Journal of Medicinal Plants and By-products (2021) 1: 45-52

Original Article

Variation of Main Alkaloids and Fatty Acids among Five Natural Populations of *Peganum harmala* L. from Iran

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Article History: Received: 23 October 2019/Accepted in revised form: 13 August 2020

Abstract

Alkaloids and fatty acids extracted from plant species are considered as popular ingredients in pharmaceutical products. The medicinal herb *Peganum harmala* L., native to arid and semi-arid rangeland, is known as an excellent source of β-carboline alkaloids and some important essential fatty acids. In order to extend the knowledge of phytochemical biodiversity of *P. harmala*, main alkaloids and fatty acids contents of the populations collected from five different parts of Iran were investigated. Harmine and harmaline, that are the main β-carboline alkaloids of *P. harmala* seed extract, were identified by FTIR and quantified by HPLC. Significant quantitative differences ($p \leq 0.01$) were observed in harmaline (295-354.16 mg/g dry extract) and harmine (257.91-304.66 mg/g dry extract) contents among studied populations. Furthermore, seed oil was extracted and analysis of fatty acids was performed quantitatively using GC method. The result showed that the average oil content of *P. harmala* was 15.82±1.06%, with no significant difference ($p \leq 0.05$) among the samples. The most abundant fatty acid in all analyzed samples was linoleic acid (C18:2) (54.33-60.53% of the total fatty acids). Statistical analysis revealed significant differences ($p \leq 0.05$) among the populations for linoleic acid (C18:2) (58.21±2.70%) and palmitic acid (C16:0) (9.99±1.19%) contents. Additionally, all the samples were rich in unsaturated fatty acids and β-carbolines, harmaline and harmine. These data indicate that the amounts of major alkaloids and fatty acids vary considerably among the *P. harmala*'s natural populations, which could be potential sources of these important phytochemicals for pharmaceutical and industrial purposes.

Keywords: *Peganum harmala* L., Fatty acids, β-carboline alkaloids, Harmine, Harmaline

Introduction

Peganum harmala L. (family Nitrariaceae), commonly known as Syrian Rue, is an important perennial medicinal plant widely distributed and native to arid and semi-arid areas of north Africa, Mediterranean, the Middle east, Pakistan, India and Iran. It was also introduced in parts of the southwest America and Australia [1-3]. This wild herbaceous species, with short creeping root, grows spontaneously in steppe areas and sandy soils, and can reach 30-100 cm in height [4].

Phytochemical analysis of *P. harmala* revealed the presence of alkaloids, fatty acids, steroids, flavonoids, anthraquinones, amino acids, and polysaccharides in its seeds and other parts of the plant [5-8]. The seeds consist of 2.5 to 4% mixed β-carboline alkaloids [9] such as harmine, harmaline, harmol, harmalol and tetrahydroharmine [10,11], which are known as harmala's alkaloids, because they were originally isolated from *Peganum harmala* [12,13]. The main alkaloids of *P. harmala* are harmaline (harmidine) and harmine (banisterine). Harmaline ($C_{13}H_{14}N_2O$), with chemical name of 7-Methoxy-1-methyl-3,4-dihydrobeta-carboline, is the major alkaloid of this plant, and harmine ($C_{13}H_{12}N_2O$), with chemical name of 7-Methoxy-1-methylbeta-carboline, pharmacologically resembles harmaline in its action but with less toxicity [4,14].

Several reports indicate that *P. harmala* extract has a wide range of pharmacological and biological properties such as anticancer [15], hypothermic and hallucinogenic [16], antiparasitic and anti-HIV [17], anti-microbial [18],

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anti-oxidant [19] and monoamine oxidase (MAO) inhibition [20] activities. In most cases, these effects are caused by alkaloids of type β-carboline [21].

Furthermore, *P. harmala* has been one of the sources of edible oil [22]. In a recent study, *P. harmala* seed has shown the potential as a source of omega-3 essential oil (α-linolenic acid, 14.79%) and omega-6 oil (linoleic acid, 10.61%). Also, saturated fatty acids in the seed oil (palmitic acid, 48.13% and stearic acid, 13.8%) were considered to be used in the soaps, cosmetics, lubricants and softening industry [8]. Apostolico *et al*., [23] have recently found that the composition of the essential oil of *P. harmala* seeds from five countries in northern Africa was quite varied and they concluded that the external environment can affect the metabolic pathway of the same plant.

Generally, there is little information about the metabolic profile of plants at the population level [24]. Although the main alkaloids of *P. harmala* were reported, few studies on the fatty acids of *P. harmala* were carried out [8]. Nevertheless, until now, the variation of the alkaloids and fatty acids of *P. harmala* in different locations has not been investigated. In general, the objective of this study was to evaluate *P. harmala* seeds from five selected sites of Iran in order to carry out a quantitative and qualitative analysis of the major alkaloids and fatty acids.

Material and Methods

Plant Materials

For this study, the seed samples of *Peganum harmala* L. were collected from five different sites of Iran. This sampling was done based on information from local Agricultural Extension Agencies and producers throughout Iran during 2017 (Fig. 1). The salient characteristics of the regions are reported in Table1. The plant materials have been identified and deposited in National Institute of Genetics Engineering and Biotechnology (NIGEB), Tehran, Iran.

β-carboline Alkaloids Extraction

The powdered dry seeds of *P. harmala* (5g) were mixed with 50 mL acetic acid (30% (w/w)) at room temperature for 24 hours. Afterwards, the mixture was filtered using Whatman 0.45 mm filter paper, while it was washed twice more with 10 mL acetic acid (30% (w/w)). The filtrate was transferred to a separatory funnel, and then washed thoroughly with 100 ml of petroleum ether: ethyl acetate (1:1). The aqueous layer was collected and basified with sodium hydroxide 10 M and transferred to another separatory funnel and extracted three times with 50 mL chloroform. Eventually, the solvent of organic layer was evaporated to dryness on a rotary evaporator at 45 °C [25].

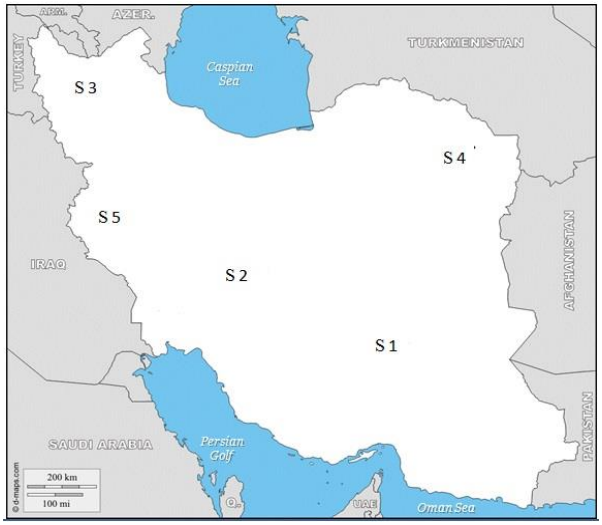


Fig. 1 The geographical sites of the *Peganum harmala*'s populations; S1: Kerman, S2: Isfahan, S3: Tabriz, S4: Mashhad and S5: Kermanshah

FTIR Analysis of β-carboline Alkaloids

The IR spectra were recorded on a Fourier transform infrared spectrophotometer (FT-IR, BRUKER, Germany). One mg of dried extracts and standards ,Harmaline (286044-1G- Sigma) and Harmine (51330-1G- Sigma), were ground with 150 mg KBr powder and pressed into pellets for infrared spectrometry analysis. The instrument was operated under dry air purge, and the scans were collected at scanning speed of 2 $mm.s^{-1}$ with resolution of 4 cm^{-1} in the frequency range of 4000 to 500 cm^{-1}.

Table 1 Main characteristics of selected sites of *Peganum harmala* **L.**

Site (code)	Region of Iran	Latitude	Longitude	Altitude (m asl*)	Climate
S1	Kerman	30°16' °N	57°04' °E	1765	arid
S2	Isfahan	32°59' °N	51°03' °E	1991	arid
S3	Tabriz	38°04' °N	46°17' °E	1542	semi-arid
S4	Mashhad	36°18' °N	59°36' °E	1070	semi-arid
S5	Kermanshah	34°18' °N	47°03' °E	1392	semi-arid

Annual mean temperatures in the arid and semi-arid climates were 24º – 27 ºC and 27º – 30 ºC, respectively. Annual mean rainfalls were 104 – 181 mm and 205 – 308 mm in the arid and semi-arid climates, respectively.

* metres above sea level (m asl)

HPLC Analysis of β-carboline Alkaloids

High performance liquid chromatography (HPLC) analysis was conducted using a Cecil 1100 series (Cecil Instruments Ltd., Cambridge, England). The mobile phase composition was 10 mM potassium phosphate buffer (pH 7) and acetonitrile (50 : 50 , v/v) with the flow rate of 1.5 mL.min^{-1} at room temperature (25 ˚C).

Stock solutions were prepared individually in methanol for harmaline and harmine standards (Sigma, USA) at five concentrations of 100 – 1000 µg.mL^{-1} and used to establish standard curves of harmaline standard (Figure 4a) and harmine standard (Figure 4b). Four mg of each extract was dissolved in 1ml Methanol and filtered through a 0.45 µm polypropylene filter prior to direct injection into the HPLC column. Volumes of 20 µl of prepared stocks and sample solutions were subsequently injected into a 4.6 mm × 250 mm ODS-3 C18 column under the same conditions and the chromatograms were recorded at maximum absorbance wavelength of 330 nm.

Oil extraction and preparation of fatty acid methyl esters (FAME)

Oil extraction was conducted from ground dry seeds (5 g) using *n*-hexane as the solvent in a Soxhlet apparatus for 1 h at 40 – 60 ºC. Oil content of the samples was expressed as a percentage of the oil in dry weight.

The fatty acid methyl esters (FAMEs) were prepared by converting them to methyl esters by the addition of 0.2 mL of 2N methanolic potassium hydroxide to 0.1 g of the extracted oil, heating in a water bath at its boiling temperature for 10 minutes, followed by the addition of 1ml of *n*-hexane and incubated at room temperature for 1 hour [26]. A 1 µl aliquot of the superior phase containing FAMEs was injected and analyzed by gas chromatography. The fatty acid composition of the samples was specified using gas chromatography as FAMEs according to European Regulations (EEC 2568/91).

Gas Chromatography Analysis

The gas chromatographic analysis of FAMEs was carried out by an ACME 6100 Younglin Capillary Gas chromatograph equipped with a FID (VICI, Valco, Houston, TX, USA). A fused-silica capillary column (TRB-5, 60 m ×0.32 mm ×0.5 µm film thicknesses, TR-120563, Teknokroma, Barcelona, Spain) was used. The injector, detector and oven temperatures were set at 240, 250, and 185 °C, respectively. The carrier gas was helium with the flow rate of 1 mL min^{-1} and the split ratio of 1: 50. The amount of each fatty acid was expressed as a percentage of the total fatty acids.

Statistical Analysis

All experiments were carried out in triplicate and all data were represented as means± standard deviation (SD). Statistical comparisons were performed using an analysis of variance (ANOVA) according to Duncan's test through the SPSS 18.0 Software. The least significant differences were statistically calculated at p-values ≤ 0.05.

Results

Evaluation of Alkaloids Contents

The seed extracts of *Peganum harmala* were characterized and two major β-carboline alkaloids, harmaline and harmine, were identified by FTIR. Figure 2 compares the typical infrared spectrum of *P. harmala* seed extracts with harmine and harmaline standards. Moreover, quantitative determination of the major alkaloids in crude extracts were analyzed using HPLC. A typical HPLC chromatogram of the extracts compared with the standards was exhibited in Figure 3. The retention times for harmaline and harmine were observed to be at 3.36 and 5.42 minutes, respectively.

The alkaloid contents in the extracts of *P. harmala* was quantified using the corresponding linear calibration curves with correlation coefficient (R^2) of ≤ 0.99, as shown in Figure 4. Accordingly, the amounts of harmaline and harmine (based on dry extract) varied significantly among the samples collected from five different regions ($p \leq 0.01$) (Table 4). In the all examined extracts, the average contents of harmaline and harmine were 332.23±22.24 mg/g and 284.31±15.62 mg/g, respectively. On average, the highest content of harmaline (354.16±1.01 mg/g) was related to the samples of Isfahan (S2), and the highest amount of harmine (304.66±1.84 mg/g) was related to the samples of Kermanshah (S5). Meanwhile, the total concentration of the two alkaloids in dry extracts varied among the populations ($p \leq 0.01$) (Table4), and the largest amount (640.00±2.78 mg/g) was detected in the samples collected in Kermanshah (S5), whereas the samples from Tabriz (S3) presented the lowest total alkaloid concentrations (583.00±2.50 mg/g), as shown in Table 2.

Evaluation of Fatty Acids Contents

The results of oil quantification in Table 3 showed that *P. harmala* seed oil (15.82±1.06% on average) ranged from 15.32±1.27% (S2) to 16.14±1.09 % (S1), and there is no significant difference ($p \leq 0.05$) among the samples from different localities.

After esterification of the fatty acids to the fatty acid methyl esters (FAMEs), they were determined by GC analysis. The two major fatty acids were linoleic acid (C18:2) (58.21±2.70%) and oleic acid (C18:1) (27.99±2.42%), followed by palmitic acid (C16:0) (9.99±1.19%) and stearic acid (C18:0) (2.69±0.46%). The *P. harmala* seed oils also contained linolenic (C18:3) and palmitoleic (C16:1) acids, but in small quantities (0.12–0.38%) (Table 3). Among the unsaturated fatty acids

(USFAs), linoleic acid (C18:2) was significantly varied ($p \leq 0.05$) from 54.33±2.24% (S2) to 60.53±0.95% (S1). Significant differences ($p \leq 0.05$) were also found among the populations for total unsaturated fatty acids, which ranged from 82.12±5.02% (S2) to 88.04±0.31% (S3). In addition, palmitic acid (C16:0) was the only saturated fatty acid (SFA) found to be different ($p \leq 0.05$) from 8.99±0.06% (S3) to 11.60±1.63% (S2). No significant difference in the other fatty acids among different sites was found (Table 4).

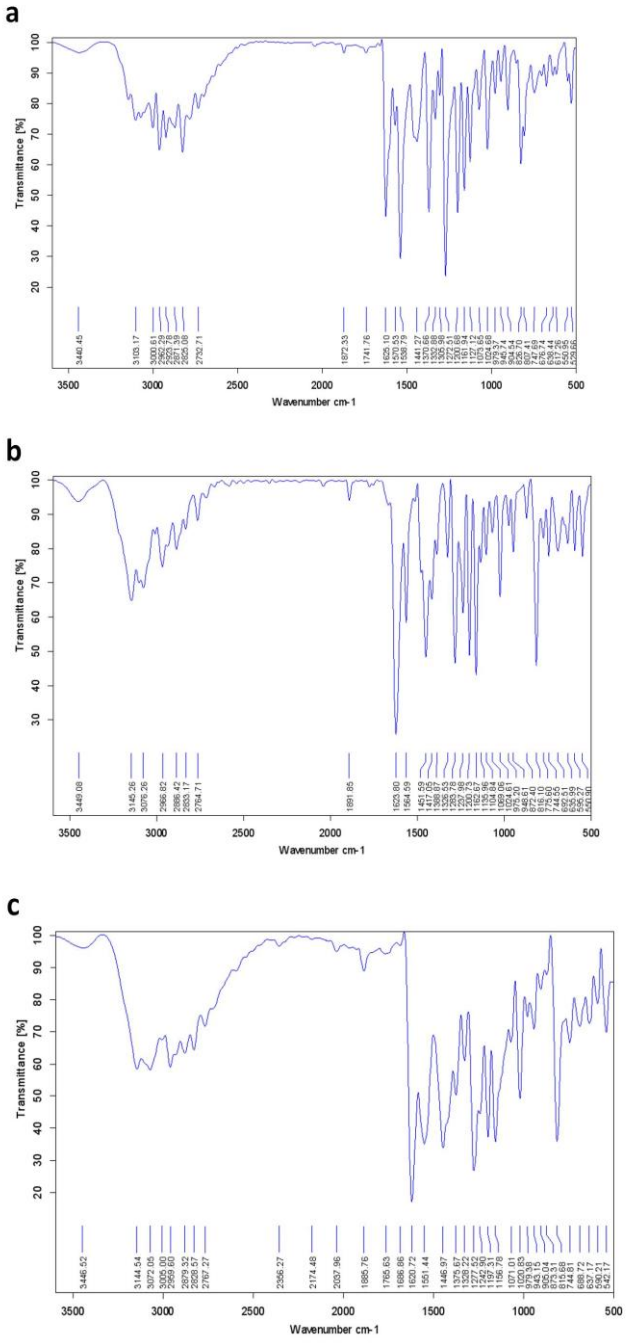


Fig. 2 FT-IR spectra in the region of 4000 – 500 cm^{-1} for (a) Harmaline standard, (b) Harmine standard and (c) *P. harmala* seed extract

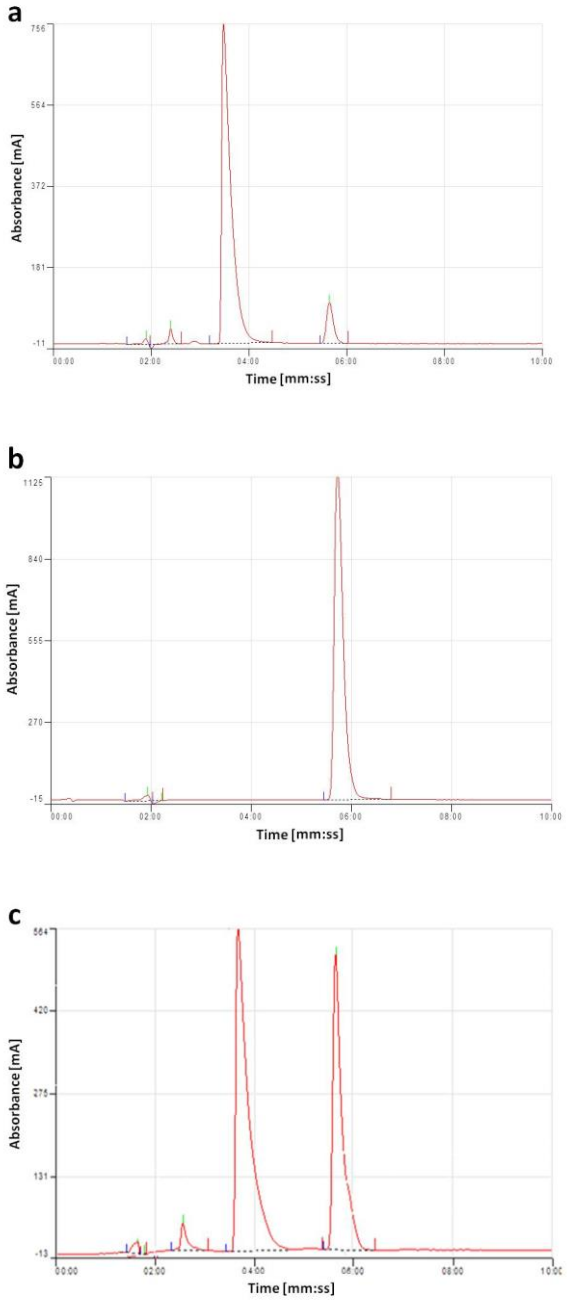
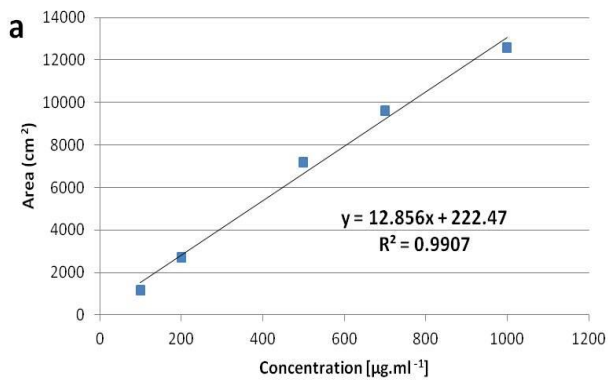


Fig. 3 HPLC chromatograms at absorbance wavelength of 330 nm for (a) Harmaline, (b) Harmine and (c) *P. harmala* seed extract



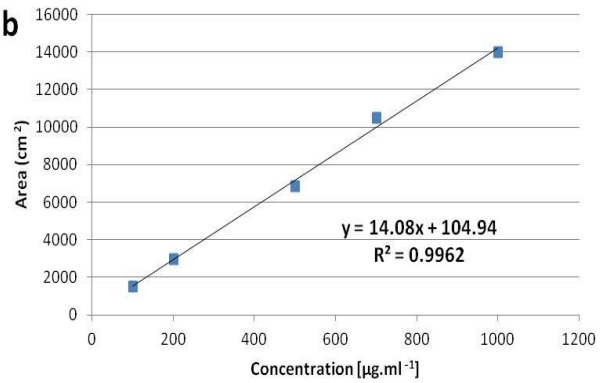


Fig. 4 Linear calibration curves for (a) Harmaline and (b) Harmine

Discussion

Medicinal plants have been proposed as potential sources of pharmaceutical natural products. In terms of active components, harmine and harmaline, which are two major β-carboline alkaloids in the *P. harmala* extract, have exhibited various types of pharmacological and biological activities. What is more, the proportions of β-carboline alkaloids change sharply with the stage of development of seeds and finally harmine and harmaline are stored in high concentrations in ripe seeds [20, 21]. Therefore, in this study, the dry seeds of *P. harmala* were subjected to acidic/basic extraction to obtain the alkaloids, and then harmine and harmaline were detected as the major β-carboline alkaloids of the extracts via FTIR. According to comparison of infrared absorption of *P. harmala* seed extracts with the standards of alkaloids (Fig. 2 a and b), it could be found that in frequency region of 4000 - 500 cm^{-1}; the functional groups (C-H), (C=O), (C=N), (OCH3) and (C-N) were absorbed at about wave lengths, 1071, 1277, 1446, 1620 and 3072 cm^{-1}, respectively, resembling harmaline ($C_{13}H_{14}N_2O$) and harmine ($C_{13}H_{12}N_2O$) molecular formulas [25, 27].

Our study revealed that the concentrations of alkaloids (based on dry extract) quite varied among the populations of *P. harmala*. On average, harmaline content was higher than harmine content in all the samples and from each gram of the dry extract, 332.23±22.24 mg harmaline and 284.31±15.62 mg harmine were determined by HPLC (Table 2). The relative contents of the β-carbolines seemed to be low, but they were 4-5 times higher than those previously reported in Iran, which identified that each gram of *P. harmala* seed extract contained 79.0 mg and 55.5 mg of harmaline and harmine, respectively [28]. Moreover, the average of harmaline content (332.23mg/g) was nearly similar to the amount of harmaline (325 mg in 1.20 g of crude extract) obtained by Wang *et al.*, [29], but the average of harmine content (284.31 mg/g) was lower than their data with the value of 554 mg in 1.20 g of crude extract. These differences might be related to the different geographical origins with different environmental conditions. Indeed, the concentration of plant secondary metabolites (PSMs) could change considerably due to the influence of several biotic and abiotic factors [30]. In this regard, it has been shown that alkaloid content of medicinal plants such as *Corchorus depressus* [31], *Papaver somniferum* L. [32] and *Tribulus terrestris* [33] were affected by environmental and spatial factors.

Besides the PSMs, the Fatty acids composition of plant oils is also influenced by a lot of factors such as genotype, ecology, morphology, physiology and agronomic practices [34]. In this study, linoleic acid (C18:2) was the most abundant fatty acid (58.21±2.70%), which was significantly different ($p \leq 0.05$) among the populations, followed by oleic acid (C18:1) with average value of 27.99±2.42% (Table 3).

Table 2 Alkaloid amounts of *P.harmala* seeds from five different sites of Iran

Site (code)	Location	Harrmaline	Harrmine	Sum of alkaloids
S1	Kerman	351.58±2.12 a	286.33±1.18 bc	637.91±1.04 a
S2	Isfahan	354.16±1.01 a	284.08±1.25 c	638.25±2.25 a
S3	Tabriz	325.08±1.28 c	257.91±1.28 d	583.00±2.50 b
S4	Mashhad	295.00±1.75 d	288.58±0.52 b	583.58±2.09 b
S5	Kermanshah	335.33±1.01 b	304.66±1.84 a	640.00±2.78 a
Mean of means	-	332.23±22.24	284.31±15.62	616.55±28.18

The values are expressed as (mg/g dry extract); mean ± standard deviation (n = 3). Mean values within each column followed by the same lower-case letter are not significantly different at $p \leq 0.01$ level.

Table 3 Oil content and fatty acid composition of *P. harmala* seeds from five different sites of Iran

Site (code)	Location	oil content (% of dry weight)	Fatty acid composition (% of total)							
			C16:0	C18:0	SFAs	C16:1 n7	C18:1 n9	C18:2 n6	C18:3 n3	UFAs
S1	Kerman	16.14±1.09 a	9.95±1.08 ab	3.01±0.62 a	12.97±1.69 ab	0.13±0.02 a	26.10±2.22 b	60.53±0.95 a	0.26±0.05 ab	87.03±1.60 a
S2	Isfahan	15.32±1.27 a	11.60±1.63 a	2.86±0.28 a	14.46±1.52 a	0.15±0.04 a	27.26±3.41 ab	54.33±2.24 b	0.38±0.13 a	82.12±5.02 b
S3	Tabriz	15.63±1.42 a	8.99±0.06 b	2.91±0.32 a	11.91±0.28 b	0.17±0.01 a	27.68±1.28 ab	59.92±1.20 a	0.26±0.08 ab	88.04±0.31 a
S4	Mashhad	16.09±0.71 a	9.51±0.54 b	2.53±0.33 ab	12.05±0.59 b	0.18±0.05 a	27.92±0.08 ab	59.59±0.67 a	0.27±0.09 ab	87.96±0.52 a
S5	Kermanshah	15.94±1.39 a	9.90±0.18 ab	2.12±0.18 b	12.02±0.32 b	0.12±0.01 a	31.02±1.59 a	56.70±1.38 b	0.17±0.01 b	88.02±0.22 a
Mean of means	-	15.82±1.06	9.99±1.19	2.69±0.46	12.68±1.35	0.15±0.04	27.99±2.42	58.21±2.70	0.27±0.097	86.63±3.10

SFAs: saturated fatty acids. UFAs: unsaturated fatty acids. All values are mean ± standard deviation (n = 3). Mean values within each column followed by the same lower-case letter are not significantly different at $p \leq 0.05$ level.

Table 4 Analysis of variance for alkaloid and fatty acid contents in five populations of *Peganum harmala* L.

		Alkaloids			Fatty acids							
Source of variation	df	Harmaline	Harrmine	Sum of alkaloids	C16:0	C18:0	SFAs	C16:1 n7	C18:1 n9	C18:2 n6	C18:3 n3	UFAs
Between sites	4	1726.91**	850.05**	2767.29**	2.87*	0.39^{ns}	3.51^{ns}	0.002^{ns}	10.02^{ns}	20.68*	0.016 ^{ns}	19.64*
Error	10	2.25	1.65	4.91	0.83	0.14	1.14	0.001	4.16	1.95	0.007	5.64

SFAs: saturated fatty acids. UFAs: unsaturated fatty acids.
** $p \leq 0.01$, * $p \leq 0.05$ and ns non-significant.

Interestingly, the range of omega-3 (n3) and omega-6 (n6) fatty acids of *P. harmala* oil obtained in this study were more than those reported in the previous study [8]. The same result was reported by Nehdi *et al*., [35] that recommended the seed oil of harmal (*Rhazya stricta*) with oil content of 13.68% as dietetic oil, because it was high in linoleic acid (59.03%) and oleic acid (27.01%). However, in this study the oil content of *P. harmala* seeds was 15.82±1.06% on average, and we found that more than four fifths of the fatty acids from total lipids of *P. harmala* were unsaturated fatty acids (UFAs) with an average value of 86.63±3.10%. Therefore, *P. harmala* seed oil could be considered as a rich source of unsaturated fatty acids. In contrast, the average amount of saturated fatty acid (SFAs) in total lipids were only 12.68±1.35%. Palmitic acid (C16:0) was varied among the sites ($p \leq 0.05$), and there was a lower percentage of stearic acid (C18:0, averaging 2.69±0.46%) relative to palmitic acid (C16:0, averaging 9.99±1.19%). The contents of C16:0 (9.99±1.19%) and C18:3 (0.27±0.097%) in our samples (Table 3) were much lower compared with those in the previous report on *P. harmala* from Saudi Arabia [8]. These differences in fatty acids composition in the same plant, might be due to sample collection from different areas. To date, no literature has reported on the geographical variation of fatty acid composition in *P. harmala* seed oils. However, other studies regarding variation in fatty acids of some other plant oils such as olive oil [36], sunflower oil [37] and apricot kernel oil [38] revealed significant differences among different geographical regions for C16:0 , C18:2 and other fatty acids. In general, the relative proportion of the C18 unsaturated fatty acids, including oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3), determines final use of the crops oil. Hence, in the food industry, the oils which contain high level of linoleic acid (more than 60%) are being utilised in polyunsaturated oils and margarine production. Moreover the low content of linolenic acid (C18:3) improves the flavour stability of the product [39]. Thus, the optimal features of the *P. harmala* oil in all the populations were the high content of linoleic acid (C18:2) and the low content of linolenic acid (C18:3).

Conclusion

The present study indicated that Iranian *P. harmala*, containing considerable amounts of unsaturated fatty acids in the oil and β-carbolines in the extract, can be considered as a good source for the extraction of these phytochemicals. This investigation revealed that *P. harmala* seed oil has a relatively high content of C18:2 and low C18:3 acids. Furthermore, the contents of two fatty acids, C16:0 and C18:2, and two β-carboline alkaloids, harmaline and harmine, in this medicinal plant from five different sites of Iran was quite varied; this could be attributed to the geographical distribution of the plant. The natural plant populations assessed might be potential candidates for selecting favourable chemotypes aimed at cost-effective production of these herbal compounds for pharmaceutical and industrial uses in the future. However, further studies are required to evaluate the effects of genetic and environmental factors on these valuable phytochemicals.

Acknowledgement

This study received financial support from National Institute of Genetic Engineering and Biotechnology (NIGEB).

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