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Research Article

Preparation of Oxymel (Sekanjabin-e) Buzuri Syrup as Vascular Opener (Mofatteh) Product and Its Standardization

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ABSTRACT

Background: Sekanjabin-e buzuri consisting extracts of *Chicorium intybus* L. (Kasni), *Cuscuta chinensis* Lam. (Koshus), *Apium graveolens* L. (Karafs) and *Pimpinella anisum* L. (Anison) seeds is a Traditional Persian Medicine product. These drugs have many applications in traditional medicine, but they are more effective in opening vascular obstructions and related functions, especially in cardiovascular system.

Purpose: In this study we prepared a proper Sekanjabin-e buzuri and developed a HPLC method for analysis of chlorogenic acid (CGA), as an herbal marker compound, for quality control and standardization in both Sekanjabin-e buzuri syrup and its ingredient sources.

Methods: Sekanjabin-e buzuri is a group of oxymels that have many different formulations. A proper formulation has chosen from literature (from Gharabadin-e-salehi) and prepared. For standardization of Sekanjabin-e buzuri we developed a method for detecting chlorogenic acid content. A reversed phase MZ C18 column (150*3.0 mm, 5µm) using a mixture of acetonitrile-phosphoric acid 0.1% with gradient elution program for 20 minutes with flow rate of 1.5 ml/min with UV detection at 330 nm.

Results: The chlorogenic acid R_t =5.1 minutes and linear over the range of 0.2-1.5 µg/ml, (R^2 = 0.9996). The calculated LOD and LOQ of chlorogenic acid were 0.02 and 0.06 µg/ml, respectively. The concentration of chlorogenic acid was 7.69, 10.37, 2.25, 2.88 and 22.86 µg/ml for *Chicorium intybus* L., *Cuscuta chinensis* Lam., *Apium graveolens* L. and *Pimpinella anisum* L. seeds and Sekanjabin-e buzuri syrup, respectively.

Conclusion: This standardized Sekanjabin-e buzuri syrup will be used as a vascular opener (Mofatteh) complementary product for opening internal organs obstruction e.g. promoting cardiovascular health.

Introduction

Sekanjabin (oxymel) is one of the most famous and well-known syrups in Persian medicine. In addition to its great therapeutic effects, it is very useful for maintaining health. Razi quotes Galen that he believes the best syrup for hygiene in all ages and temperaments is Sekanjabin [1]. If healthy people drink it continually, their health increases, digestion becomes good and liver and spleen obstruction does not occur [2]. Both simple Sekanjabin and its derivatives with other ingredients are used to treat many diseases. Some of their most important effects are relieving thirst, opening internal organs obstructions (as a mofatteh drug), diuretic, relieving fever and effectiveness in stomach, liver, spleen, and lung diseases [3]. Based on the application purpose, its composition can be different.

This syrup is also known in current medicine so that squill oxymel which is a famous kind of oxymel in traditional Persian medicine is included in

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British Pharmacopeia [4]. Sekanjabin-e buzuri is a group of Sekanjabins that has many different formulations. These drugs have many applications in traditional medicine, but they are more effective in opening obstructions and related functions [5, 6]. Various medicinal herbs are added to simple sekanjabin to produce various types of Sekanjabin-e buzuri. Some of these medicinal herbs are *Chicorium intybus* L. (Hendba, Kasni, Chicory), *Foeniculum vulgare* Mill. (Razianaj, Fennel), *Apium graveolens* L. (Karafs, Celery), *Cucumis sativus* L. (Khiar, Cucumber), *Portulaca oleracea* L. (Khorfe, Purslan), *Cuscuta chinensis* Lam. (Koshus), *Pimpinella anisum* L. (Anison, Anise), *Cucumis melo* L. (Kharboze), *Nardostachys jatamansi* (Nj.Cr) DC. (Sonbol-e tib, Nardin), *Rheum palmatum* L. (Ravand, Rhubarb), *Capparis spinose* L. (kabar, caper), *Cymbopogon schoenanthus* (L.) Spreng (Ezkhar, Camel grass), *Berberis vulgaris* L. (Arghis, Zereshk, Barberry) etc. [5, 7, 8].

Phenolic compounds are abundant secondary metabolites with many health effects especially due to their antioxidant and anti-inflammatory properties. The chemical structure of phenolic compounds such as the hydroxyl groups in the molecule highly influences their mechanisms of action [9, 10]. The caffeoyl quinic acid (CQA), usually known as chlorogenic acid (CGA), is one of the important polyphenols found in many herbs. The CGAs show very valuable actions including antioxidant, anti-inflammatory, radical scavenging, antimicrobial, inhibiting mutagenesis, and carcinogenesis, etc. effects and have been considered to be beneficial for human health [11]. This compound has antidiabetic, hepatoprotective and antiobesity effects by regulating lipid and glucose metabolism and antioxidant, anti-inflammatory activities [11-17]. CGA can improve human vascular function, protect vessels against oxidant-induced damage, has direct vasorelaxant effects through endothelium-dependent actions and cause an anti-hypertension activity [11, 18-20]. All these functions are consistent with the cardiovascular protective effects of chlorogenic acid. Miao *et al*. reported chlorogenic acid at high dosage has best results on the focal cerebral ischaemia reperfusion injury rat model by several mechanisms [21].

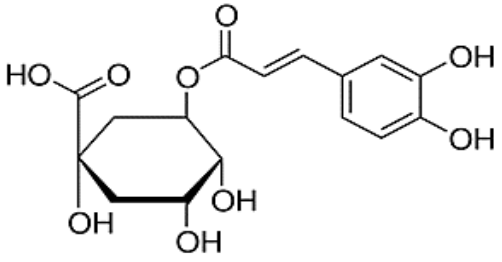

Figure 1: Chemical structure of chlorogenic acid.

Nowadays, trend towards traditional drugs has increased. But what matters about these medications is that high-quality products should be available. For standardization and chemical evaluation of herbal formulations quality, the active component or a marker substance should be chosen for analytical purpose [22]. In this study for quality control of the Sekanjabin-e buzuri, we choose the formula from Gharabadin-e-salehi and then developed a validated Reversed-Phase high-performance liquid chromatography (RP-HPLC) with UV detection for quantification of chlorogenic acid as the marker compound of the syrup [7]. Chlorogenic acid (Figure 1) is known from the literatures as one of the main compounds in the herbs used in this kind of Sekanjabin-e buzuri [23-27]. Also, this metabolite has been chosen as an active marker in Chinese traditional medicine products [28, 29]. Therefore, it is one of the major secondary metabolites which can be considered as an active marker for quality control of herbs and prepared formulations.

Cardiovascular diseases (CVDs) and atherosclerosis (vascular obstruction), One of the most common types of CVDs, are currently the most common cause of death in the world [30]. Based on the Persian medicine (PM), atherosclerosis could be comparable with the vessel obstruction "sodde orooq" due to abnormal humor accumulation [31]. As mentioned Sekanjabin-e buzuri is suggested to be effective in opening obstructions. For evaluation of the formulated syrup effectiveness clinical trial should be carried out. In clinical trials, there needs a standard method for evaluation of the syrup, which is the HPLC method developed in this study.

Materials and Methods

I Herbs and Chemicals

Seeds of *Chicorium intybus* L. (Kasni), *Cuscuta chinensis* Lam. (Koshus), *Apium graveolens* L. (Karafs) and *Pimpinella anisum* L. (Anison) were purchased from a reliable herbal market (Tehran, Iran). The seeds were identified and verified at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences (Tehran, Iran) with the voucher numbers of PMP-1631, PMP-784, PMP-1630 and PMP-1629, respectively. Standard chlorogenic acid was purchased from Sigma Aldrich chemical Co. (Germany). All solvents were of HPLC grade; water, ethanol, acetonitrile, and phosphoric acid were provided by DR. MOJALLALI Industrial Chemical Complex Co. (Iran).

II Preparation of Sekanjabin-e Buzuri

To prepare the syrup, 70 g of Kasni and 35 g of each of other herb's seed (Koshus, Karafs, Anison) were powdered coarsely and soaked in 1350 ml vinegar (with 4.1% acidity) and 150 ml water for 24h. Then it was boiled until the volume was halved and filtered. The extract was concentrated with adding 5 L sugar solution and boiling (as the formula in Gharabadin-e-Salehi) [7]. Samples from each of the individual herbs were also prepared with the method described for the syrup.

III Instruments and HPLC Condition

An Azura model HPLC (Knauer, Germany) equipped with pump P6.1 L, auto sampler AS-1, column oven CT2.1, Detector UVD 2.1L and a Clarity Chrome Software was used for this study. The column was a reversed phase MZ C_{18} (150*3.0 mm, 5µm). The mobile phase was a gradient elution of acetonitrile (A)- phosphoric acid 0.1% (B) for 20 minutes starting with A: B (10:90) for 13 min, changing to A: B (22:78) for 1 min, A: B (10:90) for 6 min. The flow rate was 1.5 ml/min and the injection volume for all samples and standard solutions was 100 µl. The UV detector was set at 330 nm at 35°C. All samples were filtrated before injection. The run time for standards and samples was 12 min.

IV Preparation of the Standard and Sample Solutions

The stock solution of standard was prepared by dissolving 10 mg of chlorogenic acid in 100 ml distilled water in volumetric flask. The stock solution was diluted for preparation of standard solutions in the range of 0.2-1.5 µg/ml. For preparing sample solutions 4.5 ml of the syrup was diluted with 100ml water: ethanol (50:50).

V Validation

The method for analysis of chlorogenic acid was evaluated regarding the validation parameters including selectivity, linearity, precision, accuracy, detection limit and quantitation limit according to the guidelines of the International Conference on Harmonization [32].

i Selectivity

Analysis of resolution and peak purity parameters represents the specificity of the method for chlorogenic acid. The blank sample was injected in duplicate for checking the interference of sample matrix with the analyte peak.

ii Linearity

The linearity of the HPLC method was evaluated by the relationship between the concentration and peak area. Different concentrations of chlorogenic acid standard solution at the range of 0.2-1.5 µg/ml were analysed for linearity evaluation.

iii Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements. For assessing this factor, the standard chlorogenic acid was prepared at 3 concentrations, 3 replicates in one day for repeatability and three consecutive days for intermediate precision. Also, six samples of the drug were analysed by the developed method at the same day for repeatability (intra-day) and at three consecutive days each day three samples for intermediate precision (inter-day). Finally, Relative standard deviations (RSD %) were calculated.

iv Accuracy

Accuracy of the method expresses the closeness between the assay value and the true value. Known three different concentrations of standard chlorogenic acid were added to the blank syrup in triplicate. The difference between the results of the assays was compared with the expected results. Accuracy should be reported as percent recovery.

v Limit of Detection and Quantization

Detection limit represents the lowest amount of analyte in a sample which can be detected from the noises in the baseline. Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Limit of detection (LOD) and limit of quantification (LOQ) values were calculated by intercept standard deviation (σ) and slope (S) obtained from the linearity, as follows:

$LOD = 3.3 * \sigma / S$

$LOQ = 10 * \sigma / S$

VI Quantification of Chlorogenic Acid in Samples

All samples, syrup, and individual plants were injected to the HPLC instrument with the same condition of the standard. The chromatogram of each sample was obtained. The peak area related to chlorogenic acid was recorded. Using the equation regression of calibration curve, the concentration of chlorogenic acid in these samples was calculated.

Results

Chromatograms obtained from injection of blank and syrup indicated that there was no interference between the matrix and the desired composition, and the peak at retention time R_t= 5.1 min refers only to chlorogenic acid. The resulting peak of chlorogenic acid had a good peak symmetry and was well separated from adjacent peaks (resolution=1.935) (Figures 2-4). So, the separation technique was selective for analysis of chlorogenic acid. The calibration was tested over the range of 0.2-1.5 µg/ml chlorogenic acid. The parameters of the calibration curve in the specified range exhibited the linear relationship between the selected concentrations and the responses of the HPLC detector (Table 1). The regression equation was Y= 245.7X - 42.5 with regression coefficient (r^2) of 0.9996. The next evaluated validation parameter was precision. Tables 2 & 3 shows the precision of the method using the standard chlorogenic acid at 3 concentrations, 3 replicates in one day and 3 consecutive days. Tables 4 & 5 show the repeatability and intermediate precision using the product. The low value of % RSD in both standard and drug represents the repeatability and intermediate precision of the analytical procedure.

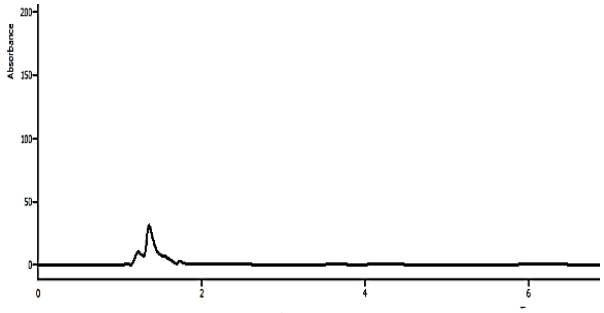

Figure 2: HPLC chromatogram of blank syrup.

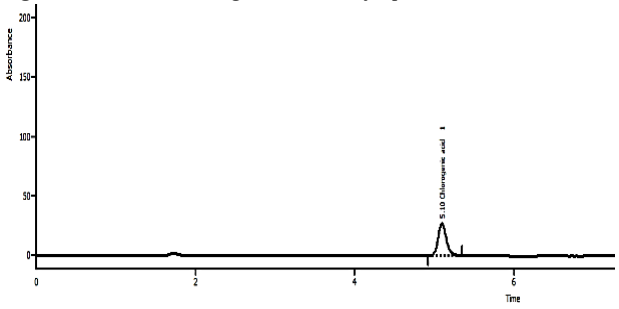

Figure 3: HPLC chromatogram of standard solution with 1 µg/ml Chlorogenic acid (R_t=5.1min).

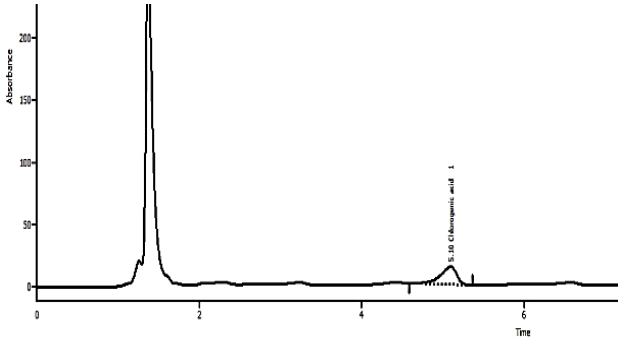

Figure 4: HPLC chromatogram of Sekanjabin-e buzuri (22.86 µg/ml chlorogenic acid in sample at R_t=5.1min, 1:20 diluted).

Table 1: Linear regression data for the calibration curve of standard chlorogenic acid in the range of 0.2-1.5 µg/ml.

Regression equation	Y = 245.7X - 42.5		
Slope	245.7	Intercept	-42.5
SD slope	1.7	SD intercept	1.6
R^2	0.9996	St. error	2.119
F	19887.97	D_f	7
SS Reg	89317.59	SS Res	31.437

Table 2: Intra-day precision of standard chlorogenic acid determination with HPLC proposed method (n=3).

Chlorogenic acid concentrations (µg/ml)	Intra-day precision	
	Mean ± SD	%RSD
0.8	0.806±0.007	0.85
1	1.003±0.006	0.58
1.2	1.167±0.006	0.49

Table 3: intermediate precision of standard chlorogenic acid determination with HPLC proposed method (n=9).

Chlorogenic acid concentrations (µg/ml)	Intermediate precision	
	Mean ± SD	%RSD
0.8	0.799±0.011	1.35
1	1.006±0.007	0.72
1.2	1.182±0.014	1.18

Table 4: Intra-day precision of chlorogenic acid determination in the syrup with HPLC proposed method (n=6).

Chlorogenic acid concentrations (µg/ml)						Mean ± SD	%RSD
S_1	S_2	S_3	S_4	S_5	S_6		
21.41	21.44	21.43	21.45	21.58	21.5	21.47±0.06	0.3

S: sample

Table 5: Intermediate precision of chlorogenic acid determination in the syrup with HPLC proposed method (n=9).

Day	Chlorogenic acid concentrations (µg/ml)			Mean ± SD	%RSD
	S_1	S_2	S_3		
1	21.83	21.85	21.87		
2	21.03	21.96	21.96	21.89±0.08	0.35
3	21.81	21.91	21.8		

S: sample

The results of recovery have been shown in (Table 6). The recovery rates are within the acceptable range of 97-103% which indicates the accuracy of the procedure. The calculated limit of detection and limit of quantification were 0.02 and 0.07 µg/ml, respectively. Our results indicate that this validated method can be used for detection and quantification of low amounts of chlorogenic acid. After method validation, the content of chlorogenic acid in Sekanjabin-e buzuri syrup and the seeds oxymel syrup was measured by the proposed HPLC method. The concentration of chlorogenic acid was 7.69, 10.37, 2.25 and 2.88 µg/ml for *Chicorium intybus* L. (Kasni), *Cuscuta chinensis* Lam. (Koshus), *Apium graveolens* L. (Karafs) and *Pimpinella anisum* L. (Anison) seeds syrup, respectively. The chlorogenic acid content was found to be 22.86 µg/ml in the Sekanjabin-e buzuri syrup.

Table 6: Accuracy of the HPLC method for the determination of chlorogenic acid in Sekanjabin-e buzuri blank syrup.

level	Added concentrations (µg/ml)	Mean concentration founded (µg/ml) (n=3)	%recovery	%RSD
80%	0.77	0.76	98.99	1.08
100%	1.02	0.99	97.54	0.53
120%	1.15	1.14	98.67	0.33

Discussion

Although Sekanjabin-e buzuri is a well-known syrup in traditional medicine according to our information there is no validated method for preparing and quality control and standardization of it. From different validation parameters the calculated limit of detection and limit of quantification with this validated HPLC method (0.02 and 0.07 µg/ml, respectively) were much lower than the reported limit in previous studies [33-36]. Wang *et al*. obtained 0.069 and 0.218 µg/ml for LOD and LOQ respectively, using a gradient elution procedure with the same mobile phase on Hypersil C18 column (200*4.6 mm i.d., 5 µm particle size) [35]. Low amounts of chlorogenic acid can be detected and quantificated by this method in compare with other methods.

Also, it is important to mention that different factors such as genotype, environmental conditions, harvesting time, storage etc. can affect the plants phytochemical composition, generally [37-39]. The chlorogenic acid content obtained in this study for each seed was, 27.5 mg (*Chicorium intybus* L.), 74.1 mg (*Cuscuta chinensis* Lam.), 16.1 mg (*Apium graveolens* L.) and 20.6 mg (*Pimpinella anisum* L.) in 100 g dried weight seeds. Available literatures report the chlorogenic acid content of these seeds but due to different methods of extraction and lack of transparency in reporting, the studies are not comparable with each other [40-45].

Conclusion

In this study, after preparation of Sekanjabin-e buzuri, an HPLC method with UV detection was developed and validated for analysis of chlorogenic acid as a marker in our formulation. The results showed that the proposed HPLC method was relatively fast, simple, and reliable for determination and quantification of chlorogenic acid according to the validation parameters including selectivity, linearity, precision, and accuracy, limit of detection and limit of quantification for both plant seeds and formulations. Therefore, this method can be used for standardization and quality control of Sekanjabin-e buzuri and similar herbal drugs with this secondary metabolite and after it could be administered it in clinical trials. As mentioned Sekanjabin-e buzuri is suggested to be effective in opening obstructions. For evaluation of the formulated syrup effectiveness clinical trial should be carried out. In clinical trials, a standard method for evaluation of the syrup is needed, which is the HPLC method developed in this study.

Conflicts of Interest

None.

Abbreviations

CQA: Caffeoyl Quinic Acid
CGA: Chlorogenic Acid
RP-HPLC: Reversed-Phase High-Performance Liquid Chromatography
CVDs: Cardiovascular Diseases
UV: Ultraviolet
RSD: Relative Standard Deviations
LOD: Limit of Detection
LOQ: Limit of Quantification
O: Intercept Standard Deviation
S: Slope

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