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A NOVEL STUDY ON GENETIC DIVERSITY OF IRANIAN MUSTARD SEEDS AND SOME CHEMICAL PROPERTIES OF THEIR OILS

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ABSTRACT

This novel study aimed to investigate the genetic diversity of Iranian mustard seeds and some chemical properties of their oils. Genetic diversity was carried out using trnH-psbA sequences. A phylogenic tree was constructed after sequencing. The chemical compositions of seeds and analytical characteristics of seed oils were determined. Results indicated that PCR products of samples had different patterns and their sequences (MK328472, MK328473) didn't have 100% identity with related sequences. Samples contained 32.08% and 33.98% oil, respectively. The protein content of samples was in the range of 29.87% and 30.43%. Although mustard seed samples were collected from two different regions of Iran, their investigated chemical compositions were similar (P> 0.05). Acid values of oils were 0.70 and 0.55 mg KOH/g oil, with peroxide values of 2.92 and 3.27 meq O2/kg oil, respectively. The oxidative stability indices were 4.12 and 4.32 hours (p>0.05). The types and amounts of fatty acids in the oil samples were similar (p>0.05) and they were rich in unsaturated fatty acids (88.06% and 89.13%), and low contents of saturated fatty acids (10.87% and 11.94%) were observed. Despite the similarities in chemical properties, two samples were not the same in molecular specifications. It seems that they probably belonged to new species/subspecies of the Brassica spp. As future work, it is recommended to investigate the application of Iranian mustard seed or its derivatives in food products.

Keywords: Iranian mustard, Seed oil, Chemical composition, Genetic diversity

INTRODUCTION

Mustard is cultivated in various parts of the world since olden day. Total mustard seed production reached 532,769 tonnes in 2021 in the World according to the food and agriculture organization corporate statistical database (**FAO, 2021**). In the recent years, due to high yield, drought and disease-resistant properties, the mustard seed has attracted considerable interest; so it can be a suitable candidate to be used by farmers in the dry lands of Iran. Mustard belongs to the genus Brassica of the Brassicaceae or Cruciferae family (**Yu *et al*., 2003**). It is a source of plant-based oil production (**OECD, 2011**). Also, mustard as a popular spice or flavoring agent is used to enhance the taste and flavor of foods, marinades, sauces, salad dressings, sausages, and other food products (**Kumar *et al*., 2011; Adeli Milani *et al*., 2014**). Iranian mustard is cultivated in southern areas of Iran and used traditionally as natural preservative. In the previous research, the antibacterial activities of Iranian mustard essential oils were demonstrated. The higher quantities of allyl isothiocyanate (75.87 and 80.07%) were found in Iranian mustard seeds, compared to previously reported values. This high quantity may occur due to the differences in genetic factors and climatic or geographical conditions (temperature, rainfall, altitude, hours of sunshine, type of fertilizers, harvest time and post-harvest conditions), which can affect the functionality and bioactivity of mustard seeds (**Adeli Milani *et al*., 2019**). Also, in another research the novel bioactive coating enriched with nanoemulsion of mustard essential oil were used to improve the quality and extend the shelf life of turkey meat (**Adeli Milani *et al*., 2020**).

Mustard consumption patterns vary in different countries according to local food habits. Mustard oil is widely used in India for cooking purposes (**Abul-Fadl *et al*., 2011**). Mahyawah is a traditional fermented fish sauce widely used in the southern provinces of Iran. This traditional food is prepared from fresh or dried Sardinella sp. or anchovies (Stolephorus sp.), mustard seeds, water, and other additives made locally. After thorough mixing, the jars are placed in the sun until the desired taste and aroma are achieved (**Zarei *et al*., 2012**).

There are several reports on the chemical compositions of the various mustards grown under different ecological conditions. The three famous species of the mustard genus are known as white or yellow mustard (*Sinapis alba L.*), black mustard (*Brassica nigra*), and brown or oriental mustard (*Brassica juncea L.*) (**Yu *et al*., 2003; Abul-Fadl *et al*., 2011; Sharif *et al*., 2017; Singh *et al*., 2017; Sharma *et al*., 2018**). Mustard species are not limited to these three ones. Developments in molecular techniques have provided researchers with the DNA barcoding to identify species. This method is accurate, sensitive, reliable, and capable enough to distinguish between closely related cultivars (**Kress and Erickson, 2007; Lo and Shaw, 2018**). The choice of gene region for DNA barcoding is the most crucial issue for the accuracy of the method. The gene region shall be a short universal gene sequence, and available in reference sequence data. Also, it is taken from a standardized portion of the genome and contained sufficient variations to discriminate between species. The intergenic spacer trnH-psbA is one of the most variable non-coding plastids used as a barcode marker for identifying probable discrimination between species. The trnH-psbA is the best plant barcode which separates individual species in smaller groups. It is also advantageous because of the availability of the datasets and playing a critical role in the development of plant DNA barcodes (**Nguyen *et al*., 2015**). The main issue of this research was to identify two mustard species grown and cultivated in Iran accurately. The hypothesis of this research was "Two Iranian mustard seeds samples have a similar chemical composition and belong to Brassica juncea. Therefore, the specific objective of this study was using DNA barcoding in order to identify two mustard seeds cultivated in the southern regions of Iran. Also, the characteristics of both seeds and extracted oils of samples were investigated.

MATERIAL AND METHODS

Materials, chemicals and reagents

Two mustard seeds samples (B1 and B2) were prepared from two southern regions of Iran named Hajiabad (Hormozgan Province, Iran, N28° 18' 39.594'', E55° 54' 8.83') and Larestan (Fars Province, Iran, N27° 40' 26.906" , E54° 20' 8.824"). All of the chemicals and reagents used were analytical grades and purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA).

Methods

Morphological characteristics

Morphological characteristics of the samples were evaluated in the department of botany of Payame Noor University in Fars province, Iran.

Preparation of mustard seed powder

Dry and clean mustard seed samples were ground into the powder using blade-carbide grinding (Sanyo, Japan) for 2 min and passed through 40 mesh sieve (400 µm) and kept in polyethylene containers in a cool place.

Amplification of trnH-psbA for barcoding

The total DNA from the samples was extracted by the CTAB method. The DNA content was measured using spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA) and samples run on 1% agarose gel. DNA dilutions (25 ng/µl) were prepared for PCR reaction **(Lo and Shaw, 2018; Nguyen *et al.*, 2015)**. The total DNA (1 µl) was used as a template for PCR reaction to amplify genes. The trnH-psbA genes of samples were amplified using PCR with Taq DNA polymerase (Sigma, USA), Qiagen (0.2 µl), dNTP 10 mM (0.3 µl), buffer MgCl2 10X (2 µl), water (15.5 µl), specific primers (0.5 µl) psbAF (5'-GTTATGCATGAAC GTAATGCTC-3'), and trnHR (5'-CGCGCATGGTGGATTCACAATCC3').
PCR amplification was conducted in thermal cycler (Bio-Rad T100™, USA) using the thermal program at 94ºC for 5 min, 30 cycles consisted of 94 ºC for 1 min, 55ºC for 1 min, 72ºC for 1.5 min, and a final extension step of 72ºC for 7 min (**Kress and Erickson, 2007; Nguyen *et al*., 2015**). Amplified bands were visualized by 1% (w/v) agarose gel electrophoresis at 120 V and viewed on a gel documentation system (**Bio-Rad GDS, USA**). Bidirectional sequencing of purified and diluted PCR products (only sharp band of 300bp) was performed using the Sanger method with the ABI system by Macrogene. The sequences were submitted to the NCBI server (**www.ncbi.nlm.nih.gov**) as accession number MK328472 and MK328473, then the sequences were aligned using the Clustal W program (**Larkin *et al*., 2007**). UPGMA Neighbor-Joining tree based on the trnH-psbA gene was constructed from distances with 100 bootstrap runs, consensus trees with cut off=75. Finally, MEGA 6.0 (**www. megasoftware.net**) was used to visualize the phylogram trees and cluster (**Tamura *et al*., 2013**).

Chemical composition of mustard seeds

The proximate compositions of the mustard seed samples were analyzed following the standard methods of the Association of Official Analytical Chemists (**AOAC, 1999**): moisture by vacuum oven (method 925.10), total fat by Soxhlet (method 920.39), crude protein content by Kjeldahl (method 979.09), total ash by ignition (method 923.03), acid insoluble ash (method 941.12), crude fiber (method 920.169), mineral such as Fe, Cu, Ca (method 999.10). Carbohydrate content was estimated by difference of mean values, that is, 100- (sum of percentages of moisture, protein, lipid, and ash) (**AOAC, 2006**).

Extraction and chemical analysis of the mustard seed oils (MSO)

The oils of mustard seeds were extracted using the Soxhlet method with petroleum ether (b.p. 60-80°C). The solvent was removed using a rotary vacuum evaporator (IKA, Germany) at a temperature bellow 40 °C. The preparation of fatty acid methyl esters was performed according to AOCS (method Ce 1i-07). The fatty acids compositions of mustard seeds oils were determined as methyl ester using gas-liquid chromatography (Varian GC Model CP3800/450-GC, Netherlands). The GC system was loaded with software Class GC-10 (version-2.00), equipped with a Flame Ionization Detector and stainless steel column (dimensions 20 m×0.15 mm) packed with 5 % DEGS-PS. The helium was used as a carrier gas with a flow rate of 1.0 ml/min. The operating conditions were as follows: injection port temperature, 200 °C; column temperature, 230 °C: detector temperature, 260°C (**AOCS, 2007**). Three samples of the mustard oil were analyzed for their chemical and quality characteristics using the following official methods of the American Oil Chemists' Society: peroxide value (method Cd 8–53), acid value (method cd 3d-63), saponification value (method cd 3-25), iodine value (method Cd 1-25), and oil stability index (method Cd 12b-92).

RESULTS AND DISCUSSION

Morphological characteristics

The mustard seed samples were small, spherical, and brown. Because of morphological similarities, they were recognized as Brassica juncea by the botanist.

Amplification of trnH-psbA for barcoding

After electrophoresis of PCR products through 1% agarose gels, the distinct patterns were observed which represented the differences between two samples. The sizes of PCR products amplified from trnH-psbA primer were about 300 bp with the sharp band, 350 and 500 bp with the weak band in sample B1 and 300 bp with the sharp band in sample B2 (Fig 1). After repeating PCR in the different conditions such as annealing temperature, the concentration of reaction components, and thermal program, three bands were observed again in sample B1. In these studies, different molecular markers had been used for plant identification. The alignment was performed by blast the sequenced 300 bp sharp band between two samples. According to the results, the B1 sequence (MK328472.1) had 98% identity, 93% query cover, E value of 7e-118, Max score of 405, and total score of 485 with B2 sequence (MK328473). The nucleotide sequences of investigated samples were compared with the two reported sequences in the NCBI database by Mega Blast.

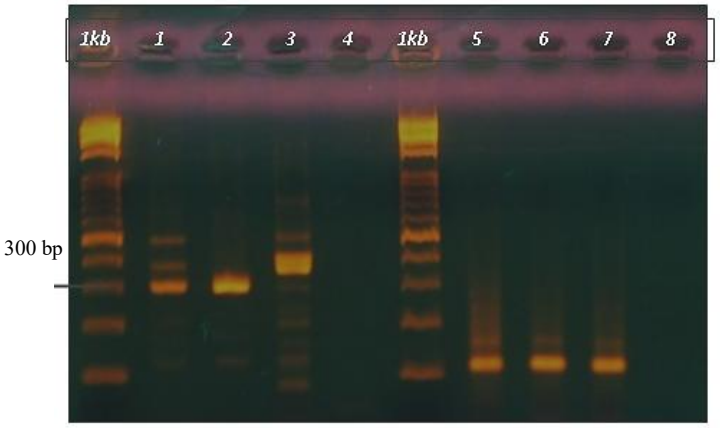

Figure 1 Gel electrophoresis of PCR products from amplification of trnH-psbA: Ladder100 bp DNA Marker, Samples 1: Brassica spp. B1, 2: Brassica spp. B2, 3: canola, 4: negative control, Ladder100 bp DNA Marker, 5: 18SrRNAB1, 6: 18SrRNA B2,7: positive control, 8: minus template (water)control18srRNA.PCRproducts were resolved on 1% TAE agarose gel

Accurate morphological identification of plant materials highly relies on the botanist's skills and cannot be implemented to discriminate between closed related species and identify an unknown plant sample to a given taxonomic rank. The sizes of PCR products (300bp) were similar to the results of **Nguyen *et al.*, (2015)**, but smaller than the reported results of Kress et al. (size of PCR products: 450 bp) **(Kress and Erickson, 2007)**. Results showed that B1 and B2 sequences had 99% identity with B. nigra chloroplast DNA, trnH-psbA intergenic spacer (KT878383) and B1 and B2 sequences had 97% and 95% identities with B. tourneforti chloroplast DNA and trnH-psbA (AB669908), respectively. B1 and B2 samples were identified as unknown queries, located in a separate cluster of other Brassica in the phylogenic tree (Fig 2).

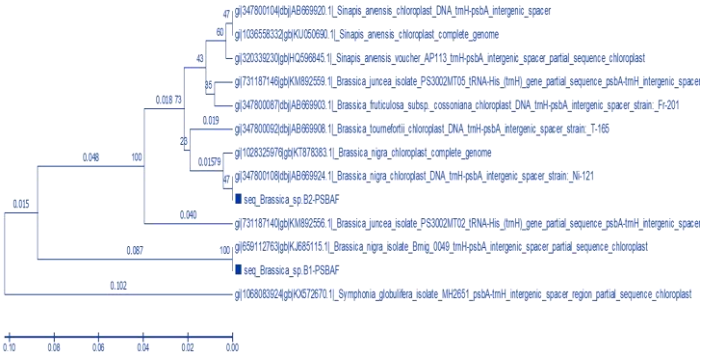

Figure 2 UPGMA Neighbor-Joining tree based on the trnH-psbA gene constructed from distances with 100 bootstrap runs and consensus trees cut off=75.

The chemical composition of investigated mustard seeds

The results of the chemical characteristics of the two mustard samples are presented in Table 1. Samples contained 32.08% and 33.98% oil, respectively. The protein content of samples was in the range of 29.87% and 30.43%. Total carbohydrates ranged between 20.58 % and 21.37% for both B1 and B2 samples, respectively. Crude fiber was 6.82% for sample B1 and 7.02% for sample B2. The values of minerals such as Fe and Ca found in the samples were in the range of 107.13 to 111.55 ppm and 27.53 to 35.37 ppm, respectively. Both samples had about 4.8% moisture. The amounts of total ash and acid– insoluble ash were 4.20-

3.91% and 0.81-0.86%, respectively. The samples had similar chemical composition (p>0.05).

Table1 Chemical composition of Iranian mustard seeds on dry weight basis (Mean± SD) [a].

Components	Sample B1	Sample B2
Moisture (%)	4.89 ± 0.06	4.81 ± 0.07
Ash (%)	4.20 ±0.03	3.91 ±0.04
Acid- insoluble Ash (%)	0.81 ± 0.43	0.86 ±0.25
Protein (%)	30.43 ±0.75	29.87 ± 0.30
Oil (%)	32.08 ±0.49	33.98 ±0.058
Fiber (%)	7.02 ± 0.20	6.82± 0.25
Total Carbohydrates (%)	21.37±0.36	20.58±0.41
Cu (ppm)	nd [b]	nd
Fe (ppm)	111.55± 0.00	107.13± 0.00
Ca (ppm)	27.53± 0.00	35.37± 0.00

[a] Values presented are means of triplicate determinations ± standard deviation (SD)
[b] nd: not detected

Although mustard seed samples were collected from two different regions of Iran, their chemical compositions were similar (P> 0.05). The mustard seed is rich in protein. The protein is of excellent nutritional quality, being rich in lysine with sufficient amounts of sulfur-containing amino acids in most of the cereals and oilseed proteins (**Abul-Fadl *et al.*, 2011**). The mustard seed samples contained adequate levels of important minerals such as Ca and Fe. Minerals are very important in the human diet and function as co-factors of many metabolic activities. The chemical characteristics of mustard seeds such as oil, protein, carbohydrate and mineral contents depend on many factors like the nature of the plant, genetic factors, cultivars, agro-ecological conditions include cultivation sites and crop management systems, nitrogen fertilizer application, etc **(Sharifet *et al.*, 2017)**. According to the requirements for mustard seeds, maximum mass loss at 103 °C is 10% (m/m). The amounts of total ash and acid–insoluble ash were lower than the maximum values determined for mustard seeds (6.5 and 1% m/m on the dry basis, respectively). The total ash and acid– insoluble ash are important indicators for the quality and purity of herbal foods **(Sharif *et al.*, 2017)**. The results of this study were compatible with the results of the other researchers. **Abul-Fadl, El-Badry, and Ammar (2011)** reported that the range of protein content was between 32.48 to 36.37% and the oil content was between 31.78 to 36.32% in investigated brown and yellow mustard seeds.

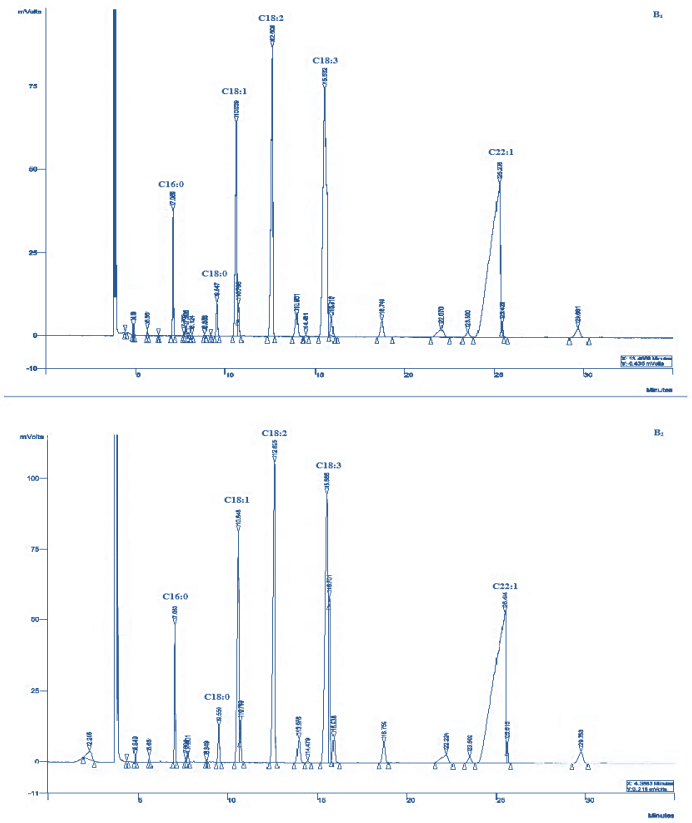

Figure 3 Gas chromatograms of Iranian Mustard seed oils (B1 and B2).

The specification of MSO

The gas chromatograms of mustard seed oil samples are presented in Fig. 1. According to Table 2, the types and amounts of fatty acids in the samples oil were similar. The oil samples were rich in unsaturated fatty acids (88.06% and 89.13%), and low contents of saturated fatty acids (10.87% and11.94%) were observed. The unsaturated fatty acids included palmitoleic acid (0.17% and 0.18%), oleic acid (9.28% and 9.38%), linoleic acid (14.12% and 14.56%), linolenic (24.35% and 24.57%) and erucic acids (39.03% and39.22%) while the saturated fatty acids were consisted of palmitic acid (2.93% and 3.7%), and stearic acid (1.22% and 1.28%). Total polyunsaturated fatty acid/total saturated fatty acid ratios were 3.59 and 3.22 in B1 and B2 samples, respectively. The acid values in mustard samples were in the range of 0.55 and 0.70 mg/g. The high PUFA/SFA ratio is an indication of unsaturation level and the high tendency of oils towards oxidative reactions. The findings of this study are similar to the results of other researchers **(Abul-Fadl *et al.*, 2011; Chowdhury *et al.*, 2014; Antova *et al.*, 2017)**. **Chowdhury *et al.* (2014)** reported that mustard oil contained 86.80±3.07% unsaturated fatty acids. According to **Antova et al., (2017)** the average amounts of saturated and unsaturated fatty acids in five accessions, Sinapis alba L. seeds were 9.32% and 90.68%, respectively. Factors such as variety, environment, and extraction technique can effect on the fatty acid compositions of seed oils of different origin. The FFA is the primary qualitative attribute for edible grade oil/fat. The Purified Food and Adulteration Act specify a maximum acceptable limit of FFA as 3% (oleic acid) for mustard oil. The peroxide value is considered as an indicator of the quality and stability of oils and fats. The results for both samples were in the range of 2.92 and 3.27 meqO2/kg and reflected the relatively good quality of the samples and were in accordance with the permissible limit mentioned in the AOCS. The induction periods of fresh mustard oil samples ranged from 4.12 to 4.32 hours. The oil is considered rancid at a peroxide value above10 meq/kg **(AOAC, 2010; Gunstone, 2008)**. PV and AV are associated with total hydroperoxide compounds and the rancid taste induced by hydrolysis of oils and edible fats. Hydroperoxides are the primary product of lipid oxidation, which, due to their low stability, are rapidly decomposed into secondary products like carbonyls. Carbonyl compounds are responsible for the well-known rancid tastes of oils and oxidized fats **(Endo *et al.*, 2001)**.

Table 2 Main fatty acids profile (%) of Iranian Mustard seed oils.

Fatty Acids	RT± 0.5 [a] (min)	Relative content (%)	
		B1	B2
Palmitic acid (C16:0)	7.063	3.07	2.93
Stearic acid (C18:0)	9.550	1.28	1.21
Oleic acid (C18:1)	10.648	9.38	9.28
Linoleic acid (C18:2)	12.625	14.55	14.12
Linolenic acid (C18:3)	15.555	24.56	24.35
Erucic acid (C22:1)	25.484	39.22	39.02

[a] RT: Retention Time

Saponification value (SV) represents the average molecular mass of the fatty acids in the oil. The lower saponification values suggest that the mean molecular weight of fatty acids or the number of ester bonds is low. The observed values support the reported values by **Khan *et al.*, (2013)** that saponification values of the extracted mustard oils were determined more than 170 **(Khan *et al.*, 2013)**.

Table 3 Some specification of Iranian Mustard seed oils (Mean ± SD) [a].

Specification	Results	
	Sample B1	Sample B2
Total Saturated FA (TSFA) (%)	10.87± 0.05	11.93± 0.04
Total Unsaturated FA (TUFA) (%)	89.13± 0.06	88.06± 0.06
Total Mono Unsaturated FA (TMUFA) (%)	50.00± 0.08	49.58± 0.02
Total Poly Unsaturated FA (TPUFA) (%)	39.13± 0.04	38.46 ± 0.06
TSFA/TUFA ratio	0.12±0.00	0.13±0.00
Linoleic acid/Oleic acid ratio	1.521	1.552
TPUFA/TMUFA ratio	0.78±0.00	0.77±0.00
TPUFA/TSFA ratio	3.59±0.01	3.22±0.00
Peroxide value(meq/Kg)	3.27± 0.05	2.92 ± 0.03
Acid value(mg/g)	0.70 ± 0.03	0.55± 0.02
Saponification value(mg/g)	176.19 ± 0.12	172.60 ± 0.13
Oxidative Stability Index(OSI)(h)	4.12 ± 0.02	4.32 ± 0.03

[a] Values presented are means of triplicate determinations ± standard deviation (SD)

The oxidative stability index (OSI) (determined by Rancimat test) is the required time for the development of measurable rancidity in oils and fats. Oxidative rancidity is due to oxidation of the double bond of fatty acid with the formation of aldehydes, ketones, and acid of lower molecular weight rather than the initially present fatty acid. The detected time to the secondary reaction products is called induction time. It characterizes the oxidation stability of oils and fats. The polyunsaturated oils are desirable for health, but are highly susceptible to oxidative deterioration. **Antova et al. (2017)** reported that the oxidative stability indices of

five accessions Sinapis alba L. seed oils were 14.8 to 25.1 hours, whereas Jham et al. (2009) reported the oxidative stability about 7 hours for wild Brazilian mustard (Brassica Juncea) seed **(Antova *et al.*, 2017; Jham *et al.*, 2009).**
The characteristics of the mustard seed oils were within the ranges prescribed in the international standards recommended for edible Brassica seed oils **(FAO and WHO, 2021)**.

CONCLUSION

Physicochemical properties and molecular specification of Iranian mustard seeds are unique. Based on the results, although two samples had many similarities in chemical properties, they were not the same in the molecular specification. Different patterns of PCR products were obtained using trnH-psbA primer for two samples and there was not 100% identity between the sequences of PCR products of samples and the other mustard reported in NCBI. The location of B1 and B2 sequences were unknown query in the phylogenic tree. The results of the blast displayed that there were the genetic distances not only between these two samples, but also between these samples and Brassica juncea. Therefore, they probably belonged to new species/subspecies of the Brassica spp. However, further research is needed on the taxonomy of Iranian mustard. Regarding the chemical composition of seeds, remarkable values of oil, the oil quality indicators such as the peroxide value, oxidation stability and the presence of unsaturated fatty acids such as oleic acid, linoleic acid, Iranian mustard seeds have the potential and ability to be used as the source of spice or vegetable oil. This work aimed to identification and presentation the seeds and seed oil of Iranian mustard in order to explore and promote its potential uses and consumption or its development and trade from limited local communities to international markets. Iranian mustard seed or its derivatives properties make it suitable to be used in food industry, the pharmaceutical industry and in cosmetics preparation. Therefore, according to the results of our research, further studies are recommended to investigate the possibility of using Iranian mustard in the food industry.

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