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Determination of Damsin, Glycyrrhizin, Khellin and Azulene in the Traditional Egyptian Medicinal Preparation Sekem Renal Herbal Tea by High Performance Liquid Chromatography

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High-performance liquid chromatography was employed to determine the contents of several marker substances such as damsin, glycyrrhizin, khellin and azulene in Sekem renal herbal tea. The separation was performed on a microbondapack C18 column with 10 μn particle diameter and 30 cm length using methanol-water (93 : 7) as a mobile phase at flow rate 0.25 mL/min with detection at 254 nm; also this condition was used to develop fingerprint chromatogram for the alcoholic extract of the renal tea to be used as a rapid method for its quality control. The accuracy and precision (relative standard deviation) of the method were in the ranges 68–100% and 1.5–8.8% respectively.

Key words: Determination, damsin, glycyrrhizin, khellin, azulene, Egyptian tea, HPLC.

INTRODUCTION

The use of herbal medicine has been practised since ancient times as is evidenced by records of Chinese, Egyptian, Greek and Roman origins that it goes as far back as 5000 years[1]. A large number of ancient Egyptian physicians *routinely* used plants as medicines[2].

Most of Egyptian remedies are composed of many herbs which contain complicated chemical constituents, hence proper methods for quality control are needed to ensure the rational use of the remedies and guard against potential hazards[3].

Although many high-performance liquid chromatographic (HPLC) methods have been developed for the determinations of one or two constituents in crude drugs or preparations[4–8], there have been a few reports on the simultaneous determination of multiple constituents in preparations. In order to establish rapid and simple HPLC methods for routine quantitative analysis, we have tried to develop a method to assay multiple constituents in the preparations simultaneously.

In this study the herbal remedy used contains seven plants (*viz.*, *Ammi visnaga, Ambrosia maritima, Achillea millefolium, Cymbopogon proximus, Glycyrrhiza glabra, Cichorium intybus* and *Mentha piperita*). Four marker substances, damsin (present in *Ambrosia maritima* herb), glycyrrhizin (present in *Glycyrrhiza glabra*

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radix), khellin (present in *Ammi visnaga* fruits) and azullene (present in *Achillea millefolium* herb) were selected for the analysis. HPLC , method was developed for the simultaneous determination of the contents of the four markers by using methanol-water as eluent and for development of fingerprint chromatogram used for the determination of the percentage of the seven plants and this is achieved by composing standard herbal mixture formed of the same type and percentage of the plants present in the marketed preparation. Comparisons were done between the chromatograms of both standard and marketed herbal extracts and the method was validated.

EXPERIMENTAL

Samples of different batch number are used for the chosen preparation which is recognized in the market under the name "Sekem renal" where each box contains 10 filter bags of 1.5 g each consisting of *Glycyrrhiza glabra* root (20 g), *Mentha piperita* (20 g), *Achillea millefolium* herb (5 g), *Ammi visnaga* fruits (20 g), *Cymbopogon proximus* herb (20 g), *Ambrosia maritima* herb (10 g) and *Cichorium intybus* (5 g).

Standard herbal mixture was prepared from the same previous plants with the same percentage. These plants were kindly supplied by Sekem Company, Cairo, Egypt.

Structures of the market substances are shown in Fig. 1.

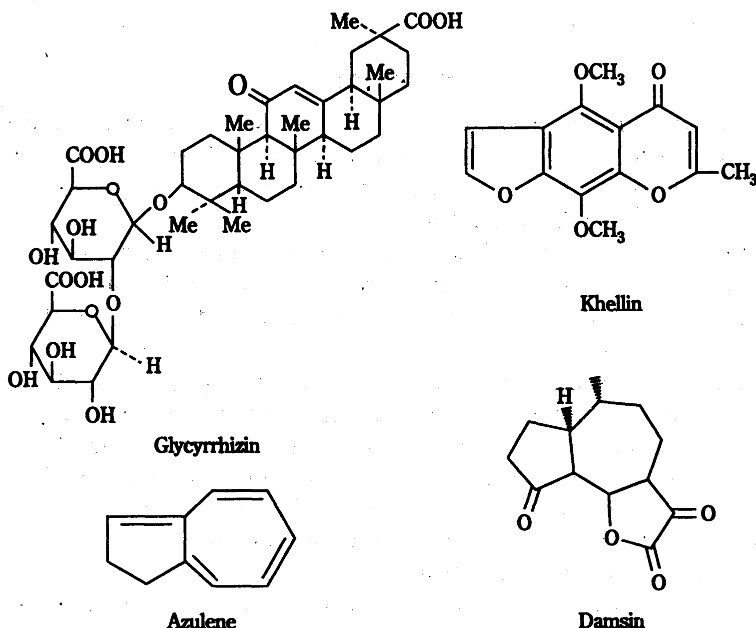

Fig. 1. Structure of marker compounds

Damsin was supplied by Faculty of Pharmacy, Helwan University, Cairo, Egypt. Glycyrrhizin was supplied by Faculty of Pharmacy, Al-Azhar University,

Cairo, Egypt. Khellin was supplied by NODCAR and azulene was purchased from Sigma Company. Methanol and 95% alcohol (HPLC grade) were purchased from BDH (England). 18 ohms deionized water was used

HPLC instrument was Bio-tek, Kontron (HPLC 500 system compatible with Kroma system 2000). Concentration under reduced pressure was carried out using rotary evaporator (Buchi R-14), Switzerland. The mobile phase was composed of methanol-water (93 : 7). The separation was performed on a microbondapack C_{18} reversed-phase column (30 × 4.6 mm I.D.) and 10 μn particle diameter. The flow-rate was 0.25 mL/min with UV absorbance detection at 254 nm. The operating temperature was maintained at room temperature.

Preparation of standard solution: Damsin, glycyrrhizin, khellin and azulene were accurately weighed and dissolved in methanol-water (93 : 7) to give serial concentrations in the ranges 0.02–0.06, 0.01–0.04, 0.005–0.02 and 0.002–0.005 mg/mL respectively.

Markers solution contained a mixture of azulene 10 μg/mL, khellin 10 μg/mL, glycyrrhizin 40 μg/mL and damsin 40 μg/mL. Calibration graphs were plotted using peak area *vs.* concentration.

Sample preparation for HPLC: Standard and marketed herbal mixtures were prepared as follows: 1 g each of standard and marketed herbal mixtures were separately macerated in 15 mL 95% alcohol for 2 h with occasional shaking, then boiled in a boiling water bath for 5 min.

Both extracts were cooled, filtered and volume was adjusted to 25 mL in a volumetric flask using 95% alcohol.

About 0.25 mL of each of the previous solutions was diluted to 5 mL using the mobile phase.

20 μL of both solutions were injected separately and the chromatograms obtained (using the previous mentioned conditions) were compared.

Recovery test: 4 g of powdered *Ammi visnaga*, *Achillea millefolium*, *Ambrosia maritima* and *Glycyrrhiza glabra* were separately macerated in 95% alcohol for 1 h with occasional shaking, heated in boiling water bath for 5 min, cooled and each was divided into 4 equal portions (one as a control) and each portion (excepting the control) was spiked with standard solutions to introduce various concentrations of damsin (3, 6 and 10 mg) to *Ambrosia* extract, glycyrrhizin (2.25, 5 and 10 mg) to *Glycyrrhiza* extract, khellin (2.5, 5 and 10 mg) to *Visnaga* extract and azulene (0.5, 1 and 2 mg) to *Achillea* extract.

The previous solutions were filtered and volume made up to 25 mL using 95% alcohol.

Aliquots of 0.5 mL of the previously prepared solutions were diluted in a volumetric flask, to 10 mL with the mobile phase, 20 μL from each was injected in HPLC apparatus using the same conditions of the experiment.

RESULTS AND DISCUSSION

The chosen marker substances in this study belong to sesquiterpenoids, triterpene saponins and furanochromons. As mentioned in the introduction, many HPLC methods have been reported for determining one or two constituents in crude drugs of preparations.

In this study, the four markers were effectively separated using methanol-water (93 : 7) as mobile phase at flow rate 0.25 mL/min using microbondapack C18 column (30 cm × 4.6 I.D.) with 10 μm particle diameter using 254 nm as the detection wavelength. This condition gave the separation of the markers as follows: damsin R_t 6.2, glycyrrhizin R_t 8.1, khellin R_t 12.9 and azulene R_t 14.4.

By running the standard and marketed herbal extracts using the same conditions, both gave peaks corresponding to the marker compounds and the concentration of these markers in the marketed mixture fitting the range (90–110%) of the concentration of the markers in the standard herbal mixture (Table-1). [Concentration of the markers was calculated from standard calibration curves established for each marker.]

TABLE–1
CONCENTRATION OF THE MARKERS IN STANDARD AND MARKETED HERBAL MIXTURES

Marker	Retention time		Peak area		Concentration	
	Standard mixture	Marketed mixture	Standard mixture	Marketed mixture	Standard mixture	Marketed mixture
Damsin	6.7	6.4	153	139	51.80	47.10
Glycyrrhizin	8.6	8.5	70	63	0.90	0.80
Khellin	13.5	13.2	357	351	1.54	1.52
Azulene	14.6	14.4	350	348	2.10	2.10

Also it is clear from the chromatograms of both mixtures gave the same number of peaks at relatively the same R_t with peak areas of the marketed extract fitting the range between (80–110%) of peak areas of the standard extract. So both chromatograms could be considered as fingerprints (Table-2) [i.e both mixtures contain the same type of plants with the same ratio] and could be used as rapid methods for quality control of this renal mixture.

TABLE-2
RETENTION TIME AND PEAK AREA OF STANDARD AND MARKETED HERBAL CHROMATOGRAMS

Spot No.	Retention time		Peak area	
	Standard mixture	Marketed mixture	Standard mixture	Marketed mixture
1.	6.1	5.9	9.1	9.8
2.	6.7	6.4	153.0	139.0
3.	8.6	8.5	70.0	63.0
4.	11.2	10.9	85.0	65.0
5.	11.9	11.5	2.9	2.4
6.	13.5	13.2	357.0	351.0
7.	14.6	14.4	350.0	348.0

Accuracy and precision of this method were checked by running standard solutions of damsin, glycyrrhizin, khellin and azulene at concentrations of (20, 40, 60), (10, 20, 40), (5, 10, 20) and (2, 3, 5) μg/mL respectively and it is clear from Table-3 that damsin gave accuracy up to 99% with C.V. 8.8, glycyrrhizin gave 100% with C.V. 1.5, khellin gave 100% with C.V. 5 and azulene gave 95% with C.V. 7.3.

TABLE-3
ACCURACY AND PRECISION OF CHOSEN MARKERS

Marker	Concentration μg/mL	Accuracy (%)	C.V.
Damsin	20	103	2.7
	40	89	6.5
	60	99	8.8
Glycyrrhizin	10	80	3.7
	20	100	1.5
	40	86	4.5
Khellin	5	100	5.0
	10	105	3.14
	20	100	7.3
Azulene	2	105	2.9
	3	86	5.3
	5	95	7.3

TABLE-4
PERCENTAGE OF RECOVERY OF CHOSEN MARKERS

Marker	Amount of plant taken (mg)	Amount of marker present (mg)	Amount of added marker (mg)	Total amount of marker present (mg)	Total marker found (mg)	% recovery	C.V.
Khellin	1040	15.00	2.50	17.50	17.50	100	3.5
	1010	14.95	5.00	19.95	15.96	80	5.0
	1000	14.90	10.00	24.90	18.68	75	7.5
Azulene	1020	0.50	0.50	1.00	0.78	78	2.8
	1000	0.49	1.00	1.49	1.12	75	5.5
	1050	0.052	2.00	2.52	1.76	70	7.0
Glycyrrhizin	1020	2.25	2.25	4.50	4.005	89	3.2
	1010	2.23	5.00	7.23	5.93	82	1.7
	1040	2.26	10.00	12.26	9.56	78	5.2
Damsin	1000	89.75	3.00	92.75	99.50	99.5	3.0
	1010	89.85	6.00	95.85	92.00	92	6.2
	1050	90.00	10.00	100.00	85.00	85	7.5

Percentage of recovery of the markers was determined and the results compiled in Table-4 which shows that the mean recoveries of khellin were 75–100%, azulene 70–78%, glycyrrhizin 78–89% and damsin 85–99.5%. The results showed that the recoveries of azulene were lower; this may be due to the highest volatility of azulene which causes great loss during the extraction procedure.

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