The Journal of Medicinal Plants and By-Products

Original Article

Phytochemical Analysis of Normal and Natural Mutant Types of *Juniperus polycarpos*

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Article History: Received: 26 October 2024/Accepted in revised form: 24 November 2024

ABSTRACT

After conducting a field survey and observing different phenotypes in natural habitats, we investigated the composition of hydro-distilled essential oils from leaves of two distinct phenotypes of wild-growing *Juniperus polycarpos*. These specimens were collected from the Zagros Mountains range in Fars Province, Iran, and analyzed utilizing Gas Chromatography-Mass Spectrometry (GC-MS). The essential oil content in the standard and naturally mutated phenotypes of *J. polycarpos* ranged between 1.18 and 0.97% v/w, respectively. Phytochemical analysis of the extracted oils revealed significant differences in the essential oil quality of the two phenotypes of *J. polycarpos*. The main compounds identified in the studied types included α-pinene, α-cedrene, β-cedrene, γ-cadinene, δ-cadinene, and cedrol. The observed variation in the essential oil profiles is attributed to differences in constituent proportions and the presence of unique components. Concerning the main constituents, the essential oils of *J. polycarpos* types were categorized into two chemotypes: I) α-pinene in the typical type and II) α-pinene-cedrol in the mutant type. Due to similarities in climatic conditions, the observed variations are attributed to genetic differences induced by natural mutation. Considering the reported results, the wild populations of *J. polycarpos* can be suggested as valuable sources of α-pinene and cedrol.

Keywords: Cedrol, Chemotype, Essential oil, Monoterpene hydrocarbons, Natural mutant

INTRODUCTION

The *Juniperus* genus, belonging to the Cupressaceae family, is divided into three groups: *Juniperus, Caryocedrus*, and *Sabina* [1]. More than 60 species are reported within the *Juniperus* genus, which are widespread in the northern hemisphere [2]. Essential oil is the main extracted phytochemical compound from the leaves of *Juniperus* species, which is widely used in various pharmaceutical, food, and cosmetic industries due to its various medicinal and biological effects [3]. The essential oils of juniper leaves are a rich source of monoterpenes such as α-pinene, sabinene, limonene, camphor, and sesquiterpenes like cedrol, caryophyllene, elemol, cadinenes, and muurolenes [4- 6]. Studies have demonstrated that *Juniperus* essential oils exhibit antioxidant, antibacterial, antifungal, anticancer, anti-inflammatory, and herbicidal properties [7- 9].

Juniperus polycarpos is a native juniper species of Iran, naturally dispersed from northern to southern Iran, especially in the Alborz and Zagros mountain ranges [10]. As a dioecious evergreen shrub, it is one of the rare conifers that typically grows in regions of Iran at elevations of 1500 to 2500 m above sea level [11]. This species has a comprehensive range of local applications, including herbal medicine, food additives, and raw materials for cosmetic applications [12, 13]. Adams confirmed, based on four DNA regions, that samples from the northwest, northeast, and southwest of Iran are *J. polycarpos* [2, 14]. Hojjati reported that the species of *J. polycarpos* is poorly distinguished by morphological traits due to its phenotypic diversity [10]. The essential oil composition of *J. polycarpos* leaves has been previously studied. In the northeast of Iran, α-pinene (51.21%), germacrene–B (4.80%), and δ-cadinene (2.56%) were identified as the main constituents [15]. In the same region, Moghaddam also reported α-pinene (41.8–75.4%), germacrene-B (1–5.8%), camphor (0.7–4.5%), and α-cadinol (0.4–4.8%) as predominant compounds [16]. In the northwest of Iran, the main compounds in the male and female leaf oils of *J. polycarpos* were *α*-pinene (59.90-32.72%), β-pinene (0-15.83%), 1,4-cineol (6.79-6.50%) and limonene (9.73-7.02%) [17]. Cedrol was not found in the essential oil of the leaves in these samples, but Adams classified oils from southern Iran into two groups: high cedrol (23.6%) and low cedrol (0-0.2%) [4]. The reported variations

in the occurrence and concentrations of oil compositions of juniper are common [10] and may be due to differences in geographical conditions, genetic status, sampling time, and age of plants, as well as harvesting and extraction processes [12, 18]. In this study, we report on the phytochemical characteristics of two phenotypes of *J. polycarpos* populations in the Zagros mountain range in Fars province, Iran, including a unique morphological mutant. While the essential oil quantity and quality of different populations of *J. polycarpos* from various parts of Iran have been previously reported [4, 15- 17], the present study is the first to report on the oil composition of a natural mutant type of *J. polycarpos* in the Zagros mountains, Iran, which significantly differed from its normal type in terms of phenotype and phytochemical traits.

MATERIAL AND METHODS

Plant Materials and Collection Site

Sepidan County in the Zagros mountain range, Fars province, Iran, is a natural habitat of *Juniperus polycarpos* covering approximately 700 ha of this species. Within the wild-growing *J. polycarpos* population in Sepidan County, there are some trees of this species that are phenotypically different from their natural samples, considered mutant types (Fig. 1). The present study was aimed to assess the essential oil content and composition of the observed mutant types of *J. polycarpos* and compare them with the phytochemical profile of usual types. The *polycarpos* species in the Sepidan region were previously identified by molecular analysis based on four DNA regions [2, 14]. The voucher specimens (voucher number of typical type: 55142 and mutant type: 55143) are deposited in the herbarium of the Research Center for Plants Sciences at the University of Shiraz, Fars province, Iran.

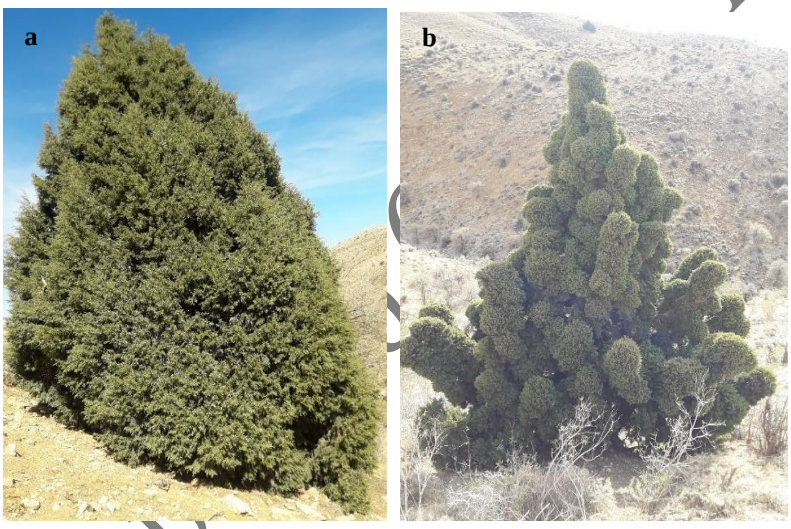

Fig. 1 The normal (a) and mutant (b) types of *J. polycarpos* in Sepidan County, Iran

The studied site is located at an altitude of 2183-2830 m above sea level with slope degrees from 0 to 73°. According to data from the Meteorological Organization, the studied area has a moderate climate with temperatures averaging 12-13°C and precipitation ranging from 500-550 mm. The physio-chemical analysis of 60 soil samples from the area revealed that the soil of the studied area is sandy-clay with a pH of 7.42-7.96, electrical conductivity 0.14-0.94 dS/cm, and organic matter content of 0.18-9.74%.

To compare the phytochemical properties of *J. polycarpos*, leaf samples from both normal and mutant types were collected in early October 2020 from the middle shoots *and* transferred to the laboratory in paper bags to dry at room temperature and extract essential oil.

Isolation of the Essential Oils

For essential oil extraction, the collected dry leaves of *J. polycarpos* were subjected to hydrodistillation for four hours using a Clevenger–type apparatus. The extracted essential oil was collected and dried using anhydrous sodium sulfate, then stored in sealed dark glass at 4ºC until chemical analysis [19].

Gas Chromatography and Mass Spectrometry (GC/MS)

The GC/MS was used to analyze the essential oils of *J. polycarpos.* The analysis was performed using a gas chromatograph (Agilent Technologies 7890 A) coupled to a mass selective detector (Agilent 5975 C) and quadruple EI mass analyzer (Agilent Technologies, Palo Alto, CA, USA). An HP–5MS column with dimensions of 30 m length × 0.25 mm ID and 0.25 µm film thickness was used as the stationary phase. The temperature was set from 60 to 210ºC with 3ºC increasing per min then raised to 240ºC with 20ºC/min rate and maintained at 240ºC for 8.5 min. The temperatures of both the GC/MS interface and injector were kept at 280ºC. The mass spectra were recorded at 70 eV a with range of 50-480 *m/z*. The ion source temperature was 230, and the temperature of the detector was maintained at 150ºC. The used carrier gas was helium with a flow rate of 1 mL/min. The ratio of split injection was 1:50. 0.1 µL of the essential oil samples were directly injected.

Identification of the Components

Identification of the components in the essential oils was done by comparing their retention index (RI) with reported RIs of known constituents in the literature, as well as comparing the mass spectra with those recorded in NIST 08 (National Institute of Standards and Technology) and Willey (Chem Station data system) [20]. The peak area of each constituent was quantified based on generated curves of standard compounds used for calibration.

Statistical Analysis

Comparing the essential oil content and composition of the two types of *J. polycarpos* was conducted based on a randomized complete block design with three replications using SPSS software (version 16).

RESULTS AND DISCUSSION

Ontogenetic and genetic variations and ecological conditions are considered the main factors affecting morphological and phytochemical traits of junipers [21- 23]. In this study, the essential oil content obtained by hydro-distillation varied significantly between normal and natural mutant types of *J. polycarpos*. The extracted essential oil of the normal type was 1.18% v/w, higher than that of the mutant type, which was 0.97% v/w, indicating a 21.65% difference between the two types of *J. polycarpos* (Table 1). Photosynthesis capacity, structure of oil glands, type, and intensity of biotic and abiotic stresses influence the biosynthesis pathways of terpenoids. These characteristics that determine the content of accumulated essential oils are closely affected by environmental conditions, genetic factors, and their interaction effects [24- 26].

The components identified through GC/MS analyses and the content of the individual constituents are reported in Table 1, based on their elution order on an HP–5MS column. In the normal type, volatile constituents make up 99.50% of the total oil composition, while in the mutant sample, this value was 98.524% of the total essential oil content. The results indicated that the essential oils of the mutant type of *J. polycarpos* were simpler than those of the normal type. Additionally, the number of compounds in the essential oils of normal and mutant types was 50 and 47, respectively. Significant differences were observed in the quantity and quality of the identified constituents in the two types of *J. polycarpos*. A comparison of essential oils from normal and mutant types revealed differences in the occurrence and concentration of various constituents. The results in Table 1 showed that α-pinene was a common compound in the essential oils of both samples studied. The content of the identified α-pinene, as the main constituent in the extracted essential oils from the leaves of the *J. polycarpos* typical type, was 46.68%. The essential oils of the mutant type mainly consisted of α-pinene (33.21%) and cedrol (26.50%). The extracted essential oil from the normal type of *J. polycarpos* had a low cedrol content (0.56%).

Therefore, two chemotypes were observed in the studied essential oils of *J. polycarpos* I) α-pinene in the normal type and II) α-pinene-cedrol in the mutant type. In addition to α-pinene and cedrol, γ-cadinene (2.36-4.45%), δ-cadinene (1.79-5.07%), α-cedrene (0.37-7.45%) and β-cedrene (0-5.60%) were the other main identified components in the studied essential oil samples.

The essential oils of *J. polycarpos* have been studied by several researchers. There was a similarity in the essential oil composition primarily in the main compounds of *J. polycarpos* from Iran. Mehdizadeh, Moghaddam, and Khani reported that α-pinene was the main component of leaf essential oil of *J. polycarpos* in different values [15, 16, 27]. Adams found that the leaves of *polycarpos* species of *Juniperus* in southern Iran were chiefly composed of α-pinene (48.7-62.5%) and cedrol (23.6%) [4]. α-Pinene, in a range of 2.71–17.31% was reported as the main component of leaf essential oils of different species of Juniperus from the Tibet Plateau [6].

Table 1 Qualitative and quantitative composition of essential oils extracted from normal and mutant types of *J. polycarpos* in Sepidan County, Iran*

NO.	RI	Components	Normal type	Mutant type
1	850	(Z)-3-Hexenol	-	0.23 ± 0.18
2	922	Tricyclene	0.48 ± 0.24	0.34 ± 0.17
3	933	α-Pinene	46.68 ± 1.42	33.21 ± 0.71
4	948	Camphene	1.55 ± 0.38	0.97 ± 0.12
5	953	Thuja-2,4(10)-diene	0.17 ± 0.09	0.03 ± 0.02
6	971	Sabinene	0.01 ± 0.07	0.01 ± 0.08
7	977	β-Pinene	1.23 ± 0.24	0.99 ± 0.17
8	990	Myrcene	2.80 ± 0.14	2.45 ± 0.39
9	1006	α-Phellandrene	0.11 ± 0.21	0.05 ± 0.01
10	1010	ρ-Mentha-1(7),8-diene	2.71 ± 0.41	-
11	1016	α-Terpinene	0.10 ±0.07	0.08 ± 0.02
12	1024	ρ-Cymene	0.74 ±0.10	0.34 ± 0.12
13	1028	Limonene	2.73 ± 0.18	2.63 ± 0.22
14	1030	1,8-Cineole	0.01 ±0.10	0.01 ± 0.02
15	1036	(Z)-β-Ocimene	0.08 ±0.08	0.06 ± 0.04
16	1046	(E)-β-Ocimene	0.02 ± 0.11	0.01 ± 0.01
17	1057	γ-Terpinene	0.71 ± 0.17	0.83 ± 0.14
18	1088	Terpinolene	2.27 ±0.20	1.33 ± 0.25
19	1099	Linalool	0.71 ± 0.33	0.39 ± 0.11
20	1105	Isopentyl isovalerate	0.11 ± 0.15	0.03 ± 0.01
21	1113	endo-Fenchol	0.06 ± 0.02	-
22	1125	α-Campholenal	0.08 ± 0.05	-
23	1138	*trans*-Pinocarveol	0.15 ± 0.19	-
24	1143	Camphor	0.92 ±0.26	0.32 ± 0.10
25	1164	Borneol	0.23 ± 0.21	-
26	1190	α-Terpineol	0.12 ± 0.14	0.07 ± 0.01
27	1205	Verbenone	0.13 ± 0.18	-
28	1241	Hexyl isovalerate	0.52 ± 0.34	0.13 ± 0.06
29	1285	Bornyl acetate	1.35 ± 0.20	0.11 ± 0.07
30	1337	δ-Elemene	0.55 ± 0.29	0.21 ± 0.11
31	1349	α-Cubebene	0.41 ± 0.21	0.08 ± 0.02
32	1375	α-Copaene	0.85 ± 0.19	0.20 ± 0.12
33	1387	Hexyl hexanoate	0.84 ± 0.24	0.31 ± 0.16
34	1391	β-Elemene	0.75 ± 0.18	0.41 ± 0.23

Adams reported two cedrol chemotypes in the population of *J. polycarpos* from Fasa, Iran: chemotype I with low cedrol content (0.0-0.1%) and chemotype II with a high amount of cedrol (10.1-13.8%). Moreover, only trace amounts of cedrol in *J. polycarpos* from Turkmenistan were reported [11].

The literature survey showed unidentified compounds in some studies, reported as the main compounds by others [4]. As observed in the present study, α-pinene was the main component in the essential oils of both studied types of *J. polycarpos*. However, in the mutant type, in addition to α-pinene, cedrol was another major constituent of essential oil, which was a minor compound in the normal type (Table 1). The leaf essential oils of *J. polycarpos* in the two normal and mutant types displayed differences in their compositions, as shown in Table 1. The observed changes in the essential oil composition of the two *J. polycarpos* types were due to the relative proportions of constituents and the presence of new constituents.

In addition to the differences in the main compounds of normal and mutant types that led to the identification of the α-pinene chemotype in the normal type and α-pinene-cedrol chemotype in the mutant type, there were significant variations in the quantity and quality of the identified minor constituents in the essential oils of the studied samples. These differences have a direct impact on the quality of the obtained essential oils.

Table 1 (Cont.)

NO.	RI	Components	Normal type	Mutant type
35	1410	α-Cedrene	0.37 ± 0.19	7.45 ± 0.69
36	1418	(E)-Caryophyllene	1.84 ± 0.12	-
37	1420	β-Cedrene	-	5.60 ± 0.51
38	1428	*cis*-Thujopsene	0.26 ± 0.20	0.82 ± 0.11
39	1433	δ-Elemene	2.59 ± 0.27	1.01 ± 0.07
40	1457	(E)-β-Farnesene	0.14 ± 0.21	0.57 ± 0.19
41	1466	α-Acoradiene	-	0.28 ± 0.18
42	1480	Germacrene D	1.28 ± 0.33	0.58 ± 0.19
43	1494	δ-Selinene	1.56 ± 0.20	-
44	1499	α-Muurolene	1.30 ± 0.35	0.47 ± 0.23
45	1506	β-Bisabolene	-	0.49 ± 0.28
46	1514	γ-Cadinene	4.45 ± 0.39	2.36 ± 0.34
47	1523	δ-Cadinene	5.07 ± 0.30	1.79 ± 0.10
48	1537	α-Cadinene	1.70 ± 0.45	0.41 ± 0.09
49	1549	Elemol	1.60 ± 0.26	0.47 ± 0.21
50	1556	Germacrene B	1.56 ± 0.33	0.64 ± 0.41
51	1574	Germacrene D-4-ol	0.67 ± 0.34	0.36 ± 0.31
52	1583	Caryophyllene oxide	-	1.28 ± 0.28
53	1599	Cedrol	0.56 ± 0.28	26.50 ± 1.32
54	1640	epi-α-Cadinol	2.39 ± 0.49	0.83 ± 0.28
55	1654	α-Cadinol	2.01 ± 0.13	0.82 ± 0.23
		Total	99.50	98.52
		Oil content (%v/w)	1.18 ± 0.37	0.97 ± 0.23

* (Data are mean ± SE

Besides α-pinene, in the analysis of the extracted essential oils from the normal type of *J. polycarpos*, γ-cadinene (4.45%) and δ-cadinene (5.07%) were characterized in high amounts compared to the other constituents. However, these compounds were in low values (2.36 and 1.79%, respectively) in the essential oils of the mutant type. On the other hand, α-cedrene and β-cedrene in the essential oil of the mutant type were 7.45 and 5.60%, respectively, but they were in low (0.37%) and unidentified amounts in the typical type.

The occurrence of ρ-mentha-1(7),8-diene (2.71%), endo-fenchol (0.06%), α-campholenal (0.08%), *trans*-pinocarveol (0.15%), borneol (0.23%), verbenone (0.13%), (e)-caryophyllene (1.84%), and δ-selinene (1.56%) was only observed in the essential oils of the normal type. However, β-cedrene (5.60%), α-acoradiene (0.28%), β-bisabolene (0.49%), and caryophyllene oxide (1.28%) were identified in the extracted essential oils of the mutant type of *J. polycarpos*. Comparing the identified constituents in the leaf essential oils of the two different types of *J. polycarpos* confirmed considerable differences in their essential oil quality. Significant variations in the essential oil composition from each *J. polycarpos* population were reported by Adams [4]. These variations can be related to differences in the climatic conditions of the collected regions of leaf samples of *J. polycarpos*, harvesting time, procedure of extraction with regard to the apparatus used, plant materials, type of solvent, and extraction time [24, 25, 28]. The expression of genes involved in the biosynthesis of monoterpenes through the methyl erythritol phosphate and sesquiterpenes by mevalonic acid pathways due to environmental and genetic factors may differ between and within conifer species [29]. The observed variation in essential oil content and quality between the two *J. polycarpos* samples can be related to differences in gene expression of enzymes and intermediates involved in the synthesis pathway of essential oils in glandular trichomes [26]. In the present study due to stable geographical and edaphic conditions for both *J. polycarpos* types, the observed variations can be attributed to genetic differences. Also, the variation in the terpenoid profile of conifers in the samples of a plant population might be due to hybridization or naturally induced mutation [30, 31].

In the overall terpene profile, the essential oils of the two studied types varied considerably in the amount of different monoterpene and sesquiterpene compounds (Fig. 2).

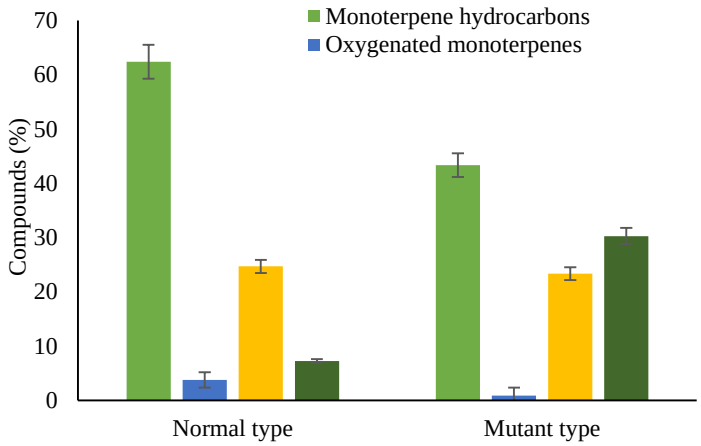

Fig. 2 The variations of different monoterpene and sesquiterpene compounds in the essential oils of normal and mutant types of *J. polycarpos* in Sepidan County, Iran

The values of monoterpene hydrocarbons in both normal and mutant types were 62.38% and 43.33%, respectively. These values were represented by α-pinene, myrcene, limonene, and terpinolene as the main component groups of essential oils. The concentrations of these compounds in the normal type were 43.96% higher than those in the natural mutant type. The sesquiterpene hydrocarbons contents were 24.67% and 23.35%, respectively, in the normal types of *J. polycarpos*, and were found to be similar. δ-Cadinene and γ-cadinene were the main sesquiterpene hydrocarbons in the essential oils of the normal type, while α-cedrene and β-cedrene, as sesquiterpene hydrocarbons, were identified in the highest amount in the mutant type. The amounts of oxygenated compounds, especially sesquiterpenes considerably differed between the normal and mutant types. Oxygenated monoterpenes, represented by linalool, camphor, and bornyl acetate, in the essential oils of the normal type, were about four times higher than those in the mutant type. The differences in essential oil quality of the studied samples were more pronounced regarding oxygenated sesquiterpenes represented by cedrol, leading to the categorization of essential oils of normal and mutant types into two chemotypes. The content of these compounds in the essential oils of *J. polycarpos* normal type (7.23%) was over 4 times lower than the oxygenated sesquiterpenes content in essential oils of the mutant type (30.26%). According to the results of several research studies, monoterpene hydrocarbons were the main components of essential oils of *Juniperus* species [15, 16, 31]. Ghorbanzadeh reported that the essential oils of different populations of *J. excelsa*, *J. communis*, and *J. sabina* from Iran chiefly consisted of monoterpene hydrocarbons [31]. The findings of Moghaddam showed that during the growing season of *J. polycarpos*, monoterpene hydrocarbons were the dominant compounds of essential oil [16]. In the present study, the assay of monoterpenes to sesquiterpenes content ratio showed interesting results. The contents of monoterpenes (represented by α-pinene) in the normal type were higher than sesquiterpene compounds, however in the essential oils of the mutant type, the content of sesquiterpene (represented by cedrol) was higher than monoterpenes. The ratios of monoterpene to sesquiterpene compounds rates in the essential oils of the normal and mutant types were approximately 2:1 and less than1:1, respectively (Fig. 3).

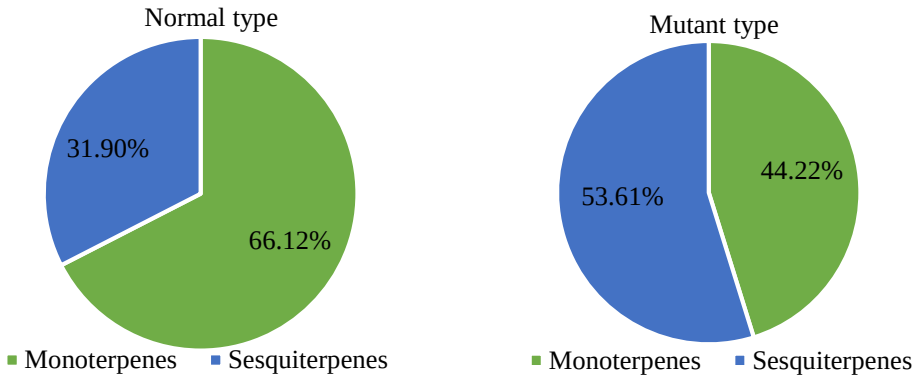

Fig. 3 Differences in the ratio of monoterpenes to sesquiterpenes in the essential oils of normal and mutant types of *J. polycarpos* in Sepidan County, Iran

These results clearly showed the different quality of extracted essential oils from the two studied samples. Being less volatile than monoterpenes ($C_{10}H_{16}$) and having stronger odors, sesquiterpenes ($C_{15}H_{24}$) have different applications in the medicine and cosmetic industries [32]. Therefore, each type of *J. polycarpos* can be produced and processed based on the type of usage.

CONCLUSION

In the present study, two distinct phenotypes of *J. polycarpos* were investigated with regard to the quantity and quality of essential oils extracted from their leaves. The results indicated significant differences in essential oil yield and composition between the phenotypes. The differences in the main compounds of the normal and mutant types lead to the identification of two chemotypes: I) α-pinene chemotype in the normal type and II) α-pinene-cedrol chemotype in the mutant type. Also, there were significant variations in the quantity and quality of the identified minor constituents regarding the concentrations and occurrences in essential oil profiles. Due to the similarity in climatic conditions, the observed variations are attributed to genetic differences induced by natural mutation. Genetic differences between studied samples are revealed in the plant physiological and biochemical processes that affect the plant photosynthesis capacity and structures of glandular trichomes and consequently determine the biosynthesis and accumulation of different terpenoid compounds. By the consideration of the reported results, the wild populations of *J. polycarpos* can be suggested as good sources of α-pinene and cedrol.

Declarations

Conflict of Interest

All authors declare that there is no conflict of interest.

Authors' contributions

All authors contributed to the performed experiments. RSH conceptualized the idea, performed the experiments, chemical and statistical analysis, interpreted the results wrote, reviewed, and edited. AR conceptualized the idea and performed the experiments. All authors approved the final manuscript.

Herbal Ethics Statement

The authors state that the experiment complies with relevant institutional, national, and international guidelines and legislation. The authors state that all the samples used in the research have been collected in coordination with the Natural Resources and Watershed Management Organization (NRWMO) -Fars province- and all necessary approvals have been obtained in this regard. The voucher specimen (voucher number of normal type: 55142 and mutant type: 55143) is deposited in the herbarium of the Research Center for Plants Sciences, University of Shiraz, Fars province, Iran.

Data Availability

The data presented in this study are available on request from the corresponding authors.

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