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ORIGINAL ARTICLE

Investigating the compounds present in the essence of flowering branches of four different types of Staychs plant, considering its taxonomic aspect in the North-Western part of Iran

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Field and purpose: Plant systematic discussion, and taxonomy, in turn, as an important tool in studying various types of morphological characteristics, tries to better characterize different types of plants. Unlike previous classification systems, the current ones not only depend on the morphological characteristics, but also implement other biological characteristics of the plants in their classification. In this regard, taking into account their evolutionary aspects, genetic affiliations and chemical structure is more important. This study investigates the compounds present in the essence of flowering branches of four different types of Staychs plant, considering its taxonomic aspect in the North-Western part of Iran.

Methodology: The essence of four species - *Stachys Inflate, Stachys schtschegleevii, Stachys byzantine*, and *Stachys lavandulifolia* - was measured using Clevenger distillation system and water-essence solvent. The compounds present in the essence of each species was identified using GC and GC/MS techniques.

Findings: The highest and lowest amount of substance m-Bis (m-phenoxyphenoxy) was observed in *Stachys Inflate* (87.91%) and Naphthalene (0.27%), respectively. The highest and lowest subtance present in *Stachys schtschegleevii* was phenol (59.16%) and octanne (1.15%), respectively. The highest and lowest subtance present in *Stachys byzantina* was phenol (68.97%) and octanne (0.54%), respectively. The highest and lowest subtance present in *Stachys lavandulifolia* was octanne (29.20%) and linalool (1.002%), respectively.

Discussion and conclusion: The results of this study showed that significant changes in the type and amount of chemical substances present in the essensial of the plants under study in different regions indicated the effect of environmental and ecological factors on the quality of the substances comprising the essence of a given plant.

Keywords: Stachys species; chemotaxonomy; gas chromatography; mass spectrometry

Introduction

In terms of its geographic location, the broad land of Iran is considered as the crossroads of the five phytogeographic vegetation areas, and it has diverse climates due to being located in an arid region and being mountainous. Prevailing such conditions has resulted in hight species diversity in Iran's plant communities, so that the botanists have identified more than 8,000 Flora Plant Elements, which is one of the richest vegetable communities in the world (Moghimi, 2005). Medicinal plants compose the major fraction of this rich flora.

The human being has always used medicinal plants for treatment, so that taking advantage of plants was always inevitable throughout the history. Even in the scriptures, such as the Gospel and the ancient Bible of India (Veda), using some plants are suggested to cure diseases. The history of using medicinal plants does not show its decreasing trend of usage in today's modern world. On the contrary, using traditional medicine and medicinal plants for health preservation is highly significant in the industrial societies and in many developing and developed countries due to increased people's trust in using these plants (Khosroshahi, 2008).

The essence is an aromatic compound found in different parts of plants. The essence is, in fact, a mixture of different substances, having completely different chemical structure, with very penetrating smell. The essence evaporates into air in the ambient temperature. That's why it is kept in volatile oils (Sanz et al., 2000). One of the valuable medicinal plants is the mountain tea or medicinal. This species of the Lamiaceae family has about 300 sub-species, broadly scattered around the world, and has created a type of species and sub-species diversity (Evans, 1989). So far, 34 species of this type is found in Iran, which are scattered in many areas, from the northern to central part of Iran (Mozafarian, 1978). Some species around the

world, in European, Asian, and African countries, are known ad medicinal plans. Having medicinal and antioxidant properties, they have antispasmodic, anti-septic and wound healing effects (Ghahraman, 1988).
Taking into account the general principle of taxon single-ancestor in plans systematic sheds light on the importance of knowledge and application of the plant evolution science to achieve evolutionary affinities and lineages at different levels of the dynastic system. Since today's classification systems are based on a number of properties, the morphology alone does not form the foundation of a given class. So, all morphologic properties do not necessarily have equal value in classification and description of taxon, and have a unique application in each case.
Given the fact that morphologic properties are mainly used in classification and identification of plan species and types in the classic taxonomy, using experimental properties facilitates a new attitude towards improved classification as the appearance is not efficient in determining the exact range of taxonomic units.
Given what mentioned above, this study investigates the compounds present in the essence of flowering branches of four different types of Staychs plant, considering its taxonomic aspect in the North-Western part of Iran. The species under study are:

1. *Stachys Inflate*, which is a stable plant with long stem from Lamiaceae family, having warm nature. It is also called Stachys grass or Stachys purple (Ghahraman, 1994).
2. *Stachys schtschegleevii*, which is a stable, bushy, with rhizome and having 30 cm height, covered with white fur hairs, having dense leaves at the thick lower end, stood up, with numerous clusters of short branches, oval or triangular leaves with very short petiole, or almost without petiole, with distinguished nervure. This plant is scattered in Mazandaran, Azarbaijan and Semnan province (Ghahraman, 1994).
3. Stachys Byzantina
4. *Stachys lavandulifolia*, which is a plant with numerous stems, covered with fur, stood up, having 6-25 cm height, with simple branches. Some are fertile and flowered, leading to inflorescence, some are short and infertile, with rather clustered leaves (Ghahraman, 1988).

There are different morphologic characteristics among these specious. However, given the importance of essence index in this group of plants, decision has been made to use chromatographic decomposition of the essence existing within these plants as the taxonomic index, i.e., chemotaxonomy, for better phylogenic characterization of these plants.
General speaking, essence is a colorless compound, especially is it is fresh and pure. Its color gets darker by time due to oxidation. This substance is miscible in alcohol, while it is immiscible in water due to its non-polar characteristic. However, it dissolves in water to a certain extent and transfers its scent to it (like herbal distillates, e.g., rose water). The main materials present in the essence change by heating, which is one of the reasons for complexity of essence extraction procedure. Because on one hand, one requires heat to extract the essence from the desired plant, while on the other hand, this heat is harmful for the important compounds within the essence, so that the valuable essence of a plant, with standard factors in an ideal essence, is very expensive. However, this is not the only reason for huge cost of many essences. Other factors such as little amount of essence in the plants, great dependency on climatic conditions, and lack of research, and knowledge and scientific-practical achievements in turn, also contribute to this problem, especially in Iran.
Importance and application of the essences: The essences have a broad application in today's human life. The main application of essences is in food, perfume, cosmetic, and pharmaceutical industries due to being aromatic and having unique and pleasant taste.
The value and application of essences lies in the properties of their chemical compounds to be responsible for their comprehensive use in pharmacology around the world, which is used as medicine in terms of pharmaceutical industry or in perfume and cosmetics, like essence of Peppermint and Thymus or their major components such as Menthol and thymol (Zargari, 1990).
The necessity of using more practical methods to extract essence oil with higher quality due to their significant use in industry is of great importance. Nowadays, with the new methods of volatile oil extraction, the scientists are looking for increasing essence stability and increasing their solubility in solvents with low alcohol concentration in food and water, as well as decreasing the storage and transportation costs of essence oils. Therefore, the purpose of this study is to investigate the compounds present in the essence of flowering branches of four different types of Staychs plant, considering its taxonomic aspect in the North-Western part of Iran.

Materials and methods

Sample collection and preparation

The samples studied in this research wre taken from flowering branches of a number of different species of Staychs plant, namely *Stachys Inflate*, which is scattered in central parts of East Azarbaijan, as well as Espiran region and marl hills within the path of Tabriz to Espiran from 1,500 m height, during May to September 2013. The species associated with this sample are Thyme, mountain ridge, Artemisia, and Vetch, to name a few. The medicinal part of *Stachys Inflate*, which is scattered in the northern part of the province and north western part of the country, especially Sahand Mountain range and mainly in Arasbaran region, in the path from Kolaleh to Aghdash village was taken from 1,300 m height in a rocky area. The species associated with this type of plant are Vetch, Teucrium, Thyme and mountain ridge. The samples were dried separately in shadow and far from light. The help of Department of Botany in Natural Resources Research Center of East Azarbaijan Province was used to name the plants in order to be as accurate as possible in naming. Then, the plats were dried, crushed and milled in a porcelain mortar.

Taking essence

The essence of the plants mentioned above was taken by distillation method with distilled water, using Clevenger device, after 3 hours from boiling. The main parts of Chouf and Clevenger device are:

- Chouf balloon
- Balloon
- Clevenger (condenser, cooling tube, and drain valve).

It has to be noted that glycerin is used to softem the powder within balloon. The Clevenger will be ready to install after mounting balloon on the Chouf, i.e., taking essence can start. After 3 hours, the distillate and water will be at the lower part, while the essence taken will be at the upper part. Water drainage will be done after finishing the essence taking process. Hexane solvent is used to collect the essence. When the essence taken reaches 0.35 mm and it is made colorless, the four species will be prepared for analysis, i.e., counting the compounds, separately and at different times. After water drainage, the essences are kept in the refrigerator (4 °C temperature) until injection to the gas chromatography device (Zargari, 1990; Antonelli et al., 1997; Kheiri, 2006).

Chromatography by gas chromatography device - mass spectrometry

The essences taken were injected to gas chromatography and gas chromatography device connected to mass spectrophotometer with the following specifications in order to identify the compounds.

Gas chromatography device connected to GC-MS mass spectrophotometer, or Gas Chromatography/ Mass Spectroscopy, was used to separate and identify the essences within these plants.

Gas chromatograph model Varian Star 3400cx, equipped with DB 5 capillary columns of 0.25 mm inner diameter, 30 m height, and 0.25 µm film thickness, with Helium carrier gas of 2 mm/min velocity.

In each case, after injecting a little amount of essence, the chromatogram resulted and the mass spectrums of different compounds within it were analyzed. Spectrum identification was done based on mass information bank, retention time, Kovats index calculation, studying the mass spectrum of each compound within the essence and investigating their refraction pattern, comparing with standard spectrum and using valid references. The relative composition percentatge of each compound in the essence was also determined by the area under the curve and comparing it with the total area under GC curve of one of the chromatogram peaks in the curve (Adams, 2001; Mirza & Ahmadi, 2000).

It has to be noted that in the first peaks in the chromatogram are associated with the bigger molecules, while the last peaks are related to smaller molecules.

In other words, the essence sample was injected to the device and entered the device column filled with attractive material. After the moving phase, helium gas was passed through the column and the essence compounds were separated from the column by pressure at different times, based on their combination desire. These different times are called retention time. The general spectrum showing the peaks associated with different materials and their separation time in the column is known as chromatogram (Figure 1).

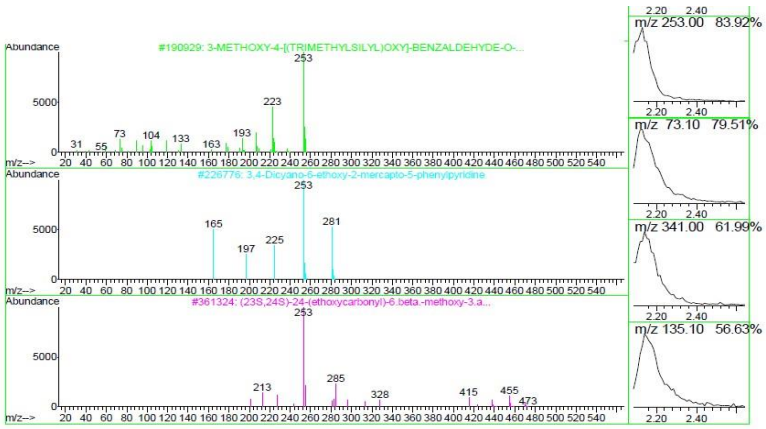

Figure 1. A sample chromatogram obtained from the Stachys species studied.

Each compound separated from the GC device was connected to mass spectrophotometer device. These compounds were crushed within the MS at high temperatures and shown in the plot based on their mass/electrical charge ratio. This is called mass spectrum, or MS.

Results

The research finding analysis was done in the framework of the research three main hypotheses as below.

First hypothesis:

The essence compounds of Stachys species collected from the north western part of Iran are different.

Descriptive statistic, including composition percentage, mode, and Chi-square test were used to analyze the data related to this hypothesis. The data related to this test is reported in Table 1.

Table 1. Composition oercentage of different compounds in four species of Stachys.

Species	***Stachys***	***Stachys***	***Stachys***	***Stachys***

Compound	*lavandulifolia* (%)	*byzantine* (%)	*schtschegleevii* (%)	*inflate* (%)
Heptane	5.3	0	0	0
Octane	29.202	0.544	1.155	0
Alpha- Pinene	3.263	0	8.41	0.68
Beta-Pinene	1.004	0	0	0
Beta -Myrcene	1.367	0	0	0
Decane	7.214	1.063	0	0.323
Sabinene	3.441	0	0	0
Linalool	1.002	0	0	0
Dodecane	3.005	0	0	0
Phenol	1.482	68.969	59.164	0
alpha.-Cubebene	3.803	0	0	0
Tetradecane	1.455	0	0	0
Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methyle	1.94	0	0	0
Germacrene D	5.951	0	1.203	0
bicyclogermacrene	3.112	0	0	0
Delta -Cadinene	4.494	0	0	0
Allospathulenol	1.642	0	0	0
Gamma -Terpinene	0	6.204	11.719	0
Benzene	0	11.862	9.819	0
m-Bis (m-phenoxyphenoxy) benzene	0	7.617	0	89.717
3-Methoxy-4-[(Trimethylsilyl)Oxy]-Benzaldehyde	0	4.828	0	0
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene	0	0	1.743	0
dl-Limonene	0	0	1.19	0
1,8-Cineole	0	0	1.688	0
Carvacrol methyl ether	0	0	1.219	0
trans-Caryophyllene	0	0	1.294	0
5.alpha.-Cholestan-19-oic acid, 2.beta.-methoxy	0	0	0	7.417
Sabinene	0	0	0	0.326
beta.-Cubebene	0	0	0	0.544
Naphthalene	0	0	0	0.274
beta.-cadinene	0	0	0	0.719

The results of Table 1 shows that there are at least 31 chemical compounds in four different species of the plant Stachys, whose composition percentage in the plants of these four species is as below.

The three compounds Octane (29.202%), Germacrene D (5.95%) and Heptane (5.30%) have the highest composition percentage in the species *Stachys lavandulifolia*. Having 17 types out of the 31 compounds available, it has the greatest diversity of chemical compounds existing in the species under study. In other words, it has a greater chemical compound diversity than thre other species (Figure 2).

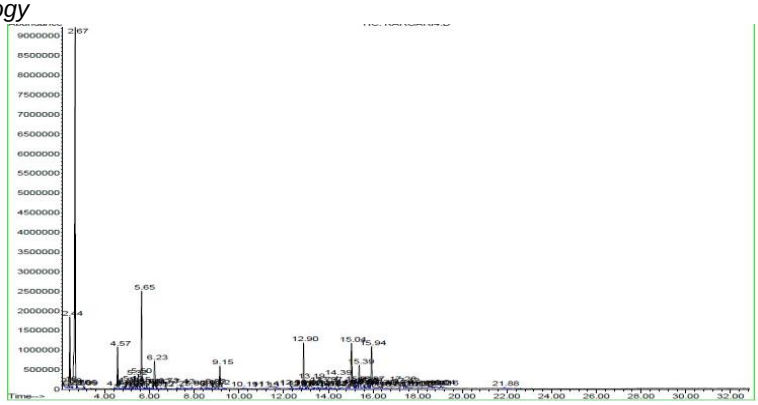

Figure 2. Chromatogram related to essence analysis of the species *Stachys lavandulifolia*.

Having 7 types of compound in its essence, the species Stachys byzantina has the lowest compound diversity among the four species studies, in which Phenol (68.969%), Benzene (11.86%), and m-Bis (m-phenoxyphenoxy) benzene (7.62%) have the highest composition percentage (Figure 3).

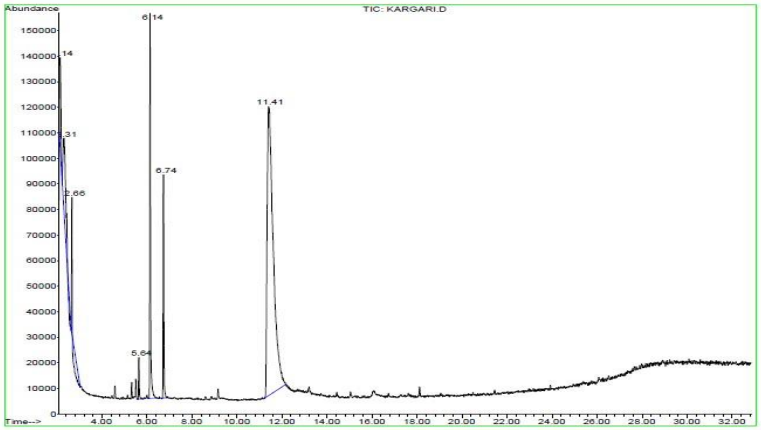

Figure 3. Chromatogram related to essence analysis of the species *Stachys byzantine*.

There are 11 types of chemical compounds in the essence of the species *Stachys schtschegleevii*, in which Phenol (59.164%), Gamma –Terpinene (11.72%), and Benzene (9.82%) have the highest composition percentage (Figure 4).

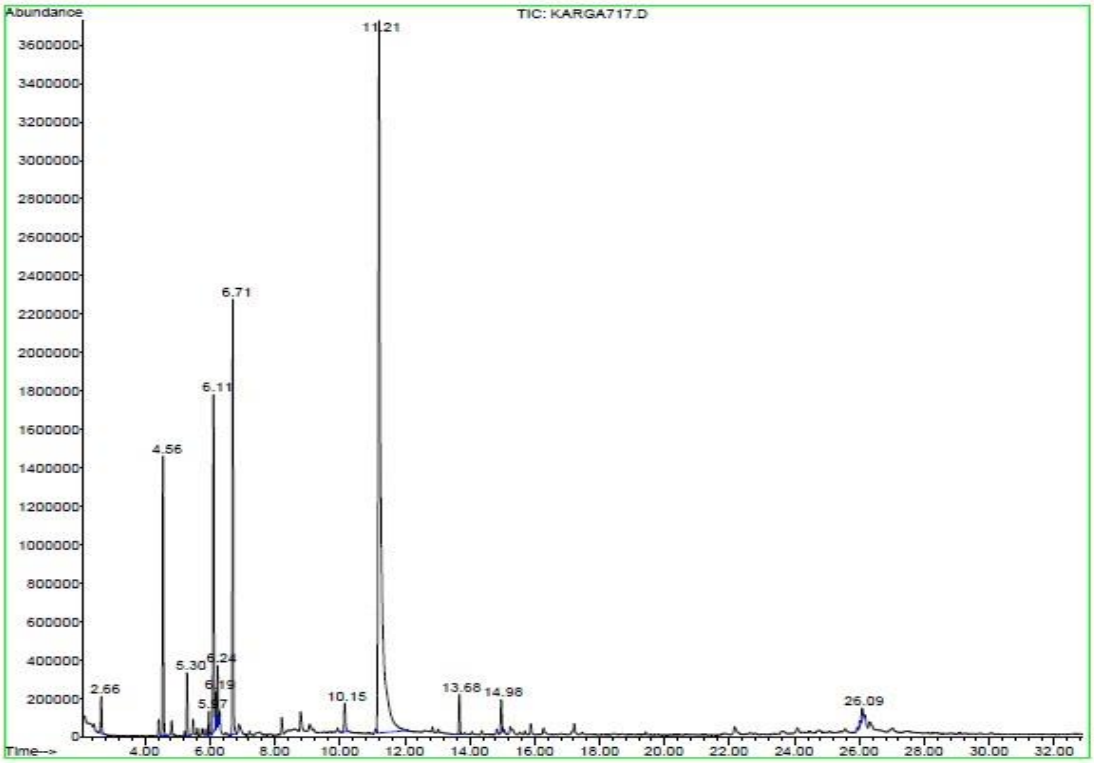

Figure 4. Chromatogram related to essence analysis of the species *Stachys schtschegleevii*.

Finally, having 8 types of chemical compounds extracted from its essence, the species *Stachys Inflate* has the highest amount of m-Bis Benzene (89.72%), as well as 7.42% 5.alpha.-Cholestan-19-oic acid, 2.beta.-methoxy and 0.72% beta.-cadinene (Figure

5).

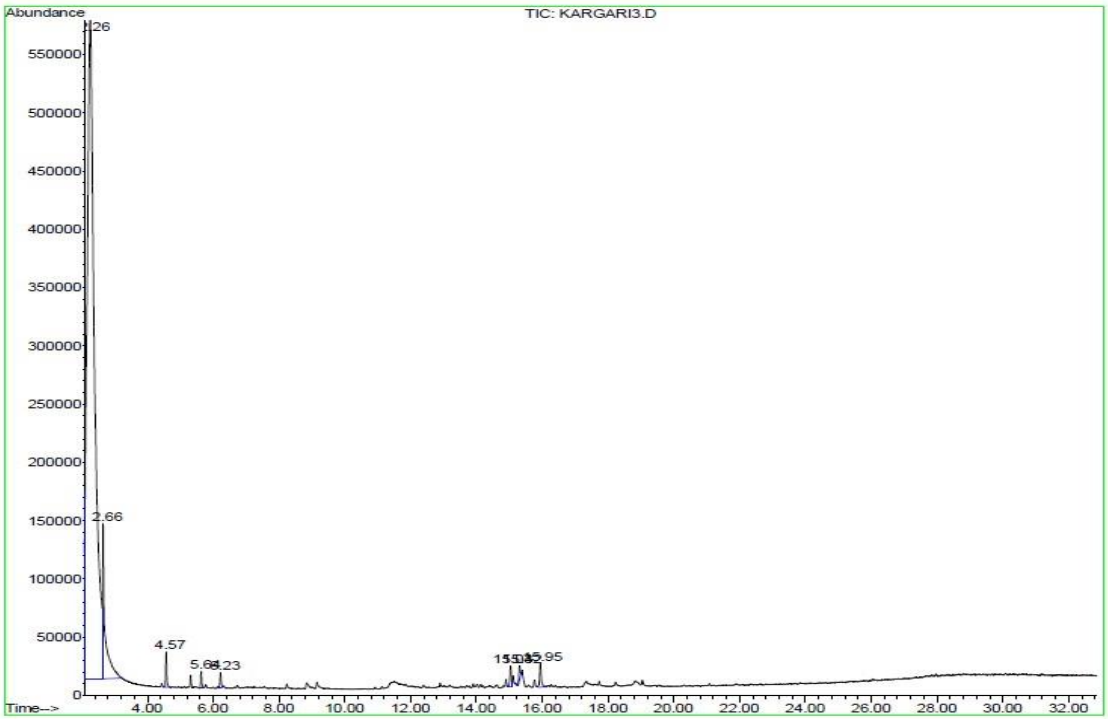

Figure 5. Chromatogram related to essence analysis of the species *Stachys Inflate*.

According to the results, there are 31 compunds existing in the four species, in which m-Bis Benzene and Nephtaline have the highest and loswet composition percentage, equal to 89.717% and 0.274%, respectively.

Second hypothesis:

The essence materials can be used as a factor to determine the similarities and differences between different species of Stachys.

The physical and chemical nature of substances in the plant essence compounds can be used as a factor to determine the similarities, and sometimes the differences, in different species of a plant, namely the species studied in this research. In this regard, one can use Kovats coefficient or retention time of each chemical compound. The data related to this index are reported in Table 2.

Table 2. Retention time of different chemical compounds in the four species of Stachys.

Species **Compound**	***Stachys lavandulifolia*** **(%)**	***Stachys byzantine*** **(%)**	***Stachys schtschegleevii*** **(%)**	***Stachys inflate*** **(%)**
Heptane	2.44	0	0	0
Octane	2.673	2.66	2.651	0
Alpha- Pinene	4.571	0	4.56	4.571
Beta-Pinene	5.314	0	0	0
Beta -Myrcene	5.503	0	0	0
Decane	5.647	5.64	0	5.636
Sabinene	6.224	0	0	0
Linalool	7.434	0	0	0
Dodecane	9.154	0	0	0
Phenol	11.406	11.41	11.207	0
alpha.-Cubebene	12.893	0	0	0
Tetradecane	13.193	0	0	0
Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methyle	14.391	0	0	0
Germacrene D	15.046	0	14.98	0
bicyclogermacrene	15.39	0	0	0
Delta -Cadinene	15.945	0	0	0
Allospathulenol	17.276	0	0	0

Gamma -Terpinene	0	6.74	6.701	0
Benzene	0	6.15	6.113	0
m-Bis (m-phenoxyphenoxy) benzene	0	2.31	0	2.263
3-methoxy-4-[(trimethylsilyl)oxy]-benzaldehyde	0	2.14	0	0
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene	0	0	5.303	0
dl-Limonene	0	0	6.18	0
1,8-Cineole	0	0	6.246	0
Carvacrol methyl ether	0	0	10.152	0
trans-Caryophyllene	0	0	13.681	0
5.alpha.-Cholestan-19-oic acid, 2.beta.-methoxy	0	0	0	2.662
Sabinene	0	0	0	6.224
beta.-Cubebene	0	0	0	15.046
Naphthalene	0	0	0	15.312
beta.-cadinene	0	0	0	15.945

The results of Table 2 shows that among different compounds extracted from the species *Stachys lavandulifolia*, the compound Allospathulenol and Heptane has the highest and lowest retention time, equal to 17.276 min and 2.440 min, respectively (Figure 6).

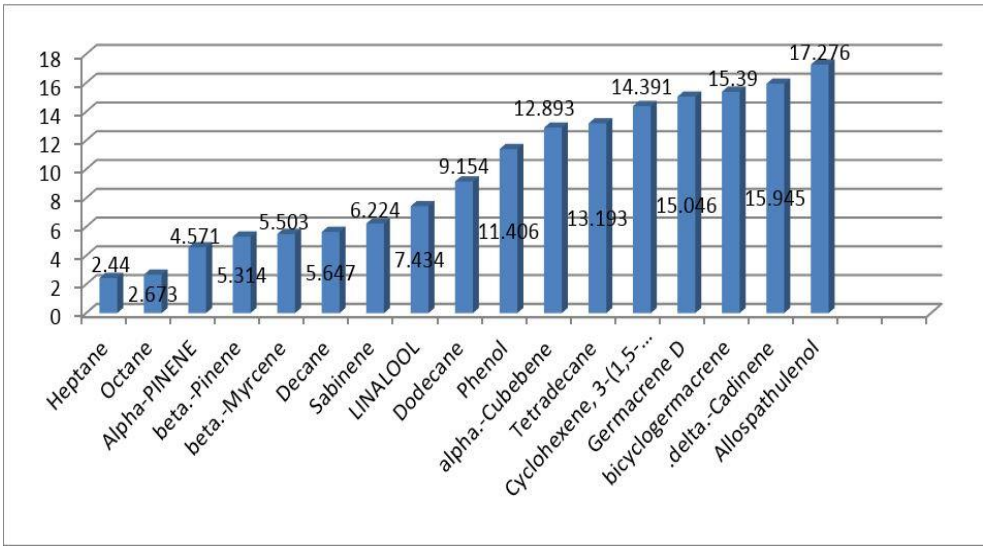

Figure 6. Retention time of essence compounds in the species *Stachys lavandulifolia*.

In the species Stachys byzantina, the compound Phenol and 3-Methoxy-4-[(trimethylsilyl)oxy]-benzaldehyde had the highest and lowest retention time, equal to 11.41 min and 2.14 min, respectively (Figure 7).

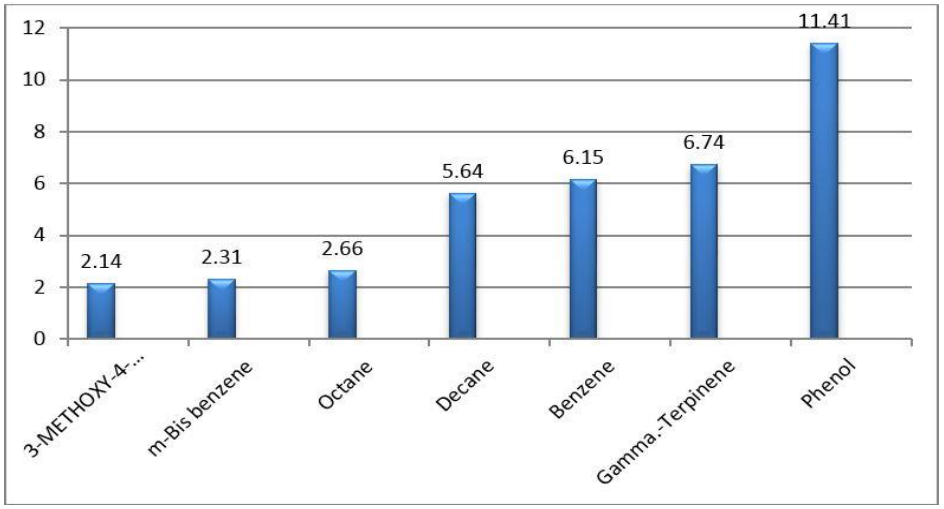

Figure 7. Retention time of essence compounds in the species *Stachys byzantine*.

In the species *Stachys schtschegleevii*, the highest and lowest retention time was associated with the compound Germacrene D and Octane, equal to 14.980 min and 2.651 min, respectively (Figure 8).

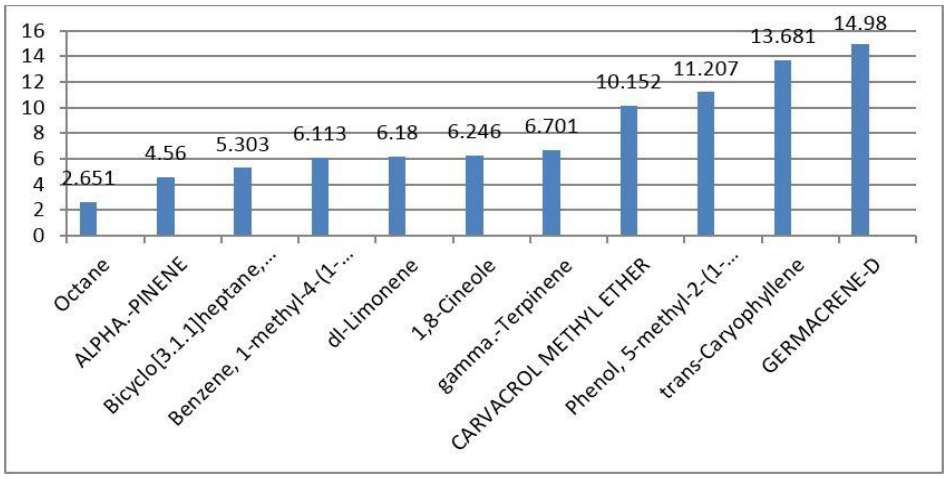

Figure 8. Retention time of essence compounds in the species *Stachys schtschegleevii*.

Finally, in the species *Stachys Inflate*, the highest and lowest retention time was associated with the compound beta.-cadinene and m-Bis benzene, equal to 15.945 min and 2.263 min, respectively (Figure 9).

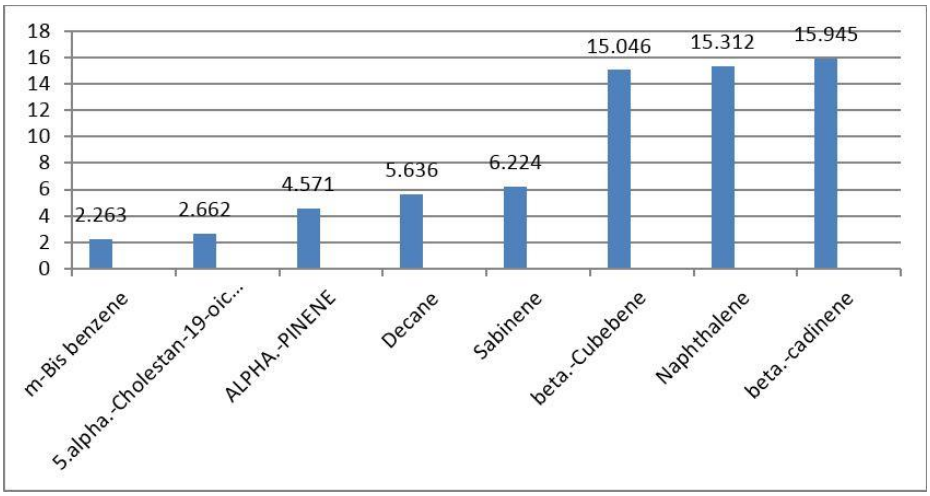

Figure 9. Retention time of essence compounds in the species *Stachys inflate*.

Generally, among all compounds extracted, the highest and lowest retention time was associated with Allospathulenol (17.276 min) and 3-Methoxy-4-[(trimethylsilyl)oxy]-benzaldehyde (2.14 min), respectively.
The point to be mentioned is that the compounds Decan, Octane, Alpha-Penine, and Phenol, seen in three different species, have almot equal retention time (Figure 10).

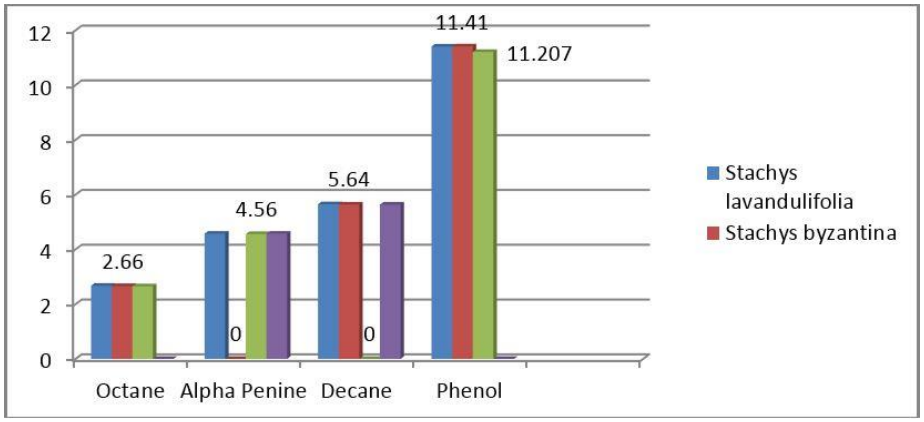

Figure 10. Comparing the retention time of some compounds existing in all four species of Stachys.

Thirs hypothesis

The phylogenetic properties different species of Stachys, collected from the north wastern part of Iran, are different in

taxonomic and morphologic terms.
As it was shown in Tables 1 and 2, despite existence of simmilar compounds in all 4 species of Stachys collected from the north wastern part of Iran, there is no similar compound with the same composition percentage and retention time in them. Therefore, although all species under study belong to one family, their philogenic properties are different.

Discussion and conclusion

According to the research findings and based on the hypotheses, besides the philogenic similarities between different speicies of Statchys studied, they have other similariries in terms of compounds. They have also similarities in terms of retention time. However, the compound composition percentage, and sometime the composition of compouns itself, differ based on the region, climate, and even the plat habitat. Comparing the results of this research with that of other researchers is discussed below.
During their reseach in 2009 on the plant *Stachys schtschegleevii*, collected from theheights of Kalibar City, East Azerbaijan Province, Tajalli et al. reported the main chemical compound in this plant: α-Pinene, β-Pinene, Germacrene-D, Limonene, Ocimene, Carene<delta-2->, Myrcene, α-Thujene, and α-Phellandrene, which is consistent with the compunds obtained from the species studied in this reseach, although their composition percentage was different. The main reason for the similarity between the compounds is geographic similarity of the region and the climate type (Tjalli & Sadeghipour, 2010).
Noorani et al. (2011) studied the essence compunds of Stachys Byzanthina, collected from Kojoor-Noshahr, Mazandaran province, and identified the components forming the essence of this plant, the major ones were: Isodorn (74.22%), Alpha-longy pitten (72.12%), 2-ethylhexyl base, phthalate (48.10%), Germacrene-d (98.5%), P-simen (71.5%), (1,5-dimethyl-4-hexenyl)-4-methyl benzene (72.4%), Luminol (31.4%), 1,4- trimethyl-2pentadecanone (58.2%).
It is clear that the species under study by them was different from the sample studied here, taken from the north western part of Iran, in terms of composition. The main reason might lies in the difference in geographic region and climatic type (Noorani et al., 2011).
In a simillar research conducted in 2012 in Baladeh-Mazandaran on the plant *Stachys lavandulifolia* Vahl., Mahzooni et al. reorted the essence composition as:
Hexadecanoic acid (13.9%), α-pinene (13.7%), germacrene-D (8.9%), β-pinene (7.0%), myrcene (4.5%), β-phellandrene (5.7%), Z-ocimene (3.4%), spathulenol (3.5%), δ-cadinene (2.0%) and α-cadinol (2.6%).
As it is clear, their studied species is different from the sample studied here, taken from the north western part of Iran, in terms of composition. However, there is a significant difference in the composition percentage, which is mainly due to the difference in geographic region and climatic type (Mahzooni-kachapi et al., 2012).
In another research conducted by Amiri et al. on the species of the plant *Stachys lavandulifolia* in Yazd region, the main components identified were Myrcene (20.9%), α-Pinene (16.3%), and α-Terpinene (20%). Although their research was mainly on antimicrobial effects of this species, their methodology was similar to this study and the composition obtained was also consistent with this inquiry (Amiri et al., 2008).
In a research conducted in 1985 on the essence of the plant S.Lavandulifolia, collected from Turkey, the most abundant compositions were octane (17.3%) and 1,8-Cineol (8.6%) (Akcicek et al., 2012). The results of this study is in accordance with our results, in both of which octane was the main compound present in the essence of this species.
According to what mentioned above and analyzing different masses of medicinal plants, namely Satchys, the major compound in the plant of each region can be identified and introduced as chi-tips. Given the major compound in each mass, this information can be used in breeding programs, standardization of pharmaceutical products and cultivation. Increased essence in a given agricultural conditions might be due to prevailing of optimal growth conditions and longer growth period, which calls for further research. Finally, given the importance of phylogenic investigations on different species of the plant Stachys in systematic and chemotaxonomic terms, it is suggested that besides this kind of investigation, it is suggested to simultaneously use genetic investigation methods and molecule and gene indices. In this case, the results obtained would be more accurate. Using different types of genetic indices in this regard would be greatly helpful.

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