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Research Article

Colorimetric Determination of Flavonoids in *Citrus medica* L. Peel Traditional Medicinal Oil

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ABSTRACT
Herbal oil preparations are widely administered in Persian folk medicine. Among those, citron (*Citrus medica* L. from Rutaceae) oil is traditionally prepared via extraction of peels in heated sesame oil and administered for musculoskeletal, gastrointestinal and nervous system ailments. Till now, no certain evaluation has been performed on standardization of such traditional oil. Current study aimed to introduce a simple method for determination of active components in citron oil. Following preparation, qualitative and quantitative determination of flavonoids were performed using thin layer chromatography and Aluminium chloride colorimetric method. The extraction of flavonoid fraction was performed using two different solvent systems as *n*-Hexane and acetone. Using an established calibration curve by eight series of serially diluted Quercetin in methanol (4.4-35.2 mg/l), total flavonoid content n-Hexane and acetone solvent systems were determined as 207.88 and 199.80 µg/ml, respectively. Current study introduced a simple and applicable method for the determination of flavonoids in traditional herbal oil samples. This method can be simply applied for standardization of traditional herbal oil dosage forms.

Keywords: *Citrus medica* L., Flavonoid, Colorimetric method, Herbal oil, Standardization

INTRODUCTION

Since antiquity, natural medicaments have being applied traditionally for a variety of disorders. These medicines were either used in the form of single or compound formulation via a specific dosage form[1,2]. Medicinal oils are often mentioned as one of the most widely used pharmaceutical dosage forms from long time ago. Traditional knowledge has indicated a 3000 years antiquity for the medicinal oils[1]. Those herbal oils and extracts have been extensively applied for medical and pharmaceutical purposes in traditional Chinese, Indian and Egyptian medical systems[3].

As for other traditional medical systems, medical and pharmaceutical manuscripts authored by outstanding Persian scholars during the medieval periods have provided numerous information about herb and plant's dosage forms[4]. Preparation of herbal medicinal oils in Traditional Persian Medicine (TPM) encompasses four main categories[1]. In two ways of preparation, oil bearing plants were being subjected to either direct compression or hydrodistillation to yield the fixed or aromatic oil[1,5]. Unconventionally, early Persian scholars have prepared the oil dosage forms of non-oily dense herbal organs via boiling the water extract of those parts in common oils such as almond, sesame or olive oil. On the other side, they have employed maceration techniques to extract the fleshy parts of a plant into an oil vehicle[6].

Herbal oil dosage forms are still widely prepared and administered in Iranian folk medicine. One of those famous and considerable herbal oils is Citron (*Citrus medica* L. from Rutaceae). This oil which is prepared by extracting the peels essential oil in heated sesame oil is being traditionally applied for various ailments such as musculoskeletal, gastrointestinal and nervous system[7]. Current experimental and pharmacological investigation has shown antifungal, anti-inflammatory, antitumor, hypoglycemic and acetyl cholinesterase inhibitory effects from citrus peels[8-11].

Despite the extensive use of this traditional oil in addition to many other herbal oils by local practitioners and healers, no certain evaluation has been performed on the standardization of citron traditional oil. Accordingly, current work aimed to introduce a simple but useful method to determine the active components of citron traditional oil.

MATERIALS AND METHODS

Collection and preparation of the citron traditional

Citron fresh fruits along with the twigs and leaves was purchased from gardens around Darab city located at southwest of Fars province (south of Iran). The sample was authenticated by the botanist of Department of Phytopharmaceuticals, School of Pharmacy, Shiraz University of Medical Sciences with a specified voucher

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Table 1: The read absorbance data of Quercetin as standard concentrations

Concentration (µg/ml)	1st absorbance	2nd absorbance	3rd absorbance	Mean ± SD
4.4	0.23	0.21	0.24	0.23 ± 0.01
8.8	0.43	0.41	0.45	0.43 ± 0.02
13.2	0.54	0 .57	0.53	0.55 ± 0.05
17.6	0.83	0.89	0.80	0.84 ± 0.05
22	0.94	0.90	0.97	0.94 ± 0.03
26.4	1.26	1.31	1.23	1.28 ± 0.05
30.8	1.48	1.56	1.46	1.50 ± 0.05
35.2	1.63	1.71	1.61	1.65 ± 0.06

Table 2: Absorbance values of oil and methanol fractions of citron oil against respective blanks

Sample	Day	Absorbance		
		Mean ± SD (intra-day)	Mean ± SD (inter-day)	RSD (%) (inter-day)
Oil phase	1	0.93 ± 0.02	0.91 ± 0.02	2.19
	2	0.89 ± 0.01		
	3	0.91 ± 0.01		
Methanol phase	1	0.54 ± 0.01	0.54 ± 0.02	3.70
	2	0.52 ± 0.02		
	3	0.56 ± 0.01		

Table 3: Flavonoid determination in oil and methanol phases extracted by *n*-Hexane solvent system

Sample	Day	Content		
		Mean ± SD (intra-day)	Mean ± SD (inter-day)	RSD (%) (inter-day)
Oil phase	1	11.82 ± 0.31	11.93 ± 0.44	3.67
	2	11.59 ± 0.34		
	3	12.38 ± 0.39		
Methanol phase	1	20.34 ± 0.43	20.02 ± 0.24	1.19
	2	19.67 ± 0.21		
	3	20.05 ± 0.08		

number.

The oil was prepared in accordance with the procedure of citron oil preparation, described in most popular pharmaceutical manuscripts of Persian medicine (7). To this, adequate amount of 100 g isolated fresh peels was soaked in sesame oil up to 1000 g. The mixture was then kept under sunshine for 3 days (weather temperature ≈ 38-40ºC). Extracted peels were renewed for 2 additional times with the same condition and time in the sesame oil. The mixture was subsequently filtered and kept in the fridge (4ºC for further steps).

Qualitative determination of flavonoids in the sample

Diagnostic qualitative tests such as Thin Layer Chromatography (TLC) and Pew tests were employed to ensure the presence of flavonoids in the sample[12]. For TLC analysis, 1 ml of the prepared oil as well as sesame oil as blank was individually diluted in *n*-Hexane (1:5). A spot of each sample was applied on the TLC and developed by Toluene/Ethyl acetate (93/7). The developed TLC for each sample was treated by natural product reagent (Sigma-Aldrich) and visualized at 366 nm UV light. For Pew's test, 1 ml of citron oil as well as sesame oil was individually added to methanol (99.5%) with 0.05 g metallic zinc and few drops of concentrated hydrochloric acid. Appearance of red to brownish color was a confirmation for the presence of flavonoids[13].

Quantitative determination of flavonoids in the oil sample using n-hexane solvent system

Aluminium chloride colorimetric method was employed to determine the amount of flavonoids in the oil sample. Firstly, eight series of serially diluted Quercetin (the standard flavonoids, Sigma-Aldrich; analytical grade) in methanol (4.4 - 35.2 mg/l) was prepared. Subsequently, Aluminium chloride (2%- Sigma-Aldrich) was added to each Quercetin samples (1:1). The absorbance was read at 450 nm following 10 minutes. A calibration curve was then established in relation with concentration and absorbance[14]. Afterward, 1 ml of oil sample was dissolved in 5 ml of *n*-Hexane. Approximately, 6 ml of Aluminium chloride (2%) in methanol was added to the previous mixture. A separatory funnel was then used to separate the methanol phase from oil phase. In parallel, same procedure

Table 4: The yield (%) of transferred flavonoid methanol phases extracted from citron oil (acetone as solvent system)

Sample	Day	Content		
		Mean ± SD (intra-day)	Mean ± SD (inter-day)	RSD (%) (inter-day)
Methanol phase	1	74.02 ± 3.60	76.90 ± 3.60	4.68
	2	78.38 ± 3.51		
	3	78.31 ± 2.84		

Table 5: The yield amount of total flavonoid in the methanol phases extracted from citron oil (acetone as solvent system)

Sample	Day	Content		
		Mean ± SD (intra-day)	Mean ± SD (inter-day)	RSD (%) (inter-day)
Methanol phase	1	55.64 ± 2.40	57.98 ± 2.91	5.02
	2	59.89 ± 2.57		
	3	58.41 ± 2.76		

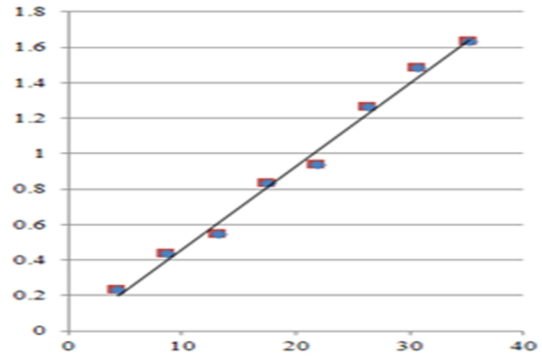

Figure 1: The established calibration curve for Quercetin

was undertaken on sesame oil alone without citron components as a blank. The absorbance of each oil and methanol phases of citron oil was then read against the blank following 10 min after adding Aluminium chloride (2%)[14].

Quantitative determination of flavonoids in the oil sample using acetone solvent system

Approximately, 10 ml of citron oil was mixed with acetone and subsequently was added to 10 ml ethanol. The polar phase was then separated from the oil phase. The yielded phase was calculated in milliliter. In parallel, same procedure was performed for sesame oil as blank. Simultaneously, 1 ml of Aluminium chloride (2%) was added separately to both methanol phases yielded from citron and sesame oil samples. The absorbance of citron methanol phase was then read against sesame oil methanol phase (blank) following 10 min[14].

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

In terms of description, the LOD is determined as the lowest concentration of an analyte in a sample which can be detected. LOQ is the known as the lowest concentration of an analyte in a sample which can be calculated with precision and accuracy[15]. To determine the LOD value, 1 mg of Quercetin was dissolved in 5 ml of methanol. In two consecutive steps, the solution was diluted until the theoretical amount of Quercetin reached to 16.67 µg/ml (stock solution). Approximately, 1 ml of Aluminium chloride (2%) was added to 1 ml of the stock solution. The absorbance was then read against pure methanol (blank) in 10 min. By serial dilution with pure methanol, subsequently, the concentration was decreased and the absorbance was respectively read until the detection was not possible. The LOD was determined as the latest concentration which was readable by spectrophotometer. In this study, LOQ was considered as the lowest Quercetin concentration employed in the standard curve.

RESULTS AND DISCUSSION

Qualitative detection of flavonoids in citron oil resulted in the appearance of a pale purple color based on PEW test. Table 1 represented the read absorbance values in triplicate for Quercetin in accordance with the respective concentrations. Accordingly, the respective calibration curve with the equation of $y = 0.046x - 0.009$ and 0.99 coefficient of determination was established (Figure 1). The quantitative amounts for Quercetin in methanol and oil phases of citron oil (Intra-day and inter-day precision values) were determined in accordance with the respective absorbance values (Table 2 and 3).

According to the Table 3, the total flavonoid content in the oil and methanol phases extracted from the citron oil were determined as 11.93 ± 0.44 and 20.02 ± 0.24 µg/ml, respectively. With reference to the primary volume of citron oil employed for the determination and amount of each phases, the total flavonoid content in 1 ml of citron oil was determined as 207.88 ± 3.68 µg/ml.

On the other hand, to determine the total flavonoid content in citron oil extracted with acetone solvent system, firstly the yield of flavonoid transferred from citron oil into the methanol phase was calculated. The yield was consequently determined as 76.90 ± 3.60%. Subsequently, quantitative determination of flavonoids in the methanol phase was determined (Table 5). As seen in the respective table, mean amount of the flavonoid was determined as 57.98 ± 2.91 µg/ml for every 1 ml of the methanol phase. In this study, the total volume of methanol phase in the extraction with acetone was calculated as 26.5 ml. Hence, the total flavonoid content in the methanol phase as 1536.5 ± 77.12 µg. At first, the methanol phase was extracted from 10 ml of citron oil. Therefore, the total flavonoid content in citron oil could be calculated as 153.65 ± 7.71 µg/ml.

With regard to the extraction yield calculated from methanol phase of the citron oil (76.90 ± 3.60%), the total amount of flavonoids in citron oil (in both polar and non-polar phases) is calculated as 199.80 µg/ml.

In this investigation, values of LOD and LOQ were determined as 0.03 and 0.39 µg/ml, respectively.

The main volatile components of citron peel are usually limonene, isolimonene, and citral[16-18]. On the other hand, fatty acids and flavonoids may also be appeared in the oil[19]. Current study aimed to qualitatively and quantitatively determine the flavonoids in a traditional dosage form, citron oil which is prepared and administered by traditional practitioners in Iran. The oil was firstly prepared in accordance with the method presented in traditional medical and pharmaceutical literatures. In two simple ways (*n*-Hexane and acetone systems), polar and non-polar extracts were prepared from the primary citron oil. Via a colorimetric method, quantitative amount of flavonoids in citron oil was determined in a methanol fraction extracted by mentioned *n*-Hexane system. According to the respective table, the total amount of flavonoids was found as 208 µg/ml in citron oil.

The employed method was an Aluminium chloride colorimetric assay, and most likely, $AlCl_3$ is not completely transferred in to the oil (polar) phase. Hence, the calculated absorbance in the oil phase may be incorrect. Therefore, the second solvent system method (acetone) was used. In that method, firstly the extraction yield of flavonoids in methanol phase was determined using another solvent (acetone). With reference to the extraction yield, the total flavonoids transferred in to the methanol phase and subsequently in the citron oil was determined. In this method, the amount of flavonoids in the oil phase was not determined concurrent with the determination in the methanol phase. However, in the previous solvent system (*n*-Hexane), determination in two phases were undertaken concurrently. The acetone solvent system method was employed to validate the *n*-Hexane method. It is accepted that solubility of Quercetin in acetone phase is higher than that in the methanol and also solubility of acetone in methanol is higher than that in *n*-Hexane[20]. Therefore, it was concluded that acetone can dissolve and transfer the flavonoids of citron oil in the methanol phase, more perfectly.

CONCLUSION

Current study introduced a simple and applicable method for the determination of flavonoids in traditional herbal oil samples. Validation of the primary applied solvent system, *n*-Hexane was confirmed by the second solvent system method, acetone. In this regard, both introduced methods can be simply applied for the standardization of traditional herbal oil dosage forms.

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