Chemical composition of *Ammi visnaga* L. and New Cytotoxic Activity of its Constituents Khellin and Visnagin

Amira Mohammed Beltagy  
Department of Pharmacognosy,  
Faculty of Pharmacy,  
Damanhour University, Damanhour,  
Egypt.

Doha Mohammed Beltagy  
Department of Biochemistry,  
Faculty of Science,  
Damanhour University, Damanhour,  
Egypt.

Abstract—Khellin and visnagin from *Ammi visnaga* L. are furanochromones known many years ago for their different medicinal uses. Despite of this, their cytotoxicity still not investigated yet. Thus, the aim of this work was to isolate them and assess their antitumor activities together with the whole plant methanolic extract against four human cell lines, Hela (cervical carcinoma cell line), Hep-G2 (liver carcinoma cell line, HCT 116 (colon carcinoma cell line) and MCF7 (breast carcinoma cell line) using SBR assay. Also, to compare IC50 values with Doxorubicin, the potent widely used anticancer drug that was used as the positive control. From another point of view, determination of the total flavonoidal content in the plant whole methanolic extract was another goal and was assessed as 1.2 ± 0.3 mg rutin equivalent/g of dry sample. The highest cytotoxic activities were shown against Hep-G2 cell line with IC50 = 10.9 ± 0.68 μg / ml and 13.3± 0.78μg / ml for Visnagin and khellin respectively. Visnagin was also found to show higher potency than khellin against HCT 116 and Hela cells with IC50 = 12.3± 0.94 and 35.5± 1.2μg / ml respectively. On the other hand, Khellin showed slightly higher potency against MCF7 cells than visnagin with IC50 = 13.3± 0.94 and 13.7± 0.942 μg / ml respectively. Unexpectedly, the total methanolic extract exhibited slight activities with much higher IC50 values. It was also obvious that both compounds showed lesser activities than Doxorubicin.

Key Words - *Ammi visnaga* L., Apiaceae, khellin, visnagin, cytotoxicity. Flavonoidal content

1. INTRODUCTION

*Ammi visnaga* L. is an annular plant grows to approximately 120 cm height and belongs to Family Apiaceae. The plant is endogenous to Egypt and other regions in the Middle East. It is known as Khella, has a slight aromatic odor and a very bitter taste. The fruits of *A. visnaga* have been used in folk medicine in Egypt many years ago to relief kidney stones by drinking teas of powdered fruits [1, 2]. The fruits have been also used for management of angina [3]. Khella extract showed marked antimicrobial activity against gram- positive and Candida species [4]. An ethnobotanical survey including 130 respondents reported khella to be 1 of 16 species of Israeli medicinal plants used for diabetes [5]. However, no clinical trials support this hypoglycemic action.

Khellin and visnagin (Fig. 1) are furanochromones isolated from *Ammi visnaga* L. and is widely used as spasmylytic agent for kidney stones. A recent in vitro research [6] indicated that an aqueous extract of *Ammi visnaga* L., as well as the main compounds khellin and visnagin could prevent cell damage caused by oxalate crystals in the renal epithelial cells. The extract as well as the isolated compounds could therefore play a potential role in the prevention of stone formation associated with hyperoxaluria. However, one safety concern regarding the use of khellin or visnagin as alternative treatment in nephrolithiasis could be that based on their furochromone structure both compounds could intercalate with DNA molecules. Also, the photodynamic properties of khellin and visnagin in their photoreaction with DNA have been studied [7] and khellin does not induce detectable DNA mutations [8]. Constituent khellin from *Ammi* fruit parts inhibited the mutagenicity of certain promutagens in *Salmonella typhimurium* [9]. A literature survey has revealed that new polycyclic compounds derived frombenzofurans and furochromones exhibited antitumor activities [10].

In addition, new heterocyclic benzofuran derivatives obtained from naturally occurring visnagin were assessed against HEPG2 (liver cancer) cell line and showed promising activity [11]. In addition, a recent study [12] revealed that khellin and some related semisynthetic compounds were obtained by condensation of khellin showed cytotoxicity in HEPG2 (liver cancer) cell line by SRB (Sulphorhodamine B) assay. Thus, the aim of this work was to isolate khellin and visnagin from *Ammi visnaga* L. and to invito assess cytotoxicity of the methanolic plant extract together with the isolated compounds against four common human cancer cell lines. Also to compare IC50 results of them with Doxorubicin the potent cytotoxic drug hopping that this may be a safe source of anticancer drug.

**Figure 1**
2. Results

2.1. Isolation and structure determination of the compounds

The total methanolic extract of *Ammi visnaga* L. was subjected to silica gel column chromatography, repeated crystallization and preparative TLC. Two furanochromones were isolated Khellin 1 (5.6 g) as a white-buff crystals has melting point 155-156°C and Visnagin 2 (3.2 g) as thread-like needles from water having mp 142-145°C.

Structure (Fig. 1) of the isolated compounds was determined using spectral methods, such as IR, 1H NMR and 13C NMR. Khellin 1 has melting point 155-156°C; UV (ethanol, λmax): 247, 281 and 331 nm. That is characteristic for chromones. IR spectrum as KBr disc showed the strong band of conjugated carbonyl stretching characteristic for chromones. The IR spectrum of the isolated compound and that of an authentic sample of khellin were superimposed. The 1H NMR (DMSO-d6) spectrum of 1 [Table 1] displayed the signals at δ H 8.05 (d, 1H, J=2.4), 7.16 (d, 1H, J=2.4), 6.01 (s, 1H), 4.07 (s, 3H), 3.91 (s, 3H), 2.33 (s, 3H). It was obvious that the compound has two methoxy groups each corresponds to three protons at δ H 3.91 and 4.07 corresponds to the methoxyl attached to C9 and C4 respectively. 13C NMR (75 MHz, DMSO-d6): δ 176.48, 164.05, 146.22, 116.33, 111.63, 110.06, 105.2, 94.76, 61.11, 19.21. Anal. calc'd. For C13H10O4 (%): C, 67.80; H, 4.38.

Visnagin 2 has melting point 142-145°C; UV (ethanol, λmax): 243, 275, 322 nm. That is conjugated, the aromatic =C–H stretching max): 243, 275, 322 nm. IR spectrum as KBr disc showed λVisnagin 113.04, 104.9, 61.6, 61.12, 19.36. Anal. calc'd. For C13H10O4 (%): C, 67.87. A At 300 MHz in DMSO-d6. b At 75MHz in DMSO-d6 s: singlet, d: doublet, br. Broad, J: coupling constant

Determination of the total flavonoidal content of the plant may help to give a clear idea about this plant as these compounds possess broad spectrum of chemical and biological activities depending on their antioxidant activities. The methanolic extract of *A. visnaga* L. fruits showed flavonoidal total content of 1.2 ± 0.3 mg rutin equivalent/g of dry sample.

2.2. Assaying the total flavonoidal content of the fruit extract [13]

Determination of the total flavonoidal content of the plant 

2.3. Cytotoxic activities of the total fruit extract and the isolated compounds

Studying the cytotoxic activity was done for the total methanolic extract together with the isolated compounds; khellin and visnagin. Cytotoxic activities of khellin are summarized in (Fig. 2) and that of visnagin in (Fig. 3). Doxorubicin was used as the positive control and determination of IC50 values depending on five different doses for each was also done. Activities were assessed on four different human cancer cell lines; Hela (cervical carcinoma cell line), Hep-G2 (liver carcinoma cell line, HCT 116 (colonic carcinoma cell line) and MCF7 (breast carcinoma cell line) using SBR assay. Total methanolic extract showed no activity against Hela (cervical carcinoma cell line), and showed slight antitumor activity against Hep-G2 (liver carcinoma cell line, HCT 116 (colonic carcinoma cell line) and MCF7 (breast carcinoma cell line) expressed by higher IC50 values ranging from 88± 1.99 μg / ml and 97± 2.05 μg / ml. Visnagin showed higher activity against Hep-G2 cell line with IC50 = 10.9 ± 0.68 μg / ml followed by HCT-116 with IC50 = 12.3± 0.94, then MCF7 cells with IC50= 13.7± 0.942μg / ml and finally against Hela cell line with IC50 = 35.5± ± 1.2μg / ml. Khellin exhibited only highest cytotoxic activity than visnagin against MCF7 cell line with IC50 = 13.3± 0.94 μg / ml. Other IC50 values were and 13.3± 0.78, 14.9± 0.97 and 40.9 ± 1.28 μg / ml respectively.

Beside being the positive control, Doxorubicin was tested in the concomitant experiment for its IC50 values against the four tested cell lines and results are shown in (Fig.4). cytotoxicity was the highest against Hela cells followed by MCF-7 then Hep-G2 and finally HCT-116 with IC50 = 3.68, 4.13, 5.18 and 5.3μg / ml respectively. Comparison between IC50 of Doxorubicin with the isolated compounds; khellin and visnagin against the four human cancer cell lines are shown (Fig.5).

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<th>No.</th>
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<td>10.5(br.s.</td>
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<td>7.18 (s, 3H)</td>
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Table 1: 1H NMR and 13C NMR data of khellin and visnagin from chloroformic fraction of the total ethanolic extract of *Ammivisnaga* L.

3. DISCUSSION

$^1$H NMR of khellin 1 indicated the presence of two methoxy groups each corresponded to three protons at δ H 3.91 and 4.07 ppm corresponded to the methoxy attached to C 9 and C4 respectively. Whereas Visnagin 2 showed only one methoxyl group assigned for three protons at δ H 4.02 ppm. $^{13}$C of compound 1 showed the signals of 14 carbon atoms, the most downfield one corresponds to the carbonyl at C5. One methyl carbon signal corresponded to C 7 found at δ 19.36 ppm. Two C-O signals at δ 61.1 & 61.62 ppm ensured the presence of two methoxyls. Demethylation of compound 2 was also confirmed by the presence of only 13 carbons in $^{13}$C NMR spectrum, only one C-O signal at δ 61.0 ppm and the upfield shift of C9 from 129.26 to 116.33 ppm. NMR data of khellin was compared with that mentioned previously [12].

UV spectra showed that khellin spectrum was shifted to the longer wave lengths UV (ethanol, $\lambda_{max}$): 247, 281 and 331 nm than visnagin UV (ethanol, $\lambda_{max}$): 243, 275, 322 nm as a result of the positive auxochrome which is capable of resonating with the conjugated ketone structure. IR spectra of both compounds were superimposed with that of authentic samples.

Total flavonoidal content of the fruits were assessed for the first time and exhibited small values as expected to cramocarpic fruits and assessed to be 1.2 ± 0.3 mg rutin equivalent/g of dry sample. The individual flavonoids were previously studied on Amm. visnaga L. grown in Iraq [13] while quercetin and kampferol were isolated and confirmed as determination of flavonoidal content were done using Spectrophotometer (Optima SP-300, Japan). The $^1$H and $^{13}$C NMR spectra were recorded in DMSO-d6 using TMS as internal standard, on Varian- Mercury BB 300 NMR.

Cytotoxicity assays were measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise). Germany. TLC analysis was done on precoated silica gel 60 F254 plates(Germany).UV detection of the TLC plates was done using Camag, Switzerland UV Lamp. Column chromatographic separations were performed on silica gel (70-230 mesh) (E- Merk, Germany). Khellin (97% purity), visnagin (97%) purity, Rutin (purity>98%) was obtained from Sigma-Aldrich.

All other reagents were from Sigma (St. Louis, MO, USA) and were of analytical grade.

4. EXPERIMENTAL

4.1. General experimental procedure

Melting points were determined using Fisher- Johns melting point apparatus. Solvent evaporation and concentration was performed on Rotavapor Heidolphv 2000 and measurement of UV spectra in methanol as well as determination of flavonoidal content were done using Spectrophotometer (Optima SP-300, Japan). The $^1$H and $^{13}$C NMR spectra were recorded in DMSO-d6 using TMS as internal standard, on Varian- Mercury BB 300 NMR. Cytotoxicity assays were measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise). Germany. TLC analysis was done on precoated silica gel 60 F254 plates (Germany).

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4.2. Plant material

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All other reagents were from Sigma (St. Louis, MO, USA) and were of analytical grade.

4.3. Fruit extraction of Ammi visnaga L.

The powdered fruits of Ammi visnaga L. (750 gm) were extracted with hot methanol in a soxhelet three times till complete exhaustion with 3L methanol and then filtered. The methanolic extract was evaporated under reduced pressure to give 80 g. The yield of methanol extract of Ammi visnaga L fruit was 80 g (10.7 %) of greenish brown residue that had very bitter taste. Residue sample (Five grams) was kept in refrigerator for determination of total flavonoidal content and also investigation of the cytotoxic activity against the four human cell lines.

In conclusion, the cytotoxic activities of the two drugs may open a new branch of potent anticancer drugs of relatively known safety than the already used drugs.
4.4. Isolation of compounds from the chloroformic fraction of total methanolic extract [12]
The remaining residue (75 g) was suspended in distilled water and defatted with petroleum ether (40-60) two times then extracted three times with chloroform. The chloroformic soluble fraction (1.5 L) was evaporated to dryness under reduced pressure to give 50 g of brownish green residue of very bitter taste. TLC analysis of the chloroformic extract on silica gel F 254 showed five spots using solvent system; ethylacetate: chloroform (60:40) and CHCl₃: MeOH (9:0.5) after spraying plates with 5% ethanolic KOH and heat at 120°C for 10 min, and also with anisaldehyde/ H₂SO₄ and heat for 10 min at 120°C. The chloroformic residue was submitted to silica gel column (520 g, Kieselgel 60, 70–230 mesh, Merck) and eluted with petroleum ether followed by petroleum ether-ethyl acetate in increasing polarity. Fractions eluted with petroleum ether-ethyl acetate (70%) were concentrated and was purified by repeated crystallization from methanol to obtain Compound 1. The methanol mother-liquor after separation of crystals was evaporated to dryness under vacuo. The resulting residue is taken up in benzene and treated subsequently with petroleum ether until a distinct turbidity is accomplished. As the reaction mixture is cooled, small quantity of the white–buff crystals of compound 1 was separated out which is removed by filtration. To the filtrate, further addition of light petroleum initiated the process of separation of compound 2 as thread-like needles. The isolated crystals were dissolved in a mixture of CHCl₃/MeOH and subjected to TLC analysis in the previous two solvent systems. The developed chromatograms were visualized under 245 and 365-nm UV light and the spots resulted from the solvent systems. The developed chromatograms were subjected to TLC analysis in the previous two solvent systems. The developed chromatograms were visualized under 245 and 365-nm UV light and the spots resulted from the solvent systems. The developed chromatograms were subjected to TLC analysis in the previous two solvent systems.

4.5. Determination of total flavonoid concentration in the alcoholic extract
The content of flavonoids in the total methanolic extract of Ammi visnag a was determined using spectrophotometric method [15]. The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% AlCl₃ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax = 415 nm. The mean value of absorbance of three readings was determined. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

4.6. Determination of cytotoxicity
4.6.1. Human tumor cell lines
Non small human colon carcinoma (HCT116), epitheliodcervixcarcinoma (Hela), human hepatocellular liver carcinoma (HepG2), human breast carcinoma (MCF-7) cell lines were obtained in frozen state under liquid nitrogen (-180°C) from the American Type Culture Collection. The tumor cell lines were maintained by serial sub-culturing in the National Cancer Institute, Cairo, Egypt.

4.6.2. Culture media
The cells were suspended in RPMI 1640 medium (SIGMA ALDRICH) supplemented with 10% fetal calf serum (SIGMA, USA) in presence 1% antibiotic antymycotic mixture (10.000 U/ml K-penicillin, 10.000μg/ml streptomycin sulphate and 25 μg/ml amphotericin B) and 1% L-glutamine (all purchased from Lonza, Belgium).

4.6.3. Assay method for cytotoxic activity
The cytotoxicity against HCT 116, Hela, Hep-G2 and MCF-7 cells were tested in the National Cancer Institute, according to the SRB (Sulfonhodamine B) assay (3-(4, 5-dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide) method by Skehan et al [16], Adriamycin® (Doxorubicin) 10 mg vials Pharmacia, Sweden) was used as the reference drug. Briefly, cells were seeded in 96-multiwell plates at densities of 10⁴ cells/well in a fresh media and incubated under normal growth condition for approximately 24 h before treatment with the tested sample to allow attachment of cells to the wall of the plate. Then, 200 μl aliquot of serial dilution with DMSO (100%) of methanolic extract of Ammi fruits and the isolated compounds; Khellin and visnagin (0, 1, 2.5, 5 and 10 μg/ml) was used and the plates were incubated for 24, 48 and 72 h at 37°C in a humidified incubator containing 5% CO2 in air. Control cells were treated with vehicle alone. Three wells were prepared for each individual dose. Following 24, 48 and 72 h treatment, cells were fixed, washed and stained with Sulforhodamine B stain (Sigma, USA). Color intensity was measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise, Germany). The relation between the mean of surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified plant extract. Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested drug. Doxorubicin (1.0 μg/ml) was used as the positive control. Also, determination of IC50 of Doxorubicin for all the tested cell lines was also so as done for direct comparison with the tested extracts. The experiment was performed triplicate with DMSO at 1% and Doxorubicin as negative and positive controls respectively.
4.7. Statistical analysis

All the aforementioned experiments were conducted in triplicate trials. Data were expressed as mean ± standard deviation (SD). Data were analyzed by using one-way ANOVA followed by Duncan's multiple range tests using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Results were considered significant if the probability of error was < 0.05.

Figure 2: Cytotoxicity of khellin against four human cell lines

Figure 3: Cytotoxicity of visnagin against four human cell lines
Figure 4: Cytotoxicity of Doxorubicin against four human cell lines

Figure 5: Comparison between the mean IC₅₀ values khellin and visnagin isolated from Ammi visnaga L. fruits and Doxorubicin against, MCF-7, Hep-G2, HCT 116 and Hela cell lines.

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