Introduction
Pomegranate (Punica granatum L., Lythraceae) is an important commercial horticultural fruit. Native to an area from Iran to India, it is also cultivated in Turkey, Afghanistan, Spain, Italy, France, Egypt, and further parts of North Africa, China, and the United States [1]. Iran is one of the main origin centers of pomegranate and is the largest producer in the world with an annual production of over than 800,000 tons [2–4]. Pomegranate is adapted to a wide range of climatic and soil conditions such as tropical and subtropical areas and temperate climate [2–4].

Pomegranate presents a remarkable ecotypic variation in fruit characteristics, including color, size, and taste. Even pomegranate trees with the same genotype, but growing in different geographical regions, show ecotypic variation [5–7]. Total anthocyanin, flavonoid, phenolic acid, and tannins contents also vary in different ecotypes of pomegranate. Pomegranate is rich in antioxidants, including phenolic acids, tannins, flavonols, and anthocyanins [8]. The potential of these naturally available phytonutrients has been investigated in the context of cardiovascular diseases [9], hypertension [10], many types of cancers [11–13], hyperlipidemia [14], inflammation [15], diabetes [16–18], Alzheimer’s disease [19], and ageing [20]. These compounds also showed antibacterial, antifungal [21], and antiviral [22, 23] properties. Pomegranate juice contains 3-fold more antioxidants than red wine and green tea [24] and can be used as a homemade remedy to treat dysentery and anemia [25].
Chemical characterization of organism ecotypes has attracted much attention in recent years. The metabolite profile of an organism or a tissue thereof can be characteristic for ecotypes growing in different geographical areas. Various techniques have been used for the comprehensive analysis of plant metabolites and, among them, NMR spectroscopy is particularly popular in the field of metabolome analysis [26]. The features of NMR spectroscopy, including non-destructivity, non-selectiveness and simple sample preparation, make it a method of choice for the profiling of a broad range of metabolites [27].

The objective of the present study was to investigate and compare the metabolic profile of pomegranate ecotypes that were grown in eight geographical origins of Iran (Fig. 1). The results of the present metabolomics analysis should help to characterize ecotypes that may be used either for cultivation or for the improvement of health-promoting pomegranates in reproduction programs. This information would also be valuable for food manufacturers and the fruit juice industry to select chemotypes which might have the most suitable characteristics for pomegranate-based products. Due to the high demand for pomegranate juice and other processed products, adulteration of pomegranate juice has become a major issue. Therefore, the metabolic profile revealed in this study could be used for quality control of industrial products and for preventing adulteration of pomegranate products.

In this study, the metabolic profile of pomegranate juice has been investigated by using 1D $^1$H-NMR spectroscopy associated with additional 2D NMR techniques. Multivariate statistical analyses, principal component analysis (PCA), and orthogonal partial least squares-discriminant analysis (OPLS-DA) were applied to reveal differences and ecotypic diversity among eight geographical origins of Iran.

Result and Discussion

Proton NMR analysis of various methanol pomegranate juice extracts dissolved in DMSO-$d_6$ provided spectroscopic metabolic profiles of different geographical origins. A typical $^1$H-NMR spectrum of a sample of pomegranate juice extract is shown in Fig. 2a. In $^1$H-NMR spectra, characteristic chemical shifts are associated with the occurrence of various types of metabolites, including amino and organic acids ($\delta$ 0–2.5) (Fig. 2b), glycosides ($\delta$ 2.5–5) (Fig. 2c), and phenolic compounds ($\delta$ 6.5–10.5) (Fig. 2d). The spectra were dominated by glucose, sucrose, and glycosides, but the most particular part of each $^1$H-NMR spectrum was in the $\delta$ 6.5–10.5 range, which corresponds to the aromatic rings of anthocyanins. 2D NMR techniques, such as HMBC and HSQC, were also used for resonance assignments. The $^1$H NMR chemical shifts of the identified metabolites are listed in Table 1. The amino acids valine and proline and glutamic acid were identified in pomegranate juice extracts by comparison of their $^1$H-NMR chemical shifts with literature data [28–30]. The HMBC correlation between H-2 and the carbon resonances at $\delta$ 171 and 174 confirmed the identity of valine and glutamic acid, respectively. A number of signals were assigned to organic acids such as succinic, fumaric, citric, malic, and tartaric acids. Citric acid is the predominant organic acid, and detailed analysis of 2D NMR (HSQC and HMBC) experiments confirmed the assignment. The $^1$H NMR signals at $\delta$ 2.64 (d, $J$ = 15.4 Hz) and $\delta$ 2.75 (d, $J$ = 15.4 Hz) and the corresponding $^1$C resonance at $\delta$ 43.2 were assigned to H-2 in the HSQC spectrum. This was further confirmed by the correlation between H-2 and the carbons signals at $\delta$ 172.5, 178.2 (carbonyl groups), 74.8 (carbon atom carrying the hydroxy group), and 43.2 (methylene groups) in the HMBC spectrum. Succinic acid and malic acid were successfully identified by 2D NMR spectra.

Proton resonances of free sugars and the glycosidic part, anthocyanins, were observed in the region between $\delta$ 3.00 and 5.50. The anomeric protons of $\beta$-glucose at $\delta$ 4.34 (d, $J$ = 7.6 Hz), $\alpha$-glucose at $\delta$ 4.98 (d, $J$ = 3.3 Hz), fructose at $\delta$ 3.88 (d, $J$ = 3.1 Hz), and sucrose at $\delta$ 5.26 (d, $J$ = 3.5 Hz) were identified in this region.

Proton resonances of phenolic compounds including gallic acid, ellagic acid, protocatechuic acid, and catechin were identified in the aromatic region (Fig. 2d). The $^1$H-NMR chemical shift of gallic acid was observed at $\delta$ 7.04 (s) while a sharp singlet signal at $\delta$ 7.47 was assigned to ellagic acid. Catechin and the tannins $\alpha$- and $\beta$-punicalagins were characterized using 2D NMR spectra and the comparison of their $^1$H-NMR chemical shifts with those reported in the literature [31]. Polyphenolic compounds are a major class of phytochemicals that are extractable from almost all parts of the pomegranate tree. Among polyphenols, anthocyanins are particularly abundant in fruits [24].

Pomegranate juice is an important source of anthocyanins. The main anthocyanins in pomegranate fruits include 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin [32]. Due to the positive charge, the signal of H-4 (C ring) is observed in the downfield region of the NMR spectrum.
din, cyanidin, and pelargonidin 3,5-diglucosides were identified by comparison of their $^1$H-NMR chemical shifts with those reported in previous studies [33–35].

PCA as the unsupervised method is one of the most common multivariate data analysis methods and is applied to reduce the dimensionality of a multivariate data set [36]. Therefore, it enables the indication the relationships between samples in a multidimensional space. In this study, the variables were the integrated NMR spectral regions (bucket table). The PCA score plot of all pomegranate juice extracts clearly separated Mazandaran samples from other groups on the first two components (Fig. 3). However, the other groups were not well discriminated from each other by this analysis method.

Mazandaran samples were clustered on the negative side of PC1 and the positive side of PC2 while the other groups were distributed in the center and the remaining three parts of the PCA score plot.

The application of OPLS-DA as the supervised method is considered to be the next step in multivariate statistical analyses. This multivariate statistical analysis has better classification efficiency than the PCA model through system noise filtering and the extraction of variable information. The $^1$H-NMR data set from the hot desert climate of central Iran (Yazd and Tabas pomegranate) and the wet region South of the Caspian Sea (Mazandaran) were first subjected to the OPLS-DA model. The resulting score plot (Fig. 4a) of the OPLS-DA model showed clear discrimination and differences in metabolic ecotypes between the pomegranate juice samples from the two climatically different geographical origins. The resulting values of R2Y(cum) and Q2(cum) of 0.966 and 0.885, respectively, gave an indication of good fitness and predictability of the OPLS-DA model. The loading plot (Fig. 4b) was useful to identify the spectral signals responsible for the clustering and discrimination among the samples. The assignments of these signals led to the identification of compounds accountable for the separation on the score plot.

The Mazandaran sample corresponds to a wild type of pomegranate that grows in the forests of Mazandaran and is considered by the local population as a natural immune system booster. The major organic acids in pomegranate fruit are citric and malic acids. Mazandaran samples have a sour taste and the highest citric acid content. Other pomegranate samples did not possess the same NMR profile. By examining the loading plot (Fig. 4b), it was evident that Mazandaran extracts show high signal intensities for citric acid, while Tabas and Yazd pomegranate juice were characterized by higher intensities for malic acid. Similar findings regarding the correlation between sourness and citric acid content, and between sweetness and malic acid content in samples originating from diverse countries have been previously reported [37–41]. The content of succinic acid was relatively higher in Mazandaran samples than in that of Yazd and Tabas pomegranates. The obtained OPLS-DA score and loading plots from the comparison of metabolic profile belonging to Mazandaran and Shiraz pomegranates revealed similar results (Figs. 1S and 2S, Supporting Information).

$^1$H-NMR data sets related to the pomegranate extracts from two geographic regions of the East (Bajestan, Kashmar, Ferdows...
Table 1 ¹H-NMR chemical shifts of the assigned compounds from the pomegranate juice extracts.

<table>
<thead>
<tr>
<th>Identified compounds</th>
<th>δ_H</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Glucose</td>
<td>4.98 (d, J = 3.3)</td>
</tr>
<tr>
<td>β-Glucose</td>
<td>4.34 (d, J = 7.6), 2.98 (dd, J = 16.4, 8.3)</td>
</tr>
<tr>
<td>Citric acid</td>
<td>2.64 (d, J = 15.4), 2.75 (d, J = 15.4)</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>2.11 (s)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>7.04 (s)</td>
</tr>
<tr>
<td>Malic acid</td>
<td>2.48 (dd, J = 15.6, 7.1), 2.63 (dd J = 15.6, 4.1)</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>4.30 (s)</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>7.47 (s)</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>6.18 (s)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5.26 (d, J = 3.5)</td>
</tr>
<tr>
<td>Fructose</td>
<td>3.88 (d, J = 3.1)</td>
</tr>
<tr>
<td>α-Punicalagin</td>
<td>7.21 (s), 7.01 (s), 6.88 (s)</td>
</tr>
<tr>
<td>β-Punicalagin</td>
<td>7.24 (s), 7.05 (s), 6.92 (s)</td>
</tr>
<tr>
<td>Pelargonidin-3,5-di-O-glucoside</td>
<td>8.93 (s), 8.14 (d), 6.99 (s), 6.96 (s)</td>
</tr>
<tr>
<td>Delphinidin-3-O-glucoside</td>
<td>8.95 (s), 7.91 (s), 6.88 (d, J = 1.5), 6.71(d, J = 1.5)</td>
</tr>
<tr>
<td>Delphinidin-3,5-di-O-glucoside</td>
<td>8.57 (s), 7.09 (s), 6.81(s), 6.62 (s)</td>
</tr>
<tr>
<td>Cyanidin-3,5-di-O-glucoside</td>
<td>9.25 (s), 8.85 (d, J = 7.8), 8.10 (d, J = 2.0), 6.91(s)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>6.22 (s), 6.40 (s), 7.41 (d, J = 8.4)</td>
</tr>
<tr>
<td>Valine</td>
<td>1.05 (d, J = 7.0), 1.1 (d, J = 7.0), 2.19 (m), 3.59</td>
</tr>
<tr>
<td>Catechin</td>
<td>2.68 (m), 4.02 (m), 5.63 (d, J = 2.3), 5.66 (d, J = 2.3), 6.71 (J = 8.1)</td>
</tr>
<tr>
<td>Proline</td>
<td>1.96 (m), 2.30 (m), 4.10 (m)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.01 (m), 2.32 (m), 3.65</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>6.94 (d, J = 7.0), 7.23 (dd, J = 8.1, 2.0)</td>
</tr>
</tbody>
</table>

Fig. 3 Score plot (PC1 vs. PC2) of PCA results obtained from all pomegranate juice extracts. Group 1: Mazandaran (M), Group 2: Kashmar (K), Group 3: Bajestan (B), Group 4: Ferdows (F), Group 5: Tabas (T), Group 6: Shiraz (Sh), Group 7: Yazd (Y), and Group 8: Paveh (P).
from Khorasan Razavi and South Khorasan provinces) and center of Iran (Yazd) were subjected to OPLS-DA to highlight the differences and similarities among the samples. The OPLS-DA score plot (Fig. 3S A, Supporting Information) showed very tight clustering among the pomegranate juice extracts of Khorasan provinces that were separated from Yazd pomegranate samples. The overall appearance of the NMR spectra related to these origins showed a difference not so much in the absence or presence of certain metabolites but rather in the levels of the metabolites. Thus, the corresponding loading plot (Fig. 3S B, Supporting Information) showed significant contents of glucose, fructose, and anthocyanin glycosides and a slight increase in amino acids in Khorasan pomegranates compared with the Yazd pomegranate. In addition, Khorasan pomegranate extracts showed higher signal intensities for malic acid (Fig. 3S B, Supporting Information) than pomegranate samples from central Iran (Yazd).

The discriminant analysis was carried out between two other geographical origins with different climates from mountainous areas in the West of Iran (Paveh pomegranate from Kermanshah province) and semiarid regions in the East of Iran (Khorasan provinces). These two origins were not significantly separated from each other in the score plot of the OPLS-DA model (Fig. 4S, Supporting Information).

A significant variation in total phenolic content was found among the pomegranate ecotypes. The differences in phenolic compounds could be due to genetic diversity [42], environmental
conditions, and geographical origins [43]. In this study, it was possible to discriminate the different geographical origins using only the aromatic part (δ 6.5–10.5) of the NMR spectra. A good separation was observed between Mazandaran samples and Yazd pomegranate juice, which is shown in the score plot of the OPLS-DA model (Fig. 5S, Supporting Information). By investigating the loading plot, it was clear that Yazd pomegranate showed high signal intensities for ellagic acid and had a higher content of anthocyanins than other pomegranate samples (Fig. 5).

The amount of cyanidin was measured on the basis of the mean peak areas of the characteristic signal of this compound at δ 9.24 (Fig. 6S, Supporting Information). The obtained results showed that Mazandaran pomegranate contained the lowest anthocyanin content, while Bajestan, Ferdows, and Yazd had the highest amount of cyanidin-3,5-di-glucoside.

A detailed investigation about the three pomegranate origins of East of Iran, including Kashmar, Bajestan (from Khorasan Razavi province), and Ferdows (from South Khorasan province), revealed the higher content of phenolic compounds in Bajestan and Ferdows, two connected geographical origins with relatively similar climates, than that of Kashmar. The 1H-NMR profile of the aromatic region plays a key role for the separation of Kashmar pomegranate from the two others. The amino acid and sugar regions of the 1H-NMR spectrum were similar to one another and not responsible for the discrimination of Bajestan, Kashmar, and Ferdows samples. The score and loading plots of this analysis are shown in Fig. 7S, Supporting Information. These ecotypes with a high content of polyphenolic compounds may be considered for the preparation of food supplements.

In conclusion, our NMR-based metabolomics approach proved to be a good and reliable method for the ecotype discrimination of pomegranate from different geographical origins. With the use of different multivariate data analyses, such as PCA and OPLS-DA, associated to 1H-NMR, some ecotypic differences among the different origins have been detected. The different ecotypes of pomegranate mainly differ in their sugar profile, along with significant fluctuations in organic acid content and phenolic profile. Pomegranates of Khorasan provinces (Kashmar, Bajestan, and Ferdows) differ in the metabolic profile of the phenolic compounds. Among these, the concentration of phenolic compounds was higher in Bajestan and Ferdows pomegranate juice extracts than in those of Kashmar. Overall, Bajestan, Ferdows, and Yazd pomegranates had a higher level of phenolic compounds, including anthocyanin and ellagic acid derivatives, than those of other pomegranate samples. Mazandaran samples were clearly different from the other pomegranate ecotypes due to the higher content of citric acid and succinic acid.

Materials and Methods

Solvents and standard
Dimethyl sulfoxide (DMSO2) (purity 99.0%) and DMSO-d6 were purchased from Sigma. Methanol (analytical grade) was purchased from Daejung.

Pomegranate ecotypes
Fruits of the pomegranate ecotypes Bajestan and Kashmar (from Khorasan Razavi province, East of Iran), Tabas and Ferdows (from South Khorasan province, East of Iran), Taft, Meybod, Bahabad, Mehriz, and Ashkezar (from Yazd, center of Iran), Shiraz (from South of Iran), Mazandaran (from Mazandaran, North of Iran, a wild type of pomegranate), and Paveh (from Kermanshah, West of Iran) were collected in 2017 during the last 10 days of September and the first 2 weeks of October. Voucher specimens (Mazandaran No. 13263 a), Paveh (No. 13263 b), Shiraz (No. 13263 c),
Yazd (No. 13263 d), Tabas (No. 13263 e), Kashmar (No. 13263 f), Bajestan (No. 13263 g) and Ferdows (No. 13263 h)); were identified by Prof. Emami and Ms. Souzani (Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Sciences) and were deposited at the Herbarium of the Mashhad University of Medical Sciences, School of Pharmacy, Mashhad, Iran. These samples are representative of the main pomegranate cultivars in Iran. For each sample, 1 kg of pomegranates (at least 3 samples from each geographical region) at the maturity stage was collected. The geographical origin of the collected ecotypes is shown in Fig. 1.

Preparation of raw pomegranate juice
Following peeling, the edible part of the pomegranate was manually obtained from whole fruits. The fruit juice was obtained by mixing the edible fruit part in a household blender. The whole content was then filtered to remove the seeds and the raw pomegranate juice was freeze-dried. The resulting powder was kept at −21°C until analysis.

Extraction and sample preparation for NMR analysis
Samples for NMR analysis were prepared by the addition of 5 mL of methanol to 0.5 g of freeze-dried powder. The mixture was stirred at room temperature for 60 min and filtered to remove the insoluble residue. After evaporation of the methanol, the filtrate was freeze-dried again. Next, 140 mg were dissolved in 400 microliters of DMSO-d_6 and 5 mg of DMSO-d_2 (dimethyl sulfone as a reference) were added to each sample. For NMR analysis, the samples were transferred to 5 mm NMR tubes.

NMR spectroscopy

1H-NMR spectra were recorded at 25°C on a 300-MHz Bruker AVANCE III-300 spectrometer operating at a proton NMR frequency of 300.81 MHz. DMSO-d_6 was used as the internal lock. Each 1H-NMR spectrum consisted of 512 scans with a spectral width of 6000 Hz, an acquisition time of 5.45 s, and a relaxation delay of 1 s per scan. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the H_2O frequency during the recycle delay. The pulse angle and the pulse width were 50° and 15°, respectively. The spectra were Fourier transformed after multiplying the FIDs by an exponential weighting function corresponding to a line broadening of 0.3 Hz.

Data analysis and statistics
The preprocessing of spectra, including normalization, alignment and bucketing, was carried out using MesReNova (version 12.0.2-20910). The calibration of the spectra was carried out with the internal standard (DMSO-d_2) peak at δ 3.02. Spectral intensities were normalized to the internal standard peak (DMSO-d_2) by applying the probability quotient normalization (PQN) algorithm. This method is based on the calculation of a most probable dilution factor by looking at the distribution of the quotients of the amplitudes of a test spectrum by those of a reference spectrum [44]. The NMR spectra were binned using a uniform bucketing method with an equal width of 0.04 ppm between the regions of δ 0.5–10.5. The matrix size contained 229 variables. The exported data were subsequently mean-centered and Pareto-scaled prior to multivariate statistical analysis to enhance the contribution from medium-sized features without inflating the noise from quiet areas of the spectrum [45]. PCA and OPLS-DA with scaling based on Pareto were performed with SIMCA software (version 14.1, Umetrics).

Supporting information
OPLS-DA score plot and loading column plot related to the discrimination of juice samples between Mazandaran and Shiraz pomegranates are described in Figs. 15 and 25. Tight clustering among the pomegranate juice extracts of Khorasan provinces is shown in Fig. 35. OPLS-DA score plot of pomegranate juice extracts from the mountainous areas in the West of Iran (Paveh pomegranate from Kermanshah) and semiarid regions in the East of Iran (Khorasan provinces) is available in Fig. 45. The discrimination of the aromatic region (δ 6.5–10.5) between Yazd and Mazandaran pomegranate juice extracts is shown in Fig. 55. Relative quantification of cyanidin-3,5-diglucoside based on the mean peak area of the signal at δ 9.24 is presented in Fig. 65.

Acknowledgements
This work was supported by grants from the Mashhad University of Medical Sciences Research Council (951316) and the National Institute for Medical Research Development (965403).

Conflict of Interest
The authors declare that they have no conflict of interest.

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