	**DEPT-135**	**Literature** (Tesemma et al., 2013; Mekoya, 2007)	
				^{1}H-NMR	**^{13}C-NMR**
1		37.2	37.2		37.2
2		31.5	31.5		31.6
3	3.53 (broad m,1H)	71.8	71.8	3.52	71.8
4	2.27	45.8	45.8		45.9
5		140.7			140.7
6	5.35 (t, 1H)	121.7	121.7	5.34	121.7
7		29.7	29.7		29.2
8		31.9	31.9		31.9
9		50.1	50.1		50.2
10		36.5			36.5
11		21.1	21.1		21.1
12		39.8	39.8		39.8
13		42.3			42.3
14		56.8	56.8		56.8
15		24.3	24.3		24.3
16		26.0	26.0		26.2
17		56.0	56.0		56.1
18	0.68 (*s,* 3H)	19.0		0.65	19.0
19	0.93 (*s,* 3H)	11.9		0.98	11.9
20		36.2	36.2		36.1
21	0.92 (dd, 3H)	18.8		0.91	18.7
22		33.9	33.9		33.9
23		28.3	28.3		28.2
24		23.1	21.1		23.1
25	1.83	19.4	19.4		19.4
26	0.83 (d, 3H)	19.8	19.8	0.84	19.8
27	0.84 (*dd*, 3H)	11.9		0.87	11.9

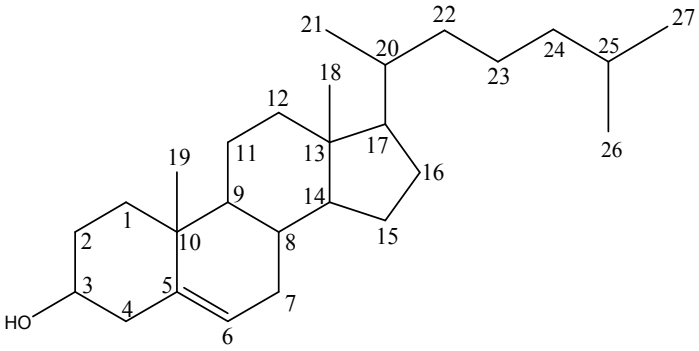

Fig 2. Structure of compound **1** (cholest-5-en-3-ol)

Compound **2** was obtained as a yellow amorphous powder (12mg) from the methanol extract with R_f value of 0.35 under chloroform/methanol (90:10) solvent system. The UV spectrum (Appendix 6) showed in absorbance peak (λ_{max}) (methanol) at 345 nm attributed to π-π* conjugation. The IR spectrum (Appendix 7) showed sharp and broad absorption bands at 1740 cm^{-1} and 3406cm^{-1} attributed to ester carbonyl and hydroxyl functional groups, respectively. The bands at 2845 and 2924cm^{-1} also revealed the presence of methyelenes (sp^2) and methyls (sp^3) C-H stretching vibrations. The medium bands at 1679cm^{-1} and 1057cm^{-1} indicate the presence of aromatic C=C and C-O stretching vibrations, respectively. Moreover, the bands at 1443cm^{-1}, 1364 cm^{-1} and 760 cm^{-1}attributed to CH_2 bend, symmetrical $CH_3$and CH_2out-of-plane bending, respectively.

The ^{1}H-NMR spectrum (DMSO-d_6, 400H_Z) (Appendix 8) showed signals for the hexyl chain including methyl protons at δ0.84 (t, 3H, H-6'), six methylene protons at δ1.79-1.97 (m, 2H, H-2'), 1.30-1.45 (m, 2H, H-3'), 1.17-1.22 (m, 2H, H-4'),1.13-1.15 (m, 2H, H-5'), while the signal for oxygenated methylene protons appeared at δ4.07 (t, 2H, H-1'). The signal for the methoxyl group was observed as singlet at δ3.99. The meta-coupled aromatic protons appeared at δ 7.54 (d, J=2.1 Hz, 1H, H-6) and 7.75 (d, J= 2.1 H_z, 1H, H-2). The signals of a glucopyranose moiety appeared at δ5.28 (d, J =7.4 H_Z, 1H, H-1"), 3.48-3.50 (m, 1H, H-2"), 3.39-3.42 (m, 1H, H-3"), 3.34-3.37 (m, 1H, H-4"), 3.43-3.46 (m, 1H, H-5") and oxygenated methylene protons at δ3.60 (dd, J=11.5, 3.0 H_Z, 1H, H-6") and 3.70 (dd, J=11.5, 4.9 Hz, 1H, H-6"). The anomeric proton of the glucopyranose moiety appeared at at δ4.1.

The ^{13}C-NMR (DMSO-d_6, 150H_Z) (Appendix 9) and DEPT-135 (Appendix 10) spectra (Table 3) showed 20 carbon signals with two methyl at δ14.4 (C-6') and δ56.4 (C-8), six methylene at δ66.2 (C-1'), 31.2 (C-2'), 29.5 (C-3'), 29.2 (C-4'), 22.6 (C-5') and 62.1 (C-6a"), seven methine at δ112.3 (C-2), δ105.1 (C-6), δ102.2 (C-1"), δ73.7 (C-2"), δ79.5 (C-3"), δ70.0 (C-4") and δ76.9 (C-5") and five quaternary carbons. The most downfield signals appearing at δ160.2 attributed to the ester carbonyl, whereas the quaternary carbons appearing at δ154.1, 153.8 and 139.8 were assigned to the oxygenated aromatic carbons. The signal for the anomeric carbon of glucopyranose moiety appeared at δ102.5. Thus, based on the above spectral data the structure of compound 2 was found to be phenolic glucoside given trivial name mutiniside in literature (Ali *et al* 2009) (Figure 3).

Table 3. ^{1}H (DMSO-d_6, 400MHz) and ^{13}C-NMR (DMSO-d_6, 150MHz) data of compound **2** and reported literature

Position	^{1}H-NMR	^{13}C-NMR	DEPT-135	Literature (Ali *et al* 2009)	
				^{1}H-NMR	^{13}C-NMR
1					128.5
2	7.75(d, J= 2.1 Hz, 1H)	112.3		7.32(d)	111.6
3		154..1			151.6
4		139.8			132.3
5		153.8			145.1
6	7.54(d, J= 2.1 Hz, 1H)	105.1	105.1	7.00(d)	105.1
7		160.2			166.2
8	3.99(s)	56.4	56.4	3.82(s)	50.5
1′	4.07(t)	66.2	66.24	4.12(t)	68.1
2′	1.79-1.97 (m, 2H)	31.2		1.78-1.81(m)	30.1
3′	1.30-1.45 (m, 2H)	29.5	29.5	1.32-1.36(m)	29.2
4′	1.17-1.22 (m, 2H)	29.2		1.18-1.20(m)	26.7
5′	1.13-1.15 (m, 2H)	22.6		1.15-1.17(m)	22.5
6′	0.85(t, 3H)	14.4		0.9(t, 3H)	14.0
1″	5.28(d)	102.2	102.2	5.02(d)	101.5
2″		73.7	73.5	3.48-3.50(m)	74.3
3″		79.5		3.39-3.42(m)	77.0
4″		70.0	70.0	3.34-3.37(m)	70.0
5″		76.9	76.9	3.43-3.46(m)	76.5
6″	3.70 (dd, 1H)	62.1	62.1	3.6(dd)	62.0

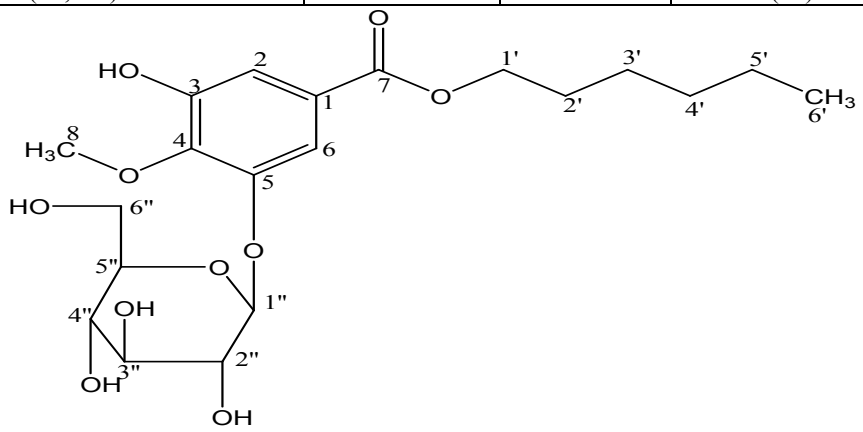

Fig 3. Structure of compound **2**

Conclusion and Recommendation

This study is one of the few attempts to isolate phytochemical constituents from the roots of *Combretum paniculatum* of Ethiopian flora. The qualitative preliminary phytochemical screening of the methanol roots extract revealed the presence of steroids, alkaloids, terpenoids, phenols, flavonoids, saponins, tannins, glycosides and absence of anthraquinones. Silica gel column chromatographic separation of the methanol extract gave cholest-5-en-3-ol (**1**) and mutiniside (**2**). In agreement with the previous study, the wide traditional use of the plant may be attributed to its rich steroids and phenolic constituents. The finding of these pharmacologically important secondary metabolites from root extracts brings the attention of experts to look more on the medicinal importance of the plant. To the best of our knowledge, this is the first report on the presence of such kinds of compounds in the root of *Combretum paniculatum*.

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