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Optimization of Solvent Systems for the Extraction of Vitexin as the Major Bioactive Flavonoid in *Prosopis farcta*

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Abstract

Prosopis farcta, a plant belongs to the mimosoideae, is characterized by a very wide spectrum of various bioactive and medical constituents. Vitexin, the marker flavonoid found in *Prosopis*, has potent and broad antitumour efficacy in preclinical models. Many studies had been done for the isolation of flavonoids (vitexin) by completely different chromatographically methodology. During this study, vitexin was isolated from *Prosopis farcta* by 6 different extraction methods in which parameters as the type, concentration and pH of the extracting solvents considered. Among different solvent systems used, methanol-water (40%, containing acetic acid 0.5%) was found to be the best solvent generating the highest yield (0.554 $mg \cdot g^{-1}$ DW) from *Prosopis* leaves. The present work suggests an efficient method for estimation the greatest content of vitexin analyzed by HPLC technique and introduces *Prosopis farcta* as a suitable source of this flavonoid with several pharmacological properties.

Keywords

Prosopis farcta, Vitexin, Solvent System, HPLC Technique

1. Introduction

The genus *Prosopis* (Leguminosae, subfamily Mimosoideae) includes about 50 species. Species of *Prosopis* are often spiny trees or shrubs predominantly well adapted to hot, arid climates. These plants have been commonly distributed in northern Africa, Southwestern Asia, West to the Middle East, and in the USA. *Prosopis farcta* is a native of Asia, distributed from India to Iran [1]. *P. farcta* has been used as a traditional medicine for treatment of some diseases and disorders.

For example, it may be used in the treatment of inflammation, measles, diabetes [2] [3] and also reduce cardiac or chest pain [1]. Antitumour activity [4], antioxidant capacity [5] and antimicrobial activity [6] have been reported for *Prosopis* species.

The medicinal value of these plants can be observed from the chemical agents they possess which may alter certain physiologic actions in the human body. The most important of these bioactive constituents of *Prosopis* species are L-arabinose, Lectin, alkaloids, flavonoids and phenolic compounds [7] [8]. Recently, flavonoids have attracted interest due to the discovery of their pharmacological activities [9] [10]. Vitexin, a nature-derived lignin compound (**Figure 1**), is found in *Prosopis farcta* [8]. It has been shown to have broad anti-tumor activity, activate caspases and induce cell apoptosis [11]. Additionally, vitexin inhibits an analgesic effect in a variety of inflammatory pain models by preventing the decrease of reduced glutathione levels and modulating cytokine production [12].

Extraction is the first crucial step in preparation of active ingredients from plant materials. Optimizing the extraction process with high yield may be the result of a synergistic combination of fundamental factors as extraction technique and efficacy of solvent extraction [13]. HPLC method as the main choice for fingerprinting study, has gained increasing importance for the analysis of plant extracts [14]. The efficacy of solvent extraction is influenced by many parameters such as the type of solvent, solvent concentration, time, temperature, pH and liquid-to-solid ratio [15]. Thus, the main objectives of this research were aimed 1) to introduce the best extraction method to provide for the maximum yield of vitexin from *P. farcta* and 2) to report this species of *Prosopis* as a considerable source of antinociceptive and antioxidant flavonoid.

2. Materials and Methods

2.1. Reagents and Chemicals

High performance liquid chromatography (HPLC)-grade solvents (Merck, Germany): acetic acid, ortho-phosphoric acid, n-hexan, methanol, ethanol and acetonitrile. All solvents were filtered through 0.45-µl filters before injection into HPLC. Standard chemical: vitexin (49,513 Fluka).

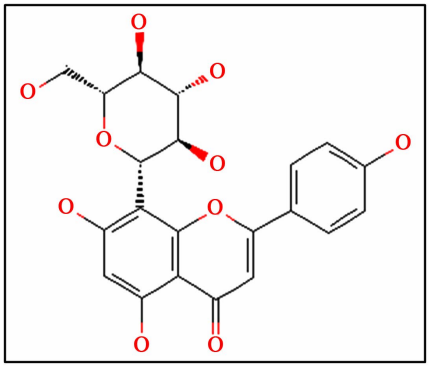

Figure 1. Structure of vitexin compound.

2.2. Plant Materials and Growth Conditions

Seeds of *Prosopis farcta* were collected in western Ilam province, IRAN. The healthy seeds of uniform size were selected and scarified with 98% sulfuric acid for 13 min and surface sterilized with 2% sodium hypochlorite solution, followed by repeated washings with distilled water, and germinated by placing in a Petri dish with two layers of water-saturated filter paper. Germinated seedlings with 20 mm-long roots were then transferred into plastic containers with 2.5 dm^3 of Hoagland nutrient solution (pH 6.8). The seedlings were left to grow in a growth chamber under a cycle of 16 h light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 25˚C/27˚C day/night temperature and 60% - 80% air humidity. After three weeks, leaves of plants were collected, dried at 60˚C and used for vitexin extraction.

2.3. Extraction Methods

2.3.1. Method 1: 85% Ethanol Extraction

The extraction was carried out using 2 g of powdered leaves with 30 ml of 85% ethanol in an ultrasonic extraction device for 30 min, repeated twice. The extract was collected and filtered, the filtrate was dried at 50˚C in a rotary evaporator. The dried extract was dissolved in the methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. After filtering through a filter paper and a 0.45 μm membrane filter (Millipore), the extract was injected directly into the HPLC [16].

2.3.2. Method 2: 70% Methanol Extraction

The extraction was carried out using 2 g of fine powder with 20 mL of 70% methanol. This process involved 2 min of vigorous vortexing, 30 min of sonication and incubation with shaking either for 3 h at room temperature. After the mixture had been centrifuged for 10 min at 1900 g, the supernatant was taken and filtered through a 0.45 μm membrane filter. After partitioning with 20 mL of n-hexane to remove lipids, 1 mL aliquots of the extract were vacuum dried for 2 h at room temperature. The dried aliquot was then resuspended in 10% methanol, and used for HPLC [17].

2.3.3. Method 3: 40% Acidic Methanol Extraction

The dried leaves (2 g) transferred to a tube with 15 mL of 40% aqueous methanol, containing 0.5% acetic acid. After shaking for 4 h, samples were centrifuged (12 min, 13,000 rpm) and the supernatant was used for HPLC [18].

2.3.4. Method 4: 94% Ethanol Extraction

2 g of powdered leaves were incubated with 15 ml of 94% ethanol in a petri dish for 1 h. The extract was evaporated to 500 μl and transferred into a centrifuge tube. The petri dish was subsequently washed three times with 2 ml of methanol and the extract and washings were combined and evaporated. The extracts were then dissolved into 100 μl of methanol. After adding 100 μl H_2O, the extracts were centrifuged in eppendorf tubes at 14,000 × g for 3 min. The supernatants were used for HPLC analyses [19].

2.3.5. Method 5: 100% Methanol Extraction

2 g of powdered tissue was extracted (3 h) with 10 ml methanol (×3). Further extraction was carried out with organic solvents including methanol/water (50:50). The methanol extracts were pooled, and the solvent was evaporated in vacuo at 35˚C. The residue was suspended in acetonitrile (50 mL) and extracted three times with hexane (20 mL) to remove residual lipid components. The hexane extracts were discarded, and the acetonitrile solution was dried over anhydrous magnesium sulfate. The acetonitrile was removed in vacuum (35˚C), and the dried residue was suspended in methanol, prior to separation of the components by HPLC [20].

2.3.6. Method 6: 53% Acetonitrile Extraction

0.2 g of powdered leaves and 2 mL of solvent 53% acetonitrile/H_2O were mixed and shaken at 250 rpm for 2 h at ambient temperature and then centrifuged at 7000 × g for 5 min. The supernatant was used for phenolic compound analysis [21].

2.4. HPLC Analysis

The analytical HPLC system employed comprised an Agilent 1100 Series high-performance liquid chromatograph equipped with an Agilent 1100 Series diode-array detector (Agilent Technologies, Germany). The HPLC pumps, column oven, and diode-array system were monitored and controlled using the HP Chem Station computer program (Agilent). The analyses were conducted using C18 chromatographic column Eclipse XDB (ZORBAX, USA), sizes 4.6 × 250 mm, 5 μm. Vitexin was separated using the method of [22] with some modification. The gradient mobile phase contained 0.5% ortho-phosphoric acid in water (A solvent) and acetonitrile (solvent B), and the UV detector was set at 350, 280 and 354 nm. The elution was facilitated by program as follows: 0 - 30 min 18% B, 30 - 60 min 67% B, 60 - 65 min 18% B, 65 - 70 min 18% B. Chromatography was performed at 25˚C at a flow rate of 0.8 ml/minute. The presence of vitexin in the samples was verified by comparison of the retention time and UV spectral peaks of the sample with those of an authentic sample (**Figure 2** and **Figure 3**).

Five concentrations of vitexin were subjected to linear regression analysis to calculate the calibration equation. Calibration for vitexin ranged from 0 to 100 ppm, with a regression line equation: $Y = 43.628X$; n = 4; r = 0.9949 (**Figure 4**).

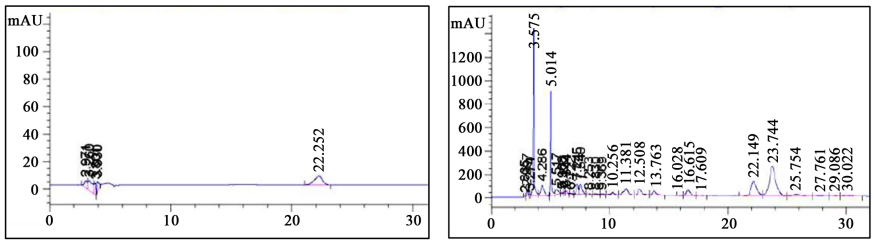

Figure 2. Chromatogram of vitexin standard (left) and a chromatogram of showing separation of vitexin in prosopis sample (right).

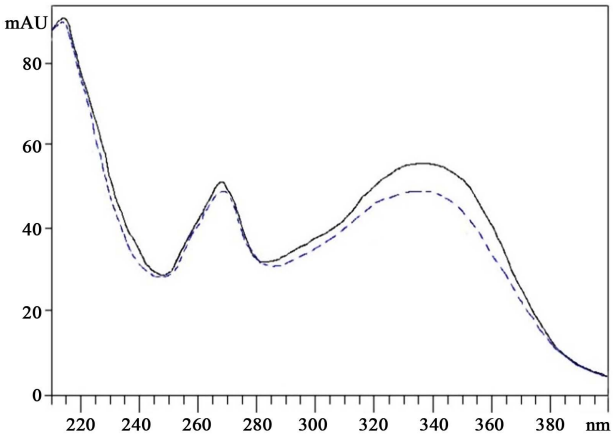

Figure 3. Vitexin spectrum in standard (Solid line) and sample (dashed line).

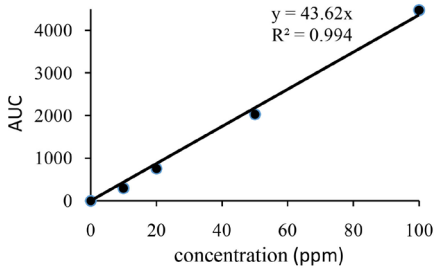

Figure 4. Vitexin calibration curve using HPLC. Each value is the mean of three replications.

2.5. Statistical Treatment

All data were analyzed using MSTAT-C software. Duncan's multiple range tests were used to measure statistical differences between treatment methods and controls. P = 0.05 or =0.01 was considered significantly different.

3. Results and Discussion

Vitexin peaks were identified in the extracts by comparing the retention time with that obtained from the standard solution (**Figure 2**), by spiking the standard solution into the sample solution and by a comparison of its UV spectra with that of the standard (**Figure 3**). The retention time of vitexin peak in the extract of six methods varied from 22.05 to 22.40, compared to 22.25 min in case of vitexin standard. Our study showed the absorption maxima of vitexin obtained from *Prosopis* leaves ranged as 350 > 254 > 280 respectively. Also, UV spectra of vitexin between 210 and 400 nm is shown in **Figure 3**.

Sample preparation is the most critical point for a successful and reliable analysis. Many sample preparation methods have been developed to extract phenolic compounds from this wide range of samples [23]. We compared the effectiveness of the different reported methods for the extraction of flavonoid vitexin from *Prosopis* leaves [16]-[21]. As can be seen in **Figure 5**, the best results were obtained by using method 3, 0.554 $mg \cdot g^{-1}$, followed by the method 2 and 5, 0.498 and 0.463 $mg \cdot g^{-1}$, respectively.

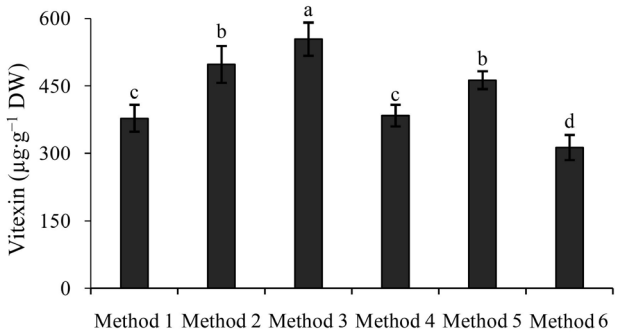

Figure 5. Comparison of vitexin values extracted by different methods. DW, dried weight. Data are means ± SD. Values within rows followed by the same letter(s) are not significantly different at ($P < 0.05$) level. method 1: 85% ethanol extraction, method 2: 70% methanol extraction, method 3: 40% acidic methanol extraction, method 4: 94% ethanol extraction, method 5: 100% methanol extraction, method 6: 53% acetonitrile extraction.

In all the extraction methods acetonitrile/H_2O (method 6) had the lowest extractability, resulting in a smaller amount of marker flavonoid, 0.313 $mg \cdot g^{-1}$. Numerous reports indicate that the amount of phenolic compounds varies with the conditions during extraction. For example, 80% methanol and acetonitrile/HCl solution were used to extract isoflavones from soybeans and soyfood respectively [24] [25]. Other Studies showed the optimized extraction solvent for phenolic compounds is to be 70% ethanol or 58% acetonitrile [26] [27].

The present study divides different extraction methods into three solvent systems: methanol, ethanol and acetonitrile. Methanol system extracted higher amounts of vitexin than ethanol and then acetonitril systems. Moreover, the ratio of water in the solvent system played an important role in the amount of the phenolic compounds extracted [25]. [28] reported that the highest extraction of phenolic compounds from plant material with methanol-water was achieved during the first stage of extraction. As shown in **Figure 5**, for vitexin extraction, methanol concentration of 40% (method 3), is better than 70% and 100% (methods 2, 5). It appears that alcohol mixed with up to 50% water gives the best blend for vitexin extraction. As shown in **Figure 5**, no significant differences were found when using extraction methods 1 and 4 while their dried extracts (prepared for injection) was dissolved in different solvent systems (methanol-acetonitrile-water (40:15:45, v/v/v) and waret-H_2O (50:50) respectively). It may show the fact that solvent systems used to the first stage of extraction are relatively important than ones in consecutive procedures [28]. Moreover, it is important to take account the structural diversity of polyphenols because these compounds may have multiple hydroxyl groups that can be conjugated to sugars, acids or alkyl groups [29]. Hydrolysis of flavonoids, frequently used to remove the sugar moieties from glycosides, may be acidic, basic, or enzymatic [23]. Numerous papers have been cited that the glycosides of flavones and flavonols were hydrolysed by refluxing in 1 - 2 M HCl in 50% MeOH-H_2O [23]. Probably, the other effective factor in the selection of method 3 as the best one

can be acidic pH of the extracting solvents or in other words acidic hydrolysis of vitexin by acetic acid.

4. Conclusion

Taken as a whole, our results show that the extraction method [methanol/H_2O (40%) containing acetic acid] combined with determination by HPLC is an accurate method to extract more amount of vitexin in plant material such as 21-day old *Prosopis* seedlings. Additionally, the amount of vitexin extracted using this method in this work (0.554 mg/g DW) suggests that *Prosopis farcta* constitutes a promising biotechnological system for producing this anti-tumor agent. When comparing our vitexin production with others previously reported [30] [31], we can infer that *Prosopis* cultures should be optimized to be competitive at an industrial level.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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