Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 6, 2015

Original Article

SIMULTANEOUS ESTIMATION OF CAFFEIC AND CHLOROGENIC ACID CONTENT IN *AMMI MAJUS* SEED BY TLC AND HPLC

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Received: 27 Mar 2015 Revised and Accepted: 28 Apr 2015

ABSTRACT

Objective: The present investigation formulated in order to identify and quantify of caffeic and chlorogenic acid content in *Ammi majus* seed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Methods: In the present study identification and quantification of caffeic and chlorogenic acid in *Ammi majus* seeds were investigated. Identification of these phenolic acids were done by using thin layer chromatography performed on a sheet of aluminium plates of silica gel as a stationary phase, suitable solvent systems which is Toluene: Ethyl acetate: Formic acid: Water (15:90:5:5), also use UV light and 5 % $FeCl_3$ for detection. This identification was further augmented by using high performance liquid chromatography and then quantified by using C18 column, isocratic mobile phase (acetonitrile: water (0.5% orthophosphoric acid) 15:85) and UV/Visible 320 nm detector.

Results: Thin layer chromatography analysis of seed extracts revealed the presence of caffeic and chlorogenic acid in *Ammi majus* seed. The HPLC method was validated for linearity, accuracy, precision and limit of detection and quantification. The percentage of caffeic acid 0.031% in higher quantity than chlorogenic acid. Calibration curve was linear over concentration 5-200 µg/ml with correlation coefficients 0.998 and 0.999 for caffeic and chlorogenic acid. The relative standard deviation of the method was less than 2% for intra and inter-day precision assay and the average recoveries were between 97.47-101.92%. The proposed method was found to be linear, accurate, and precise.

Conclusion: This study confirms the presence of caffeic and chlorogenic acid in *Ammi majus* seed extracts and the percentage of caffeic acid higher than chlorogenic acid.

Keywords: *Ammi majus*, TLC, HPLC, Caffeic acid, Chlorogenic acid.

INTRODUCTION

Ammi majus belong to family Apiaceae is annual winter weed growing mainly in side of channels, fields and gardens is a 0.30 to 0.60 meter high was found in Asia [1], Iraq [2], Egypt and widely distributed in Mediterranean region, Abyssinia, Europe, West Africa, freely grows in the Nile Delta region [3]. That has been commonly used for skin disorders such as psoriasis and vitiligo, leprosy, kidney stone, urinary tract infection [4]. *Ammi majus* is one of the richest known sources of coumarin and furocoumarins. This furocoumarins such as methoxsalen, 8-methoxy psoralen, ammoidin, heraclenin acts as bactericidal, fungicidal, mollscicidal, nematicidal, insecticidal, and viricidal. An infusion is used to calm the digestive system, whilst it is also used in the treatment of asthma and angina, also some isolated coumarin compounds such as 6-hydroxy-7-methoxy-4-methyl coumarin and 6-hydroxy-7-methoxy-4-methoxy coumarin showed anti-inflammatory and antiviral activity [4,5]. *Ammi majus* show antioxidant effect and their use in diabetic nephropathy and myocardial injury due to the presence of different active constituent such as quercetine, luteolin, kaempferol [6], marmesinin, and other compounds that inhibit cytochrome p450 such as xanthotoxin bergapten, imperatorin and isoimpinellin. Also used for treatment of liver disorders [1], previous research show from phenolic compound such as xanthotoxin, imperatorin, quercetin, hydroquinone, catechol, resorcinol, salicylic acid and vanillic acid are found in *Ammi majus*. Salicylic acid, resorcinol and quercetin in high concentration showed stronger inhibitory activity against two gram positive bacteria *Staphylococcus aureus, Staphylococcus epidermidis* and three gram negative bacteria *Escherichia coli, Proteus mirabilis and Proteus vulgaris* [7], the drug β-methoxypsoralen from *Ammi majus* is used to treat T-cell lymphoma [8]. And the literature survey on this plant showed that there was no research on simultaneous estimation of caffeic and chlorogenic acid content in *Ammi majus* seed. The aim of the present study was planned to perform thin layer chromatography and to develop a simple, precise and accurate high performance liquid chromatography method for identification and quantification of caffeic and chlorogenic acid content in *Ammi majus* seed as per ICH guidelines.

MATERIALS AND METHODS

Plant material

Ammi majus seeds were collected from orchards during July. The seeds were cleaned, dried under shade for 15 days, coarsely powdered and stored in bottles until used. The identity of the plant was confirmed by the department of Pharmacognosy, College of Pharmacy, Hawler Medical University (Voucher No.2).

Sample preparation

The accurately weighed dried seed powder 1 gm was extracted with ethanol 80% using ultrasonic (LUC-405, Korea) assisted extractor for 1hr at 40 °C [9] then filtered and which was after drying dissolved in 10 ml (5N) HCl and refluxed for 1hr, liquid-liquid fractionation using ethyl acetate (10 x 3 ml) resulted an organic fraction on drying in vacuum that used for evaluation of phenolic acid constituents. The organic extract was used for TLC and HPLC.

Thin layer chromatography for identification of phenolic acid

TLC is a chromatographic technique which is used for the separation of the mixture of compounds. TLC is performed on a sheet of aluminium plates of silica gel, which are commercially available 60 F254 (Merck). Prepared ethyl acetate fraction of seed extracts spotted onto the TLC plate as a single spot with capillary tubes and compare with standard phenolic acid which are caffeic (Chroma Dex, USA) and chlorogenic acid (Chroma Dex, USA), by using suitable solvent systems which is Toluene: Ethyl acetate: Formic acid: Water (15:90:5:5). Two different detection methods, first by using, UV light wave length 254 nm and 366 nm and second by spraying with 5% $FeCl_3$. Qualitative evaluation of separated substance was carried out by calculating the retardation factor (R_f) values.

Qualitative and quantitative estimation of phenolic acid by using HPLC technique

Qualitative and quantitative estimations of caffeic and chlorogenic acid were done by using Knauer/Germany High Performance Liquid Chromatography (HPLC) in which identifications were made by compares of retention time obtained at identical chromatographic conditions of analyzed samples and authentic standards with Eurospher 100, C18 column (4.6 mm i.d. x 250 mm, 5 mm) and UV/Visible detector. The flow rate of the mobile phase for caffeic and chlorogenic acid was kept at 1 ml/min. Mobile phase A was acetonitrile (Scharlauchemie, S. A., Europian Union) and B water (Scharlauchemie, S. A., Europian Union) containing 0.5% O-phosphoric acid the isocratic conditions were as 15% A and 85% B. The temperature of column was controlled at 25 °C. Injection volume was 20 µl. The detection wavelength 320 nm. The retention time values for standard compounds were determined 10 min. prior to each run, the HPLC-UV/Visible system was allowed to warm, and the baseline was monitored until it was stable before sample analysis.

Method validation [10, 11]

Linearity

One mg each of caffeic and chlorogenic acid was separately weighed into a 5 ml volumetric flask, caffeic and chlorogenic acid dissolved in acetonitrile: water (1:1) filled up to volume for preparing stock solutions. Standard solutions were prepared for each compound at five different concentrations (5, 10, 50, 100, and 200 µg/ml) levels in 5 ml volumetric flasks for the establishment of calibration curves. The prepared dilutions were injected in series, peak area was calculated for each dilution, and concentration was plotted against peak area.

Accuracy

Accuracy was determined by the standard addition method for the three concentrations (50, 100, and 200 µg/ml) and the recovery was calculated by comparison of the found amounts with the added ones. The experiment was performed in triplicate and recovery (%) was calculated for each concentration.

Precision

Precision was determined as both repeatability and intermediate precision in accordance with ICH recommendations. Repeatability of sample injection was determined as intra-day variation and intermediate precision were determined by measurement of inter-day variation. For both intra-day and inter-day variation, standard solutions at three different concentrations (50, 100, and 200 µg/ml) were determined in triplicate.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by the standard deviation (Sy/x) method. Blank samples were injected in triplicate and the peak areas of the blanks were calculated. LOD and LOQ were determined from the slope (S) of the calibration plot and the standard deviation of the response for the blank sample, Sy/x, by use of the formulae LOD = 3 × Sy/x/S and LOQ = 10 × Sy/x/S.

RESULTS AND DISCUSSION

The determination of phenolic acids is important both for their characterization in the drug and to facilitate more efficient uses of the important plant resources. There are several methods for the determination of phenolic acids in herbal drugs such as TLC, HPLC and capillary electrophoresis [12-14]. The most reliable and applicable methods are TLC and HPLC methods.

Identification of phenolic acid by TLC

The results of TLC for ethyl acetate fraction was obtained from seed of *Ammi majus* showed presence of number constituents in which the R_f value and colour of two of these constituents were similar to the caffeic acid 0.5, chlorogenic acid 0.09 standard and identified as caffeic acid, chlorogenic acid, the results were represented in table 1 and fig. 1.

Table 1: Showed the Rf values of caffeic acid and chlorogenic acid and their standards in suitable solvent systems

Ethyl acetate fraction	Rf Value	Standard	Rf Value	Spot colour		
				UV 254	UV 366	FeCl3 5%
Ea1	0.088	Chlorogenic acid	0.09	Dark spot	Blue	Dark blue
Ea2	0.5	Caffeic acid	0.5	Dark spot	Light blue	Dark green

Ea1: Ethyl acetate first constituent; Ea2: Ethyl acetate second constituent

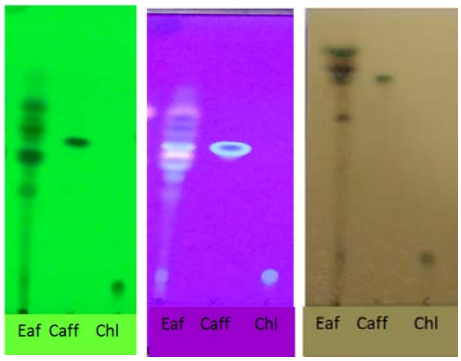

Fig. 1: TLC of ethyl acetate fraction seed extracts of *Ammi majus* detection by UV-light at (A) 254 nm, (B) 366 nm, (C) $FeCl_3$. (Eaf: Ethyl acetate fraction; Caff: Caffeic acid standard; Chl: Chlorogenic acid standard)

Qualitative and quantitative estimation of phenolic acid by HPLC

In the present study qualitative and quantitative analysis of caffeic and chlorogenic acid content in ethyl acetate fraction of *Ammi majus* was performed for the first time by HPLC. The presence of phenolic acids is supported by previously recorded data [7], but no previous study on HPLC validation of caffeic acid and chlorogenic acid presence in *Ammi majus* seed.

The peak of caffeic acid and chlorogenic acid in seed extract were identified by comparing the retention time (3.9 and 2.6 min respectively) and UV spectra with those of standard and the results were shown in table 2 and fig. 2. The content of caffeic acid and chlorogenic acid quantified in seed extracts and the results revealed that seed contain caffeic acid 0.031% in higher quantity than chlorogenic acid 0.028% were shown in table 2.

Table 2: Quantitative study of caffeic and chlorogenic acid in seed extract by HPLC

Compound name	RT	Concentration % *Ammi Majus*
Caffeic acid	3.9	0.031
Chlorogenic acid	2.6	0.028

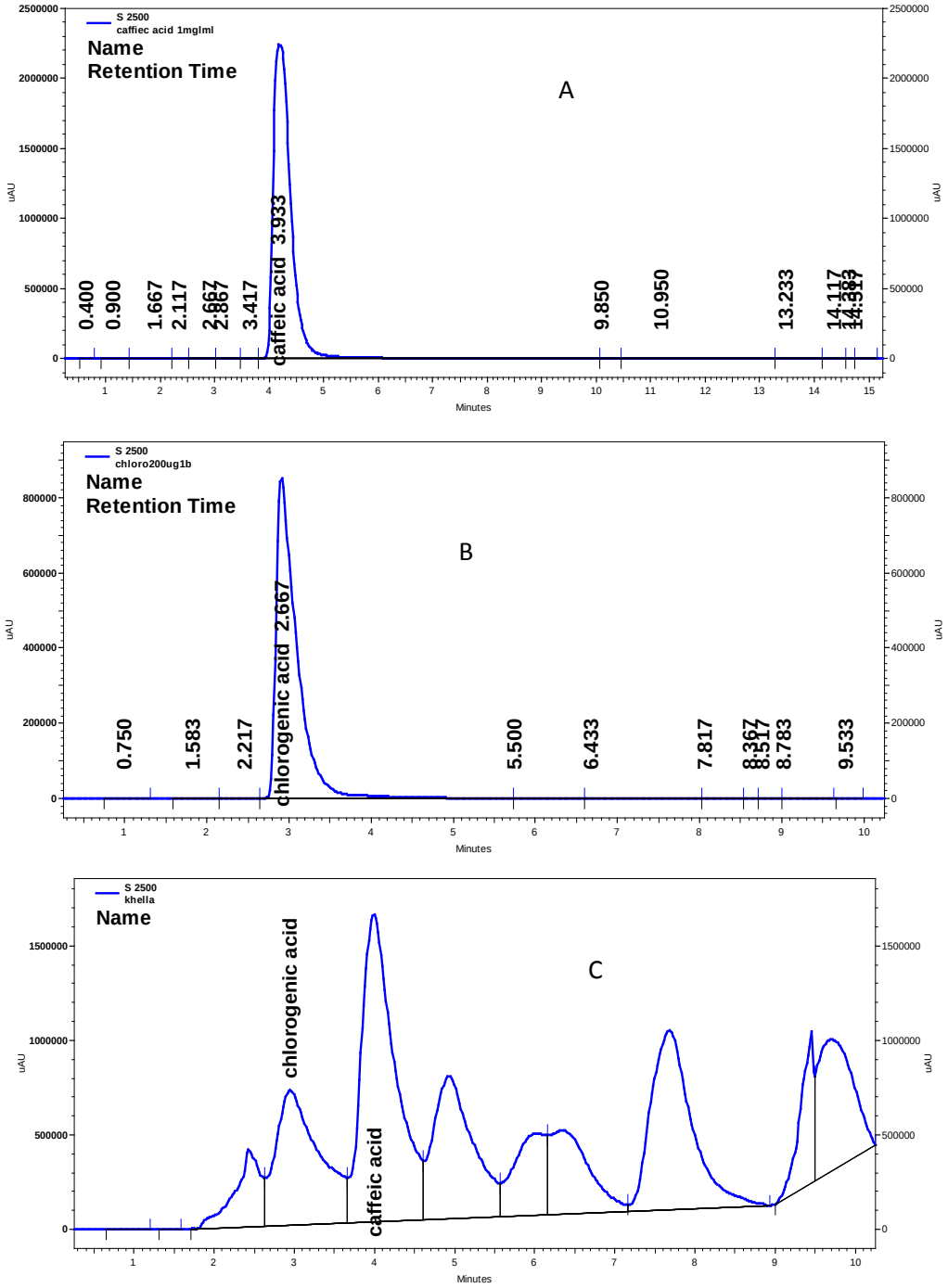

Fig. 2: HPLC chromatogram of A-Caffeic acid standard B-Chlorogenic acid standard C-Seed extract

Method validation

The developed HPLC method was validated for estimation of caffeic acid and chlorogenic acid content in *Ammi majus* seed by determining the parameters including linearity. LOD, LOQ accuracy and precision according to the ICH guidelines [10, 11]. The validation studies for were performed on C18 column with mobile phase A was acetonitrile and B water containing 0.5% O-phosphoric acid the isocratic conditions were as 15% A and 85% B, addition of O-phosphoric acid in a mobile phase composed of acetonitrile and water is found to be good for sharpening peak shapes and improving analytical sensitivity and resolution for the HPLC analysis of phenolic compounds.

The flow rate was kept at 1 ml/min. The temperature of column was controlled at 25 °C, detection wavelength 320 nm and the retention time values for standard compounds were determined 10 min.

Linearity, LOD and LOQ

Five standards solutions of caffeic acid and chlorogenic acid in acetonitrile: water with concentrations of 5, 10, 50, 100, and 200 µg/ml were prepared. The calibration curves linear for caffeic acid and chlorogenic acid were plotted peak area of chromatograms against five different concentrations was shown in table 3, fig. 3 and fig. 4 in three replicates (n=3).

Table 3: Calibration data for the proposed HPLC methods

Compounds			
Caffeic acid		**Chlorogenic acid**	
Concentration (µg/ml)	**Peak area 10^5(mAU)**	**Concentration (µg/ml)**	**Peak area 10^5(mAU)**
5	8.83	5	5.46
10	17.16	10	10.63
50	78.66	50	46.06
100	156.76	100	95.73
200	312.33	200	178.8

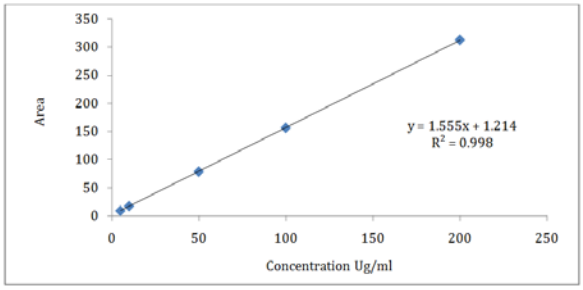

Fig. 3: Linearity of caffeic acid standard

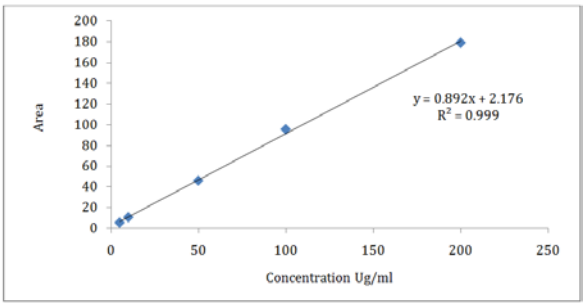

Fig. 4: Linearity of chlorogenic acid standard

Regression equation and coefficient of correlation caffeic acid and chlorogenic acid were (0.998 and 0.999) revealed a good linearity response for the method developed as represented in table 4. The LOD and LOQ defined as the lowest concentration of the analyte that can be clearly detected or quantified were calculated by multiplying the signal to noise ratio by (3) LOD and by (10) LOQ. The LODs caffeic acid and chlorogenic acid were 0.932 and 14.95 µg/ml, while LOQs was found to be 2.82 and 45.31 µg/ml. This indicates that the proposed method exhibits a good sensitivity for the quantification.

Table 4: System suitability and validation parameters

Parameters	Caffeic acid	Chlorogenic acid
Linearity range (µg/ml)	5-200	
Regression equation	Y=aX-b	
Correlation coefficient(r)	0.998	0.999
Slope	1.555	0.892
Intercept	1.214	2.176
SE of intercept	0.196511	1.807838
SD of intercept	0.439399	4.042326
LOD (µg/ml)	0.932486	14.95479
LOQ (µg/ml)	2.825714	45.31756

Accuracy

The accuracy of the proposed method was expressed as the recovery at three different concentration (50, 100, and 200 µg/ml) were determined in triplicate was found to be in the range of 97.47-101.92%, indicating good accuracy of the method were shown in table 5.

Precision

A repeatability test was performed in order to estimate intra-day variation in the peak areas and retention times. Highest value for relative standard deviation (RSD) was 0.93% for caffeic acid and 0.57% for chlorogenic acid at three different concentration (50, 100, and 200 µg/ml) were determined in triplicate which proving that repeatability of the method is satisfactory.

An intermediate precision (inter-day repeatability) was determined over two consecutive days analyzing working solutions under the same chromatographic conditions were highest value for RSD was 1.20% for caffeic acid and 1.34% for chlorogenic acid were shown in table 5**.** Development of validated analysis techniques of these phytotherapeutic agents are an important practice for standardization and quality control of the herbal drugs used in clinical trials and efficacy, safety, and quality control of herbal drug preparations [15].

Table 5: Precision and recovery data of HPLC methods

Compound	Amount added (µg/ml)	Amount recovered (µg/ml)[a]	Recovery (%)[a]	RSD (%)	
				Intra day[b]	Inter day[c]
Caffeic acid	50	49.95±0.46	99.91±0.46	0.93	1.20
	100	100.11±0.53	100.11±0.53	0.53	0.37
	200	200.03±1.22	100.01±1.22	0.61	0.13
Chlorogenic acid	50	48.73±0.17	97.47±0.17	0.35	1.34
	100	101.9±0.58	101.92±0.58	0.57	0.46
	200	199.39±0.29	99.69±0.29	0.15	0.15

[a]Mean±SD (n=3)

[b]Samples were analyzed three times a day

[c]Sample were analyzed three times a day over two consecutive days

CONCLUSION

From the results of analysis of ethyl acetate fraction of seed extract by TLC and HPLC demonstrated presence caffeic and chlorogenic acid in *Ammi majus* and presence of caffeic acid in higher quantity than chlorogenic acid. A convenient and rapid HPLC-UV/Vis has been developed for the estimation of phenolic acid in seed extract. The method is fast, accurate, sensitive; provide excellent recoveries, convenient and effective for the simultaneous quantification of phenolic acid in seed extract.

ACKNOWLEDGEMENT

The authors are gratefully acknowledging the financial support of department of pharmacognosy, college of pharmacy, Hawler Medical University.

CONFLICTS OF INTERESTS

Authors have no conflicts of interest to declare

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