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Antioxidant Activity of Leaves and Fruits of *Oxystelma esculentum* var.*alpini* with a New Triterpene.

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ABSTRACT

Antioxidant activities of the leaves and fruits of *Oxystelmaesculentum* were evaluated to isolate the most active compounds. Ethyl acetate fraction of leaves exhibited to the highest antioxidant with IC_{50} of 37.5µg/mL followed by chloroform. Isolation and structure determination of ethyl acetate and chloroform fractions leads to identification of a new compound, methyl 3*β,*20*α*-dihydroxytaraxastane-28-oate (**1**) along with five known metabolites, cholesterol (**2),** cholest-22-en-3-ol, (3*β*, 5*α*,22*E*) (**3**)**,** quercetin (**4**)**,** lupeol (**5)**, 24-methylenecycloartenol (**6)**, querectine-3-*O*-α-rhamnoside.The structure elucidation was accomplished mainly by Nuclear Magnetic Resonance spectroscopic and Mass spectrometric methods.
Keywords: Antioxidant activity, triterpene, *Oxystelmaesculentum, Asir region*.

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INTRODUCTION

It is known that natural antioxidants of plant origin are more effective than synthetic antioxidant [1] and many research on medicinal plants, their extracts and constituents as a defensive for degenerative diseases caused by oxidative stress was reported [2,3]. So, there is a great need for natural potent antioxidants with fewer side harmful effects. *Oxystelmaesculentum* var. *alpini*belongs to family Asclepiadaceae which characterized by a number of biologically active phytomedicines such as indoloquinoline alkaloids, flavonoids, triterpenes, cardenolides, pregnane and C/D-cis- polyoxypregnane esters and glycosides [4-7]. The latex juice of the plant is used in leucoderma, cough, gonorrhea, and muscle pain [8]. The antioxidant effects of methanol extract is responsible for protection from Paracetamol-induced lipid peroxidation [9] and showed a significant antitumor activity which might due to its antioxidant on Ehrlich's ascites carcinoma cell line (EAC) [10]. Due to the south area of Saudi Arabia "Asir" is characterized by a unique habitant with expected new chemical structures. Therefore, the present investigation is to compare the antioxidant activities of leaves and fruits of *Oxystelmaesculentum* var. *alpini*to isolate the most active constituents from the plant grown in Asir region.

MATERIALS AND METHODS

General Material

NMR spectra (^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H,^{1}H-COSY, HMBC, HSQC and NOESY) were recorded using a Bruker 850.150 NMR. Operating frequencies were 850.15 MHz for acquiring ^{1}H-NMR and 213.77 MHz for ^{13}C-NMR spectra. Liquid Chromatography Electrospray Ionization Mass Spectra (LCESIMS) (positive mode) were measured with Bruker's unique UHR-QqTOF (Ultra-High Resolution Qq-Time-Of-Flight) mass spectrometry with > 50,000 Full-Sensitivity Resolution (FSR). Column Chromatography (CC): silica gel (40-0.063±0.2 µm, Merck, Darmstadt, Germany). Thin Layer Chromatography (TLC): silica gel (0.25 and 1 mm pre-coated plates 60 F254, Merck. Sephadex LH-20 (Sigma-Aldrich, Germany).

Plant Material

The aerial parts of *Oxystelmaesculentum* var. *alpini*was collected from in February 2017 from Abha, Asir region, Saudi Arabia and identified at Botany department, College of science, King Khalid University and deposited in herbarium of Botany department with number of OE02017.

Extraction and isolation

The air dried aerial parts of plant (2.0 kg) was exhaustively extracted by addition of organic solvents in the order of increasing polarity: petroleum ether, ethyl acetate, chloroform and 85% ethanol. Each solvent was concentrated under reduced pressure at temperature 45^{o}C till dryness.

The chloroform fraction (12.07 g) was subjected to column chromatography on silica gel and eluted with hexane, hexane/ethyl acetate, chloroform and chloroform/methanol. The eluted fractions were monitored with TLC using different solvent systems: hexane/ethyl acetate (9:1), (8:2), (7:3) and (1:1), chloroform, chloroform/methanol (8:2), (7:3) and (1:1). TLC plates were sprayed with 5% sulphuric acid/methanol. The fractions eluted with chloroform afforded two sub-fractions 1 (40.8 mg) and 2 (70.4 mg). The fraction 1 was purified on Sephadex LH-20 eluted with chloroform/methanol (1:1) afforded pure compounds (**1**)(6.0 mg). The fraction 2showed two major impure compounds which purified on Sephadex LH-20 using chloroform/methanol (1:1) as eluent to give the mixture of compound **2** and **3** (10.4 mg).

The ethyl acetate extract (17.4 g) was chromatographed on a silica gel column and eluted successively with increasing polarities of solvents (hexane, hexane: ethyl acetate, chloroform and chloroform: methanol) to give 145 fractions. The fractions eluted with chloroform divided into two sub-fractions 1 (75.3 mg) and 2 (32.0 mg). The fraction 1 showed two major compounds which further purified on Sephadex LH-20 eluted with chloroform/methanol (1:1) to afford pure compound **4** (49.7 mg). The fraction 2 was purified on Sephadex LH-20 eluted with chloroform/methanol (1:1) to afford mixture of compounds **5** and **6** (18.8 mg) and further purification on silica gel column, pure compound **5** was obtained (9.5 mg). The fraction eluted with chloroform/methanol (8:2) afforded semi pure compound **7** (12.4 mg) which further purified on silica gel eluted with chloroform/methanol 7:3 to obtain pure compound **7** (3.0 mg).

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Spectroscopic data of a new compound

Methyl-3β-20α-dihydroxytaraxastane-28-oate (**1**): HRESIMS: *m/z* 489.3938 ([M+H]$^+$, $C_{31}H_{53}O_4$, calcd. 489.3989); ^{1}H and ^{13}C NMR (Table 1).

Table 1: ^{1}H NMR and ^{13}C NMR data for compound (at 850.15 MHzin CD_3OD, □ in ppm, *J* in Hz)

Position (H)	δ_H (*J* in *Hz*)	δ_C
1	0.93 *m*, 1.70 *m*	40.1
2	1.55 *m*	28.1
3	3.12, *dd*, 4.3, 11.1	79.7
4	-	39.9
5	0.71, *br. d*, 11.1	56.8
6	1.45, 1.58, *m*	19.5
7	1.55, *m*	35.0
8	-	42.6
9	1.35, *m*	49.7
10	-	38.2
11	1.32, 1.54, *m*	23.7
12	1.35, 2.01, *m*	29.8
13	1.73, *ddd*, 4.3, 11.1, 15.3	39.0
14	-	44.6
15	1.28, 1.86, *m*	29.2
16	1.59, *t*, 8.5	26.0
17	-	49.5
18	1.8, *dd*, 4.3, 10.2	51.4
19	1.79, 1.10, *m*	40.3
20	-	73.9
21	1.69, 1.57, *m*	36.9
22	2.26, *t*, 7.7	35.02
23	0.95, *s*	28.8
24	0.75, *s*	16.1
25	0.86, *s*	16.1
26	0.83, *s*	19.8
27	0.98, *s*	15.4
28	-	177.8
29	1.09, *d*, 6.8	16.8
30	1.20, *s*	31.6
OCH_3	3.64, *s*	51.8

Determination of DPPH Free Radical Scavenging Activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activities of each fraction were valuated. 1 mL of methanolic solution of varying concentration samples (25, 50 and 100 µg/ml) were added to 1 mL of methanol solution of DPPH (60 µM). The prepared solutions were mixed and left for 30 min at room temperature. The optical density was measured at 520 nm using a spectrophotometer (UV-1650PC Shimadzu, Japan). The percent scavenging effect was determined by comparing the absorbance of solution containing the test sample to that of control solution without the test sample taking the corresponding blanks. Mean of three measurements for each compound was calculated according to the method of Matsushige et al. [11].

RESULTS AND DISCUSSIONS

DPPH radical scavenging assay of extracts

Radical scavenging activity of plant extracts against stable DPPH was determined spectrophotometerically(Figure 1). When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep violet to light violet) were measured at 520 nm on a

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UV/visible light spectrophotometer. Radical scavenging activity was measured according to the method of Matsushige et al. [11]. The antioxidant activities of leaves and fruits extracts at three different concentrations revealed that ethyl acetate of leaves showed the highest antioxidant activity with IC_{50} of 37.5 µg/mL followed by the chloroform/leave. Therefore, ethyl acetate and chloroform of leaves were subjected to chromatographic isolation to identify their chemical constituents. The previous studies investigated the antitumor effect and antioxidant of the methanol extract of the plant showed a significant reduction in tumor volume and decreased the lipid peroxidation due to the presence of antioxidant agents [9,10] while our results revealed the antioxidant constituents mainly are concentrated in ethyl acetate fraction. These difference might be due to the different in extraction procedures. Further report on the antioxidant activity against the superoxide anion radical in organic and inorganic media showed the methanol extract more active than those obtained in acetone [12].

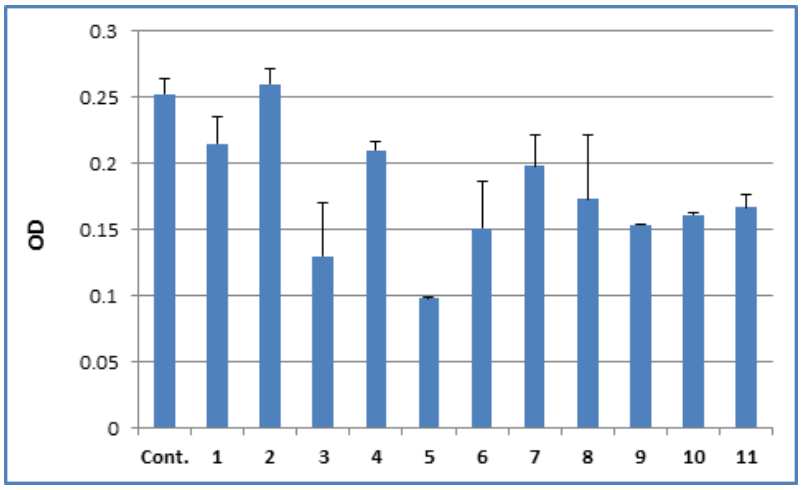

Figure 1: Free radical scavenging activity of different extracts in the DPPH radical assay. Values are expressed as mean ± SD, n = 3 at a concentration of (50 µg/ml for all tested extracts).
1: petroleum ether/leave, 2: petroleum ether/fruit, 3: chloroform/leave, 4: chloroform/fruit, 5: ethyl acetate/leave, 6: ethyl acetate/fruit, 7: methanol extract/leave, 8: methanol extract/fruit, 9: total extract/leave, 10: total extract/fruit, 11: latex

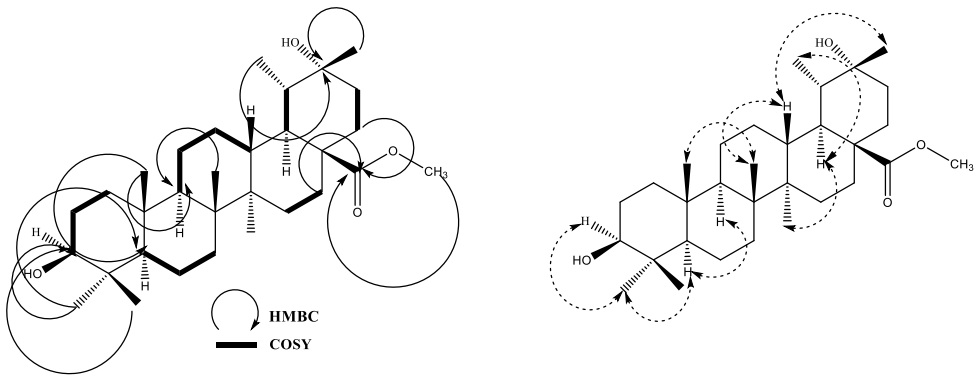

Figure 2: ^{1}H-^{1}H COSY, selected HMBC and NOESY of Compound 1

Structure determination

Compound **1** was obtained as white amorphous and gives a positive triterpeneLibermannBurchardt test. The molecular formula was deduced from HRESIMS ion peak at *m/z* 489.39383 corresponding to molecular formula of $C_{31}H_{53}O_4$ which suggested six degrees of unsaturation. ^{1}H-NMR and ^{13}C-NMR showed six singlet methyl groups at δ 1.19, 0.98, 0.95, 0.86, 0.83, 0.75 for 3H-30, 3H-27, 3H-23, 3H-25, 3H-26, 3H-24 respectively and one methyl at δ 1.09, *d, J* = 6.8 Hz, 3H-29 with corresponding ^{13}C- data at δ : 31.6, 15.4, 28.8, 16.1, 19.8, 16.1 and 16.8 respectively (Table 1). The oxymethine H-3 appeared at δ 3.12, *dd, J* = 4.3, 11.1 Hz

with corresponding carbon at *δ* 79.7. DEPT experiment exhibited to quaternary carbon bearing oxygen function at *δ* 73.9 for C-20 and carbonyl at *δ* 177.8, C-28 with five methines, five quaternary and 10 methylene groups. HMBC spectrum revealed the correlation of $^{2}J_{H\text{-}C}$ and $^{3}J_{H\text{-}C}$ of compound **1** as shown (Figure 2) with further connectivity between methoxy protons at *δ* 3.6 to carbonyl group to suggest the presence of methyl ester. After complete assignment of ^{1}H and ^{13}C NMR chemical shifts by means of ^{1}H,^{1}H-COSY, HSQC, and HMBC spectra, the relative stereochemistry of compound **1** was inferred from 2D NOESY spectrum. NOESY correlations of 18α axial proton at *δ* 1.8 and 27 axial methyl protons, 29*α* methyl protons; the correlation between 13*β* proton at *δ* 1.73 and 26*β* methyl protons, 30*β* methyl protons (Figure 2) confirmed the taraxastane skeleton. Based on the above finding, compound **1** was identified as: methyl-3*β*-20*α*-dihydroxytaraxastane-28-ote.

The other known compounds were identified by comparison of NMR spectroscopy and Mass spectrometry as well as comparison with the published data as, cholesterol **2** and cholest-22-en-3-ol, (3*β*,5*α*, 22*E*) **3** [13], quercetin**4** [14],lupeol**5** [8], 24-methylenecycloartenol **6** [15], querectine-3-*O*-α-rhamnoside (**7**) [16].

CONCLUSION

Ethyl acetate and chloroform of *Oxystelmaesculentum* leaves has the highest antioxidant activities than other plant fractions. The leaves contains high percent of quercetin which could be responsible for its antioxidant behavior. A new triterpene, methyl-3β-20α-dihydroxytaraxastane-28-Oate along with six metaboliteswere identified which confirm that Asir region of Saudi Arabia is characterized with a new chemical structure.

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