Original Research Article

Assessment of Authenticity of Market Samples of *Hypericum* Using Phytochemical Fingerprinting Tools

ABSTRACT

Aims: *Hypericum perforatum* L., known as "Hofarighun" is a widely used herbal drug in Traditional Persian Medicine (TPM). Detection of non-relevant plants, instead of this species, in the herbal market encourages the need for the establishment of it's chemical authentication and standardization, through implying rapid and efficient phytochemical techniques.
Study design: Twelve *Hypericum* samples were acquired from traditional medicine markets of different regions of Iran (Tehran, Sanandaj, Mashhad, Kerman, Bandar Abbas, Ahvaz, Yazd, Babol, Yasuj, Shiraz (Chehel Giah), Shiraz (Kazerun Gate), and Shiraz (Adloo Zerehi), based on microscopic characterization. Positive control was taken in the form of cultivated specimen of *H. perforatum*.
Place and Duration of Study: Study was performed during in Medicinal plants processing Research Center, SUMS, Shiraz in the months between February to December 2021.
Methodology: Essential oil samples were injected into a gas chromatograph (GC) and compounds were identified as per the spectra obtained. Total phenol, flavonoid and HPTLC analysis of samples were also done.
Results: α-pinene was found in highest proportion in majority of samples i.e. 35.55-63.69%. However other compounds such as 1-dodecanol (10.82%), caryophyllene (15.87%) and β-cubebene (15.14%) were also analyzed in samples and the cultivated sample respectively. Total phenol and flavonoid content among the *Hypericum* extracts were found to be between 50.31±3.22 to 262.76±8.12 mg Gallic Acid Equivalent (GAE)/g of Ext. and 13.47±1.68 to 79.26±5.78 mg Quercetin Equivalent (QE)/g of Ext., respectively.
Conclusion: The noticeable findings of present study can be used as a framework for authentication of *Hypericum perforatum* samples.The methods used were found to be feasible and efficient in detection of adultrations and may contribute to minimize the safety and efficacy concerns over the samples available in the traditional herbal pharmacies.

Keywords: Hypericum perforatum; essential oil; phenol; flavonoid; HPTLC; antioxidant.

1. INTRODUCTION

Physiological disorders including depressive disorders may serve as one of the leading cause of disability throughout the world, involving amelioration through central neurotransmitters reuptake inhibitors viz. non-selective serotonin and noradrenaline reuptake inhibitors, monoamine oxidase enzyme (MAO) inhibitors and GABA-mimetic agents [1]. One of the most popular medicinal plants used in Iranian traditional and folk medicine for the treatment of these diseases is "Hofarighun" which

has been well documented in many reference sources of medicine and pharmacy [2]. *Hypericum perfotatum* L., known as St. John's Wort, from the family Hypericaceae has been the subject of numerous scientific and clinical research studies. The medicinal parts of the plant are flowers and twigs [3]. The extracts, products and chemical components of this plant have shown anti-epileptic, anti-schizophrenic, anti-migraine, analgesic, antidiabetic, antimicrobial, wound healing and antioxidant effects in various clinical studies [4,5]. Additionally, new research have interestingly revealed its encouraging effects in the treatment of nicotine and alcohol addiction [6]. Phytochemical screening of *Hypericum* species have revealed the presence of phytocompounds such as phenolics and their aliphatic derivatives, naphthodiantrons, flavonoids, xanthones, pyrones and terpenes [7-9]. The essential oil of this plant is commonly used as a preservative in food and health products [10].
Hofarighun is widely administered by traditional and folk healers and is being supplied in the Iranian medicinal plants market [11]. But so far little research has been done on the authenticity of the species of the genus, *Hypericum* in the market that are sometimes mistakenly prescribed by local and traditional vendors and therapists in place of the main genus or species. Therefore, authentication of the samples available in the medicinal herbal market was found to be an absolutely logical need. In the present study, 12 *Hypericum* samples were collected from the herbal medicine market, regardless of the place of planting or the time of collection of the samples. During this study, the morphological characteristics, botanical features and phytochemical contents of various *Hypericum* samples were examined in order to provide a comparative model, relevant to compounds profiles and the overall differences between the samples supplied in the market. In order to compare the chemotaxonomic and morphological characteristics of these species, a sample of *H. perforatum* was grown under the standard condition and used as a control in the present study.

2. MATERIAL AND METHODS

2.1 Chemicals and Reagents

Gallic acid, ferric chloride,methanol, potassium ferricyanide, ethanol, Folin-Ciocalteu reagent and TLC silica gel 60 F_{254} aluminum plates were obtained from Merck, Darmstadt, Germany. 1,1-diphenyl, 2-picryl hydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) and quercetin were purchased from Sigma Aldrich Chemical Co USA. All other chemicals and solvents used were of analytical grade from Merck.

2.2 Plant collection and identification

Various samples of Hofarighun from the pharmaceutical market have been collected from Tehran, Sanandaj, Mashhad, Kerman, Bandar Abbas, Ahvaz, Yazd, Babol, Yasuj, Shiraz (Chehel Giah), Shiraz (Kazerun Gate), and Shiraz (Adloo Zerehi). The collected samples, were deposited to the herbarium of department of pharmacognosy, school of pharmacy, Shiraz University of Medical Sciences and characterised by plant taxonomist and ultimately, each specimen was allocated a herbarium number. Moreover, a standard sample of *H. perforatum* was used in this study. All samples were ground using an electric mill. Details of samples including place of collection and herbarium voucher numbers are given in Table 1.

Table 1. *Hypericum* samples collected from different regions

Samples	Scientific name	Herbarium No.	Place of collection
S1	*Hypericum scabrum* L.	PM 1067	Ahvaz
S2	*Hypericum elongatum* L.	PM 1068	Bandar Abbas

S3	*Hypericum elongatum* L	PM 1069	Tehran
S4	*Hypericum perforatum* L.	PM 1070	Kerman
S5	*Hypericum scabrum* L.	PM 1071	Yazd
S6	*Hypericum helianthemoides* (Spach) Boiss.	PM 1072	Sanandaj
S7	*Hypericum scabrum* L.	PM 1073	Yasuj
S8	*Hypericum scabrum* L.	PM 1074	Babol
S9	*Hypericum scabrum* L.	PM 1075	Mashhad
S10	*Hypericum elongatum* L.	PM 1076	Shiraz (Chehel Giah)
S11	*Hypericum elongatum* L.	PM 1077	Shiraz (Kazerun Gate)
S12	*Hypericum perforatum* L.	PM 1078	Shiraz (Adloo Zerehi)
S13	*Hypericum perforatum* L.	PM 1079	Control

2.3 Taxonomic and Morphological Screening

The initial step in implementing fingerprint analysis of plants is the morphological analyses. This process has an important role in identifying the macroscopic and microscopic characteristics of plant species and their relevant pharmacobotanical properties. Different parts of the *Hypericum* samples, including the stem, leaf and flower were powdered by a Chinese mortar and the powders were separately passed through a 70-mesh sieve. Each plant sample was weighed (5 g) and transferred to a test tube and 5 ml solution of 60% hydrated chlorine was added to it and then heated on a flame and centrifuged after boiling. Then the top layer was discarded and 50 ml of distilled water was added to the residue and again centrifuged. Subsequently, the bottom layer was transferred to a petri dish, and a few drops of ethanolic solution of phloroglucinol-hydrochloric acid were added followed by addition of glycerin to prevent the samples from drying out and to increase their stabilities. The slides prepared from various parts of *Hypericum* samples were then digitally photographed using a Ceti Magnum-PH Trinocular Compound Microscope. The relevant specifications observed were recorded and examined thoroughly**.**

2.4 Extraction of Essential Oil

Essential oil was isolated according to a method given in the European Pharmacopoeia [12]. Each *Hypericum* Sample (50 g) was separately crushed in a grinder. The powdered plant material was transferred into a round-bottomed flask and 500 mL distilled water was then added. The mixture was further subjected to hydrodistillation for 4 h using a Clevenger type apparatus. The essential oil samples were separately collected and dried over anhydrous sodium sulphate and stored at 4 ºC until GC/MS analysis and antioxidant assay.

2.5 Screening the Essential Oil Composition

2.5.1 GC/MS Analysis

The analysis was performed on a gas chromatograph 7890A system coupled with a mass detector 5975 C, Agilent technologies, USA. HP-5MS capillary column (5% phenyl methyl siloxane, 30 m × 0.25 mm× 0.25 µm) was used [13]. Oven temperature was adjusted to rise from 60 to 280 ºC at a rate of 10 ºC/min and held at 280 ºC for 10 min. Helium was used as the carrier gas with a flow rate of 1 mL/min.The interface temperature was 280 ºC. A volume of 1 µL of the essential oil was injected in split mode (1:50) and mass spectra were acquired in EI mode (70 eV) in a mass range of 30–600 m/z.

2.5.1.1 Identification of volatile compounds and GC/MS fingerprints

Each sample was diluted 1:5 with dichloromethane before injection. The samples were dried over sodium sulfate prior to injection and 1 µL of diluted essential oil sample was injected into the gas chromatograph. Identification and quantification of essential oil components was performed by calculating Kovats Index (KI) for each constituent. Comparison of data were made, using the information given in Wiley nl 7 library, Adams [14], NIST [15] and Pherobase [16] mass spectral sources as well as the values reported in the literature. In order to confirm the structure of each oil component, inspection of mass spectral fragmentation pattern of each compound was also performed and the results were compared with the reported values.

2.6 Preparation of Ethanolic Extract

The ethanolic extracts were prepared by adding 25 g each of powdered *Hypericum* samples to 250 ml of %96 ethanol, in separate Erlenmeyer flasks. The flasks were capped and agitated in the dark at 25 °C for 3 hours on a magnetic stirrer (IKA, Germany). The extract were concentrated under reduced pressure at 40 ºC on a rotary evaporator and further concentrated in a speed vacuum and finally freeze-dried in a vacuum freeze dryer (Christ Alpha 1- 4 LD, Martin Christ, Germany) and stored at 2 ºC pending analysis.

2.6.1 Determination of Total Phenolic Content of Extracts

Measurement of total phenolic content was performed according to the Folin-Ciocalteu method. In this assay, gallic acid was used as a standard. To prepare a calibration curve, 2 mg of gallic acid was dissolved in 10 ml of methanol, to get 200 µg/mL stock solution. From this solution, a serial dilutions were made to provide solutions containing 200, 160, 80, 40 and 20 µg/mL of gallic acid. To 500 µL of each concentration, 5 mL of Folin-Ciocalteu reagent and 4 mL of sodium carbonate solution (105.9 g/L) were added respectively. The samples were vortexed and the absorbance were recorded after 15 minutes against the blank at 765 nm using T90+ UV/VIS Spectrophotometer (PG Instrument Ltd.). Methanol was used as the blank. A calibration plot was made of absorbance versus concentration and the equation line so obtained, was used to calculate the concentration of unknown samples. Assessment of total phenolic content of extracts was performed using a methanolic solution of 0.5 g/L of ethanol extract obtained from each *Hypericum* sample [17]. All results are presented as mean±standard deviation and the phenolic content was expressed as mg gallic acid equivalent per gram of dry extract (mg GAE/g).

2.6.2. Determination of Total Flavonoid Content of Extracts

Determination of total flavonoid content of various *Hypericum* extracts were conducted using the aluminum chloride spectrophotometric method [18]. A stock solution of quercetin as a standard, consisting of 0.5 mg/mL of quercetin in methanol was prepared and then a calibration curve was generated with solutions containing 100, 80, 60, 40, 20 µg/mL of quercetin. To 3 ml of each concentration, 3 ml of 2% aluminum chloride ($AlCl_3.6H_2O$) solution was added which turns the solution yellow. To make 2% aluminum chloride solution, 5 g of aluminum chloride was dissolved in 250 ml of methanol. To measure the total flavonoid content of the samples, solutions of *Hypericum* extracts were prepared at a concentration of 0.5 mg/mL. To 3 ml of each concentration, 3 ml of 2% aluminum chloride solution was added. The mixture were vortexed and allowed to stand for 15 minutes, and the absorbance were read at 415 nm using a UV Spectrophotometer. All the tests were conducted in triplicate and their mean values were reported. Total flavonoid content of the extracts were calculated using regression equation derived from the quercetin calibration curve. Methanol was used as a blank.

2.7. High Performance Thin-Layer Chromatography Fingerprints

High-Performance Thin Layer Chromatography (HPTLC) includes four main components called Automatic TLC Sampler 4 (ATS4), TLC tank or Automatic Developing Chamber (ADC2), and Visualizer 2, which display spots in UV wavelengths (254 and 366 nm), and TLC scanner. In order to perform thin-layer chromatography, a CAMAG HPTLC system *(*Camag, Muttenz, Switzerland*)* was used in the present study [19]. Solutions of 3 mg/mL in methanol of each *Hypericum* extract was prepared, and 25 µl of each sample was loaded onto a silica gel 60 F_{254}, aluminum plate (10×20 cm). The loaded TLC plate was developed in ethyl acetate-acetic acid-formic acid-water (50:11:11:2) as the elutting solvent. The developed TLC plate was dried and then sprayed with anisaldehyde-sulfuric acid reagent and heated at 110 ^{o}C for 10 min until the spots were visualized. Anisaldehyde-sulfuric acid reagent was freshly prepared by dissolving 0.5 ml anisaldehyde in 10 ml of pure acetic acid. The solution was diluted to a volume of 85 mL by addition of methanol. Finally, 5 ml concentrated sulfuric acid was added and the resulting solution was mixed thoroughly.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Taxonomic and Morphological Characteristics

Various vegetative and reproductive parts of each *Hypericum* sample, including flowers, leaves and stems were prepared and powdered separately. The images from each plant part were carefully obtained and their microscopic features were thoroughly examined. Major variations in microscopic characteristics among various samples were observed, particularly in fruit endocarp tissues, stem and petal textures, exocarp tissue and pollen grains. Assessment of the secretory structures of vegetative organs showed the presence of type A canals in stem tissues of all *Hypericum* species, whereas the type B secretory canals were observed in subepidermic tissues of *H. perforatum*, which are in agreement with the previous reports [20,21]. All examined species including *H. perforatum* declared 1–2 layered palisade parenchyma in leaf mesophyll, as indicated in the earlier studies [22].
The outstanding anatomical characteristics such as equifacial leaves, anomocytic stomata and 1–2 layered palisade parenchyma were observed in *H. perforatum* as reported earlier [23]. Whereas dorsiventral leaves, anisocytic stomata and 2–3 layers of palisade parenchyma were distinctive features in other studied *Hypericum* samples.

3.1.2 Essential Oil Components

The detailed results of GC/MS analysis of the essential oil samples and the types of compounds present in various *Hypericum* samples are given in Table 2. Moreover, the calculated KI values for each compound, derived from the GC spectrum, are presented. In order to identify the chemical composition of each *Hypericum* essential oil sample, the retention index and the mass spectral fragmentation pattern of each compound were analyzed simultaneously. Figure 1 shows the GC chromatogram of essential oil of the aerial parts of a cultivated sample of *H. perforatum*, used as a control in the present study.

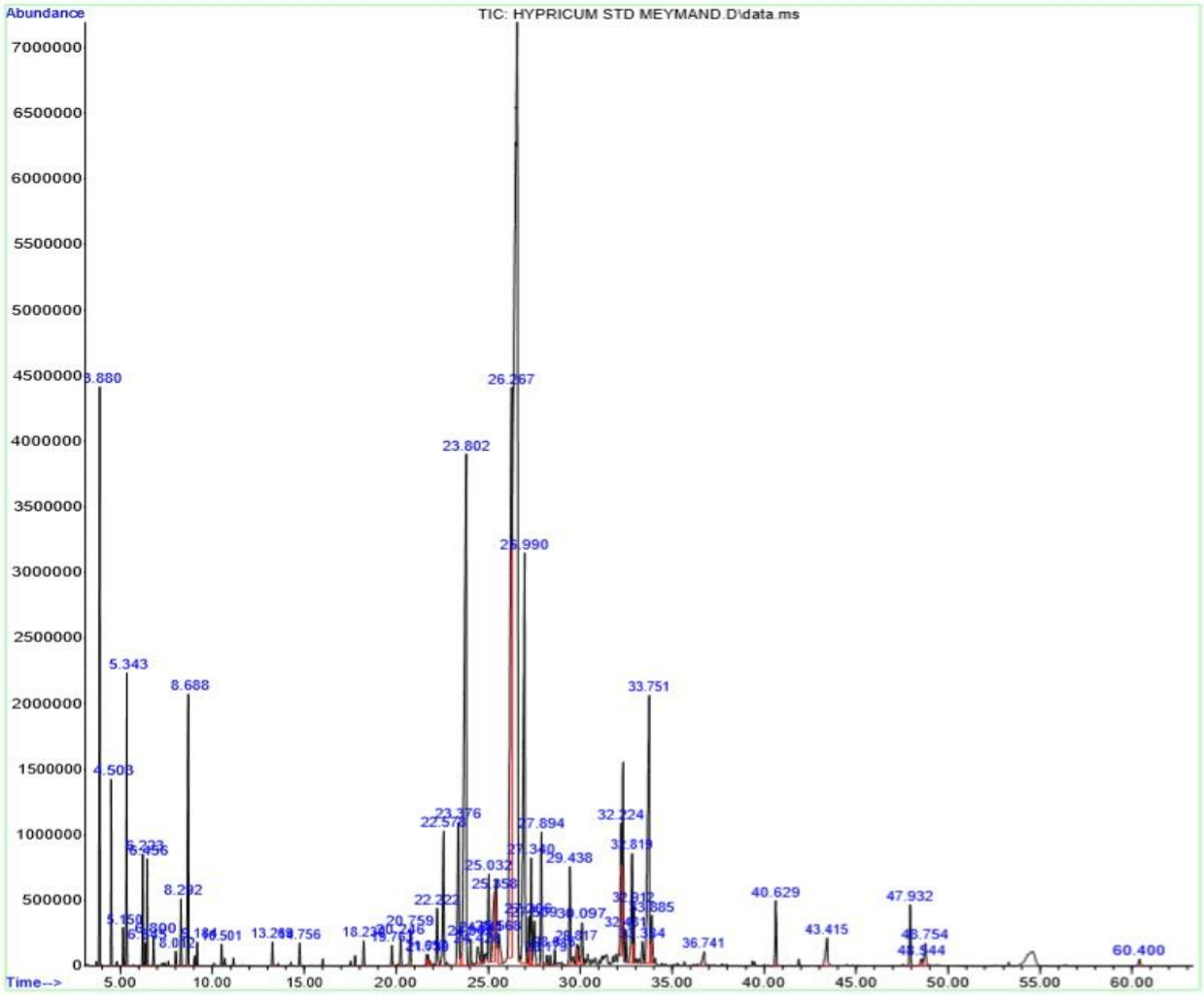

Figure 1. GC spectrum of the control sample (*Hypericum perforatum*)

Table 2. Essential oil composition of various *Hypericum* Samples

Component	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}	C	KI^{Cal}	KI^{Rep}	Ref.
Isononane	-	-	-	-	-	-	-	-	-	-	-	-	5.32	860	864	22,23
Nonane	0.63	0.58	0.88	0.20	0.83	1.58	0.75	1.44	0.71	1.16	1.06	1.77	1.33	899	900	22,23
α- Thujene	-	-	-	-	-	-	-	-	-	-	-	-	0.29	925	924	24
α- Pinene	52.08	54.48	50.10	0.86	35.55	49.39	51.77	50.70	38.81	63.69	63.68	37.48	2.37	935	938	22
Sabinene	-	-	-	-	-	-	-	-	-	-	-	-	0.19	960	961	25
Camphene	1.33	0.78	1.28	-	0.49	0.61	0.55	-	-	0.67	0.70	-	0.52	966	964	27
Verbenene	0.94	-	0.55	-	-	-	0.59	0.63	-	-	-	-	-	976	975	24
β- Pinene	1.55	2.94	1.71	0.17	1.83	2.75	2.59	3.39	1.96	3.55	3.63	1.83	0.92	989	982	25
β- Myrcene	-	0.67	-	-	0.5	0.78	-	-	-	0.94	0.95	0.5	0.26	995	995	23

Decane	0.95	0.99	1.36	1.84	3.08	0.94	1.02	1.10	1.25	-	0.85	-	0.48	999	1000	27
p- Cymene	2.61	1.69	1.69	3.67	3.89	1.2	5.18	-	-	1.2	1.41	3.89	-	1025	1027	23
Limonene	2.53	1.89	2.91	-	1.03	1.67	1.69	1.62	1.12	1.9	2.14	1.03	-	1034	1031	27
β- Ocimene	-	-	-	-	-	-	-	-	-	-	-	-	3.37	1048	1050	27
γ- Terpinene	0.6	-	-	-	1.3	0.71	2	1.42	2.33	-	-	1.3	-	1060	1060	28
Acetophenone	0.53	0.96	-	-	-	0.76	0.92	-	0.46	-	-	-	-	1068	1068	29
2-Methyldecane	-	-	-	-	-	-	-	-	-	-	-	-	0.24	1076	1076	24
Undecane	1.17	1.03	1.39	1.54	1.08	1.24	1.03	1.24	-	0.93	0.91	1.38	0.23	1099	1100	26
Linalool	0.61	-	0.59	-	-	-	-	-	2.28	-	-	-	-	1100	1101	29
α-Campholenal	3.27	-	2.77	-	0.67	1.65	3.18	3.6	1.49	2.23	-	0.67	-	1130	1130	30
trans-Pinocarveol	1.57	1.77	1.68	-	0.45	1.16	2.01	1.12	0.92	1.13	1.08	0.45	-	1141	1142	25
Camphor	1.07	0.84	-	0.28	0.41	-	-	1.7	0.44	-	-	0.41	-	1146	1148	24
Borneol	2.69	0.77	2.98	-	-	1.12	1.45	0.53	-	0.81	-	-	-	1170	1169	29
4-Terpineol	-	-	-	0.29	0.58	-	-	0.61	0.41	-	-	0.58	-	1179	1179	26
Cymen-8-ol	-	-	0.96	-	0.43	-	-	-	0.55	-	-	0.43	-	1186	1187	27
α-Terpineol	0.55	-	0.79	0.28	-	-	-	0.63	0.88	-	-	-	-	1192	1190	28
Dodecane	-	-	-	1.17	-	-	-	-	-	-	0.49	-	0.28	1200	1200	31
Verbenone	1.04	2.03	2	-	0.43	0.73	2.95	1.08	1.15	0.92	-	0.43	-	1214	1214	32
trans-Carveol	0.86	0.88	1.41	-	-	0.56	1.65	0.74	0.84	0.78	-	-	-	1220	1220	27
Linalyl acetate	0.77	-	-	1.0	-	-	-	-	4.15	-	-	-	-	1256	1257	30
Thymol	1.23	-	0.61	3.67	0.73	0.55	0.97	-	3.81	-	-	0.73	-	1293	1292	25
Carvacrol	1.37	-	0.94	2.9	0.64	0.57	0.86	-	2.17	-	1.18	0.64	-	1301	1299	22
Methyl caprate	-	-	-	-	-	-	-	-	-	-	-	-	0.27	1327	1328	32
Bicycloelemene	-	-	-	-	-	-	-	-	-	-	-	-	0.39	1335	1336	28
Eugenol	-	-	-	0.52	-	-	-	-	-	-	-	-	-	1350	1351	33
α-Copaene	0.6	1.14	0.77	1.03	1.17	1.0	0.96	0.57	0.73	0.78	0.87	1.17	0.17	1379	1376	23
β- Cubebene	-	-	-	-	-	-	-	-	-	-	-	-	15.14	1391	1391	30
β.Bourbonene	-	-	-	-	-	-	-	-	-	-	-	-	0.98	1405	1406	27
β- Elemene	-	-	-	-	-	-	-	-	-	-	-	-	2.45	1411	1410	25
(+)-β-Funebrene	-	-	-	-	-	-	-	-	-	-	-	-	3.05	1414	1415	25
Caryophyllene	0.91	0.95	0.96	1.89	1.36	2.43	0.75	0.52	1.58	2.72	2.87	1.36	15.87	1424	1423	25
Aromadendrene	0.76	1.36	0.6	-	0.8	1.05	0.74	0.59	0.6	0.91	0.79	0.8	-	1444	1443	24
1-Dodecanol	-	-	-	10.82	-	-	-	-	-	-	-	-	-	1471	1472	31
α-Muurolene	1.45	-	-	-	6.17	3.29	1.52	1.97	-	2.84	-	6.17	1.02	1484	1484	29
β-Selinene	0.83	1.5	-	5.43	5.07	0.79	-	-	-	-	-	5.07	0.82	1493	1489	32
Pentadecane	0.88	-	0.56	3.97	-	-	-	-	-	-	-	-	-	1498	1500	23
Zingiberene	-	2.54	-	-	-	-	-	-	-	-	-	-	0.23	1500	1501	24
Bicyclogermacrene	-	-	-	-	-	-	-	-	-	-	-	-	10.76	1505	1505	26
β- Farnesene	-	-	-	-	-	-	-	-	-	-	-	-	3.07	1510	1509	31
α -Amorphene	1.43	2.81	-	1.17	1.94	-	-	-	1.51	1.81	1.71	1.94	-	1518	1516	24
Nerolidol	-	-	-	-	-	-	-	-	-	-	-	-	1.53	1527	1527	33
δ -Cadinene	2.53	4.2	-	1.74	2.34	4.24	2.36	1.92	3.28	3.61	3.58	2.34	-	1531	1530	30
α- Calacorene	0.52	0.67	0.52	-	0.45	0.76	-	-	-	-	-	0.45	-	1549	1548	28
Spathulenol	1.05	2.36	0.69	2.23	1.33	2.08	0.86	0.94	1.17	1.55	1.57	1.33	-	1582	1585	27
Caryophyllene oxide	0.91	-	0.78	4.57	2.21	0.95	-	0.57	-	1.28	1.46	2.21	-	1591	1589	29
τ-Muurolol	0.55	-	0.55	-	1.0	-	-	-	-	-	-	1.0	-	1648	1648	24
α -Cadinol	-	-	-	-	-	0.88	-	-	-	-	-	-	2.29	1661	1660	27
α -Bisabolol	-	-	-	-	-	-	-	-	-	-	-	-	0.86	1703	1704	33
Myristic acid	-	-	-	-	-	-	-	-	-	-	-	-	0.42	1766	1765	25
Pentadecanol	-	-	-	-	-	-	-	-	-	-	-	-	7.36	1771	1772	30
Hexahydrofarnesyl acetone	0.66	-	-	2.19	0.66	-	-	-	-	-	-	0.75	-	1842	1843	26
Cyclohexadecane	-	-	-	-	-	-	-	-	-	-	-	-	1.05	1883	1883	24
Palmitic acid	-	-	-	-	-	0.8	-	-	-	-	-	-	0.86	1964	1964	22
Heptadecanol	-	-	-	2.65	-	-	-	-	-	-	-	0.81	0.18	1970	1969	33
Linoleic acid	-	-	-	-	-	-	-	-	-	-	-	-	0.52	2094	2095	30
Identification (%)	93.03	89.83	82.03	56.08	78.42	86.24	88.35	78.63	74.6	95.41	90.93	78.92	85.09	-	-	

S1: Ahvaz S2: Bandar Abbas S3: Tehran S4: Kerman S5: Yazd S6: Sanandaj S7: Yasuj S8: Babol S9: Mashhad S10: Shiraz Chehel Giah S11: Shiraz Kazerun Gate S12: Shiraz Adloo Zerehi C: Control

3.1.3 Phenolic Content

The ethanolic extracts of 12 *Hypericum* samples and a field-grown sample of *H. perforatum* as control were assayed for the total phenolic content. The highest phenolic content in the samples of S4, S9, S10 and S6 were found to be 262.76±8.12, 153.41±8.31, 132.64±8.5 and 113.87±4.92 mg GAE/g of dried extract respectively as listed in Table 3. Lower values of phenolic content were detected to be 98.80±10.83, 80.88±4.18, 74.72±2.06 and 70.5±2.99 in a descending order for S8, S2, S5 and S1 respectively (Table 3). The lowest phenolic content was noticed in the samples were found to be 62.97±1.37, 62.54±2.46, 58.98±2.29 and 50.31±3.22 mg GAE/g of dried extracts for S3, S7, S11 and S12 respectively (Table 3).

3.1.4 Flavonoid Content

Determination of total flavonoid content of all *Hypericum* samples indicated the greatest flavonoid content in the samples S4, S10, S5, S9, and S2 (79.26±5.78, 42.37±0.3, 38.67±3.36, 38.22±2.78 and 37.83±5.11 QE/g of dried extracts respectively), while the lowest values were detected for S3, S12 and S1 (18.94±0.17, 14.97±0.62 and 13.47±1.68 mg QE/g of dried extracts respectively) as presented in Table 3. The total flavonoid content recorded for the sample S4 (79.26±5.78 mg QE/g) were the highest among all the investigated samples (Table 3). In general, some of the extracts exhibited almost close values in terms of flavonoid contents (Table 3).

Table 3. Total phenolic and flavonoid content of *Hypericum* samples

Sample	Total phenol mg GAE/g of Ext.(Mean±SD)	Total flavonoid mg QE/g of Ext. (Mean±SD)
S_1	70.5±2.99	13.47±1.68
S_2	80.88±4.18	37.83±5.11
S_3	62.97±1.37	18.94±0.17
S_4	262.76±8.12	79.26±5.78
S_5	74.72±2.06	38.67±3.36
S_6	113.87±4.92	36.31±1.08
S_7	62.54±2.46	23.35±1.28
S_8	98.8±10.83	36.47±1.99
S_9	153.41±8.31	38.22±2.78
S_{10}	132.64±8.5	42.37±0.3
S_{11}	58.98±2.29	28.67±2.12
S_{12}	50.31±3.22	14.97±0.62
C	227.49±8.27	64.39±7.47

S1: Ahvaz S2: Bandar Abbas S3: Tehran S4: Kerman S5: Yazd S6: Sanandaj S7: Yasuj S8: Babol S9: Mashhad S10: Shiraz Chehel Giah S11: Shiraz Kazerun Gate S12: Shiraz Adloo Zerehi C: Control

3.1.5 HPTLC Profile of *Hypericum* Extracts

All samples of ethanolic extracts showed almost similar thin-layer chromatographic patterns and no remarkable difference was observed between the samples. Inspection of the high performance thin-layer chromatoplate obtained from various ethanolic extracts of *Hypericum* samples and the control, declared an efficient separation of their chemical components. Comparing the profiles and the R_f

values of compounds of various *Hypericum* samples with those of the control (*Hypericum perforatum*), indicated the similarity of their chemical composition as illustrated by the chromatogram in Figure 2. However, samples S1, S4, S9 and S10, slighly differed from others and the control, in terms of number, size and intensity of the spots (Figure 2).

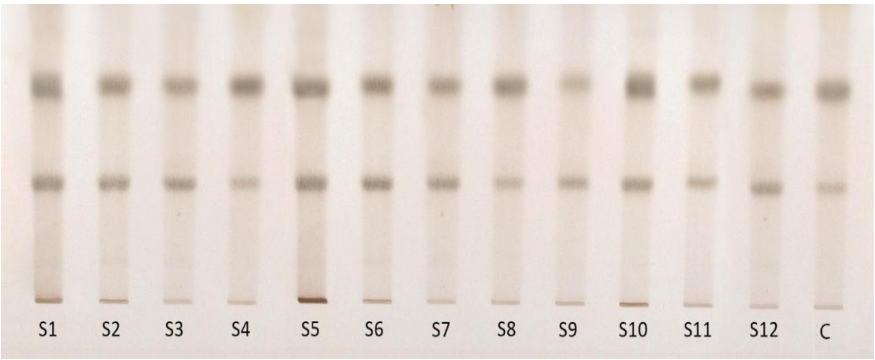

Figure 2. HPTLC of methanolic extracts visualized with anisaldehyde-sulfuric acid

3.2 Discussion

The aim of this study was to evaluate the herbal samples presented as *Hypericum* in the Iranian herbal medicine market. In practice, various specimens of this plant with close macroscopic features are available under the name of *Hypericum* in Iranian herbal pharmacies which are prescribed to control or treat inflammatory and infectious diseases, depression and mental disorders. However, lack of careful monitoring of the sources, quality or mode of cultivation could sometime hamper the selection of the right specimens in the pharmaceutical market. It is therefore mandatory to conduct regular screening of medicinal herbs available in the market. Evidence have revealed that medicinal plants are sometimes contaminated with non active or toxic plants which are sometimes intentional, but in many cases, this is supposed to be due to the incompetence of the traditional sellers or therapists in the correct recognition of the genus or species.

Considering the prescription and sales of unauthenticated or uncharacterized samples of medicinal plants, 12 samples, presented as *Hypericum* in the Iranian medicinal plant markets were collected from various cities. Each sample was then identified and their essential oil analysis and HPTLC fingerprinting were performed and finally all the samples examined for their total phenolic and flavonoid contents.

The most important approach in the systematic characterization of *Hypericum* species is their secretory tissues. These structures can be found in the stem, bark, petals, sepals and pistil. But part of these organs are lacking or show varied distribution in different species. Secretory glands are the specific microscopic features present in all species, but they are different in terms of number, type and position. Secretory vesicles are also present in all species, but they differ in terms of diameter, cavity and the location among the species and different varieties. The dark nodules were observed in certain organs of some *Hypericum* species. This feature is considered as a specific microscopic characteristic among *Hypericum* species [23].

The results of present study confirmed that all samples collected from different markets, belong to the genus *Hypericum*. Based on the taxonomic characterisation, samples S1, S5, S7, S8, and S9, were confirmed to be *H. scabrum*, while S2, S3, S10 and S11 were identified as *H. elongatum*. The sample S4, was characterized as *H. perforatum*, whereas, S6 was identified as *H. helianthemoides*.

The results of essential oil analysis showed a significant correlation between the volatile components of different *Hypericum* samples (Table 2). α-Pinene, a bicyclic monoterpene showed the contribution

of greater than 5% and was the dominant constituent in all samples of essential oil, except S4 and control. This finding is consistent with the results of previously reported studies, that introduced α-pinene, as the major component of the essential oil of *Hypericum* species in southern France, Turkey and Italy [23-25]. Caryophyllene oxide and β-cubebene were the dominant constituents of the control. This is in close agreement with the results of earlier studies, conducted on different *Hypericum* samples in Lithuania and Croatia, which showed the oxygenated sesquiterpenes, such as caryophyllene oxide and β-cubebene, as the most dominant constituents of essential oil in all tested samples, while a small contribution of oxygenated monoterpenes were detected [26,27]. The main component of the essential oil of S4 sample was recognized to be 1-dodecanol. This compound has been detected as one of the major components of the essential oil of French *H. perforatum* var. *perforatum*, which corroborates the results of previous studies [28].

As can be seen in Table 2, most of the essential oil samples revealed high content of α-pinene, which is consistent with the values given in the Iranian herbal pharmacopoeia for *H. perforatum* [29]. While the sample S4, was found to be rich in 1-dodecanol, which is different from other specimens in terms of major components of essential oil. It is noteworthy to point out that, factors such as contamination with other plants, methods of drying and storage, moisture and light, phenological stages, collection area, method of processing and seasonal changes and particularly the genetic characteristics of the species might have affected the essential oil yield and composition, [30,31]. Consequently, the variations observed in volatile constituents of some of the *Hypericum* samples may thus in part or totally be attributed to the above mentioned factors.

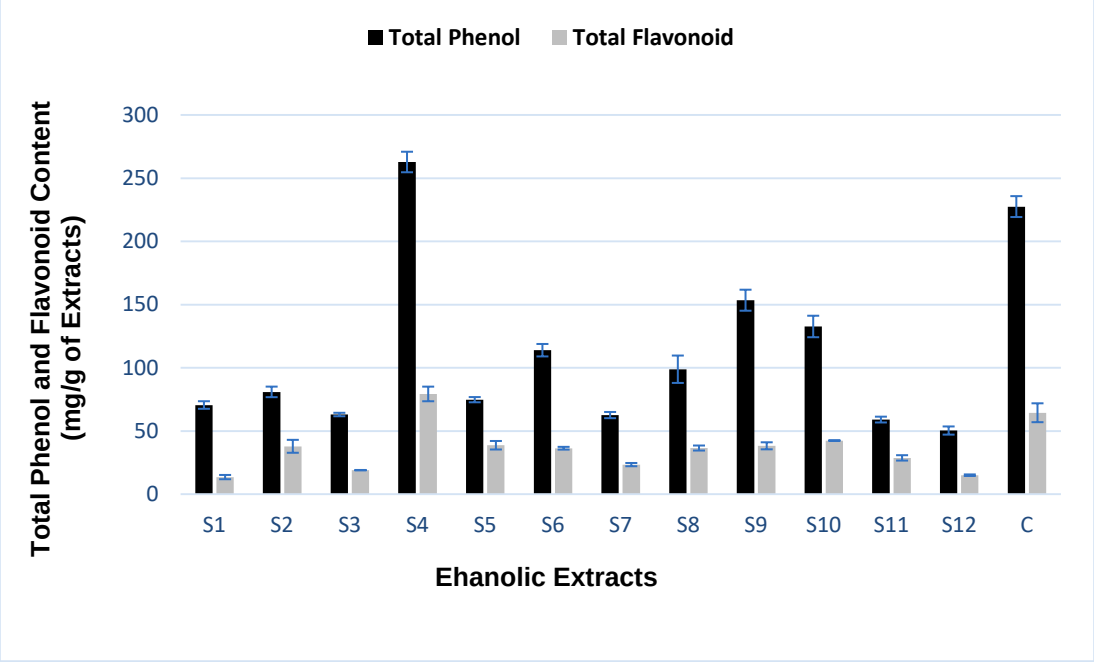

Figure 3. Comparison of phenolic and flavonoid contents of *Hypericum* samples

Beside essential oil, *Hypericum* species contain a non volatile group of phenolic constituents including hypericin, a natural polyphenolic polycyclic quinone, and hyperforin a terpene ketone. These compounds and their structural analogs have already shown synergistic antioxidant effects and anti lipid peroxy radical properties [32,33]. The phenolic and flavonoid content of *Hypericum* specimens undergo significant quantitative alterations during the vegetative stages [34-36]. The total phenolic content of the samples S6, S9 and S10 were found to be in close agreement with the average phenolic content previously reported for *Hypericum* species (150.44 mg GAE/g of dried extract), using ethanol as extraction solvent. As given in Table 3, higher values of total phenol were found in

S4 and control samples, which are consistent with the research of Öztürk et al. on *H. perforatum* in Turkey [37,38]. As presented in Table 3, the phenolic content of the remaining samples were found to be in agreement with the results of another research, reported earlier [39].

The flavonoid content of all tested *Hypericum* samples were also consistent with the results of Öztürk et al. [37]. Table 3 shows that, the samples S1, S2, 3, 5, 7, S11 and S12 were comparable in terms of phenolic and flavonoid contents, whereas the samples of S6 and S8 were close in terms of their flavonoid content. The samples S9 and S10 showed similar values for flavonoid content, while S4 (*H. perforatum*) revealed a close similarity to that of the control in terms of both phenol and flavonoid content, which could be due to their species resemblance (Table 3, Figure 3). Considering the proof of the relationship between soil salinity and increase in total phenols and flavonoids, it is also likely that, S4 sample might have collected from a place with higher degree of soil salinity, compare to other samples [40,41]. Determination of flavonoid content of *Hypericum* samples indicated that our results are consistent with those of previous studies [37,42]. In general, the extracts of *Hypericum* samples S1, S2, S3, S5, S7, S11 and S12 showed closer distribution range of phenolics and flavonoids compare to other samples (Table 3, Figure 3). While almost close ranges of phenolic and flavonoid concentrations were detected between the samples S6 and S8, S9 and S10 and the S4 and control. In order to investigate the profile of phytochemical markers in various *Hypericum* specimens, high performance thin-layer chromatography were employed. Comparison of HPTLC profiles of *Hypericum* samples, indicated similarities among the samples in terms of their polar and non polar components (Figure 2). However, the differences observed between the chemical profile of various *Hypericum* extracts with those of the control and their specific markers can be considered as a benchmark in differentiation and diagnostic characterization of various *Hypericum* samples [43]. Therefore, HPTLC fingerprinting may be considered as an efficient diagnostic tool for authentication and quality assessment of *Hypericum* species and the related herbal samples available in the market.

4. STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS software version 22.0 (IBM, Armonk, NY, USA) and the values expressed as mean±SD. Independent t test was carried to compare the data and their significance of the difference was assessed at $P < 0.05$ level.

5. CONCLUSION

To ensure the major safety and efficacy concerns arising from the use of herbal medicines available in the traditional pharmacies, there is always a need for efficient herbal authentication methods. The results of this study clearly indicate that the use of instrumental techniques of analysis can greatly help in establishing quality assurance and the management of adultrations in the market samples of *Hypericum.* These techniques could be used in detecting the variations in the chemical constituents of *Hypericum* samples obtained from different geographic regions. The reliability and simplicity of these methods also encourage their potential use in careful quality assessment of the highly traded plants like *Hypericum* species, prior to their use in drug, food and cosmetic formulations

CONSENT

It is not applicable

ETHICAL APPROVAL

The research proposal was approved by the Ethics Committee of Shiraz University of Medical Sciences, under the registration code ; IR.SUMS.REC.1397.297 on June 23, 2018.

COMPETING INTEREST

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

The Study conception and design were performed by Mohammad M. Zarshenas and Mohammad Ali Farboodniay Jahromi.
Acquisition of data was carried out by Nastaran Babaei Rizvandi.
Analysis and interpretation of data were done by Mohammad Ali Farboodniay Jahromi, Mohammad M. Zarshenas and Nastaran Babaei Rizvandi.
Drafting of manuscript was carried out by Mohammad Ali Farboodniay Jahromi and Mohammad M. Zarshenas.
The critical revision of the manuscript was made and completed by Mohammad Ali Farboodniay Jahromi.
All authors read and approved the final manuscript.

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