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Development of new isolation technique and validated HPLC method development for khellin- A major constituent of *Ammi visnaga* Lam. fruits

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Ammi visnaga Lam. (Family-Apiaceae) commonly known as khella or picktooth fruit is used for skeletal muscle relaxant activity. In this study, furanochromone khellin was isolated from its fruits by solvent extraction followed by partitioning of solvents. The isolated compound was identified by UV visible spectroscopy, IR, MASS, ^{1}H NMR, ^{13}C NMR and TLC. Reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for quantification of khellin by using C-18 column (250 x 4.6, 5-µm) with an isocratic mobile phase, acetonitrile and water with 0.1 % ortho phosphoric acid (40:60 *v/v*) at a flow rate of 1.0 mL/min. The wavelength of maximum absorption (λ_{max}) selected for quantification of khellin was 338 nm. The amount of khellin present in fruits was found to be 1.06 % w/w. The developed method has been validated for accuracy, precision and specificity and successfully applied for quantification of isolated compound in fruits. Thus, isolated khellin could be used as phytochemical markers for the quality control of this plant as well as other formulation containing khella fruits.

Keywords: *Ammi visnaga*, Apiaceae, *Khella, Picktooth*, HPLC, Khellin, Furanochromone, Quantitative estimation.

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Introduction

Ammi visnaga Lam. (Family-Apiaceae) is an herbaceous plant that grows wild in Egypt, where a decoction from it acquired a reputation in folk medicine as a diuretic that could relieve renal colic. This was common problem in Egypt arising from renal stone formation associated with schistosomiasis. A study on extract revealed its ability to relax smooth muscle, including that of the ureter and coronary arteries, in a variety of animal species. This formed the basis of a clinical investigation that confirmed that, in addition to the diuretic action, it could relieve spasm of the ureter[1, 2].

Khellin (Fig.1) (4, 9-dimethoxy-7-methyl-5H-furo [3, 2-g][1]benzopyran-5-one) is a major constituents of fruits and seeds of the plant *A.visnaga*. The fruits contain about 1 % of this compound. Pure khellin occurs in colorless, odorless needle-shaped crystals, having a bitter taste. It has also been called visammin by some authors. It is a commercial medicinal plant mainly grown in Mediterranean areas in open fields. The extract of fruits has been widely employed as herbal medicine in the treatment of coronary diseases and bronchial asthma. Khellin is used as a spasmolytic agent in the therapy of asthma and angina pectoris and recently its use has been proposed for the treatment of vitiligo and psoriasis. Studies on the photogenic and mutagenic activity of khellin have also been reported. As khellin is an important phytoconstituent present in visnaga, it is necessary to develop rapid and simple method for isolation and analysis of this phytoconstituent[3, 4].

Present study was divided in two parts, first part is to devise a simple and efficient extraction method for isolation of khellin and other part include development of rapid analytical method for detection of khellin using High Performance Liquid Chromatography and its validation according to ICH guideline[5, 6].

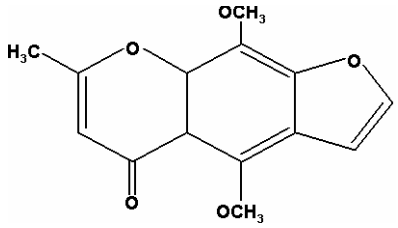

Fig. 1—Structure of Khellin

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Material and Methods

A. visnaga fruits were procured from Egypt. All the reagents and chemicals used were of analytical grade. HPLC grade methanol was obtained from Merck India. Water was double distilled. Solvents were filtered through a 0.45 µm filter (Millipore Bedford, MA, USA) and degassed in an ultrasonic bath (Remi Instruments, Mumbai, India) before use. HPLC analysis was performed with a Jasco (Hachioji, Tokyo, Japan) system consisting of an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i) equipped with a 20-µL loop, and a UV–visible detector (UV-1575). Compounds were separated on a 250 mm × 4.6 mm i.d., 5-µm particle, Hibar LiChrocart Purospher Star RP-18 endcapped column (Merck, Darmstadt, Germany).

Isolation

The air dried powdered sample (250 g) was moistened with 10 % aqueous sodium carbonate solution, allowed to dry then refluxed with methanol (1000 mL) for 1 h. Filtered under vacuum then marc was collected and again refluxed with methanol (500 mL) for 1h. Filtered and collected the extract, combined both the extracts and concentrated up to $1/4^{th}$ of the original volume. Concentrated methanolic extract was partitioned with 250 mL of petroleum ether (60-80). Petroleum ether fraction was collected; partitioning with petroleum ether was repeated 6-7 time using fresh solvent. Combined all petroleum ether fractions and concentrated up to $1/10^{th}$ of the original volume. Then concentrated fraction was allowed to stand for 1 h. Khellin get settled down at the bottom.

Melting point was determined using in open capillary tubes in a Veego (India) melting point apparatus and was uncorrected. UV spectrum was measured on a Jasco V 530 UV-Visible spectrophotometer. IR spectrum was taken on a Perkin Elmer FT-IR spectrophotometer. ^{1}H and ^{13}C NMR data were measured on a Joel 400 MHz spectrometer in $CDCl_3$. Mass spectrum was taken on Micromass Q-TOF mass spectrometer.

HPLC method development and validation

Isocratic mobile phase Acetonitrile: Water (40: 60) with 0.1 % ortho phosphoric acid at flow rate 1 mL/min had been selected for the study. The injection volume was 20 µL and detection wavelength was 338 nm. HPLC was performed at ambient temperature and data were analyzed on a computer equipped with Borwin software.

Calibration plot was constructed for compound, after triplicate analysis of each calibration solution, by plotting peak area against concentration (µg/mL) of the corresponding standard solution. To determine the limits of detection (LOD) and quantification (LOQ) standard solution was further diluted in methanol. LOD and LOQ were defined as the amounts for which signal-to-noise ratios (*S*/*N*) were 3 and 10, respectively. Precision was determined as the intra-day and inter-day variation of results from analysis of six different standard solutions. Intra-day precision was determined by triplicate analysis of solution on a single day. Inter-day precision was determined by triplicate analysis of the solution on three successive days. The relative standard deviations (RSD) of retention time (Rt) and peak area (Pa) of analyte was calculated as measures of precision and repeatability. The accuracy of the method was determined by application of the standard addition method. 0.5 mL standard of 10, 15, and 20 µg/mL concentration to the 0.5 mL of the extract solution containing 10 µg/mL were added to sample solution and analyzed in triplicate as described above. The total amount of each compound was calculated from the corresponding calibration plot and the recovery of each compound was calculated by use of following equation.

Recovery (%) =

(amount found − amount contained)/amount added × 100

Results

Isolated compound was identified by various analytical methods and chemical tests. Compound was obtained as colorless crystalline solid with melting point of 154-155°C. Its molecular formula was determined as $C_{14}H_{12}O_5$ by an ion peak at m/z 261.23 [M^+]. The UV spectrum of compound in ethanol exhibited the absorption maxima at 253 nm and 338 nm which are characteristic for a chromone structure. In the IR spectrum, prominent absorption bands were found at 2990/cm (CH_3 stretching) 1655/cm (conjugated C=O), 1349/cm (Ar-O Streching), 1120/cm (C-O stretching). ^{1}H NMR data of isolated compound are shown in Table 1. In thin layer chromatography (TLC) analysis, compound showed single spot on silica gel plate at R_f value 0.42 in solvent system toluene-ether (1:1). TLC detection was carried out under UV at 254 nm and 365 nm.

Table 1—H-1 and C-13 NMR of isolated khellin

H^1 NMR	δ values (ppm)	C^{13} NMR	δ values (ppm)
C7'H	1.71 d	C 2	140.8
C4' H, C9 H	3.50 d	C 3	107.0
C6 H	4.94 d	C 4	152.5
C3H	5.49 d	C 5	197.5
C2 H	6.49 d	C 6	94.7
		C 9	129.8
		C 4'	52.2
		C 7'	23.7
		C 9'	52.4

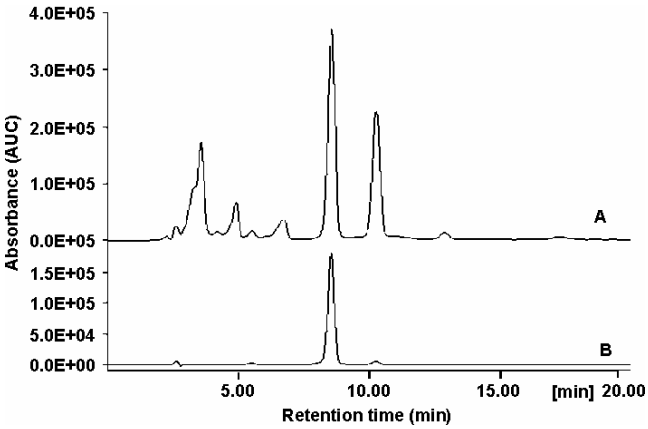

Fig.2—HPLC chromatogram of *Ammi visnaga* extract (A); Isolated khellin (B)

The compound showed characteristic yellowish green fluorescence under UV light. Spectral data and chemical properties of isolated compound were identical to those reported in earlier studies on khellin[7, 8]. The structure of isolated compound is shown in Fig. 1.

The HPLC method was optimized by varying the flow rate and the relative amounts of methanol and water (0.1 % ortho-phosphoric acid). Use of a mobile phase containing acetonitrile (40 %), water with 0.1% ortho-phosphoric acid (60 %) with flow rate of 1.0 mL/min was found to be optimum for RP-HPLC quantification of khellin. The Retention time (Rt) for khellin was 8 min and the wavelength of maximum absorption (λ_{max}) of khellin was 338 nm. Chromatograms of standard solution of isolated khellin and a sample solution (Fig. 2) revealed the presence of khellin as marker constituent in sample solutions.

The calibration plot for khellin was linear in the range of 10-60 µg/mL. The regression equation was y= 19493x-40255 with a correlation coefficient

Table 2—Precision and repeatability of khellin

Analyte	Analyte concentration (µg/mL)	Precision (RSD[a]) Intra-day (n=3) Rt	Intra-day (n=3) Pa	Inter-day (n=3) Rt	Inter-day (n=3) Pa
Khellin	10	2.12	1.29	2.34	1.38
	40	1.69	1.59	2.11	2.01
	60	1.34	1.70	1.48	0.78

Rt - Retention time, Pa- Peak area, RSD[a]- Relative standard deviation

Table 3—Percent recovery study of khellin by HPLC (n=3)

Analyte	Contained (µg)	Added (µg)	Found (µg)	Recovery (%)	Mean (%)	RSD[a] (%)
Khellin	10	10	20.89	104.45	101.37	2.41
	10	10	20.45	102.25		
	10	10	19.82	99.10		
	10	15	24.62	98.48		
	10	15	26.08	104.32		
	10	15	25.12	100.48		
	10	20	29.55	98.5		
	10	20	30.28	100.93		
	10	20	31.16	103.86		

RSD[a]- Relative standard deviation

(r^2) of 0.995. The LOD and LOQ were found to be 3.03 µg and 9.09 µg. Intra-day and inter-day RSD of retention time and peak area were less than 2 %, showing good precision (Table 2). Recovery of khellin was in the range of 98.5 and 105 % with RSD < 2 % (Table 3) indicating the accuracy of developed method. These results revealed that the method is rapid, precise, sensitive, and highly accurate for quantification of khellin. The amount of khellin found in the *A. visnaga* fruits was 1.06 mg/100 mg (1.09-1.14 % w/w; Relative standard deviation, 2.75).

Discussion

Therapeutic value of *A. visnaga* is attributed to khellin and present paper will be helpful in isolation of khellin using novel, relatively simple method. Therefore, it is possible to isolate khellin within short period of time. Method has shown good reproducibility of the yield and purity and this method can be used to get large quantity of khellin, which can be employed for different application. HPLC validation result revealed that this method enable rapid, precise, sensitive and accurate for quantification of khellin in plant source. This

method with slight modification may also be used for quantification and quality control for formulation containing *A. visnaga* fruits.

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