

Phytochemical assessments of *Astragalus hamosus* pods (*Iklil-ul-Malik*)

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Abstract

Authentication and quality control of herbs in herbal markets is an important challenge for practitioners and food and drug organizations. Rapid and simple pharmacognostic evaluations are beneficial to overcome this problem. On the other hand, there is a mist about the scientific names of some of these herbs even in scientific literatures. *Iklil-ul-Malik* or *Astragalus hamosus* L. (Papilionaceae) is one of these medicinal plants. Pharmacognostic (macroscopic, microscopic and phytochemical) properties of *Iklil-ul-Malik* pods were investigated. Different ash and extractive values were determined. The HPTLC finger prints of the herbs' fractions were prepared, using different reagents and mobile phases. The fatty acids, steroids, alkene and terpenoids of the pods were investigated by GC-MS analysis. Soluble fatty acid and soluble sugar content were determined by spectroscopic methods. Total ash, acid insoluble and water soluble ash were determined to be 75.00±11.83, 8.33±2.89 and 40.00±5.00 mg/g respectively. The extractive values for n-hexane, dichloromethane and ethanol fractions were 0.77%, 0.16% and 0.49% (w/w). Free amino acid and soluble sugar contents were respectively 3.33±0.001 and 8.83±0.004 (w/w %). Polyphenols, triterpenes, glycosides and glycolipids were detected in different fractions. The principal fatty acids were linoleic acid (48.64%), linolenic acids (25.35%), lauric acid (8.12%) and stearic acid (6.38%). By presenting the pharmacognostic values and HPTLC fingerprints in this manuscript, the herb can be authenticated. **Keywords:** *Astragalus hamosus*, *Iklil-ul-Malik*, *Nakhonak*, Milk vetch, Phytochemicals.

1. Introduction

There are increasing demands for new and effective natural medicaments. In this regard, many medicinal herbs from different origins can be selected for clinical and pharmacological investigations. One of the medicinal plants traditionally used for various medical purposes is *Astragalus hamosus* L. from the family Papilionaceae (1).

A. hamosus pod or seedpod is known as *Iklil-ul-Malik*, Milk vetch, European milkvetch, or *Nakhonak* in medical and pharmaceutical manuscripts of Traditional Persian medicine (TPM) (2, 3). Belonging to one of the largest angiosperm

genera (4), *A. hamosus* is an annual herbaceous plant growing up to 30 cm of height. The plant is a hard drought-tolerant legume with high seed productivity (5). The leaves have many leaflets and the flowers are small, white or yellow, grouped at the end of a stalk. The fruits perfectly characterize this species as they are strongly curved (3). In Iran, this plant grows in Persian Gulf islands, Kashan, Fars and Bushehr provinces. Flowers bloom in spring and pods are usually collected in summer.

A. hamosus has been applied for various diseases and complications in TPM and Ayurveda. Topical application of the extract of aerial parts has been reported to be effective in the control and treatment of headache, vertigo, strokes and dementia. Oral administration of *A. hamosus* decoc-

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tion has also been recommended for gastrointestinal upset, inflammations, respiratory discomfort and urinary complications (2).

The information on phytochemical and pharmacological properties of this plant is scant. Concerning pharmacological properties of *A. hamosus* and its isolated metabolites, limited antioxidant, anti-inflammatory, antiproliferative, antimicrobial, and hepatoprotective activities have been evaluated in animal models (6-10).

Despite the famousness of this herb, pharmacognostic and pharmacologic assessments have been confined to the aforementioned information. In this regard, current work is aimed to assess some pharmacognostic properties of *Iklil-ul-Malik*.

2. Materials and methods

A. homosus pods were purchased from a herbal market in Shiraz (Iran) and were authenticated by S. Khademian, botanist of Department of Phytopharmaceuticals (Traditional Pharmacy), School of Pharmacy, Shiraz University of Medical Sciences (Voucher number: PM-190). The plant material was powdered and kept in dark closed container for further steps.

2.1. Physicochemical analysis

Standard methods of analysis described by WHO guidelines were used to determine the total, water soluble and acid insoluble ash indices (11). For the detection of extractive values and HPTLC analysis, 100 g of pod powder was extracted with n-hexane applying Soxhlet apparatus (6 hrs.). The residuum was dried and macerated with dichloromethane and then by ethanol (two days for each). Each fraction was concentrated with a rotary evaporator and dried at 40 °C in a speed vacuum apparatus to obtain solvent free fractions. The oily upper layer of the solvent free n-hexane fraction was considered as the fixed oil of the pods and its lower sediment considered as the n-hexane fraction. The obtained dried fractions were weighed out and kept in teflon capped tubes at -20 °C.

2.2. High performance thin layer chromatography

HPTLC (CAMAG) was used to screen the primary and secondary metabolites. A portion (10 µl) of n-hexane, dichloromethane and

ethanol fractions (5 mg/ml) and 2 µl of the oil were applied on silica gel plates 60F254 (10-20 cm, Merck). The plates were run in 3 mobile phases of different polarities as mentioned below:

1) Toluene- acetone (80:20, M1); 2) Toluene- chloroform- acetone (40:25:35, M2); 3) n-butanol- glacial acetic acid- water (50:10:40, M3); 4) chloroform- acetone- water (98:1.99:0.01, M4) (12).

Chromatographic spots were visualized with and without reagents under ultraviolet (UV254, UV365 nm) or visible light.

Reagent list includes: Phosphomolybdic acid reagent, Dragendorff, 5% potassium hydroxide, Orcinol, NP (Ethanolamine Diphenyl borate)/PEG, Liebermann Burchard, 3% FeCl₃ (vis.), vanillin-sulfuric acid and anisaldehyde-sulfuric acid (13, 14). All chemicals and solvents were of analytical grade purchased from Merck (Germany) or Sigma Aldrich (United States).

2.3. Free amino acid and soluble sugar content

One gram of powdered pods was dispersed in 5 mL of 80% ethanol (v/v) and the mixture was boiled and subsequently, centrifuged at 2000 rpm each for 10 min. The supernatant was collected and the pellet was re-extracted in 5 mL of hot 80% ethanol. Supernatants were combined, and the total soluble sugar was quantified by the phenol sulfuric acid method (15), using glucose as the standard. Free amino acids were determined by Bradford assay using leucine as the standard.

2.4. Fatty acid analysis of the pods oil

In order to determine the fatty acid contents of fixed oil obtained from pods, methyl ester derivatives of fatty acids were prepared based on the procedure explained by AOAC (16). Toluene (1 ml) and sulfuric acid in methanol (1%, 2 ml) were added to 0.2 g oil or standard fatty acids, in a teflon capped test tube. Hexadecanoic acid was considered as an internal standard. The reaction media was incubated at 50 °C overnight. After the addition of 5% NaCl solution, the resulted fatty acid methyl esters were extracted with n-hexane (2×5ml). The resulted supernatant was washed with sodium bicarbonate 2% solution and dried by

anhydrous Na₂SO₄. After centrifugation (10 min, 3000 rpm) and discarding the sediments, the solvent was removed under a stream of nitrogen and kept at -20 °C. Prior to gas chromatography-mass spectrometry (GC-MS) analysis, a skirt of n-hexane (500µl) was added to dissolve the samples (17).

2.5. Isolation and TMS derivatization of sterols

After the addition of free cholesterol and cholesteryl heptadecanoate (0.2 mg) to the oil as internal standards, 300 µl of the oil was applied on a packed Silica gel column and fractionated sequentially with hexane- diethyl ether (200:1, v/v, fraction A), hexane- diethyl ether (96:4, v/v, fraction B) and diethyl ether- acetic acid (100:0.2, v/v, fraction C). Wax esters and sterol esters were eluted in fraction A, triacylglycerols in fraction B, and free sterols in fraction C. The fractions were dried under a stream of nitrogen. Saponification of sterol esters was performed using KOH (33%, 1 ml) and ethanol (96%, 4 ml), followed by incubation for 1 in 80 °C. After washing the mixture with distilled water, the cooled unsaponifiable parts were extracted with n-hexane (3ml×3). TMS-derivatives of the sterols were prepared by incubating the dried sterol containing fractions with 200 of N, O-bis (trimethylsilyl) trifluoroacetamide at 60 °C for 2 (18).

2.6. GC-MS analysis of fatty acids and sterols

Derivatized fatty acid and sterols were determined by GC-MS analysis which was carried out using a Hewlett-Packard 6890 apparatus. The gas chromatograph was equipped with an HP-5MS capillary column (phenyl methyl siloxane, 25 m; 0.25 mm i.d.). The injector temperature was 250 °C and positive ion electron impact mass spectra were recorded at ionization energy of 70 eV. Helium (1 ml/min) was used as a carrier gas. For fatty acid analysis, oven ramping program was started from 160 °C (2 min), then increased to 230 °C at a rate of 8 °C/min. The oven temperature was held at 230°C for 20 min.

For TMS derivatives of sterols, oven temperature was started at 230 °C (1 min), then increased to 275 °C at 1 °C/min. Finally, the oven was held at 275 °C for 30 min. The injector temperature was 275 °C (18).

Fatty acids and sterols were identified by comparing the mass spectra and retention times obtained, with those of reference compounds, or with mass spectra in the literature. For those compounds for which neither standard compounds nor reference spectra were available, chemical structures were postulated according to the general patterns of mass spectrometric fragmentation of dif-



Figure 1. Dried ripped seedpods of *A. hamosus* (*Iklil-ul-Malik* or *Nakhonak*).

ferent sterols.

3. Results

3.1. Physicochemical properties of the pods

Iklil-ul-Malik Pods or seedpods are indehiscent fleshy round or oval legumes similar to some fishhooks with a 1-2 cm diameter. Each pod contains 15–20 small and rectangular shaped seeds. The pods have yellow to yellowish brown color (Fig 1). In the literature, *Iklil-ul-Malik* or *Nakhonak* has been described as pods of *Melilotus officinalis* (Lam.) L. which is not correct according to the morphological differences between the two plants and their pods. In contrast to *A. hamosus*, the plant, *M. officinalis* grows up to 1.5 m and needs high humidity. *M. officinalis* has yellow flowers and small pods with a length about 5 mm (3).

The total ash for *A. hamosus* pods was determined to be 75.00 ± 11.83 mg/g. Total ash represents physiological ash derived from the pods' tissues and non-physiological ash derived from environmental contamination such as soil or sand. Acid insoluble and water soluble ash values were determined to be 8.33 ± 2.89 and 40.00 ± 5.00 mg/g, respectively. Acid soluble ash value reflects silica content of the pods.

To evaluate the quality of herbs, the extractive values representing different metabolite content were determined as 0.77%, 0.16% and

0.49% (w/w) for n-hexane, dichloromethane and ethanol fractions of pods, respectively.

Free amino acid content was 3.33 ± 0.001 (w/w %), while soluble sugar content of the pods was 8.83 ± 0.004 (w/w %).

The classes of *A. hamosus* pods' primary and secondary metabolites, which were screened by HPTLC using UV lamps and variety of chemical reagents, are shown in Fig 2.

Polyphenols were detected in dichloromethane and ethanol fractions, visualized by FeCl₃ reagent (data not shown). Several triterpenes including saponins were detected by Liebermann-Burchard reagents in hexane, dichloromethane and ethanol fractions. Some glycosides and glycolipids were detected in ethanol fractions visualized by Orcinol. We could not detect any alkaloid in the pods' fraction. The authors of the present article could not find any HPTLC finger prints for the pods of this plant to be compared with the results of other studies, but in previous investigations on the leaves of this plant, some flavonoids as hyperoside, isoquercitrin, astragalinalin and a flavonol glycoside (rhamnocitrin 4-β-D-galactopyranoside) has been detected. Also, some saponins have been isolated and identified from *A. hamosus* aerial parts (6, 19).

The fixed oil of the pods, which had a rich green color was chromatographed in M4 and sprayed with Liebermann Burchard, indicated the

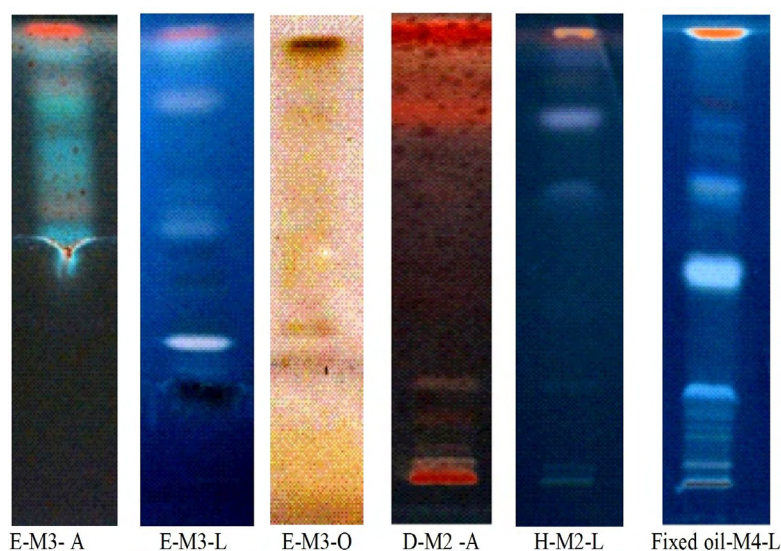


Figure 2. HPTLC fingerprints of ethanol (E), dichloromethane (D), hexane (H) and fixed oil of *A. hamosus* pods. The TLCs were run in M2, M3 or M4 mobile phases and were visualized with Liebermann Burchard (L) or anisaldehyde-sulfuric acid (A) under UV365 or Orcinol (O) in visible light.

presence of different fatty acids and steroidal triterpenes (Fig. 1C). Different polyphenolic compounds including tannins and flavonoids as well as glycosides and an alkaloid spot were identified in ethanol fraction (HPTLCs are not shown here).

3.2. Phytosterols and fatty acid profile of the pods oil

According to Table 1, principal fatty acids of the pods' oil were linoleic acid (C18: 2, 48.64%), linolenic acids (C18: 3, 25.35%), lauric acid (C12:0, 8.12%) and stearic acid (C18:0, 6.38%). Other fatty acids (1-2%) were tricosanoic acid (C23:0), 7-10-13-hexadecatrienoic acid or roughanic acid (C16:3), 9-hexadecenoic acid (C16: 1) and 13-docosenoic acid (C22:0). Cyclopropaneoctanoic acid, 2-hexyl which was detected in fixed oil (1.56%), is a cyclopropane fatty acid. This kind of Fatty acid has been found in a variety of plants (20), bacteria (21), a number of protozoa, sponges (22), Myriapoda and even in human serum and adipose tissues (23). Fenozan acid (3-(3, 5-di-tert-butyl-4-hydroxyphenyl)-propionic acid) was also detected in the pods' fixed oil with a con-

centration of 0.099%. Fenozan acid is a sterically hindered phenol which can act as a radical scavenger antioxidant (24). It has been also reported to have antiarrhythmic and vasodilatory effects (25).

This is the first report on the fixed oil constituents of *A. hamosus* pods and thus, it is not possible to compare the results with any previous data. However, in an assessment, the volatile constituents of *A. hamosus* aerial parts during fructification (leaf and pod) had terpenes, acids and hydrocarbons such as phytol, hexadecanoic acid and heptacosane (26). These findings are similar and comparable with the results of the present study, except for the detection of phytol, however another derivative of phytol, neophytadiene, was detected in the oil with the concentration of 0.419%. As a diterpenoid compound, neophytadiene has been extracted from tobacco leaves, tobacco stems and wastes, or is generated by the dehydration of phytol (27, 28).

About 1.437% of the oil consists of non-fatty acids such as alkanes, alkenes or terpenoids (Table 2). In this study, we could not detect any phytosterols by GC-MS analysis.

The total unsaturation of this oil was

Table 1. Fatty acid composition of *A. hamosus* pods.

Retention time (min)	Fatty acid name	Formula	Percentage (mean±SD)
3.502	Dodecanoic acid	C12:0	8.117±0.452
5.606	Tetradecanoic acid	C14:0	0.380±0.026
6.777	Pentadecanoic acid	C15:0	0.055±0.001
7.732	9-Hexadecenoic acid	C16:1	1.311±0.243
8.035	Hexadecanoic acid	C16:0	0.312±0.002
8.257	Fenozan acid	C17H26O3	0.099±0.002
8.795	Cyclopropaneoctanoic acid,2-hexyl	C17:0	1.557±0.132
9.143	Heptadecanoic acid	C17:0	0.051±0.003
10.109	9,12-Octadecadienoic acid	C18:2	48.639±3.839
10.184	9,12,15-Octadecatrienoic acid	C18:3	25.347±3.216
10.229	7-10-13-Hexadecatrienoic acid	C16:3	1.437±0.229
10.338	Octadecanoic acid	C18:0	6.381±0.128
11.012	9,15-Octadecadienoic acid	C18:2	0.440±0.035
12.435	11-Eicosenoic acid	C20:1	0.225±0.002
12.818	Eicosanoic acid	C20:0	0.160±0.004
14.573	Heneicosanoic acid	C21:0	0.742±0.415
16.116	13-Docosenoic acid	C22:1	1.006±0.055
16.865	Docosanoic acid	C22:0	0.121±0.002
19.86	Tricosanoic acid	C23:0	1.548±0.456

Table 2. Non fatty acid constituents of the fixed oil obtained from *A. hamosus* pods.

Retention time (min)	Fatty acid name	Formula	Percentage (mean±SD)
4.222	Hexadecane	C16H34	0.062±0.004
5.771	5-Phenyldodecane	C18H30	0.168±0.101
6.377	1-Octadecene	C18H36	0.047±0.025
6.451	Octadecane	C18H36	0.050±0.001
6.926	Neophytadiene	C20H38	0.419±0.086
13.961	Tetracosane	C24H50	1.325±0.532

determined to be about 79.01%, while the highest portion belongs to polyunsaturated fatty acids (PUFAs) such as linoleic acid and linolenic acid. PUFAs are essential fatty acids and play an important role in many biological functions. PUFAs have been reported to have cardiovascular protective, neuroprotective, anti-migraine and anti-rheumatoid properties (29, 30). It was also revealed that quality of life in Alzheimer's patients improves upon oral ingestion of essential fatty acids, (31). On the other side, in an open-label uncontrolled study, PUFAs were administered to 168 patients for a period of 6 months. According to the mentioned study, gamma-linolenic and alpha-Linolenic acids might possess prophylactic effects on migraine headaches (31). Also, they have been reported to be beneficial in the management of recurrent migraines in adolescents (32). PUFAs have also been reported to have a role in controlling sexual hormonal balances which may be effective in menopausal disorders (32).

Although there is not enough scientific evidence for the different therapeutic uses of *Iklil-ul-Malik* in traditional medicine, it is hypothetically possible that the presence of PUFAs in the pods might play a role in the efficacy of its traditional applications such as treatment of headache,

dementia or sexual hormonal diseases, in chronic administrations. In oral ingestion and even topical applications of *Iklil-ul-Malik* poly herbal oil formulations or pastes there is a high chance for PUFAs to be absorbed. However more studies are required to prove this hypothesis.

4. Conclusion

Rapid and simple pharmacognostic evaluations provided in this study can help traditional herbalists and food and drug organizations to overcome the problems regarding authentication of *Iklil-ul-Malik* (the pods of *Astragalus hamosus*). Surprisingly the pods fractions did not show significant antioxidant properties. The seedpods oil of this herb is rich in PUFAs. This may hypothetically explain some of its therapeutic applications in traditional medicine.

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Conflict of Interest

None declared.

5. References

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Azadeh Hamed *et al.*

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