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Research Article

Glycosides of *Onopordum alexandrinum* Boiss. and its Central Nervous System (CNS) and Some Biological Activities

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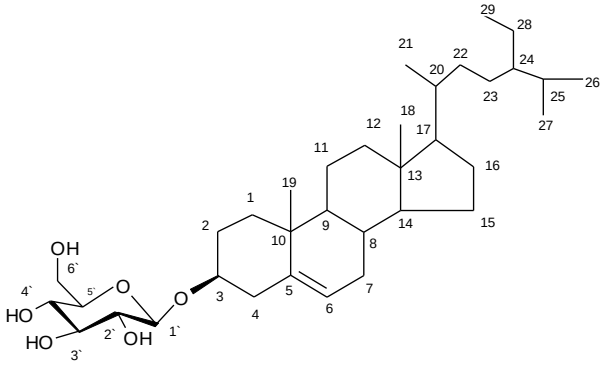
ABSTRACT
Two glycosides, were isolated for the first time from Onopordum alexandrinum. Their chemical structures were elucidated using spectroscopic methods, including 1D, 2D and HRGSMS as well as comparing with literature values, where compound (1) was identified as β-sitoserol-3-O-β-glucopyranoside and compound (2) as Ethyl β-D-fructopyranoside. The biological activities of the isolated extracts of both the roots and aerial parts were examined for their CNS and antimicrobial activities. Methanol extract of the roots and aerial parts of the plant were examined for any CNS activity using Opioid and cannabinoid receptors binding activity assay, where the results were disappointing at this stage, showing no binding affinity to any of the tested receptors. Further biological investigations of both extracts showed no acute toxicity up to 5gm/kg body weight, also no considerable cytotoxicity was observed on Huh-7 Liver cancer or A-495 lung cancer cell lines. Concomitantly, no antibacterial or antifungal activities were observed using some selected species against Ciprofloxacin and Amphotericin B respectively, for both the total methanol extracts and their hexane and chloroform subfractions.

Keywords: *Onopordum alexandrinum*, glycosides, total extracts, CNS, cytotoxicity and antimicrobial activities.

INTRODUCTION

It was noticed that *Onopordum alexandrinum* Boiss. sweet tuberous roots when eaten by natives of the western Egyptian desert, cause hallucination and death in some cases at higher doses. *Onopordum* or *Onopordon*, are early Latin names given to a group of thistles, belonging to tribe Cardueae, family Astraceae which comprise about 50 species distributed throughout the Mediterranean and semi-arid areas of Eurasia and North Africa[1]. The representatives of genus *Onopordum* are native to Europe (mainly the Mediterranean region), Northern Africa, the Canary Island, the Caucasus, Southwest and Central Asia, and Iraq, where they grow at waste places and open, sandy or stony habitats[2,3]. Surveying the biological effects of some species of the genera, *Onopordum,* they were found to exert several biological activities such as the inhibition of TNF-α and nitric oxide in murine endothelial cells[4], the augmentation of the NK cell activity[5a,5b] and the ability to change the rhythmic system by adjusting heart rate activity[6], where these beneficial effects of *Onopordum* genera were due to the presence of many secondary metabolites especially flavonoids, lignans, and sesquiterpene lactones resembling the most common constituents[7-10]. On other hand Nahed El-Najjar, *et al* found that O. cynarocephalum showed high selectivity against colon cancer cells with no apparent toxic effects on normal cells[11]. *Onopordum alexandinum* is a species present in the western deserts near Alalmain - Borg AlArab area {Alalamain} in Egypt with many synonyms as: *Onopordum alexandrinum* Boiss. var. maroccanum Rouy, and *Onopordum dissectum* Murb. of two varieties costatum Maire and lixense Maire.}. The metabolites isolated from the species of genus *Onopordum* include sesquiterpenoids, flavonoids, acetylenic compounds, steroids, triterpenes, lipids and nitrogen containing compounds[12]. An old study revealed the presence of nitrogenous bases of *Onopordum alexandrinum* Boiss. and resulted in the isolation of stachydrine and choline and the detection of flavonoid components which were: Luteolin-7-monoglucoside, apigenin-7-glucoside and quercetin[13]. While a bioactivity-guided fractionation of the ethyl acetate fraction of the flowers of *O. alexandrinum* L. yielded a new flavonoidal glycoside designated as



β-Sitosterol-3-O-β-glucopyranoside

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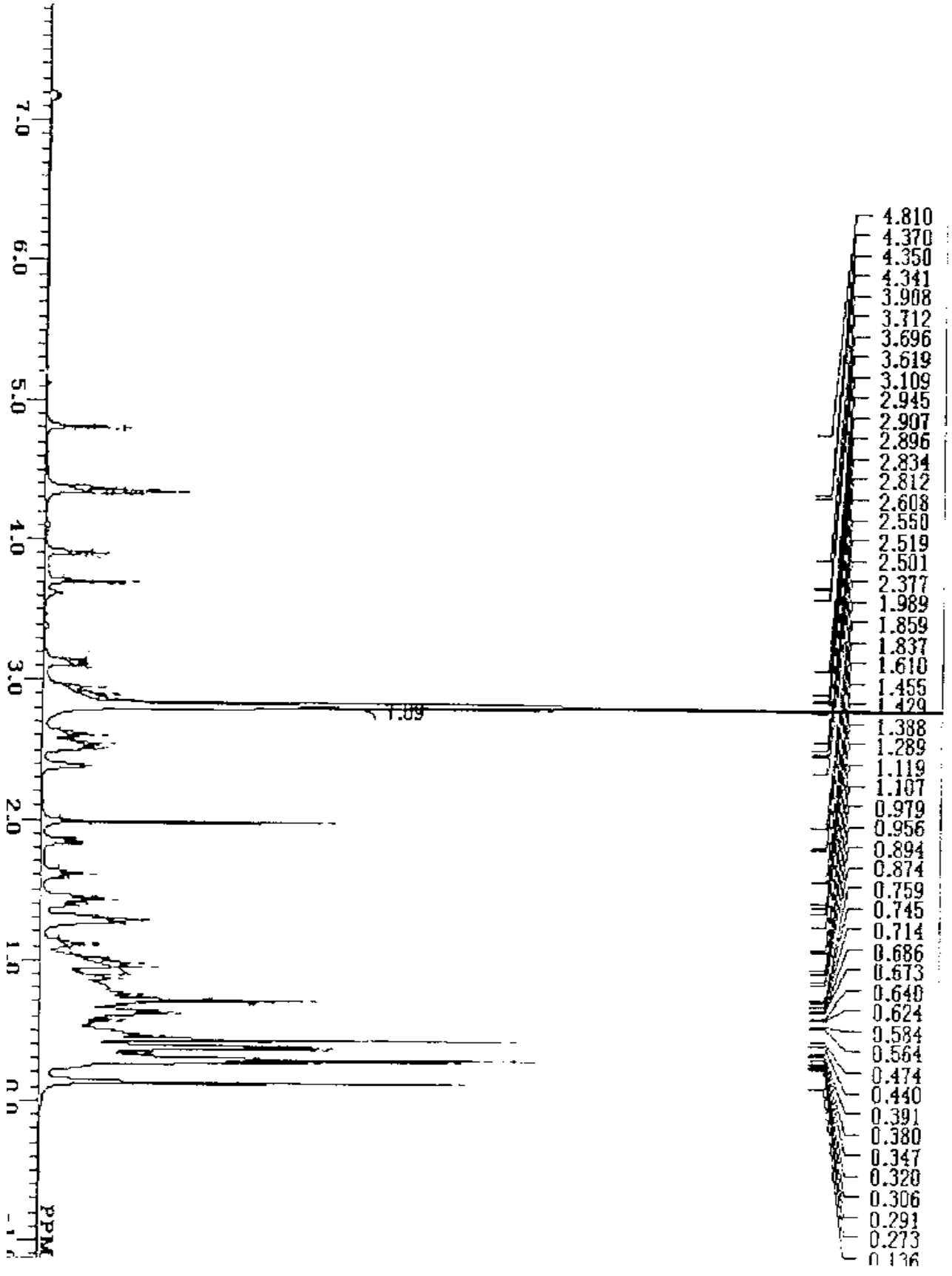


Figure 1a: ^{1}H-NMR spectrum of compound 1.

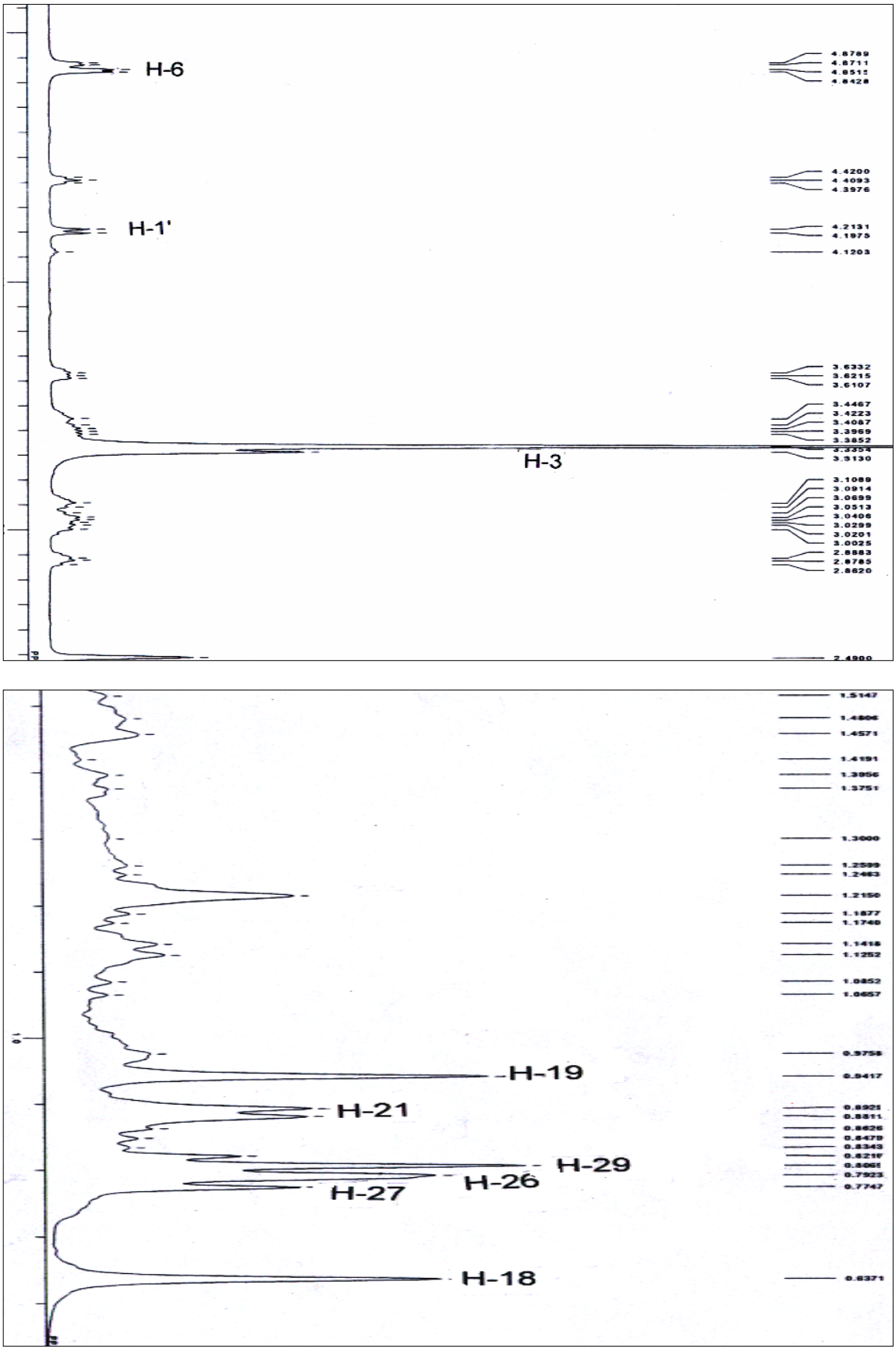


Figure 1b: Expanded [1]H-NMR spectrum of compound 1.

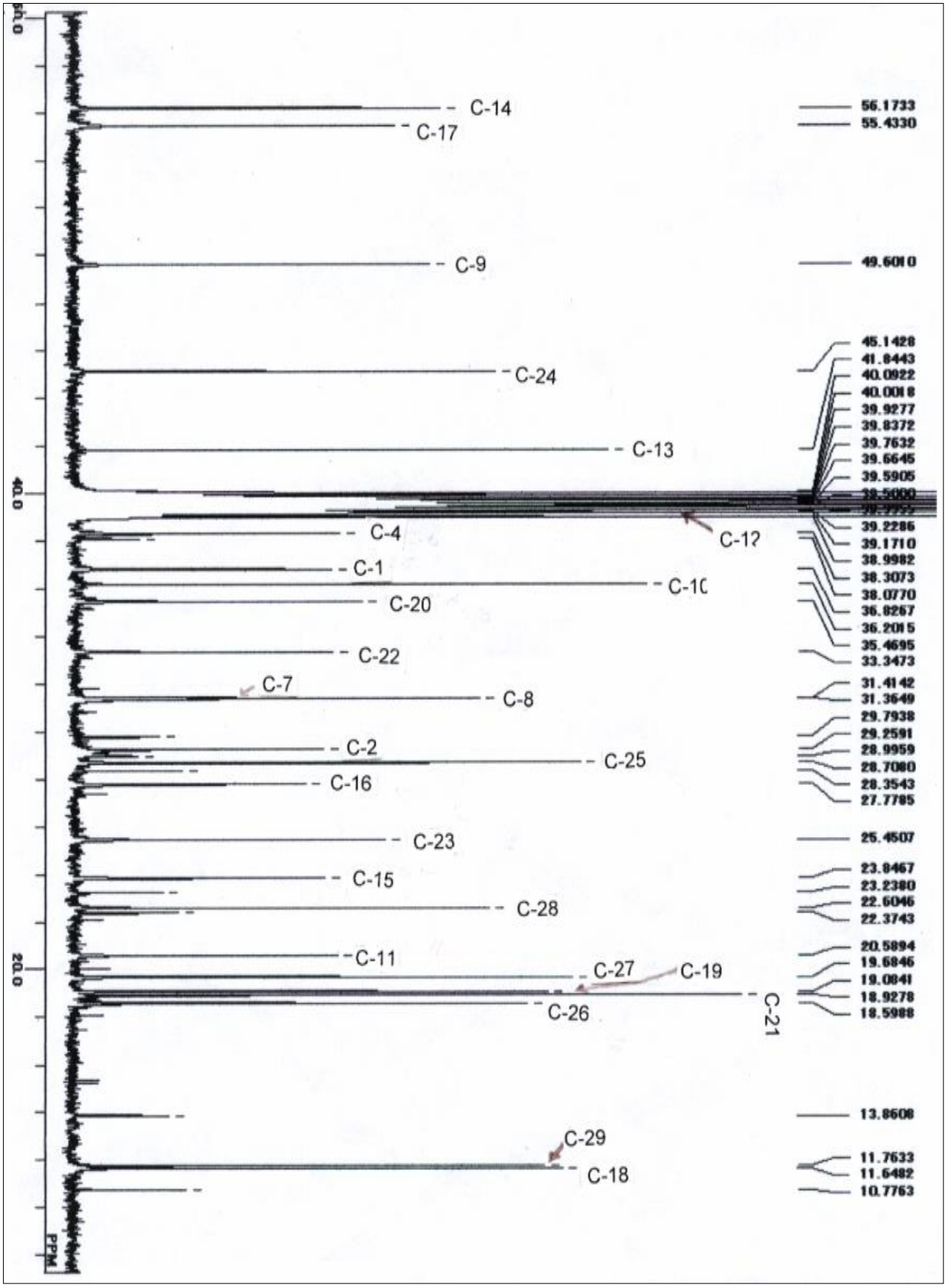


Figure 1c: ^{13}C-NMR spectrum of compound 1 .

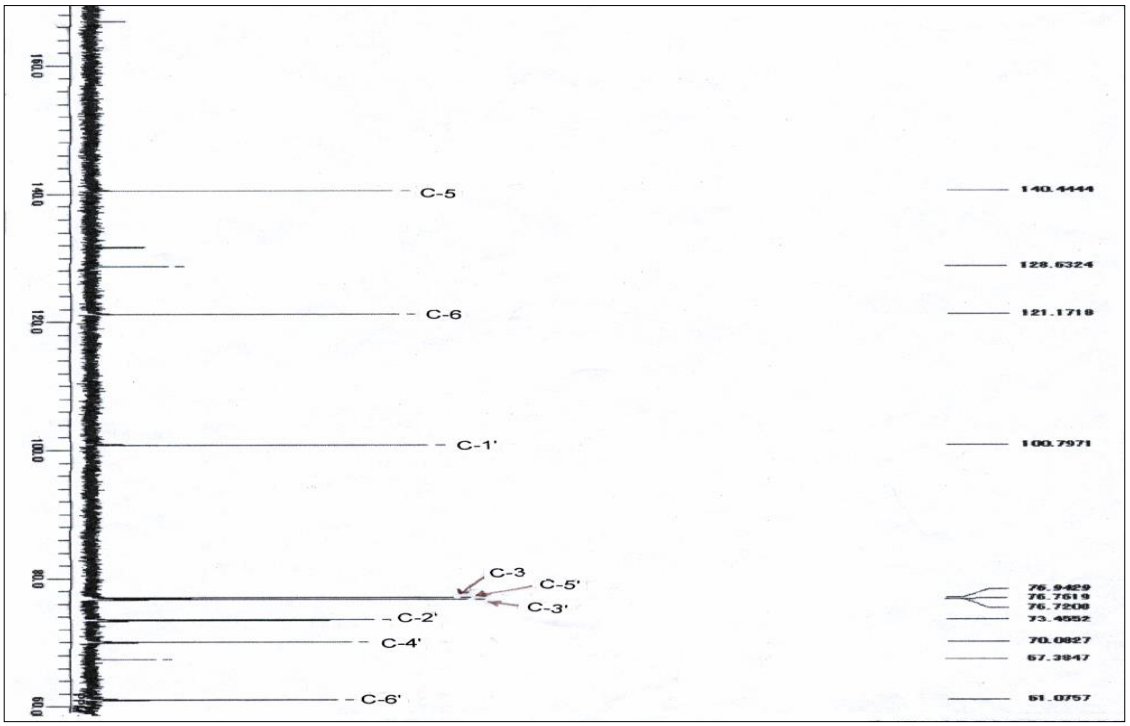


Figure 1c: {Expanded} ^{13}C-NMR spectrum of compound 1

acacetin-7-O-galacturonide, alongside with nine known flavonoids; 6-methoxy-apigenin (hispidulin), acacetin, apigenin, luteolin, kaempferol, eriodictyol, apigenin-7-O-glucoside, luteolin-7-O-glucoside and kaempferol-3-O-rutinoside. The compounds were assayed for their hepatoprotective activity against CCl4-induced hepatic cell damage in rats and free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) where acacetin-7-O-galacturonide has shown significant hepatoprotective and free radical scavenging effects[14]. Furthermore, an investigation of this plant`s seeds resulted in the isolation of many flavonoidal glycosides and phenolic acids which showed high antioxidant activity as well as hepatoprotective activity and very low toxicity on liver cells[15]. Nothing was mentioned about the activity of this plant as a hallucinogen, and none of the compounds isolated can cause this action. We were interested in investigating the truth of this matter, identifying the cause of the misuse of this plant by the natives, trying to isolate the compounds responsible for this symptom and possibly using it for treatment or even as a scaffold for the development of a new therapeutic medication for treatment of neuropathic pain[16]. Two compounds β-sitoserol-3-O-β-glucopyranoside, (1) and Ethyl β-D-fructopyranoside (2) were isolated and the structures were elucidated by spectroscopic methods, to the best of our knowledge are both first report in this genus.

EXPERIMENTAL

Plant Material

The aerial parts and roots of *Onopordum* were collected from Marsa Mattroh at the north western coastal region, Egypt in April 2013 and identified by Prof. Adel Kamel Youssef (Professor of Ecology and Phytochemistry) and Dr. Magda Mohamed Hassan (Assistant Professor of Taxonomy, Suez Canal University to whom the authors are deeply indebted. The plant organs powdered and kept in dark containers till used. A voucher specimen was kept at the Pharmacognosy department, Faculty of pharmacy, Minia University and given the code: Mn -Ph-Cog-012. Plant extracts were prepared by percolation of both the aerial parts and the roots by using methanol without the aid of any heat till exhaustion, solvent evaporated under vacuum, residues dried and weighted. For Antimicrobial activity Both aerial parts and roots methanol extracts were further exhaustively extracted with hexane, chloroform and ethyl acetate, successively and the remaining residues of each dissolved in methanol and kept for use. The following instruments were used to obtain physical and spectroscopic data: specific rotations: High resolution mass spectra were measured using a Bruker Bio Apex FT mass spectrometer. Melting points were recorded on a Thomas Hoover capillary melting point apparatus; 1H-NMR (400 MHz), 13C-NMR (100 MHZ), and 2D-NMR spectra were recorded using the residual solvent signal as an internal standard on a Varian AS 400 spectrometer. Silica gel CC was performed on silica gel 60 [(E. Merck, Darmstadt, Germany), 70-230 mesh]. Precoated silica gel 60 F254 plates (E. Merck; 0.25 mm in thickness) were used for TLC monitoring visualized by spraying with a 10% solution of H2SO4 in ethanol and heated to around 150°C on a hotplate.

Biological investigations

1-Radioligand Binding for Cannabinoid and Opioid Receptor Subtypes bioassay[17]

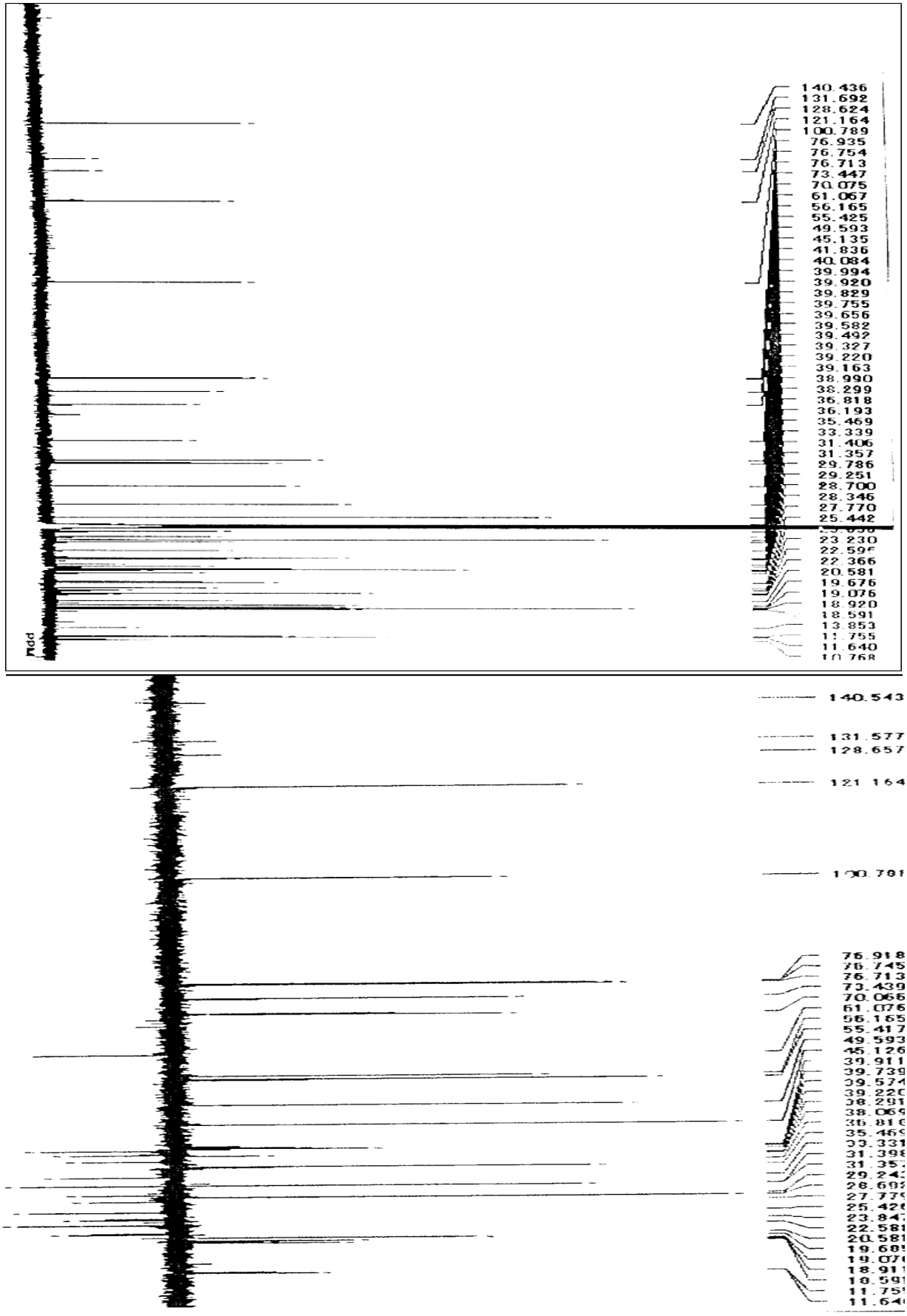


Figure 1d: DEPT ^{13}C-NMR spectrum of compound 1. DMSO.

In the primary bioassay screen, both the investigated extracts were tested at a final concentration of 10 µM for into a 96-well plate followed by 0.6 nM [3H] CP-55,940 and 10 µg of cannabinoid membrane resuspended in 50 mM Tris (pH7.4), 154 mM NaCl, and 20 mM Di-Na-EDTA supplemented with 0.02% BSA. For the opioid receptor assays, saturation experiments were performed to determine optimal radioligand [3H] enkephlin and [3H] DAMGO) and membrane concentrations. The cannabinoid assay was allowed to incubate at 37 º C for 90 min, while the opioid assay was incubated at 25 o C for 60 min. Both reactions were then terminated by rapid filtration using GF/C or GF/B filters (presoaked in 0.3% BSA) and washed with the buffer. Dried filters were then covered with scintillant and measured for the amount of radioligand retained using a Perkin-Elmer Topcount (Perkin-Elmer Life Sciences Inc., Boston, MA, USA). Nonspecific binding, which was determined in the presence of 1 µM CP-55,940 for cannabinoid receptors or 10 µM DPDPE, nor-Binaltorphimine, or DAMGO for opioid receptors, was subtracted from the total binding to yield the specific-binding values. Extracts showing competitive inhibition of the labeled ligand to bind to the receptor at 40% or greater were tested in a dose response curve with concentrations of the test compound ranging from 300 µM.

2-Acute toxicity study: Cytotoxicity assessment against Huh-7 liver cancer cells and A-495 lung cancer cell line

Cell culture

Huh-7, human hepatoma cell line and A-495 human lung cancer cell line were grown in DMEM with high glucose level 0.45% and supplemented with 10% heat inactivated FBS, 100 units/mL of penicillin and 100 mg/mL of streptomycin and maintained at 37° in a humidified atmosphere containing 5% CO2. The cells were maintained as “monolayer culture” by serial subculturing.

SRB {S B} cytotoxicity assay

Cytotoxicity was determined using SRB method as previously described by Skehan et al.[18] Exponentially growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96-well plates at 1000-2000 cells/well in RPMI-1640supplemented medium. After 24 h, cells were incubated for 72 h with various concentrations of the tested compounds. Following 72 h treatment, the cells will be fixed with 10% trichloroacetic acid for 1 h at 4 ºC. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris-HCl for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, USA).

Data analysis

The dose response curve of compounds was analyzed using Emax model.

$$\%\,\text{Cell viability} = (100 - R) \times \left(1 - \frac{[D]^m}{K_d{}^m + [D]^m}\right) + R$$

Where R is the residual unaffected fraction (the resistance fraction), [D] is the drug concentration used, Kd is the drug concentration that produces a 50% reduction of the competitive binding to the respective receptor. For the cannabinoid receptor assays, the test extracts were added maximum inhibition rate and m is a Hill-type coefficient. IC50 was defined as the drug concentration required to reduce fluorescence to 50% of that of the control (i.e., Kd = IC50 when R=0 and Emax =100-R)[19].

Antimicrobial activity

In vitro Antimicrobial Assay

All organisms used for the biological evaluation were obtained from the American Type Culture Collection (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906 and the bacteria methicillin-resistant *S. aureus* ATCC 43300 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) methods (Samoylenko et al., 2009 and referenced therein). *M. intracellulare* was tested using a modified method (Franzblau et al., 1998). Samples were serially-diluted in 20 % DMSO/saline and transferred in duplicate to 96-well flat bottom microplates. Microbial inocula were prepared by correcting the OD630 of microbe suspensions in incubation broth to afford final target inocula. Ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi are included as positive controls in each assay. All organisms were read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (*M. intracellulare, A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Percent growth was plotted versus test concentration to afford the IC50.

In vitro antimalarial activity

Antimalarial activity was determined in vitro against chloroquine sensitive (D6, Sierra Leone) and resistant (W2, Indo China) strains of Plasmodium falciparum by measuring plasmodial LDH activity[20]. Test compounds were dissolved in DMSO (2 mg/mL). A 200 µL suspension of P. falciparum culture (2 % parasitemia and 2 % hematocrit in RPMI 1640 medium supplemented with 10 % human serum and 60 µg/mL amikacin) was added to the wells of a 96-well plate containing 10 µL of serially diluted samples. The plate was flushed with a gas mixture of 90 % N_2, 5 % O_2, and 5 % CO_2 and incubated at 37 °C for 72 h in a modular incubation chamber. Plasmodial LDH activity was determined by using Malstat TM reagent (Flow Inc., Portland, OR). Briefly, 20 µL of the incubation mixture was mixed with 100 µL of the Malstat reagent and incubated for 30 min. Then, 20 µL of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was added and the plate is further incubated for 1 h in dark. The reaction was stopped by adding 100 µL of a 5 % acetic acid solution. The plate was read at 650 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont). IC50 values were obtained from the dose–response curves generated by plotting percent growth versus drug concentration.

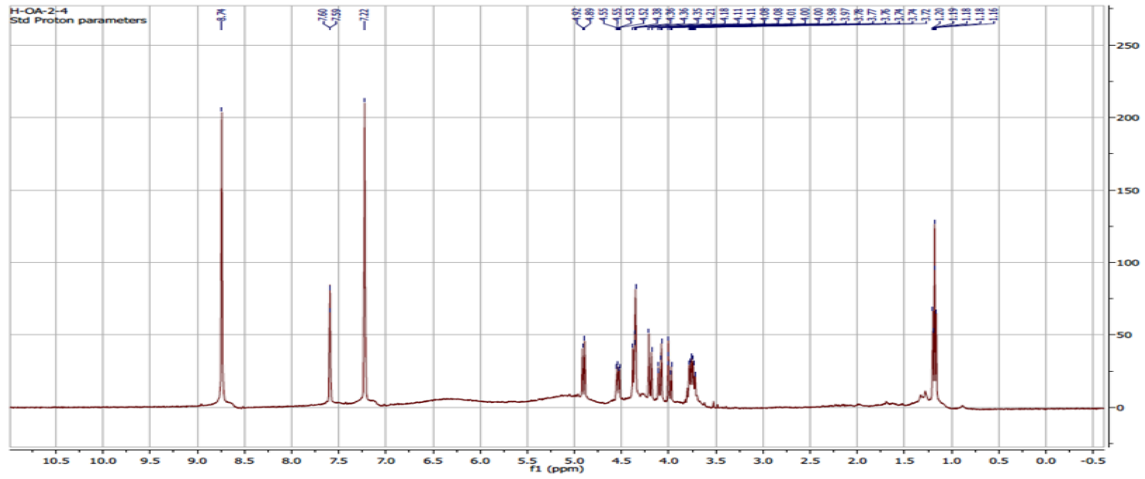


Figure 2a: ^{1}H-NMR spectrum of compound 2.

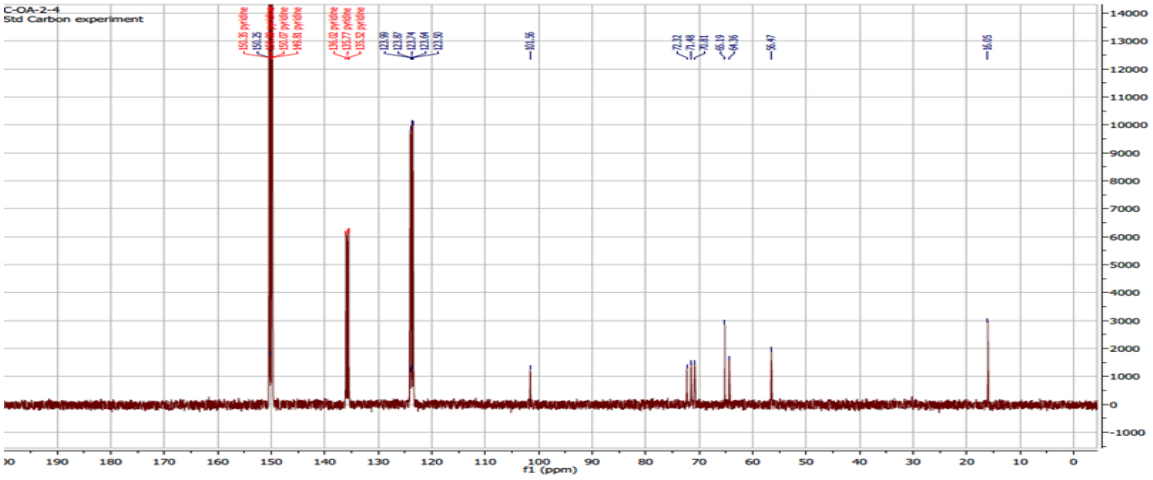


Figure 2b: ^{13}C-NMR spectrum of compound 2.

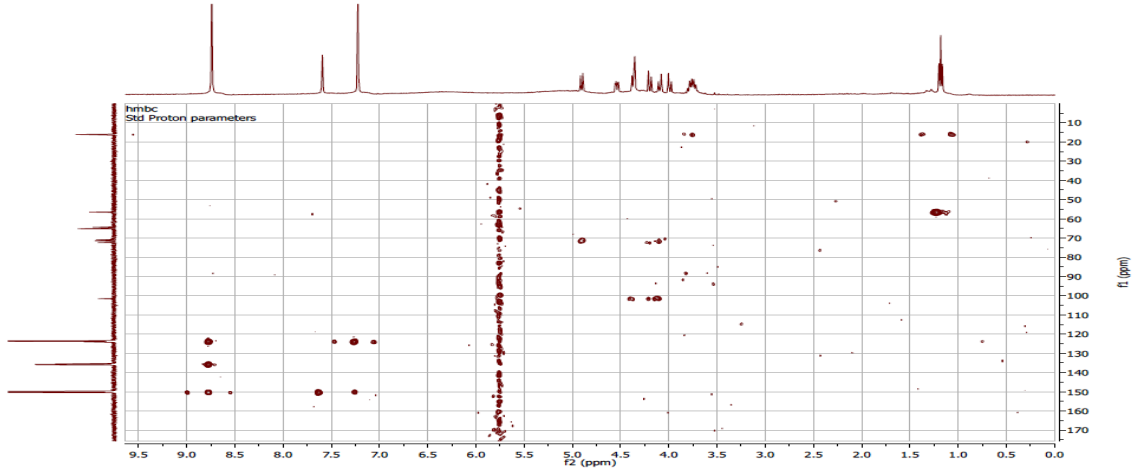


Figure 2c: HMBC spectrum of compound 2.

Chloroquine was included in each assay as positive control. DMSO (0.25 %) was used as a vehicle control.

In vitro antileishmanial activity

The antileishmanial activity of the compounds was tested in vitro against a culture of L. donovani promastigotes[21]. The promastigotes were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum (Gibco Chem. Co.) at 26 oC. A 3-day-old culture was diluted to 5× 105 promastigotes/mL. Drug dilutions were prepared directly in cell suspension in 96-well plates. Plates were incubated at 26 °C for 48 h and growth of leishmania promastigotes was determined by the Alamar blue assay as described earlier. Standard fluorescence was measured on a Fluostar Galaxy plate reader (BMG Lab Technologies) at an

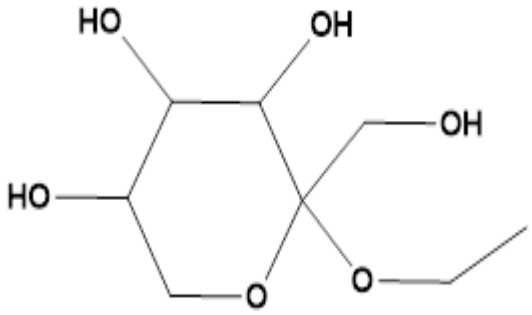
Table 1: The ^{1}H-NMR spectral data of compound 1 (DMSO-500Hz).

protons	Chemical shifts, J constant
H-3	3.33 (1H, m)
H-6	4.85 (1H, m)
H-18	0.64 (3H, s)
H-19	0.94 (3H, s)
H-21	0.89 (1H,d,*J*=5.9*Hz*)
H-26	0.79 (3H,d,*J*=8.8*Hz*)
H-27	0.77 (3H,d,*J*=8.8*Hz*)
H-29	0.81 (3H,t,overlapped)
H-1'	4.21 (1H,d, *J*=7.8*Hz*)

Table 2: ^{13}C-NMR spectral data of compound 1 (DMSO- 125MHz).

Carbons	Chemical shift (ppm)	Carbons	Chemicalshift (ppm)
C-1	36.8	C-19	19.0
C-2	28.9	C-20	35.4
C-3	76.9	C-21	18.9
C-4	38.3	C-22	33.3
C-5	140.4	C-23	25.4
C-6	121.2	C-24	45.1
C-7	31.4	C-25	28.7
C-8	31.3	C-26	18.5
C-9	49.6	C-27	19.6
C-10	36.2	C-28	22.6
C-11	20.5	C-29	11.7
C-12	*	Glucose	
C-13	41.8	C-1'	100.8
C-14	56.1	C-2'	73.4
C-15	23.8	C-3'	76.7
C-16	27.7	C-4'	70.1
C-17	55.4	C-5'	76.7
C-18	11.6	C-6'	61.1

* obscured by solvent peak.



Ethyl. β-D-Fructopyranoside

excitation wavelength of 544 nm and an emission wavelength of 590 nm. Pentamidine and Amphoterecin B were used as the standard antileishmanial agents. IC50 values were computed from dose-response curves as above.

Isolation of Compounds

Air-dried roots of Onopordon (200 g powder) were extracted three times with MeOH (5 L×3) at room temperature for one week and then concentrated. The methanol extract (15 g) was subjected to a silica gel column [0.5 kg, gradient elution using Hex 100%, Hex-EtOAc (9:1), (8:2), (7:3), (5:5), (4:6), (2:8), and EtOAc 100%] to give eight fractions. Fraction 2 (55 mg) was purified on silica gel column using Hex-EtOAc (gradient elution), to give compound 1 (20mg). Fraction 7 (40mg), was subject to Sephadex column eluted with MeOH producing 6 subfractions. Sub-fraction 4 and 5 (19 mg), were loaded on a silica gel column (0.4 g) with isocratic elution using Chloroform-Methanol-Water (15:6:1), compound 2 (4mg) was obtained. Compound 1, is present as white crystals, chemical properties revealed the steroidal and/or triterpenoidal nature of the compound.

Spectroscopic investigation of Compound 1

a-^{1}H-NMR spectral analysis

^{1}H-NMR spectral data of compound 1 are recorded in Table 1 and shown in Fig. 1a&1b as

The ^{13}C-NMR spectral analysis

The ^{13}C-NMR data of compound 1 are listed in Table 2 and illustrated in Fig. 1c, (1c expanded) &1d Preliminary inspection of ^{13}C-NMR data confirmed the presence of 35 carbons. Determination of the multiplicity was carried out by DEPT experiment, which revealed the presence of three quaternary carbons, 14 methine, 12 methylene and six methyl carbon signals, of which 29 carbon signals were for the aglycone while 6 carbon signals were for a sugar moiety. Signals appeared at δ_C 11.64, 19.08, 18.9, 18.5, 19.6 and 11.76 are corresponding to the six methyl carbons of C-18, C-19, C-21, C-26, C-27 and C-29 respectively. In^{1}H-NMR spectrum, the signals at δ_H 0.64 and 0.94 (3H, s) were corresponding to H-18 and H-19 respectively, two doublets at δ_H 0.77 and 0.79 (3H, d, *J*=8.8 *Hz*) were assignable to H-26 and H-27, respectively, the doublet at δ_H 0.89 (3H, d, *J*=5.9 *Hz*) was clear for H-21 and a signal at δ_H 0.81 (3H,t) assigned for H-29. Moreover, there was a downfield signal at δ_H 4.85 (1H, m) corresponding to the olefinic proton at C-6 and one signal at δ_H 3.33 (1H, m) which gave evidence to the glycosylation at C-3 corresponding to H-3. At the same time ^{13}C-NMR spectrum revealed the presence of the olefinic carbons at C-5 and C-6 from the signals at δ_C 140.44 and 121.17 respectively. Comparison of the chemical shifts of the aglycon moiety to those of β-sitosterol revealed that they are coincident[22] except the downfield shift of C-3 at δ_C 76.9 indicating its substitution with the sugar moiety. The chemical shifts of the sugar signals were superimposable with those reported for β-glucopyranose[22,23]. The β-configuration at C-1 of the glucosyl unit was confirmed from the coupling constant of its anomeric proton (7.8 Hz) at δ_H 4.21 in the ^{1}H-NMR spectrum[23]. The identity was substantiated by co-chromatography with an authentic sample of β-sitosterol glucoside. Consequently, the structure of compound 1 was deduced as β-sitoserol-3-O-β-glucopyranoside. Compound 2 was isolated as a viscous substance. The molecular formula, $C_8H_{16}O_6$and molecular weight , of **2** was determined by the high-resolution (HR)-electrospray ionization (ESI)-MS analysis (*m/z* 231.0698 $[M+Na]^+$, calcd 231.0635). The ^{1}H-NMR (C_5D_6N-d_4) spectrum of 2 displayed a methyl protons [δ_H 1.16 (3H, t, J=6.8 Hz)], two methylene protons [δ_H 4.39 (2H, d,

J=11.2, 11.6 Hz) and δ_H 4.08 (2H, d, *J*=12 Hz)] and two methine protons at □□4.36, 4.53 and 4.90. The ^{13}C-NMR spectral data displayed 8 resonances, three signals were corresponding to methylene carbons □c 56.47, 64.36 and 65.19, and three methines bearing an oxygen function at □c 6 and a methyl carbon at □c 70.81, 71.48 and 72.32. The remaining two signals were attributable to methyl group with □c 56.47 and one signal representing one quatornary carbon □c 56.47. The structure was determined by 2D NMR experiments where the HMBC experiment of 2 showed long-range correlations between the methyl protons (□ 1.16) and the methylene carbon at□c 56.47, and the COSY experiment represented clear correlation between C (71.48), C (72.32) and C (70.81). The structure of compound **2** was therefore determined to be Ethyl. β-D-Fructopyranoside, illustrated in Fig 2a, 2b &2c.

RESULTS AND CONCLUSION

Onopordum alexandrinum sweet tuberous roots are eaten by natives in the Egyptian western desert, and where reported to cause hallucination and death to some native Bedouins whom have eaten higher doses. Investigating the truth of this matter and the causes of these symptoms and the possibility of using these tuberous roots as hallucinogens were all performed by examining the methanol extract of the roots and aerial parts for any CNS activity using Opioid and cannabinoid receptors binding activity assay, where the results were disappointing at this stage, showing no binding affinity to any of the tested receptors. Further biological investigations of both extracts showed no acute toxicity up to 5gm/kg body weight, also no considerable cytotoxicity was observed on Huh-7 Liver cancer or A-495 lung cancer cell lines neither was any antibacterial activity or antifungal activity observed using some selected species against Ciprofloxacin and Amphotericin B respectively, on both the total methanol extracts and their subfractions from hexane and chloroform. Several spots were visualized using TLC but only two compounds were isolated, due to the scarce amounts of the powdered plant available and its use in the many biological assays. Compound 1 was identified as β-sitoserol-3-O-β-glucopyranoside) and compound 2 was Ethyl β-D-fructopyranoside, elucidated using spectroscopic methods. These two compounds are first report in this genus. Further investigation is being performed using new supplies of the plant aerial parts and roots for new trials of isolation of other new compounds and identification of the compounds responsible for the use of this drug as a hallucinogen and its possible use for treatment as a pain suppressant.

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