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HPLC Simultaneous Determination of Khellin and Visnagin in Ammi visnaga L. Fruits

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Abstract: *A rapid and accurate HPLC method was developed for determination of both Khellin and Visnagin in Ammi visnaga L. extracts. The calibration curve of Khellin was linear with correlation coefficient (r^2) = 0.9990; over a concentration range of 53.60 – 355.00 mg/l; with a retention time of 9.19 minutes. While the calibration curve of Visnagin was linear with correlation coefficient (r^2) = 0.9999; over a concentration range of 245.40 – 1533.75 mg/l; with a retention time of 10.77 minutes. The relative standard deviation (RSD) was found to be < 2. The proposed method was validated and successfully applied for simultaneous determination of Khellin and Visnagin in the extracts. The method described is quite suitable for routine analysis γ-Pyrones in Ammi visnaga L. extracts (prepared by traditional solvent extraction as well as supercritical fluid extraction).*

Key words: *HPLC, Ammi visnaga fruits, γ-Pyrones (Khellin, Visnagin), Method Validation.*

I. Introduction

Ammi visnaga, L. belongs to the family Apiaceae, known under different names including Bishop's weed, Khella and Toothpick. The fruits are used for the treatment of kidney stones depending on its γ-Pyrones (mainly Khellin and Visnagin) (1). *Ammi visnaga L.* preparations, such as tea bags, used to ease urinary tract pain associated with kidney stones and to promote stone passage (2). Khellin and Visnagin are furanochromones compounds present in *Ammi visnaga L.* fruits (1-3). *Ammi visnaga L.* and its constituents have different claims such as antibacterial, antifungal, antiviral, antidiabetic, anti-inflammatory and neuroprotective (3-6, 7). The most important effect of Ammi visnaga extract is being peripheral and coronary vasodilator and antispasmodic; which could possibly explain the usage for urolithiasis (8-13). Khan et al. reported a reduction of oxalate and calcium content in rat kidneys after administration of *Ammi visnaga L.* tea preparation (14). Solvent extraction methods were applied to extract the active ingredients (γ-Pyrones; mainly Khellin and Visnagin) from *Ammi visnaga L.* fruits. The solvent used are methanol, ethanol and hydro-ethanol. Supercritical carbon dioxide fluid extraction technology has been replacing the traditional solvent extraction methods as it gives an extract free from residual solvents and produces uniform stable extract. Supercritical fluid extraction technique is more selective, efficient, cheap, and afford mean for simple recovery of the compounds without degradation (15-21). Different analytical methods were adopted for determination and quantitation of γ-Pyrones in *Ammi visnaga L.* fruits and its extracts and preparations (22). The aim of this study was to develop and validate a new HPLC method for quantitation of Khellin and Visnagin (major γ-Pyrones) in *Ammi visnaga L.* fruits, extracts.

II. Materials& Methods

I. Materials

- *Ammi visnaga L.* fruits samples were supplied by Mepaco-Medifood Pharmaceutical Company, Egypt. Khellin and Visnagin reference standards were purchased from Sigma Aldrich, Germany. HPLC grade Methanol and Water were acquired from Merck, Germany.

II. Equipments

- **Ultra Fast liquid chromatography:** Shimadzu, Model Prominence LC-20ADXR, Kyoto, Japan.
- **Column:** Prodigy, ODS3 (250 x 4.6 mm, 5 µm, 100Å, Phenomenex, USA) column.
- **Supercritical Fluid Extraction Unit:** 20 liters Pilot scale, ASI, USA.
- **Electronic Balance:** Model AUY220, Shimadzu Instrument, Kyoto, Japan
- **Ultrasonic bath:** NSXX Sonics Model NS-A-12-7H, Germany.

III. Experimental

1. Chromatographic conditions:

The γ-Pyrones content of *Ammi visnaga L.* extracts obtained by supercritical carbon dioxide fluid extraction were analyzed by liquid chromatography (UFLC) (Shimadzu Model Prominence LC-20 ADXR), equipped with auto sampler, SIL-20ACXR, PDA detector (Shimadzu Model: SPD-M20A); equipped with a Phenomenex Prodigy, C18 (250 x 4.6 mm, 5 µm), USA). Column temperature was 40°C. The mobile phase consists of

Methanol: Water (50: 50 v/v) at flow rate 1.5 ml/min. Detection wavelength was 245 nm and the injection volume was 20 µl.

2. Method Validation:

i. Repeatability:

System repeatability was performed by performing six replicates injections of the same sample solution, solution was prepared within ± 10 % of the working concentration.

This study was conducted by performing multiple analyses on the same portion of a homogeneous sample.

The system repeatability was assessed using 6 replicates of the standard**.**

ii. Linearity:

The stock standard solution of each standard pure was prepared as follows: about 25-45 mg of each compound was accurately weighed and placed into a 20 ml volumetric flask. Methanol was added and the solution diluted to volume with the same solvent.

Calibration curves were plotted over concentration of 53.60-335.00mg/L for Khellin and 245.40-1533.75mg/L for Visnagin as shown in figure 6 and 7; respectively.

Five microliters aliquots of each standard solution were used for HPLC analysis. Triplicate injections were made for each standard solution. Each calibration curve was obtained by plotting the peak area of the γ-pyrones at each level prepared versus the concentration of the sample as shown in Table (1).

iii. Method Precision:

This study was conducted by performing multiple analysis on a suitable number of portions of a homogeneous sample. This was performed by assaying multiple aliquots with the same concentration starting from the first step to the final step of analysis. The analytical precision of the method was determined by the relative standard deviation.

iv. Selectivity / Specificity:

It provides an indication of the selectivity and specificity of the procedure. The method is to be selective, if the main peak is well resoluted from any other peak by resolution of minimum 2. This could be done injecting placebo and compare it with that of standard and test samples, then peak purity was ascertained by use of PDA.

v. Recovery:

The accuracy of the method was evaluated with the recovery test. This involved the addition of known quantities of γ-Pyrones standards to known amounts of *Ammi visnaga L.* extract. The percentage recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100; as shown in Table (2).

vi. Ruggedness:

The degree of reproducibility determined by analysis of samples from homogeneous lot of materials, under different but typical test conditions The method is to be rugged, at any item if the pooled %RSD of the total number of replicates that have been made in this item is within the acceptance criteria, 3 replicates of a single sample of powder material are used for each determination. First day: 3 replicates, on a second day: 3 replicates, then on third day: 3 replicates of freshly prepared test from the same sample are analyzed, under the same conditions.

Ruggedness of the method is conducted by the analysis of the same samples under a variety of conditions, such as different analysts, different days and different columns.

– Day to Day:

Three replicates of a single sample of reference standard was implemented in the first day and then on a second day, third day. Then three replicates of freshly prepared Echinacea extract was analyzed. The same analyst performs every test.

– Analyst to Analyst:

It determines ruggedness between different analysts. Three replicates of a single sample of reference standard are analyzed then the other person analyzes three replicates then the other person analyzes three replicates from the same sample prepared by him.

– Column to Column:

Three replicates of a single sample of reference standard are analyzed on the first column then the same sample was analyzed on another column. The same analytical method is performed on columns of the same packing material and length but of different batch number.

vii. Robustness:

Robustness is determined by observing how a method stands up to slight variations in normal operating parameters. For HPLC for instance, this could be change if slight variation in sonication time or slight variation in aliquot stability.

– **Sonication Time**:
Three replicates of a single sample (100%) are used for each determination. Three different sonication times were used to prepare the sample were analyzed.
– **Aliquot Stability:**
Three replicates of a single sample (100%) are used for each determination. Three different sonication times were used to prepare the sample were analyzed.

viii.Limits of detection and quantification:

Limits of detection (LOD) were calculated according to the expression $3.3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. Limits of quantification (LOQ) were established by using the expression $10\sigma/S$. LOD and LOQ were experimentally verified by injections of pure standard at the LOD and LOQ concentrations; as shown in Table (3).

a. Standard Preparation:

25 mg and 45 mg of each of Khellin and Visnagin standards were accurately weighed; respectively and transferred into 20 ml volumetric flask; then dissolved in methanol and sonicated for 20 minutes. The volume was completed to 20 ml with methanol. The standard solution was filtered over 0.45 µm syringe filter prior to injection.

b. Sample Preparation:

15 mg of the obtained *Ammi visnaga L.* extracts were weighed accurately and transferred into 20 ml volumetric flask. The extract was dissolved in 20 ml methanol; then sonicated for 20 minutes. The obtained solution was filtered over 0.45 µm syringe filter prior to injection

III. Tables and Figures

Tables

Table (1): Statistical analysis for the calibration curves of the standards in the *Ammi visnaga L.* extract

Compound	Linearity range (µg/ml)	Slope, a	Intercept, b	r^2
Khellin	53.60-355.00	138007.45	-934547.98	0.9990
Visnagin	245.40-1533.75	6921.17	-50017.83	0.9999

S.D. values are given in parenthesis.
a for each curve the equation is $y = ax + b$, where y is the peak area, x is the concentration of the analyte (µg/ml), a is the slope, b is the intercept and r^2 is the correlation coefficient.

Table (2): Results of the recovery test for the used standards & extract

Compound	Spiked amount (mg)	Recovery (%)	Mean (n = 5)	R.S.D. (%)
Khellin	0.621	98.88-102.01	100.23	1.08
Visnagin	0.562	98.72-101.12	100.68	1.58

R.S.D. (%) = (standard deviation/mean) ×100.

Table (3): Limit of detection (LOD) and limit of quantification (LOQ)

Compound	LOD (µg/ml)	LOQ (µg/ml)
Khellin	8.9	26.8
Visnagin	40.9	122.7

Figures

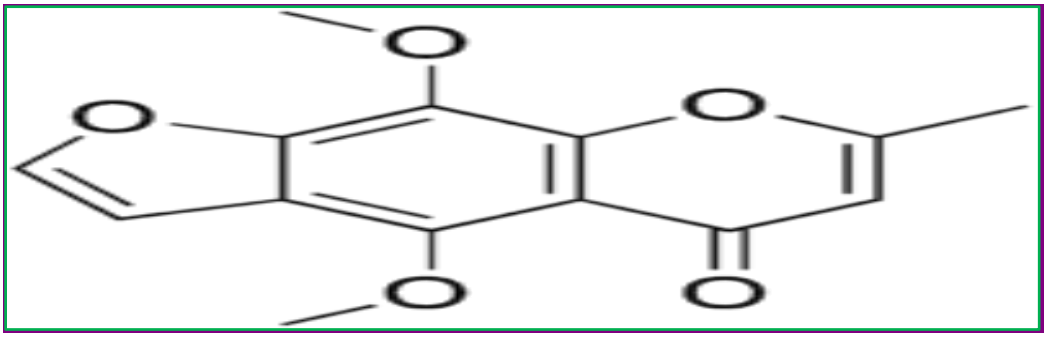

Fig. (1): Chemical structure of Khellin

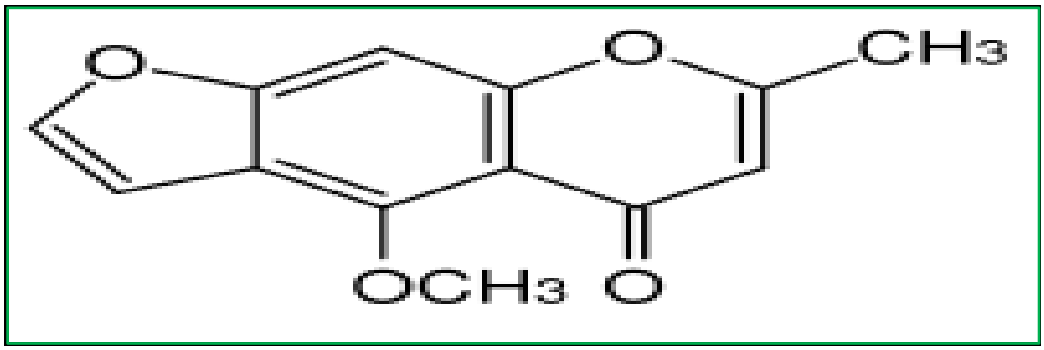

Fig. (2): Chemical structure of Visnagin

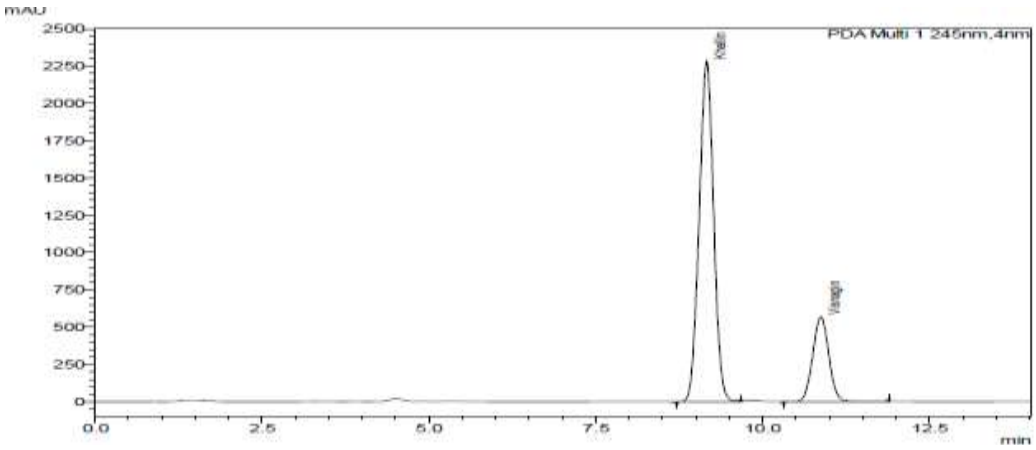

Fig. (3): HPLC Chromatogram of *Ammi visnaga* extracts

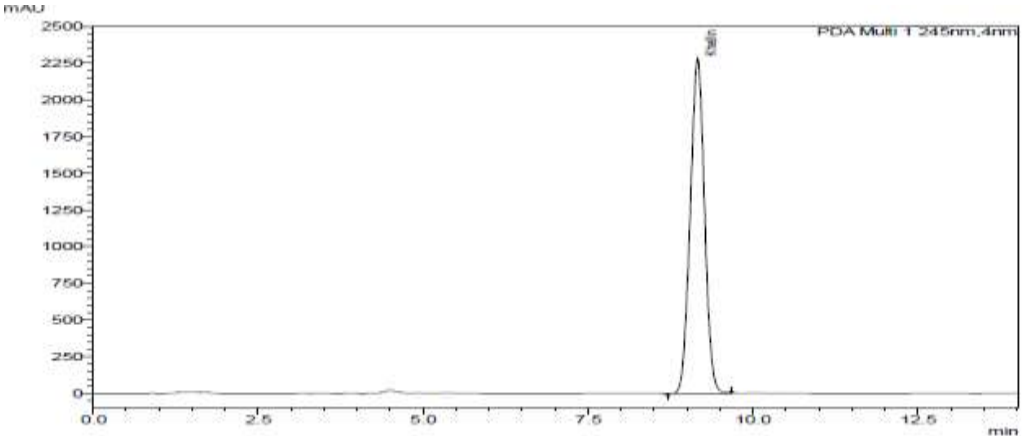

Fig. (4): HPLC Chromatogram of Khellin standard

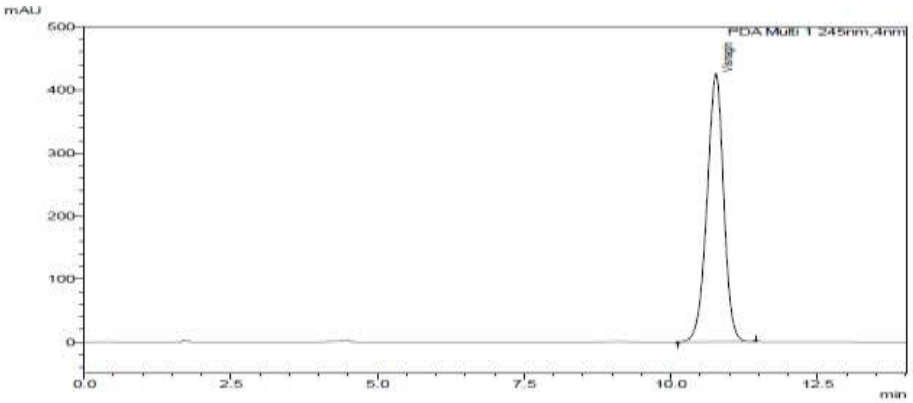

Fig. (5): HPLC Chromatogram of Visnagin standard.

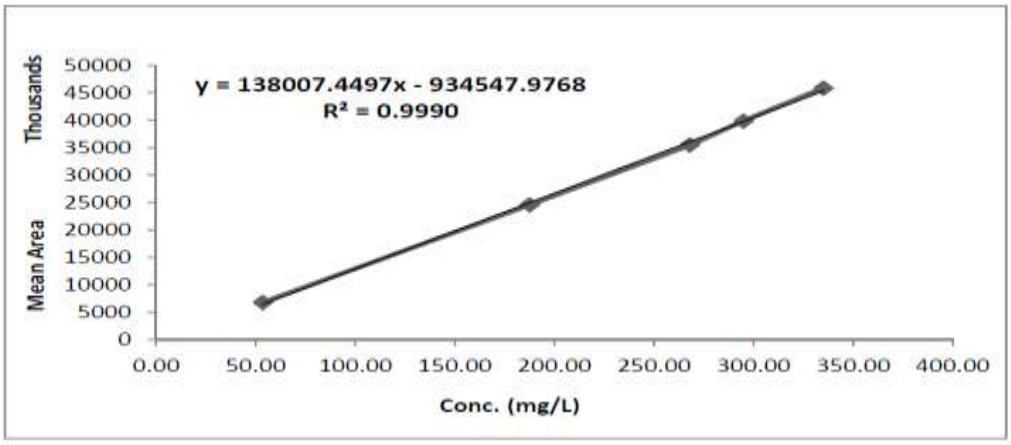

Fig. (6): Calibration curve of Standard Khellin

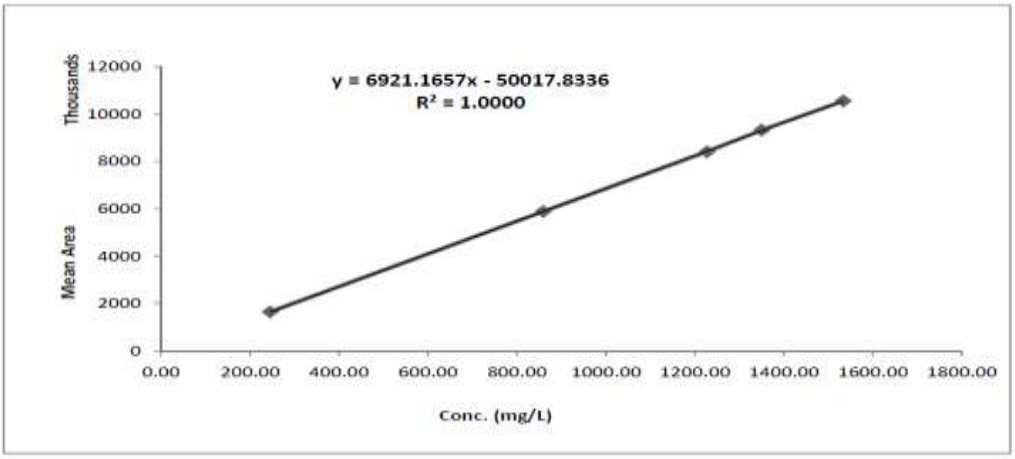

Fig. (7): Calibration curve of Standard Visnagin

IV. Results and Discussion

The used method HPLC for the analysis of *Ammi visnaga L.* extracts proved that it is an accurate, precise, fast and easy method for the quality control of complex herbal pharmaceutical products containing γ-Pyrones. From the calibration curves; we can found that the lower and upper value of linearity (53.60-1533.75 µg/ml), slope (6921.17-138007.45), intercept (-934547.98-50017.83) and the correlation coefficient (0.9990-0.9999) as shown in table (1).

The percentage recoveries table (2) range form (98.72-102.01%). Limit of detection (LOD) and limit of quantification (LOQ) table (3) where we can found the upper and lower value of LOD (8.9-40.9 µg/ml) and LOQ (26.8-122.7 µg/ml).

V. Conclusion

The validated HPLC method developed for the quantitative determination of Khellin and Visnagin in Ammi visnaga extracts was evaluated for system suitability, specificity, linearity, range, accuracy (recovery), precision (repeatability and intermediate precision), and robustness. This method enables simultaneous determination of Khellin and Visnagin because of good separation and resolution of the chromatographic peaks. As a result, the proposed HPLC method could be adopted for the quantitative routine analysis.

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