



FOXO signal activating alkaloids isolated from *Ochrosia elliptica* leaf cultivated in Egypt

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Abstract

A new yohimbine type alkaloid *N*-methylarcinine (**1**), and the known alkaloid, holeinine (**2**), have been isolated from the methanolic leaf extract of *Ochrosia elliptica* grown in Egypt. The structures of the isolated metabolites were elucidated via 1D and 2D NMR data, as well as HRESIMS spectra and the spectral data were compared with the existing data available from literature. These two alkaloids are very different in activity from previously reported class type alkaloids, where they showed weak cytotoxic activity against K-562 leukemia cells while other compounds from this class were highly toxic in nature. Interestingly, holeinine (**2**) showed unique induction of FOXO expression, a cancer signaling pathway. FOXO is involved in the expression of death receptor ligands, such as TNF apoptosis ligand, where its activation plays a critical role in tumor suppression. As several anticancer leads targeting FOXO signaling gain more attention, this compound could be a candidate for drug development after the determination of its therapeutic efficacy and more mechanistic studies need to be evaluated.

Keywords *Ochrosia elliptica* · Apocynaceae · Yohimbine alkaloid · Holeinine · FOXO

Introduction

Ochrosia elliptica Labill is a large upright tree with glossy leaves belonging to family Apocynaceae and the tree is native to Australia. *Ochrosia* is from Greek *ochros*, yellow,

apparently referring to the color of the wood of some species (the common name for the genus is “yellow wood”) while *elliptica* refers to the elliptical shape of the leaves (Hendrian 2004).

Many secondary metabolites have been isolated from different plant parts *viz.* flavonoids (El-shiekh et al. 2017), ursolic acid (Labib et al. 2016), coumarins, resinol derivatives, phenolic acids (Liu et al. 2015), and several alkaloids like ellipticine, methoxsellipticine, isoreserpiline, elliptinine, and epchrosine (Kim et al. 2011; Kuo et al. 2005; Salim et al. 2004; Carroll et al. 2008). These alkaloids showed potent cytotoxic activity. Ellipticine and its derivatives exhibited cytotoxic activity against different cell lines, e.g., human breast carcinoma (MCF-7), leukemia (HL-60 and CCRF-CEM) cells, neuroblastoma (IMR-32, UKF-NB-3 and UKF-NB-4) cells, and glioblastoma cells (U87MG) (El-shiekh et al. 2017; Kim et al. 2011; Stiborova et al. 2011; Lichota and Gwozdziński 2018). Ellipticine was found to disrupt cell cycle by binding to DNA and regulate the expression of some kinases (Lichota and Gwozdziński 2018). In addition, monoterpene indole alkaloids, e.g., 10-methoxyakuammidine, akuammidine, picrinine, and rhazimol, have been previously isolated and showed cytotoxic activity (Chen et al. 2017). Reserpiline and

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isocarapanaubine, along with ellipticine and 9-methoxyellipticine, were isolated as the main cytotoxic alkaloids from the barks of *Ochrosia elliptica* (Kuroda et al. 1999).

In a continuation to our search for compounds with potent biological activities, the methanolic leaf extract was subjected to extensive chromatographic analysis and the isolated compounds were screened for their cytotoxic activity and for their sensitivity against various cancer signaling pathways to understand their molecular mechanism.

The Forkhead box O (FOXO) is one of the members of the forkhead transcription factor family which is regulated by growth factors like insulin. FOXO plays critical role in tumor suppression in a wide range of cancers as it is involved in the expression of death receptor ligands such as TNF apoptosis ligand. FOXO has been known to interact with many pathways such as I κ B kinase (IKK) pathway, however the interactions with the Kinase of phospholipid kinase phosphatidyl inositol-3-kinase/Protein Kinase B (PI3K/AKT) pathway have been observed in different types of cancers. Targeting FOXO signaling pathway could lead to discover potential anticancer agents (Farhan et al. 2017).

Materials and methods

Plant material

Fresh leaves of *Ochrosia elliptica* Labill. Family Apocynaceae were collected in April, 2013 in El-Giza Zoo garden, Giza, Egypt. The voucher specimens of the plant material were deposited at Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt given the identity (PHG-P-OE-171).

Extraction, isolation, and identification of *Ochrosia* alkaloids

Five kilograms of the air-dried leaves were extracted via maceration with 10 L MeOH at room temperature for 1 week and filtered. The filtrate was concentrated to yield 110 g residue which was suspended in water (300 mL) and partitioned with *n*-hexane (1 L), DCM (8 L), EtOAc (4 L), and BuOH (4 L) to afford 7, 40, 12, and 38 g, respectively. BuOH fraction gave an intense orange color when sprayed with Dragendorff's reagent. Thus the BuOH fraction (20 g) was applied on top of silica gel column (300 g, 30 cm L \times 10 cm i. d.) and gradually eluted with DCM with an increasing polarity by adding MeOH to afford 14 fractions. Fraction 13 (4.46 g) gave highly dense orange color with Dragendorff's reagent, thus it was applied on an alumina column (200 g, 30 cm L \times 10 cm i. d.) to be eluted with DCM with gradual addition of MeOH to afford 218 sub-fractions. Sub-fractions (84–129, 97.2 mg) eluted with DCM:MeOH (9:1), gave

Table 1 ^{13}C NMR and ^1H data for compound (1)

Position	δC ($\text{C}_5\text{D}_5\text{N}$, 55 °C)	δH ($\text{C}_5\text{D}_5\text{N}$, 55 °C)
2	131.3 C	–
3	65.3, CH	5.88 (<i>d</i> , 10.5)
5	56.6, CH ₂	4.03 (<i>m</i>) 4.13 (<i>m</i>)
6	18.6, CH ₂	3.13 (<i>m</i>)
7	101.9, C	–
8	126.9, C	–
9	101.1, CH	7.14 (<i>d</i> , 2.5)
10	154.8, C	–
11	112.7, CH	7.06 (<i>dd</i> , 8.7, 2.5)
12	113.5, CH	7.69 (<i>d</i> , 8.7)
13	133.4, C	–
14	33.2, CH ₂	2.19 (<i>m</i>) 3.25 (<i>bd</i> , 14.5)
15	27.8, CH	3.44 (<i>m</i>)
16	108.7, C	–
17	154.8, CH	7.56 obscured
18	18.2, CH ₃	1.52 (<i>d</i> , 6.0)
19	72.4, CH	4.75 (<i>m</i>)
20	36.1, CH	2.55 (<i>m</i>)
21	62.3, CH ₂	4.24 (<i>m</i>), 4.45 (<i>m</i>)
COOMe	167.1, C	–
COOMe	51.1, CH ₃	3.68 (<i>s</i>)
10-OMe	56.0, CH ₃	3.80 (<i>s</i>)
CH ₃ -N	50.3, CH ₃	3.60 (<i>s</i>)

orange color upon spraying with Dragendorff's reagent and were purified using preparative HPLC to afford two compounds (1) (4.0 mg) and (2) (3.0 mg).

N-methylarcinine (1): Pale yellow powder; $[\alpha]_{\text{D}}^{25}$ –55.5 (c 0.18 MeOH); UV (MeOH) λ_{max} nm (log ϵ) 233 (4.33), 269 (3.93), 296 (3.71), 310 (3.60); ^1H NMR and ^{13}C NMR ($\text{C}_5\text{D}_5\text{N}$, 55 °C, 400 MHz), see Table 1; HR-ESI-MS at m/z 397.2125 $[\text{M}]^+$ (calcd. for $\text{C}_{23}\text{H}_{29}\text{N}_2\text{O}_4$, 397.21273).

Preparative HPLC

Quantitative HPLC analysis was conducted using an Agilent 1100 HPLC system equipped with a degasser (G1379A), quaternary pump (G13311A), auto sampler (G1313A), column oven (G1316A), and UV-Diode detector (G1315B) controlled by Chemstation software. The analysis was carried out on RP-C18 columns (150 \times 4.6 mm; particle size 5 μm ; Luna) and (250 \times 10.0 mm; particle size 10 μm ; Luna) with column oven temperature set at 25 °C and using the gradient system of eluent water (A) and acetonitrile (B) for the separation of the target compounds. The gradient condition was as follows: 0–2 min (5% B), 2–32 min (50% B), and 32–37 min (100% B). The flow rates of the solvent

were 1.0 mL/min for the analytical injections while 4 mL/min for the semi preparative ones and the injection volumes were 5.0 and 50 μ L for the analytical and semi preparative, respectively. All the analysis was carried out at wavelengths of 220 and 254 nm with a run time 35 min. HPLC grade acetonitrile and water solvents were used. Acetic acid was added as a modifier to achieve a final concentration of 0.1% in each solvent.

Cytotoxicity assay

K-562 from ATCC, were plated in clear 384-well plates at an initial density of 2500 cells/well in 40 μ L of growth medium (DMEM with 10% FBS and 1% Pen/step). Next day, the test agents were added at the specified concentration and treatment continued for 48 h and the cell viability was finally assessed using WST-8 assay Cell Counting KitBimake according to manufacturer's instructions (Kageyama et al. 2018). The results were measured by absorbance at 450 nm using SpectraMax M5 plate reader (Molecular Devices). Cell viability was calculated in comparison with DMSO control.

Transfection and luciferase assay

Hela cells from ATCC were plated in white opaque 384-well plates at a density of 4300 cells/well in 30 μ L of growth medium (DMEM with 10% FBS and 1% Pen/step). Next day, the medium was aspirated and replaced with DMEM containing 10% FBS. The cells were transfected with FOXO plasmid (Fork head box O1 (pFKHR-Luc) from Signosis Inc., using X-tremeGENE HP transfection reagent (Roche). After 24 h of transfection, the test agents were added to the transfected cells. After 6 h of induction, the cells were lysed by the addition of One-Glo luciferase assay system (Promega). The light output was detected in a Glomax Multi + detection system with Instinct Software (Promega) and enhanced luciferase activity by the test agent is assessed (Zaki et al. 2013).

Results and discussion

Preparative HPLC analysis resulted in the isolation of compounds (1) and (2) at retention times 14 and 15 min, respectively.

Compound (1) showed HR-ESI-MS at m/z 397.2125 $[M]^+$ which is compatible with the molecular formula $C_{23}H_{29}N_2O_4$ showing 11 degree of unsaturation. Spectral data were run in C_5D_5N at 55 $^\circ$ C (Table 1). Spectral data were run in different deuterated solvents viz. $CDCl_3$, CD_3OD , and C_5D_5N , at different temperatures. The trials that have been made to detect the structure of the isolated compounds in deuterated methanol and chloroform were not successful to elucidate the structure due to the disappearance of

some major peaks viz. C5, C14, C16, and C21 in $CDCl_3$, and C5 and C21 in CD_3OD . Thus we have used C_5D_5N at 55 $^\circ$ C.

The 1H NMR has shown the presence of three aromatic protons at δ_H 7.14 (d , $J = 2.5$ Hz, H-C9), 7.06 (dd , $J = 8.7$, 2.5 Hz, H-C11), and 7.69 (d , $J = 8.7$ Hz, H-C12) with only one methoxy attached to C-10 at δ_C 56.0 ppm which was supported by HMBC correlations which in turn confirmed the presence of monosubstituted indole ring. The shielded carbon at C-16 (δ_C 108.7 ppm) indicated the attachment to a carbonyl moiety (δ_C 167.1 ppm) having an ester linkage to a methoxy 3.68 (s , 3H) at δ_C 51.1 ppm. Two olefinic carbon moieties have been recorded. The first one resonate at δ_C 131.3 and 101.9 ppm (C-2, C-7), respectively, while the second double bond resonates at δ_C 108.7 (C-16) and 154.8 (C-17) to which a downfield proton appeared at δ_H 8.40 (s , H-17). The NMR data revealed the presence of two methyl groups, one is attached to N atom thus appearing at δ_H 3.60 (s), δ_C 50.3; while the second methyl is attached at C-19 thus appearing as δ_H 1.52 (d , $J = 6.0$ Hz) and δ_C 18.2 ppm.

The non-aromatic region showed the presence of four methine carbons at δ_C 65.3 (C-3), 27.8 (C-15), 72.4 (C-19), and 36.1 (C-20). The downfield value of C-3 and C-19 had supported their attachment to N and O, respectively. Data have shown four methylene carbons at δ_C 56.6 (C-5), 18.6 (C-6), 33.2 (C-14), and 62.3 (C-21).

Analysis of the 1D and 2D NMR including the NOESY spectrum have supported the α -configuration of H attached to C-3, C-15, and C-19 of compound (1) which represents a yohmbine type alkaloid similar to those of *Ochrosia* but lacking one methoxy function at C11 (Figs. 1 and 2). Compound (1) was identified as *N*-methylarcinine and it was isolated and identified for the first time from the genus *Ochrosia*.

Compound (2) was isolated as white amorphous powder. Its spectra were similar to compound (1) but with additional methoxy at C11 (Fig. 3). By comparing its spectral data

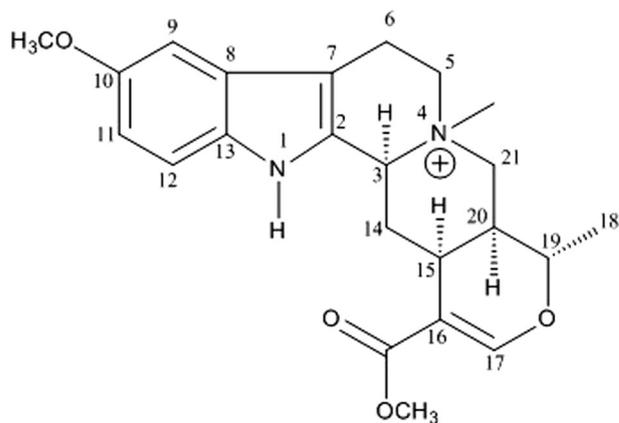


Fig. 1 Chemical structure of compound (1) isolated from *O.elliptica*

with those reported, it was anticipated that compound (2) is the known alkaloid; holeinine (Sainsbury and Webb 1972).

In a continuous search for new anticancer entities isolated from natural sources, the isolated compounds were tested for their cytotoxic activity at various concentrations in K-562 leukemia cells (Fig. 4). Both compounds showed mild cytotoxic activity (10–20%) at the highest concentration tested (100 μM). Doxorubicin and Taxol were used as positive controls for this assay. This data suggested that these compounds are less toxic compared to the previously isolated *Ochrosia* alkaloids, e.g., ellipticine, and methoxyellipticine, which showed more potent toxic activities. Interestingly, compound (2) showed very unique activation of FOXO gene expression (9.5-fold induction at 100 μM concentration, when compared with pTK vector as control) (Fig. 5). At the same concentration, this compound does not

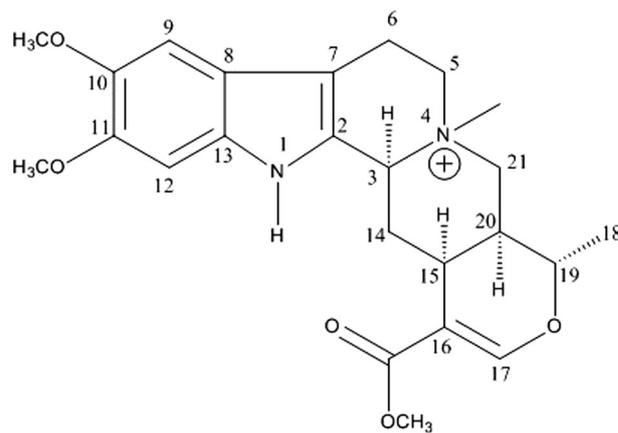


Fig. 3 Chemical structure of compound (2) Holeinine isolated from *O. elliptica*

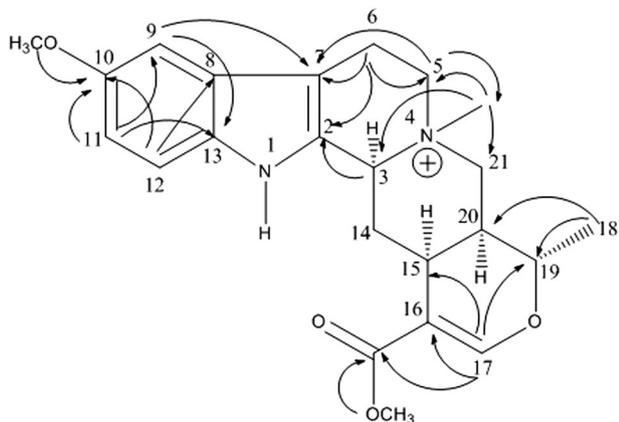


Fig. 2 HMBC correlation of compound (1) isolated from *O. elliptica*

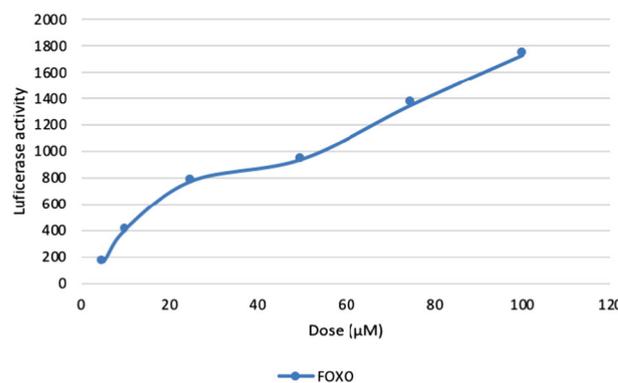
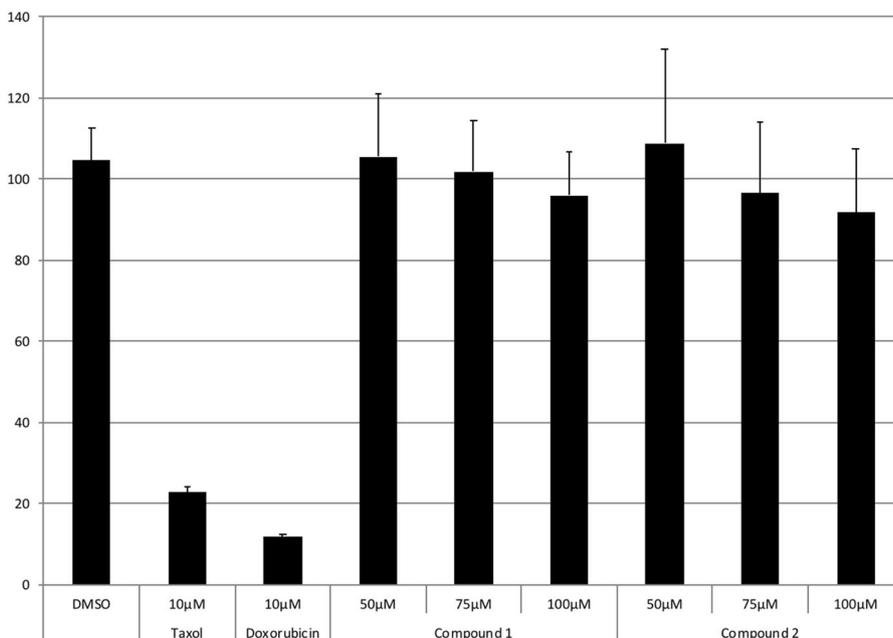


Fig. 5 Dose response effect of compound 2 on FOXO gene expression

Fig. 4 Dose response effect of the isolated alkaloids in K-562 cell cytotoxicity assay



show any activity toward other anticancer signaling pathways. FOXO plays important roles in prevention and treatment of cancer (Farhan et al. 2017). FOXOs are involved in cell fate decisions (apoptosis) and it is suggested to play a pivotal functional role as a tumor suppressor in a wide range of cancers (Greer and Brunet 2005). In recent days, the compounds that target FOXO signaling pathway have been emerged as efficacious agents against several cancers. Based on this preliminary signaling pathway screening, compound (2) could have cytotoxic potential as FOXO induction which is critical in tumor suppression thus open the window for further exploration (Zhang et al. 2010).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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