

Bioactive Terpenoids from the Roots of Cyphostemmaniveum

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Abstract
Cyphostemmaniveum (Vitaceae) is a plant traditionally used against bacteria, woundand pest in Ethiopia. In view of its traditional use and absence of scientific report, the roots of Cyphostemmaniveum were successively extracted with n-hexane, ethyl acetate and methanol to furnish 1 g (0.33%), 5.1 g (1.7%) and 20g (6.7%), respectively. Silica gel column chromatography of the EtOAc extract led to the isolation of two compounds identified as β-sitosterol and compound **2**, the later was isolated for the first time from the genus.The methanol, EtOAc extract and isolated compounds were assessed for their antibacterial activity against S. aureus**,** E. coil, P. mirabilis and K. pneumonia. Results showed that compound **2** displayed activity against all bacterial pathogens tested in this study while the methanol extract was found to be active only against S. aureus suggesting that compound **2** is responsible for the antibacterial activity of the roots of the plant.The antibacterial activity displayed by the extract and compound **2** substantiate the traditional use of this plant against bacteria.Furthermore, the antioxidant activities of the extracts and isolated compounds were evaluated using DPPH and the result revealed that MeOH extract displayed modest DPPH radical scavenging activity compared with ascorbic acid.
Keywords: Cyphostemmaniveum, Antibacterial, Antioxidant, β-sitosterol, compound 2

1. Introduction

The use of plants as medicine has played a significant role in treating humans' health problems (Belayhun*et al*., 2012). The plant-based health care continues and remains as the main alternative treatment for different ailments in developing countries. This is largely due to shortage of pharmaceutical products, prohibitive distance of the health service stations, unaffordable prices by small holder farmers and pastoralists for conventional drugs, emergence of certain diseases and appearance of drug resistant microbes and/or helminthes (Bekele, E, 2007). Focus on plant research has increased all over the world, and a large body of evidence collected show the immense potential of medicinal plants against life threatening diseases (Hanan, M, *et al*., 2014).Recently, isolation of active compounds from medicinal plants repossess more attention for the production of plant derived modern drugs (Giday, M., *et al*., 2007; Assefa, A., *et al*., 2007).

The genus *Cyphostemma*(Vitacea) comprises of about 300 species, of which only a few are succulent, taking the form of caudiciform shrubs, trees and scrambling lianas (Omoruyi, B., *et al*., 2012). Some species in this genus are exploited for their chemical constituents. For instance, the bark of *C. greveana*was reported to have lasiodiplodin, 12-hydroxy-15-oxo-selina-4,1l-diene, 1*β*,6*α*-dihydroxyeudesm-4(15)-ene, 16,18-dihydroxykolavenic acid lactone and opposit-4(15)-ene-1*β*,7-diol (Shugeng, C., *et al*., 2011). Compounds including cyphostemminA,cyphostemminB, resveratrol,parthenocissin A, e-viniferin, gnetin C, pallidol, gnetin E and ampelopsin D were also reported from *Cyphostemmacrotalarioides*(Bala, A., *et al*., 2000).

Cyphostemmaniveum, a plant belonging to the genus*Cyphostemma*and family Vitacea,is amongmedicinally significant plant in Ethiopia owing to its traditional use against wound, bacteria and killing pests. It has got very important place in traditional healing and also is widely recognized medicinal plant. Despite the traditional use of this plant by the local people for the treatment of enormous diseases, little work is so far undertaken to identify the phytochemicals present in this plant. Furthermore there is no scientific report on the antibacterial and antioxidant activities of the roots extracts of *Cyphostemmaniveum.* Therefore, this paper presents for the first time the results of phytochemical investigation, antibacterial and antioxidant studies of the roots extracts of *Cyphostemmaniveum.*

2. Materials and Methods

2.1. Plant Material

The root of *C. niveum* was collected in March, 2017 from Leman town, KersaMalimaWoreda, South West Showa Zone, Oromia, Ethiopia. The plant material was authenticated by Professor LegesseNegash and voucher specimen (BG-001) is deposited in the National Herbarium of Addis Ababa University.

2.2. Extraction and Isolation

Air dried roots of *C. niveum*(300g)was successively extracted with *n*-hexane (1.5 L), EtOAc (1.5L), and MeOH (1.5L) for 72 hr at room temperature on maceration. Each extracts were filtered and concentrated *in vacuo* at 40℃ to afford 1g hexane, 5.1g EtOAc and 20g MeOH extract. The EtOAc extract (5.1g) was adsorbed and fractionated over silica gel column chromatography using *n*-hexane: EtOAc of increasing polarities as eluent to

afford 18 fractions, each 50mL. The first two fractions were collected with *n*-hexane. Fractions 3 and 4 were collected using *n*-hexane: EtOAc (9:1) as eluent. Fractions 5-9, collected using *n*-hexane: EtOAc (4:1), furnished compound **1** whereas compound **2** (90mg) was isolated from fraction 10 which was collected using *n*-hexane: EtOAc (7:3) as eluent.

2.3. Antioxidant Activity

The antioxidant activity of each extracts and isolated compounds were carried out using DPPH radical scavenging assay (Gulcin, I., *et al*., 2010). The extracts were prepared at four different concentrations in methanol. Each was separately mixed with 0.04% DPPH solution in MeOH to furnish 100, 50, 25 and 12.5 µg/mL.The mixture was shaken and incubated in an oven at 37^{o}C for 30 min. The absorbance of the resultant solution was measured at 517 nm using UV-Vis spectrophotometry. The DPPH radical scavenging activity was calculated using the formula:

% Inhibition = $(A_{control} - A_{extract}) / A_{control} \times 100$

Where $A_{control}$ is the absorbance of DPPH solution and $\mathbf{A_{extract}}$is the absorbance of the test sample (DPPH solution plus sample) (Gulcin, I., *et al*., 2010).

3.4. Antibacterial Activity

The antibacterial activity of the extracts and isolated compounds were tested against three gram-negative bacterial strains including *Escherichiacoli*(ATCC25922), *Proteusmirabilis*(ATCC 35659)and *Klebsiellapneumoniae*(ATCC700603)and one Gram-positive bacterium(*Staphylococcusaureus*,ATCC25923) using Muller Hinton Agar (MHA)medium. All the microbial were obtained from Oromia Public Health Research, Capacity Building and Quality Assurance Laboratory Center, Adama, Ethiopia. Gentamycin and DMSO were used as positive and negative control, respectively.

3.4.1. Media preparation

Mueller Hinton Agarmedium was prepared by dissolving media Indi stilled water and was subsequently autoclaved at 121°C for15minutes. After cooledto50°C, was poured to sterile petridishes, allowed to solidify and used for the antibacterial activity test.

3.4.2. Preparation of inoculums

The test bacterial species were transferred from the stock cultures and streaked on Mueller Hinton plates. Well-separated bacterial colonies were then used as inoculums. Bacteria were transferred using bacteriological loop to autoclaved Mueller Hinton agar and mixed by gently swirling the flasks. Wells of 6 mm diameter were made in the culture medium (Whatmann No.1 filter paper) using sterile cork borers and 20 μL concentrations of the sample were placed in the wells using micropipette and the plates were kept for incubation at 37°C for 24 hr. Control for each bacterial strain before sample action was at 6 mm.

3.4.3. Preparation of test solutions

The samples were prepared by dissolving the ethyl acetate and methanol extract sin DMSO tofurnish100mg/mL and used as stock solutions. Likewise compound1 and 2 were dissolved in separate vial using DMSO to give 2mg/mL. Different concentrations (0.5mg/mL and1mg/mL)were prepared from the stock solutions of the extract sand isolated compounds. All of the prepared test solutions (samples) were used for antibacterial activities. The antibacterial activity, indicated by an inhibition zone surrounding the well containing the sample, was assessed by measuring the inhibition zone diameters in millimeters formed around the well against each test organism at different concentrations and recorded if the zone of inhibition was greater than 6mm. All experiment was carried out in duplicate and average were taken.

4. RESULTS AND DISCUSSION

The EtOAc extract showing good TLC profile was fractionated over silica gel to furnish two compounds (Figure 1). Herein is the characterization of these two compounds.

Compound **1** was obtained as a white solid melting at 138^{o}C. The TLC showed spot at $R_f$value 0.56 with EtOAc:*n*-hexane (3:2) as a mobile phase. The UV-Vis spectrum (EtOH) showed absorption maxima at 224nm indicating the absence of conjugation in the compound. The IR spectrum revealed strong band at 3480 cm^{-1} which is diagnostic for hydroxyl stretching.

The ^{1}H-NMR spectrum ($CDCl_3$) of **1** showed the presence of six methyls at δ_H0.70, 1.03, 0.95, 0.84, 0.86 and 0.88 characteristics of δ_H H-18, H-19, H-21, H-26, H-27 and H-29 of sterols, respectively (Suttiarporn, P., *et al*., 2015). The signal observed at δ_H5.36 (1H, *t*, *J* = 3.2 Hz, H-6) is due to the presence of olefinic proton in the compound. Also observed signal is at δ_H3.52 (1H, *m*, *J*= 5.2 Hz, H-3) due to the presence of proton on oxygenated carbon. The ^{1}H-NMR spectrum also displayed other signals integrating for 30 hydrogens in the region between δ_H1.45 to 1.67. The proton decoupled ^{13}C-NMR (Supporting information spectrum 1) with the aid of DEPT-135 (Supporting information spectrum 2) spectra ($CDCl_3$) of **1** revealed the presence of 29 well resolved carbon signals including six methyls, eleven methylenes, nine methines, and three quaternary carbons.

This is diagnostics for the presence of sterol nucleus. The quaternary carbon signal observed at δ_C140.7 (C-5) is due to olefinic carbon. The other olefinic carbon signal is evident at δ_C121.4 (C-6). This clearly indicates that compound 1 contains only one olefinic double bond. The carbon signal at δ_C71.8 is assignable to an oxygenated carbon (C-3). The data generated in comparison to the literature indicated that compound **1** is *β*-sitosterol(Belayneh, A., *et al*., 2010;Suttiarporn, P., *et al*., 2015). *β*-Sitosterol was reported to have anti-inflammatory, anti-pyretic, anti-arthritic, anti-ulcer, insulin releasing and oestrogenic effects and inhibition of spermatogenesis. *β*-Sitosterol is mainly known for its cholesterol lowering property (Belayneh, A., *et al*., 2010).

Compound **2** was obtained as white crystalline from the EtOAc extract of the roots of *C. niveum.* Its TLC showed spot at R_f0.70 with EtOAc:*n*-hexane (3:2) as eluent. The UV-Vis spectrum (EtOH) displayed absorption maxima at 270nm indicating the presence of conjugated chromophore. The IR spectrum showed strong band at 3541cm^{-1} duetohydroxyl stretching. The signal diagnostic of carbonyl group and C=C is evident at 1765.8 and 1674cm^{-1}, respectively.

The ^{1}H-NMR spectrum of **2** revealed the presence of four methyl singlets at δ_H 0.78, 0.87, 0.94 and 0.97 justifying the presence of four methyl groups on quaternary carbons. The multiplets signal at δ_H3.44 (1H) is ascribed to methine proton on an oxygenated carbon. The other signals observed at δ_H4.05 (1H) and 4.37 (1H) are due to methylene protons on oxygenated carbons. The signal due to an olefinic carbon *β* to a carbonyl was observed atδ_H 6.86. The other aliphatic proton signals were detected in the region between δ_H 0.78 to 0.97.

The proton decoupled ^{13}C-NMR spectrum (Supporting information spectrum 3) with the aid of DEPT-135 (Supporting information file 4) revealed the presence of 20 well resolved carbon resonances of which are five quaternary, five methine, six methylene and four methyls. The signal at δ_C 170.2, 136.3 and 126.9 are evident for the presence of *α,β*-unsaturated carbonyl group with the earlier signal accounted to the carbonyl carbon. The methineolefinic carbon was observed atδ_C136.3. Signals due to oxygenated aliphatic methine carbon and methylene carbon are evident at δ_C75.6 and 67.2, respectively. The other signals due to methylene carbons were observed at δ_C25.0, 28.3, 24.1, 34.5 and 37.4 which are assignable to C1, C2, C6, C7 and C14, respectively. The spectral data generated indicated that compound **2** is 1,2,3,4,4a,5,6,6a,7,7a,8,11b-dodecahydro-3-hydroxy-4,4,6a,11b-tetramethylphenanthro[2,3-c]furan-10(11aH) (Fig 1).

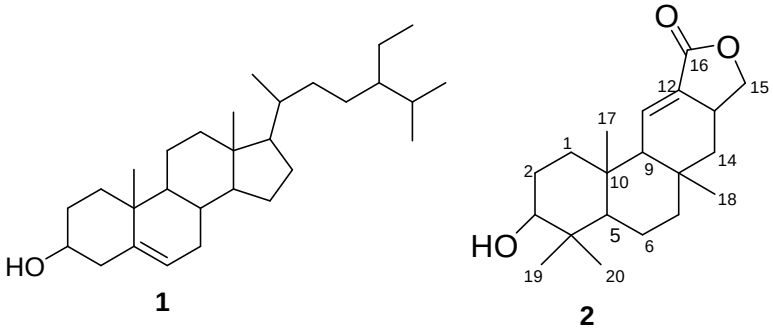


Figure 1: Compounds isolated from the roots of *C. niveum*

Antibacterial Activity

The antibacterial activities of the extracts and isolated compounds were tested against a gram positive bacteria (*Staphylococcus aureus*) and three gram negative bacteria including *Escherichia coli*, *Proteus mirabilis*, *Klebsola pneumonia.* The MeOH extract were found active against *Staphylococcus aureus* which is the only gram positive bacteria assessed in this study. This is in agreement with the antibacterial results reported in the literature for the crude extracts of *Clerodendrumviscosum*against*Staphylococcus aureus*(Oly, W.T., *et al*., 2010). On the other hand the EtOAc extract displayed activity in all bacterial pathogens tested in this study. Fractionation of the EtOAc extract trace the antibacterial activity to compound **2**which showed pronounceable activity against both gram positive and negative bacteria (Table 1). The results of our findings supports the traditional uses of this plant against bacteria.

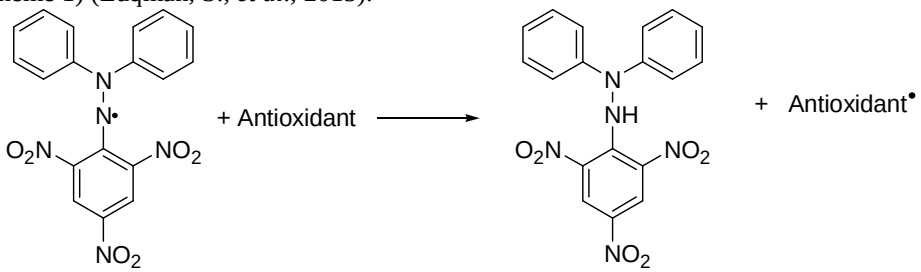
Table 1: Zone of bacterial growth inhibition (mm) for crude extract and isolated compounds from the roots of *C. niveum*at20µg/mL

Sample	*S. aureus*	*E. coli*	*Protens mirabilis*	*K. pneumonia*
Methanol extract	10	6	6	6
EtOAc extract	11	8	9	9
Compound **2**	10	9	10	11
β-Sitosterol	6	6	6	6
Gentamycin	15	15	15	15

Bacterial inhibition zone > 6mm was said to be sensitive (S), but < 6mm was taken as resistance (R) against the tested chemical substance.

Antioxidant Activity

The antioxidant activity of the extracts of *C. niveum*were evaluated using DPPH, a simple method for finding antioxidants by measuring absorbance at 517nm due to the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Gulcin, I., *et al*., 2010). When the radical is scavenged by antioxidants to produce neutral hydrazine, the absorbance at 517nm is reduced. Moreover the color turns from purple to yellow as soon as the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H (Scheme 1) (Luqman, S., *et al*., 2015).



DPPH radical (Purple in color, λ_{max} = 517 nm) DPPH-H (Yellow)

Scheme 1: The structure of DPPH radical and its product (DPPH-H)

The DPPH assay indicated that the methanol and EtOAc extracts of roots of *C. niveum*displayed modest activity at 100µg/mLwith percent DPPH inhibition of 41 and 51%, respectively compared with ascorbic acid used as positive control which inhibit the radical by 90% (Table 2). As clearly observed from the results the methanol extract showed better DPPH inhibition and IC_{50} value compared with the EtOAc extract. This is likely indicating that the antioxidant potential of this plant are polar constituents.

Table 2:Antioxidant activity of EtOAc and MeOH root extracts of *Cyphostemmaniveum*

EtOAc Extract			**MeOH Extract**		
Concentration in µg/mL	% DPPH inhibition	IC_{50}	Concentration in µg/mL	% DPPH inhibition	IC_{50}
12	13.2	40.0	12	20.8	29.0
25	19.8		25	32.1	
50	23.5		50	38.7	
100	41.5		100	50.9	

Results are averages of three replicates and ascorbic acid was used as positive control with DPPH inhibition of 90% at 100µg/mL

5. Conclusion

The EtOAc extract after silica gel column chromatography has led to the isolation of two compounds, compound 1(*β*-sitosterol) and compound **2** isolated for the first time from the genus. The extracts and isolated compounds were assessed for their antibacterial activity with compound **2** showed pronounceable activity compared with the extracts. The activity displayed by compound 2 and the EtOAc extract supports the traditional use of this plant against bacteria. The methanol extract also displayed significant radical scavenging activity compared with the EtOAc extract but modest compared to ascorbic acid.

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