Cytotoxic new furoquinoline alkaloid isolated from *Ammi majus* L. growing in Egypt

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\textbf{ABSTRACT}

Two alkaloids of the furoquinoline-type were isolated from *Ammi majus* L.; a new one and was identified as 4-hydro-7-hydroxy-8-methoxyfuroquinoline (1), and the other was isolated for the second time from nature and was identified as 4-hydro-7-hydroxy-8-prenyloxyfuroquinoline (2). The structures of the isolated compounds were established and confirmed by 1D and 2D NMR spectroscopy including \textsuperscript{1}H, \textsuperscript{13}C NMR, COSY, HSQC and HMBC, while the exact masses were confirmed by HRESI/MS. The cytotoxic activity of the isolated compounds (1 and 2) was evaluated against HepG-2, PC-3, A-549 and MCF-7 and the obtained results suggested selective antiproliferative and cytotoxic effects, with IC\textsubscript{50} = 230.2 and 326.5 μM against HepG-2 and MCF-7, respectively, for compound (1). While, compound (2) recorded IC\textsubscript{50} = 234.2 μM against MCF-7.

1. Introduction

*Ammi majus* L. (Bishop’s weed) closely resembles *Ammi visnaga* L. It belongs to the family Umbelliferae (Apiaceae). *A. majus* is an annual herbaceous plant which is widely spread in Egypt alongside the Nile and delta region (Fahmy et al. 1947). It is distributed in the Mediterranean region of Europe, western Asia and now cultivated in India (CCRUM 1987). *A. majus* is considered as one of the most important medicinal plants in Egypt (Singab 1998). It is widely used for the treatment of skin disorders such as psoriasis and vitiligo. It is used

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as an emmenagogue to regulate menstruation, as a diuretic, and for the treatment of leprosy, kidney stones and urinary tract infections (NAPRALERT, 2014). Its fruits are taken internally by the public to cure leukoderma (Fahmy et al. 1947).

Numerous reports studied the phytoconstituents of both *A. majus* and *A. visnaga*, and concluded that furocoumarines (i.e. xanthotoxin) and furanochromones (i.e. khellin and visnagin), respectively, are the major constituents which are used for numerous medicinal purposes, e.g. leukoderma (vitiligo) (Abu-Mustafa & Fayez 1961; Abu-Mustafa et al. 1971; Murry et al. 1982; Farnsworth et al. 1985; El-Gamal et al. 1991; El-Gamal, Shalaby, Duddeck, & Hiegemann 1993; Thomas et al. 1998; Badr et al. 2015). Furthermore, five flavonol glycosides were isolated from the non-fruiting aerial parts of *A. majus* (Singab 1998). Selim and Ouf (2012) reported the promising antiviral and antimicrobial activities of the isolated coumarins from *A. majus*. In addition, two adjuncts (benzofuran and chalcone derivatives) were isolated from the fruits of *A. majus* L. (El-Gamal, Shalaby, & Duddeck 1993).

To date there are no reports of alkaloidal contents of *A. majus* although it has been reported as one of the plants containing alkaloids of the family Umbelliferae (Raffauf 1996), and was previously reported by Bick (1996) to give a positive alkaloidal test. Moreover, Hartwell (1982) reported that *Ammi* species, especially *A. majus* used in the folkloric medicine for the treatment of different types of tumours, i.e. spleen, uvula and fauces and in the induration of liver and stomach. This what prompted us to investigate its alkaloidal content.

Extensive chromatography of the MeOH extract of *A. majus* successfully resulted in the isolation of two alkaloids of the furoquinoline-type; where compound (1) the hitherto unknown was identified as 4-hydro-7-hydroxy-8-methoxyfuroquinoline, while, compound (2), was isolated for the second time from nature (firstly reported by Mohammed et al. 2016) and identified as 4-hydro-7-hydroxy-8-prenyloxyfuroquinoline. The isolated alkaloids were structurally elucidated by extensive NMR spectroscopy, and then were investigated for their cytotoxicity against HepG-2, PC-3, A-549 and MCF-7 cell lines. Structure activity relationship of the biologically active metabolites was studied in order to highlight their potentials as cytotoxic candidates for new drugs that may be helpful for human and livestock diseases.

2. Results and discussion

The defatted MeOH extract of *A. majus* was subjected to successive silica gel column chromatography to afford two alkaloids of the furoquinoline-type (1 and 2).

2.1. Compound (1)

It was isolated from fraction (V) by 100% CH₂Cl₂ as an amorphous solid, with molecular formula determined to be C₁₂H₉NO₃ using HRESI/MS (+ve, S7), which revealed the presence of two quasi-molecular ion peaks at m/z 217.0733 [M + 2H]⁺ and m/z 239.0553 [M + H + Na]⁺ (calcd 217.0738 for C₁₂H₁₁NO₃ and 239.0558 for C₁₂H₁₀NO₃Na).

¹H and ¹³C NMR (CDCl₃, S1 and S2, respectively) spectra suggested a furoquinoline-type alkaloid base skeleton (Ayafor & Okogun 1982; Mohammed et al. 2016). Its ¹³C NMR (S2) spectrum revealed the presence of 12 signals, classified by DEPT (S4) and HSQC (S5) spectra into one methyl, five methines and six quaternary carbons, which confirmed the molecular formula C₁₂H₉NO₃. ¹H NMR spectrum revealed the presence of a downfield sharp singlet
signal at $\delta^H 4.27$ (3H, s, –OCH$_3$) characteristic for a phenolic methoxy group. In addition, a pair of AB doublets appeared at $\delta^H 6.80$ and $\delta^H 7.67$ (each 1H, d, $J = 2.0$ Hz; H-3 and H-2, respectively) which are characteristic for the two furan ring protons, and confirmed via a cross-peak's correlation as appeared in COSY spectrum (S3), moreover, another cross-peak was assigned to the two ortho-coupled aromatic protons, that appeared at $\delta^H 6.34$ and $\delta^H 7.75$ (each 1H, d, $J = 9.6$ Hz; H-6 and H-5, respectively). Furthermore, the sharp singlet signal that appeared at $\delta^H 7.33$ (1H, s) was assigned to H-4 based on the HMBC correlation of H-4 with C-3 at $\delta^C 106.66$ and with C-5 at $\delta^C 144.34$ (Figure S17).

The postulated structure of compound (1) was confirmed through HMBC correlations (S6) as appeared clearly in Figure S17; briefly, H-3 with C-3a and C-4, H-4 with C-3, C-3a and C-4a. H-5 and H-6 were distinguished on the base that H-5 showing HMBC correlation to C-4 at $\delta^C 112.86$, in addition, the HMBC correlation of H-5 to $\delta^C 139.40$ allowed this resonance to be assigned to C-7, which declared that C-8 resonance to be assigned at $\delta^C 145.04$. Moreover, the downfield phenolic methoxy group was confirmed to be attached to C-8 based on the HMBC correlation of the methoxy protons that appeared at $\delta^H 4.27$ with C-8 at $\delta^C 145.04$.

Thus, compound (1) was elucidated as formulated in Figure 1 and assigned to 4-hydro-7-hydroxy-8-methoxyfuroquinoline.

2.2. Compound (2)

It was isolated from fraction (III) by hexane–CH$_2$Cl$_2$ (70:30 v/v) as yellowish white powder, with m.p. 87–88 °C (CHCl$_3$). Its molecular formula was determined to be C$_{16}$H$_{13}$NO$_3$ using HRESI/MS (+ve, S14), which showed two quasi-molecular ion peaks at m/z 271.1205 [M + 2H]$^+$ and m/z 293.1020 [M + H + Na]$^+$ (calcd 271.1208 for C$_{16}$H$_{17}$NO$_3$ and 293.1022 for C$_{16}$H$_{16}$NO$_3$Na).

$^1$H- and $^{13}$C NMR spectra (CDCl$_3$, S8 and S9, respectively) of compound (2) displayed closely the same NMR data for the furoquinoline alkaloid ‘Aegelbine-B’, which was isolated from Aegle marmelos (Mohammed et al. 2016). Briefly, $^{13}$C NMR spectrum of compound (2) showed the presence of 16 signals, resolved by DEPT (S11) and HSQC (S12) into; two methyls, one methylene, six methines and seven quaternary carbons. Its $^1$H NMR and COSY spectra (S8 and S10, respectively) showed two furan ring protons appeared at $\delta^H 6.81$ and 7.69 (each 1H, d, $J = 2.4$ Hz, H-3 and H-2), respectively, two ortho-coupled aromatic protons at $\delta^H 6.36$
and 7.76 (each 1H, J = 9.6 Hz, H-6 and H-5), respectively, and H-4 appeared at δ_H 7.36 (1H, s). An olefinic proton appeared at δ_H 5.60 (1H, t, J = 6.8 Hz, H-2’), and correlated with the two oxymethylene protons appeared at δ 5.00 (2H, d, J = 6.8 Hz, H-1’), two vinylic methyl groups appeared at δ 1.71 and 1.74 (each 3H, brs). HMBC experiment (2 & 3) (S13) gave the same long range correlations of ‘Aegelbine-B’ as appeared in Figure 1. In brief, H-4 was assigned to be at δ_H 7.36 based on its correlations with C-5 at δ_C 144.22 and C-3 at δ_C 106.67. Since H-5 correlated to δ_C 143.76 suggested this resonance to be assigned to the aromatic hydroxylated C-7, which led to allocate C-8 to be at δ_C 148.56. The exact position of the prenyloxy group on the furoquinoline skeleton was confirmed to be at C-8 based on the correlation of the oxymethene protons CH2-1′ at δ_H 5.00 with C-8 at δ_C 148.56 (Figure S17).

Compound (2) was established as depicted in Figure 1 and was assigned to 4-hydro-7-hydroxy-8-prenyloxyfuroquinoline, its NMR data match well with ‘Aegelbine-B’, which firstly isolated by (Mohammed et al. 2016). 

The isolated alkaloids (1 and 2) represent a furoquinoline base skeleton with a free C-4 proton (rarely found in nature) as elucidated from HMBC correlations (Figure S17), other than the previously isolated furoquinoline alkaloids (usually methoxylated at C-4), in addition to the methoxy and prenyloxy groups at C-8 in compounds (1 and 2), respectively.

The isolated furoquinoline alkaloids (1 and 2) were evaluated in vitro for their cytotoxicity (S15), against HepG-2, PC-3, A-549 and MCF-7 cell lines using MTT assay in comparison with Adriamycin (Doxorubicin) as a reference compound. The obtained results revealed some sort of selectivity, which can be clarified as follow, compound (1) recorded the highest activity against HepG-2 and MCF-7 with IC_{50} = 230.2 and 326.5 μM, respectively, while, it showed a moderate cell inhibition against PC3 and A549. On the other hand, compound (2) recorded slight cell inhibition against HepG-2, PC-3 and A-549, while its highest activity was recorded against MCF-7 with IC_{50} = 234.2 μM.

Since furoquinoline alkaloids are classified as alkylating agents that tend to assemble covalently between the stacks of paired nucleotides in the DNA double helix, and hence lead to cell death (Wink 2007). So, activity of the isolated alkaloids in the present study may be attributed to their ability to form covalent bonds with the base pairs of the DNA (Mohammed et al. 2016), which requires the presence of hydrogen bond donors (i.e. –OH). In a former study by our group (Mohammed et al. 2016), we reported the SAR of the isolated furoquinoline alkaloids (Aegelbine-A and -B) and their cytotoxic activity. From the obtained results, we concluded that; as long as the number H-bond donors increased the activity increased, i.e. Aegelbine-A (with phenolic ortho-di-OH groups) was highly active than Aegelbine-B (compound 2 in the present study).

The presence of the four base pairs together with the unique pattern between H-bond donors and H-bond acceptors in the major groove allow greater specificity for the interaction more than in the minor groove of DNA (Rohs et al. 2010).

This fact helps to unveil the reason for the cytotoxic activity of the isolated alkaloids, even so, they both have one –OH group at C-7 (H-bond donor); compound (1) with an electron donating group ( –OCH3 at C-8) adjacent to the –OH group, helps in and stabilises the H-bond formation by rebalances the charge distribution (Li et al. 2006), while in compound (2), the presence of the bulky prenyloxy group at C-8 causes steric hindrance for the interaction with DNA base pairs, which effects on or destabilises the H-bond formation (Mohammed et al. 2016).

This comes in agreement with the previous report by Nam et al. (2005), who concluded that the presence of a single methoxy group at the C-8 position of the furoquinoline skeleton
is a critical for the inhibitory activity against human PDE5A. Moreover, the presence of a methylenedioxy group (at 6,7-positions) adjacent to the C-8 methoxy group in enhancing the inhibitory activity of the furoquinoline alkaloids against human carcinoma cell lines (Nouga et al. 2016). Furthermore, the presence of a substituted prenyloxy group at the C-7 position of the furoquinoline alkaloids with a free C-8 position revealed a significant activity against HeLa cell line (Komala et al. 2006).

3. Experimental

3.1. Plant materials

The whole plant A. majus was collected from the Delta region [along the agricultural road to Shebin El-Kom city, https://goo.gl/maps/5RkZPiN9Bu32 (30°32′49.7″N 31°06′11.2″E)] El-Menoufia governorate, Egypt in April 2010. It was kindly identified by Mrs. Theresa Labib, Head of the Taxonomy specialists at El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (No. 01/04/03) has been deposited at the Herbarium of El-Orman Botanical Garden, Giza, Egypt. The plant materials were air dried, finely powdered and used for the extraction.

3.2. General

Melting points (uncorrected) were determined on a Koffler’s melting point apparatus. NMR: Spectra were obtained using a pulse sequence supplied from Bruker AVANCE-III-400 MHz NMR spectrometer for (1D and 2D NMR). Chemical shifts were given in values (ppm) relative to trimethylsilane as an internal reference for both carbon and proton. HR-ESI/MS: as reported (Mohammed et al. 2014). All solvents used were of AR grade. Kiesel gel 60 F254 (Merck) was used for analytical TLC.

3.3. Extraction procedure

One kilogram of the air-dried powdered materials (whole plant) of A. majus were extracted by maceration process in a metal container using MeOH (4 × 5 L) at r.t. The obtained MeOH extracts were combined together and concentrated completely under vacuo to afford 60 g of dried MeOH extract. Fifteen grams of the resulted MeOH extract were subjected to a silica gel column chromatography (500 g, 60 Å, 150 × 2 cm i.d.), which eluted firstly with 100% hexane and with gradual increase of polarity till 100% CH₂Cl₂, and finally with 100% MeOH (−ve Dragendorff’s). Five main fractions (I–V) were obtained based on their TLC profile [EtOAc–MeOH (9:1 v/v)]; fraction I (100% hexane) gave hydrocarbons (discarded), and fraction II (hexane–CH₂Cl₂, 95:5 v/v) contained mainly fatty acids (grey and greyish-blue on TLC) (Mohammed et al. 2013). Fractions (III–V) (hexane–CH₂Cl₂, 70:30 v/v), (hexane–CH₂Cl₂, ‘60:40–20:80’ v/v) and (CH₂Cl₂, 100%), respectively, gave positive Dragendorff’s reagent suggested the possible presence of alkaloids (Mohammed et al. 2012). TLC comparison of the three fractions (III–V) revealed that fraction IV is a mixture of both fractions (III and V). Further chromatographic fractionation, isolation and purification of the positive fractions (III–V) using silica gel column chromatography eluted with (hexane–CH₂Cl₂) and monitored by TLC using EtOAc–MeOH (9:1 v/v) resulted in the isolation of; compound 1 (amorphous solid,
10 mg, 100% \( \text{CH}_2\text{Cl}_2 \) and identified as 4-hydro-7-hydroxy-8-methoxyfuroquinoline from fraction (V) and compound 2 (yellowish white powder, 12 mg, hexane–\( \text{CH}_2\text{Cl}_2 \) 70:30) and identified as 4-hydro-7-hydroxy-8-prenyloxyfuroquinoline from fraction (III). Fraction (IV) contained mixture of both compounds 1 and 2. The isolated compounds were examined for its purity by PTLC using the same solvent system.

3.3.1. 4-hydro-7-hydroxy-8-methoxyfuroquinoline (1)
Amorphous solid, \( ^1\text{H} \) NMR (\( \delta_H \), CDCl\(_3\), 400 MHz); 6.80 (1H, \( d \), \( J = 2.0 \) Hz, H-3), 7.67 (1H, \( d \), \( J = 2.0 \) Hz, H-2), 6.34 (1H, \( d \), \( J = 9.6 \) Hz, H-6), 7.75 (1H, \( d \), \( J = 9.6 \) Hz, H-5), 7.33 (1H, \( s \), H-4), 4.27 (3H, \( s \), –OCH\(_3\)). \( ^{13}\text{C} \) NMR (\( \delta_C \)); 146.57 (C-2, CH), 106.66 (C-3, CH), 126.07 (C-3a, C), 112.86 (C-4, CH), 116.37 (C-4a, C), 144.34 (C-5, CH), 114.56 (C-6, CH), 139.40 (C-7, C), 145.04 (C-8, C), 132.64 (C-8a, C), 160.46 (C-9a, C), 61.21 (OCH\(_3\)). Full NMR data (Table, S16, Figure S17).

3.3.2. 4-hydro-7-hydroxy-8-prenyloxyfuroquinoline (2)
Yellowish white powder, m.p. 87–88 °C (CHCl\(_3\)). \( ^1\text{H} \) NMR (\( \delta_H \), CDCl\(_3\), 400 MHz); 6.81 (1H, \( d \), \( J = 2.4 \) Hz; H-3), 7.69 (1H, \( d \), \( J = 2.4 \) Hz, H-2), 6.36 (1H, \( d \), \( J = 9.6 \) Hz, H-6), 7.76 (1H, \( d \), \( J = 9.6 \) Hz, H-5), 7.36 (1H, \( s \), H-4), 5.60 (1H, brt, \( J = 6.8 \) Hz, H-2'), 5.00 (2H, \( d \), \( J = 6.8 \) Hz, H-1'), 1.71 and 1.74 (each 3H, brs). \( ^{13}\text{C} \) NMR (\( \delta_C \)); 146.57 (C-2, CH), 106.67 (C-3, CH), 125.83 (C-3a, C), \( \delta_C \) 113.12 (C-4, CH), \( \delta_C \) 116.44 (C-4a, C), 144.22 (C-5, CH), 114.63 (C-6, CH), 143.76 (C-7, C), 148.56 (C-8, C), 131.61 (C-8a, C), 160.52 (C-9a, C), 70.12 (C-1', CH\(_2\)), 119.72 (C-2', CH), 139.40 (C-3', C), 25.77 (C-4', CH\(_2\)), 18.08 (C-5', CH\(_3\)). Full NMR data (Table, S16, Figure S17).

3.4. Cytotoxic assay
As reported previously (S15) (Thabrew et al. 1997).

4. Conclusion
The current study revealed the first isolation of furoquinoline-type alkaloids from A. majus beside that, these alkaloids represent a rarely ones with a free C-4 proton. The resulted cytotoxicity depends on their structure features; as long as the H-bond donor increased with an electron releasing group, the activity increased.

Disclosure statement
No potential conflict of interest was reported by the authors.

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