Physiological, morphological and phytochemical responses of ajowan (*Trachyspermum ammi L.*) populations to salt stress

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Abstract

Twenty-eight populations of ajowan (*Trachyspermum ammi* L.) were evaluated for morphological traits and oil yield in two consecutive years. Then, selected ajowan populations that revealed medium and high oil yield and higher seed weight in two years were selected for further studies. These were assessed for physiological traits, total phenolic and flavonoid contents and antioxidant capacity under four salt treatments control, 60, 90, and 120 mM NaCl. The essential oil composition was analyzed by gas chromatography-mass spectrometry (GC-MS) Thymol (32.7-54.29%), γ -terpinene (21.71–32.81%), and *p*-cymene (18.74–26.16%) were major components. The highest and lowest thymol were recorded for Qazvin (control) and Qazvin (Low salt concentration), respectively. Salt stress caused an increase in essential oil content of Esfahfo and Qazvin populations. The highest phenolic and flavonoid contents were found in Arak population grown in 60 mM NaCl (183.83 mg TAE g^{-1} DW) and Yazd population grown in 90 mM NaCl (5.94 mg QE g^{-1} DW). Moreover, Yazd population exhibited the strongest antioxidant activity based on DPPH (IC_{50} = 1566µg/mL) under 60 mM NaCl and the highest reducing power (0.69 nm) under 120 mM NaCl. Overall, the results revealed that low and moderate salt stress improves the phytochemicals of ajowan, being especially useful for pharmaceutical and food applications.

Introduction

A growing interest is nowadays being shown to replacing synthetic aromatic compounds with counterpart natural extracts. Distributed in semi-arid and arid parts of the world[1], ajowan, or sprague, (*Trachyspermum ammi* L.) is a medicinal and industrial plant of the Apiaceae family with white flowers and small brownish fruits. A good source of secondary metabolites, the seeds of ajowan have been used in food and pharmaceutical applications[2]. A variety of health properties was described for the ajowan seeds, which include antimicrobial, antioxidant, nematicidal, anti-inflammatory, carminative, and sedative effects[2]. Thymol, is considered to be a valuable phenolic monoterpene that is widely used in food products for its high antioxidant and antimicrobial capacity[3].Interestingly, ajowan seeds have also been found to be a good source of thymol. The seeds are suitably used for improving certain food products, especially due to their resistance to processing and their health-promoting compounds.

Phenolic compounds are among the secondary metabolites of medicinal plants that play crucial role in scavenging free radicals[4]. That means, the expression of these compounds can be regulated or induced by abiotic stress like salinity which is a major problem in the arid and semi-arid regions[5,6]. It could, therefore, be a major objective of the food industry to develop methods that would elevate the amounts of desirable metabolites in the medicinal plants.

Furthermore, physiological processes can also highly be affected by salt stress. Ajowan seed is of prime economic importance, and hence, its phenolic compounds as non-enzymatic antioxidants and enzymatic antioxidants play dual role on one hand they serve as a critical health-promoting constituents in human nutrition on the other.

A number of studies have been so far conducted on variations in the essential oil content of ajowan populations[1,2] , while a few concentrated on the polyphenols and antioxidant activity of the oils [2]. The effects of salt stress have been explored in some medicinal plants including *Myrthus communis*[6], *Mentha canadensis*[7], *Thymus vulgaris*[8], and *Salvia mirzayanii*[9], Nonetheless, There is scant information available in ajowan on the effects of salt stress on physiological and biochemical attributes.

The present study was, therefore, conducted: (1) to assess the morphological, oil and yield related traits in 28 ajowan populations in two consecutive years; (2) to evaluate the effects of salt stress on the essential oil content and compounds of four selected ajowan populations; (3) to assess the phenolic and flavonoid, antioxidant activities and physiological traits of the studied populations under salt stress.

Results And Discussion

Two year analyses in all populations. The results of two-year analysis of variance revealed significant differences among the studied genotypes for all traits. Also, the effect of year was significant for all. The interaction of G*Y (Genotype×Year) was also significant for all the studied ones (Table 1).

During the first agronomic year, plant height varied from 63.66 to 100.5 cm (Table 2). The tallest population was Khormo, while the shortest one was Farsfars population. Number of flowering branches ranged from 6.16 (Ardebil) to 20.83 (Khormo). Arak and Farsfars population recorded the lowest inflorescence diameter, while IPK2, and Khorsar population possessed the highest inflorescence diameter. Number of umbel varied considerably from 29.83 to 273.39 (Table 2). IPK3 and Farsfars possessed the highest and the lowest number of umbellule per inflorescence, respectively. Number of flowers per umbel ranged from 114 (Farsfars) to 282.67 (Yazshah). The highest and the lowest number of seeds per umbel were recorded for Yazshah (565.33) and Farsfars (228), respectively. The lowest (278.6 cm^2) and the highest (1548.7 cm^2) crown cover diameter belonged to Khorbi and Arakkho population. The two genotypes of Yazsad and Khormo exhibited the highest values for one thousand seed weight and seed yield per plant (0.96 g and 72.45, respectively), while Ardebil (0.67 g) and Tehran (9.85 g) recorded the lowest values. The highest and the lowest essential oil yield were recorded for Yazd (5.51) and Farsfars (1.20), respectively.

In the second agronomic year, plant height varied from 87.83 cm to 139.67 cm. The highest number of flowering branch was measured in the population Ardebil, while the lowest (10.5) was measured for Hamdan (Table 2). Inflorescence diameter varied from 3.3 cm to 5.5 cm. Number of umbel varied from IPK1 (76) to Ardebil (550.3). Esfahfo recorded the highest number of umbellule per inflorescence, while Khorbi recorded the lowest one. Number of flowers per umbel ranged from 164.6 (IPK1) to 510.1 (Qazvin). Qazvin and IPK1 exhibited the highest and lowest number of seeds per umbel, respectively. Crown cover diameter ranged from 489.1 cm^2 (Khorbi) to 1896.9 cm^2 (Khorsar). Yazist had the lowest one thousand seed weight (0.61 g), while Arakkho recorded the highest (0.96 g). The highest and lowest seed yield per plant were recorded for Esfahfo (315.02 g) and IPK1 (19.30 g). Finally, the highest essential oil yield was observed in Yazd (5.58) while the lowest belonged to Yazshah. Similar ranges were also reported for Indian ajowan populations[10]. High variation in two studied years can be resulted from environmental fluctuations as previously reported in other Apiaceae plants including ajowan[11], fennel[12]and cumin[13].

Hierarchical cluste analysis. The clustering patterns of the 28 ajowan (*Trachyspermum ammi* L.) populations based on their morphological data and oil yield obtained from the Ward method for two years are presented in Fig. 1 and Fig. 2. Using the analytical results of the two-year data, the genotypes were grouped into two clusters. In the first year, group 1, included Khorsar, Arakkho, Yazsad, Yazd, Esfahfo, Farsmar and Ardebil populations with the highest essential oil yield, while group 2 classified 16 populations with moderate to high range of one thousand seed weight. Five populations including Yazshah, Esfahgh, IPK3, Hamadan and Khormo were placed in group 3 was characterized mostly by high plant height.

In the second year, group 1. Group 2 consisted of 11 populations. Group 3, included Ardebil, Hamadan, Esfahgh, Araksha and Khorsa populations with high plant height. Group 4 consisted of four populations with mostly by moderate one thousand seed weight, while group 5 was characterized by high essential oil yield (Fig. 2).

As the results of two years analysis revealed high variation in respect to most of the traits. So, the major criteria for selection of populations for next treatments were the economical and industrial ones. So, for this purpose, essential oil yield and one thousand seed weight were used for selections. Accordingly, two populations that showed medium (Esfahfo and Qazvin) and two (Arak and Yazd) that revealed high amount of the mentioned traits were chosen for further experiments (Table 2).

The results of salt treatments on selected populations

Essential oil content. Based on the results obtained, the oil contents of the *T. ammi* populations were found to be considerably influenced by the salt stress treatments, While essential oil yield varied from 2.16 to 4.77% under the control condition, the highest and lowest yields were recorded for Arak and Qazvin, respectively, both of which exhibited strongly reduced levels under the examined NaCl concentrations (Table 3). However, Esfahfo and Qazvin populations recorded increases in their EO yields.

At a low salt stress (LS), the highest (4.39%) oil yield was recorded for Yazd while Arak exhibited the lowest (3.01%) yield . A trend in EO yield similar to that observed under the control conditions was observed under the moderate stress (MS) treatment such that the Arak and Esfahfo populations recorded the highest (4.22 %) and lowest (2.64%) oil yields. Under severe stress (SS) conditions, Qazvin recorded the highest (4.26%) essential oil yield while the lowest (3.77%) belonged to Esfahfo (Table 3).

Previous studies reported different ranges of essential oil yield for different ajowan populations collected from different countries. Chauhan et al.[14] reported a range of 2 to 4% for the essential oil yield extracted from ajowan seeds. These results are confirmed by Bairwa et al.[15] who also reported a range of 2–4.4% for the essential oil yield extracted by hydro-distillation from some Iranian ajowan populations. This is while higher ranges of 2.5–6.1% have also been reported for EO yields of Iranian ajowan populations[16]. In the present experiment, salinity stress was observed to decrease the essential oil yield in Yazd and Arak populations. This might have been due to the additional energy demand by plant tissues as a result of less available carbon concentration during the growth stage that results in reduced oil accumulation[7]. Furthermore, the increased production of volatile compounds in Esfahfo and Qazvin under elevated salt stress could be attributed to the elevations of oil gland density in these populations[17].

Essential oil composition. Table 4 reports all the EO constituents detected in the four studied populations under the different salt treatments. Clearly, there are high chemical polymorphisms among the Iranian ajowan populations with thymol, γ-terpinene, and *p*-cymene identified as the major components. It is seen in this Table that the amount of thymol ranged from 32.7±0.05% in Qazvin under the LS treatment to 54.29±0.02% in Qazvin under the control conditions. The highest (32.81±0.02%) and lowest (21.71±0.01%) amounts of γ-terpinene belonged to Qazvin (LS) and Esfahfo (SS), respectively. *p*-Cymene content varied from 26.16±0.02% in Esfahfo (LS) to 18.74±0.01% in Arak (C). Among the few studies reporting on the essential oil composition of Iranian ajowan populations, Moazeni et al.[18] reported γ-terpinene (23.92%), *p*-cymene (22.9%), and thymol (50.07%) as the major compounds of the essential oil from one population collected in Kazerun, Iran. Moein et al.[19] identified γ-Terpinene (48.07%), *p*-cymene (33.73%), and thymol (17.41%) as the major constituents of one population grown in Firoozabad, Iran. In Esfahan population, the most abundant components of the oil were reportedly thymol (44.5%), γ-terpinene (26.6%), p-cymene (21.6%), limonene (1.1%), and carvacrol (0.3%)[20].

The effect of salt stress on thymol content has also been reported in the genus *Thymus*[8]. This is while the populations examined in the present study showed different trends of thymol accumulation in their seeds. The differences observed between thyme and ajowan plants with respect to their thymol content might be attributed to their harvested organs. While it is the seeds of the ajowan species that are harvested for their high thymol content, the edible leaves of *Thymus* are harvested for thymol extraction.

Thymol is an aromatic and oxygenated monoterpene. Furthermore, monoterpene accumulation can be highly affected by not only phenological stages but by harvesting time as well[15]. In the case of ajowan, seeds are harvested at full maturity and monoterpenes mostly begin to increase from the full flowering stage to seed maturity[2]. Moreover, salt stress reportedly affects the biosynthesis of isoprenoids as a result of its influence on isoprene subunits.

The different trends observed in thymol accumulation in the populations examined in the present study make it difficult to draw definitive conclusions about its quantity in different populations. However, comparison of the control and severe stress treatments revealed that Arak, Esfahfo, and Qazvin showed decreases in their thymol contents in the severe stress treatment. A number of explanations have been put forth for the decrease in thymol content. One explanation claims the dissipation energy mechanism involved in isoprenoid changes under stress conditions to be responsible as the changes are attributed to the subunits available for the biosynthesis of isoprenoids or the related compounds[4]. Furthermore, it has been argued that plants subjected to severe stress (SS) prefer to use the available carbon sources for the production of carbohydrates that are necessary for grain filling[21]. Finally, the radical scavenging mechanism has also been suggested for changes in metabolites during stress conditions[4,16]. Whatever the explanation, certain compounds with high antioxidant activities might be involved in order to cope with free radicals.

Number of seeds per plant. According to results obtained, the number of seeds per plant of the *T. ammi* populations were found to be considerably influenced by the salt stress treatments. Salt stress caused a significant reduction in seed yield per plant of *T. ammi* (Table 3). A maximum reduction in seed yield was observed at the (LS) treatment. Number of seeds per plant varied from 47.66 to 68.75 g under the control condition, the lowest and highest recorded for Arak and Yazd, respectively (Table 4). At a low salt stress (LS), the highest (67.34) Number of seeds per plant was recorded for Esfahfo while Arak exhibited the lowest (40.46) number of seeds. Under the moderate stress (MS) treatment observed that the Esfahfo and Qazvin populations recorded the highest (49.50) and lowest (29.22) number of seeds. Under severe stress (SS) conditions, Arak recorded the highest (34.10) number of seeds per plant while the lowest (28.59) belonged to Qazvin (Table 3). NaCl in level modarate stress and severe stress in the growth medium caused a marked reduction in number of seeds per plant in *T. ammi*. Such an adverse effect of salinity on growth and seed yield has earlier been observed in a number crops, e.g. alfalfa[22,23], carrot[24], and cumin[25].

Total phenolic and flavonoid contents. The ajowan populations studied also showed different trends with respect to their accumulation of phenolic compounds. All the samples exhibited high TPC values ranging from 61.76 in Yazd (C) to 183.83 mg TAE g^{-1} DW in Arak (LS) followed by Qazvin (LS) (157.32 mg TAE g^{-1} DW). The accumulation of phenolic compounds in each plant is the result of such varied parameters as phenological stage, extraction process, agricultural application, and storage conditions[26]. In plants exposed to abiotic stresses, the rate of cellular oxidative damage can be controlled by the plant's capacity to produce antioxidants[27]. However, accumulation of phenolic compounds might be different in different plants as a result of salinity stress. For instance, phenolic compounds were shown to decrease in broccoli[28]. In response to salt stress, whereas NaCl treatment elevated TPC levels in maize[29] and red pepper[30].

Similarly, the ajowan populations studied exhibited substantial differences with regard to their flavonoid content. While Yazd (MS) recorded the highest TFC content (5.94 mg QE g^{-1}DW), Yazd (C) exhibited the lowest (3.48 mg QE g^{-1} DW). Moreover, a significant increase was observed in TFC under moderate salinity stress (MS) but higher salt concentrations was observed to cause diminishing levels of TFC (Table 3).

Plants are reported to employ different mechanisms for distributing flavonoids among their subcellular sections. Metabolically, plant polyphenols, such as flavonoids and phenolics, are biosynthesized through several pathways[4]. The underlying mechanism involved in flavonoid functions is based on the chelating or chipping process. Some reports evidenced the enhancement of phenolic in various plant structures and organ systems under salinity stress condition[27]. It is thought that moderate salinity stress induces the normal saline tolerance pathway via increasing flavonoid contents[30]. Hence, the variations observed in the studied ajowan populations as well as the different salt stress conditions might have led to the increase in the polymorphism in flavonoids and their accumulation.

Antioxidant activity

DPPH assay. Comparisons were made to detect variations in the scavenging of DPPH free radicals in the studied ajowan extracts (Figure. 3). The IC_{50} values were found to vary from 1566.985 µg/mL to 5889.99 µg/mL. More specifically, the extract from the Yazd population subjected to MS and SS showed the strongest antioxidant activities (1566.985 and 1657.46 µg/mL, respectively), while those from Qazvin (C) and Esfahfo (C) demonstrated the weakest activities (5889.99 and 5671.98 µg/mL, respectively). The variation in IC_{50} observed among different species might be interpreted with recourse to the diversity in their polyphenolic components[14]. Probably be suggested that plants activate metabolite biosynthesis as part of a complex antioxidant defense mechanism when they are exposed to salt stress and that the production of phenolic compounds might be part of an alternative strategy adopted by plants to respond to stressful conditions. The antioxidant capacity of *Thymus* species has been well researched. The most relevant chemotypes of *Thymus* species have been reported to be rich in phenolic monoterpenes such as thymol and carvacrol[31]. In most such studies, phenolics, due to their chemical structures that allow them to donate hydrogen to free radicals, were introduced as the major factor contributing to the antioxidant activity of the species[32]. Moreover, Tohidi et al.[33] reported that based on the observations, the highest antioxidant capacity of the recorded might be due to its high amounts of phenolic components. Studying the effect of salt stress on the antioxidant activity of Apiaceae plants, Pandey et al.[10] reported a high variation in the antioxidant activity of some Indian Apiaceae spices based on their DPPH assay results. Similarly, Akbarian et al.[34] used the DPPH method to observe a high variation in the antioxidant capacity of three *Ferula* species.

Reducing power. The reducing power of the studied ajowan populations was found to rise with increasing EO concentration (Figure. 4). Clearly, the highest antioxidant capacity in absorbanceat 700 nm was obtained in Yazd (SS) (0.84) in 500 mg/l while Arak (C) exhibited a lower activity than BHT. From among the ajowan populations, in absorbanceat 700 nm Yazd (SS), Yazd (MS), Esfahfo (SS) and Yazd (LS), recorded reducing powers of 0.84, 0.69, 0.44, 0.39, and 0.39, respectively, which were higher than those recorded for the other populations (Figure. 4). Similarly, Afshari et al.[35] reported that strongest reducing power was observed in *A. pachycephala* at concentrations of 300 and 500 mg/l. In a similar study based on the reducing power model, Vafadar Shoshtari et al.[6] found that myrtle subjected to salt stress showed elevating antioxidant activity with increasing extract concentration.

Cluster and principal component (PCA) analysis. Cluster analysis was performed using the main essential oil components, TPC, TFC to detect any similarities among the ajowan populations studied. The results are illustrated in the dendrogram in Figure 5. The analysis provided further information regarding the distribution of ajowan populations in terms of their essential oil yields and suggested diversified chemical compositions as a result of the different salt stress conditions investigated. The results obtained grouped the ajowan populations into the following four clusters. The first consisted of the two accessions of Qazvin (C) and Arak (LS). The first group consisted of the two accessions of Qazvin (C) and Arak (LS) both rich in thymol (54.29±0.02, 50.05±0.05). The second group was further divided into two subgroups. The first consisted of Qazvin (MS), Arak (MS), Yazd (LS) and Esfahfo (MS). The main components of which were TFC (5.35, 4.37) and TPC (147, 125.99). Finally, Yazd (MS) and Esfahfo (SS) with higher quantities of *p*-cymene (20.55, 19.21) was assigned to the second subgroup. The third group consisted of Esfahfo (C), Arak (SS), Yazd (SS) and Arak (C). The main components of this group were high quantities of essential oil yield (4.77, 3.22). Fourth group consisted of Qazvin (LS), Qazvin (SS),Yazd (C) and Esfahfo (LS) the main components of which

were γ-terpinene (32.81, 22.75) (Figure 6). Based on the structural similarity of thymol and carvacrol, it may be suggested that the rate of their conversion to each other may be affected by such environmental factors as salt stress[21].

PCA was also carried out to group the investigated populations in terms of their major oil components and the other studied metabolites. The PCA result revealed that the first and second components explained 69.32% of all the variation observed (Table 5) while the first component showed 44.40% of the total variation. Finally, PC2 explained 24.91% of the total variance. The first PC (PC1) had a positive correlation with *p*-cymene (0.480) and γ-terpinene (0.533), but a negative one with Thymol (-0.580). It may be noted that thymol is an isomer of carvacrol while p-cymene is considered as the precursor to both compounds[36]. Finally, PC2 showed a high positive contribution by EO content (0.405) and thymol (0.220) but negative correlation with phenol (-0.690). Comparison of cluster and PCA results showed the similar trends in most cases such as Yazd (SS), Esfahfo (C), Arak (C) and Arak (SS) possessed high amount of essential oil yield. As well as, Qazvin population in control conditions was rich in thymol. The seven population of Qazvin (SS), Yazd (C), Esfahfo (LS), Qazvin (LS) formed a single group characterized by higher of *p*-cymene and γ-terpinene. The four population of Qazvin (SS), Arak (SS), Arak (MS), Yazd (LS), Esfahfo (MS), Esfahfo (LS), and Arak (LS) formed a single group characterized by higher of TPC. Overall, the studied ajowan populations exposed to different salt level concentration and control condition were successfully distinguished based on their phytochemical traits and main EO components.

Correlations among the components. Correlation analysis demonstrated the relationships between all the measured traits and the main essential oil components under different conditions. According to Table 6, at Control condition, negative correlations were recorded between thymol and γ-terpinene (-0.961*), and DPPH and γ-terpinene (-0.990**). Under Low salt stress (LS), positive correlation was observed between DPPH and *p*-cymene (0.947*). The result of correlation analysis at moderate stress (MS) conditions exhibited the negative correlations thymol and γ-terpinene (-0.994**), while the correlation between total phenolic and thymol (0.970*), and reducing power and total phenolic (0.969*) was positive. Finally, at severe stress (SS) environment, a positive correlation was shown between γ-terpinene and *p*-cymene (0.944*), and EO content and *p*-cymene (Table 7). The γ-terpinene is the major precursor to the biosynthesis of thymol, *p*-cymene is considered as a by-product in this pathway[33]. Previous reports have shown different trends in the accumulation of these components in thyme leaves[33]. As the seeds of ajowan were used in the present study to analyze for essential oil analysis, the discrepancies observed between the results obtained for thyme and ajowan might be explained with recourse to the organs in which the oils accumulated. This is confirmed by the results reported elsewhere that highlighted changes in monoterpene frequencies based on phenological differences and harvested organs[37]. Finally, the positive correlations between the precursors and final products might be attributed to the complete transformation of the precursors.

Physiological evaluations

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). For malondialdehyde (MDA), the results of analysis of variance revealed that the main effect of populations, salinity and the interaction effect of salinity on populations were significant (Table 8). The effects of populations× salinity for MDA showed that the highest and the lowest amounts were related to Qazvin and Esfahfo populations in control conditions with 5.36 and 1.46 nmol / m leaf fresh weight, respectively (Table 8). It has been reported that leaf MDA content at 6 dS / m NaCl level has significantly increased in different *Sesamum indicum* cultivars as compared with control[38]. Unsaturated fatty acids are the main constituents of membrane lipids that are prone to peroxidation by free radicals due to salinity stress[39]. In this regard, MDA content is an indicative of oxidative damage[40].

A significant difference in hydrogen peroxide (H_2O_2) content was observed among the populations in the control environment and salinity stress treatments. H_2O_2 was decreased at salinity levels of 6 dS / m salinity level. The results of population×salinity showed that the highest and lowest values of this trait were related to Qazvin populations in control conditions with 2.40 mmol / g and Arak at 6 dS / m stress level with 0.45 mmol / g (Table 3). Hydrogen peroxide (H_2O_2) is an active signal molecule and its accumulation leads to a wide range of plant responses to environmental stresses, as these reactions are interdependent[41]. Increasing the level of environmental stresses increase the production of reactive oxygen species (ROS) such as hydrogen peroxide that lead to increase in damage to plant cells[42]. In the present study, the selected ajowan populations revealed high physiological variation against salt stress. Previous studies also highlighted this fact that different species and populations can reveal different reactions against stress and release various types of antioxidants that neutralize the effect of signal molecules and increase plant tolerance to stress[43]. Salinity tolerant cultivars have less hydrogen peroxide than sensitive cultivar. Therefore, leaf hydrogen peroxide content under stress conditions can be used as a suitable indicator for selection in salinity tolerance. This kind of variation was also observed in Apiaceae plants including *Carum carvi* L.[44]and *Foeniculum vulgare* Mill[45].

Antioxidant enzymes activity. The results of statistical analysis showed that there is a significant difference among the populations, different salinity levels and the interaction of the populations in salinity on the activity of guaiacol peroxidase and ascorbate peroxidase (Table 8). Interaction of salinity effects in populations for guaiacol peroxidase enzyme revealed that the highest and lowest values of this trait belongs to Qazvin populations at a stress level of 12 dS / m with 0.277 FW U mg^{-1} and Isfahanfo under 12 dS / m level with 0.012 FW U mg^{-1} was (Table 3). Also, the highest amount of ascorbate peroxidase enzyme was related to Arak populations in control and Arak conditions at a stress level of 6 dS / m with 0.025 FW U mg^{-1}, respectively. The lowest was related to Yazd populations at a stress level of 6 dS / m and Qazvin the stress level of 9 dS / m (Table 3).

According to ANOVA, the main effect of populations, salinity and the interaction of populations on salinity were significant for chlorophyll a and chlorophyll b (Table 8). Interaction of salinity effects in populations for chlorophyll a revealed that the highest and lowest rates of this trait were related to Esfahfo populations at a stress level of 9 dS / m with 0.36 mg /g and Yazd populations at a stress level of 6 dS / m with 0.05 mg / g, respectively (Table 3). Also, the interaction of salinity effects in the populations for chlorophyll b showed that the highest and lowest values of this trait were related to Isfahfo populations at a stress level of 9 dS / m with 0.09 mg/g and Yazd in 9 dS / m conditions (0.023 mg / g), respectively (Table 3). Due to the direct role of chlorophyll a in photosynthesis and dry matter production, this trait can also be effective increasing this difference. Most of previous reports indicated that the chlorophyll content decreases under salinity stress and the old and necrotic leaves begin to fall as the salinity period continues. Decreases in chlorophyll content as a result of salinity stress have also been reported for *cotton*[46], *pumpkin*[47]and *spinach*[48].

Carotenoids. ANOVA results showed that the main effect of populations, salinity and the interaction of populations on salinity were significant for carotenoid trait (Table 8). The effects of salinity interaction for carotenoids showed that the highest and lowest amounts were obtained in Esfahfo populations at a stress level of 9 dS / m with 0.155 mg / g and Yazd at a stress level of 9 dS / m with 0.03 mg / g, respectively (Table 3).

Protein. For malondialdehyde (MDA), the results of analysis of variance revealed that the main effect of populations, salinity and the interaction effect of salinity on populations were significant (Table 8). The effects of populations×salinity for MDA showed that the highest and lowest amounts were related to Qazvin and Esfahfo populations in control conditions with 5.36 and 1.46 nmol / m leaf fresh weight, respectively (Table 3).

Conclusion

Ajowan is an important medicinal plant with different food and pharmaceutical applications. In the present study, a two year morphological variation was performed to select some populations for slat stress study. So, the second part of study explored the responses of different ajowan populations to different salt treatments. As a result, Arak (C), Yazd (C), Yazd (LS), and Qazvin (SS) recorded superior EO yields, suggesting them as the best populations with acceptable salt tolerance for producing the highest amounts of favorable metabolites. Their main EO constituents were identified to be thymol, *p*-cymene, and γ-terpinene. It is interesting that different amounts of the main constituents can be usefully used in pharmaceutical and food applications. Moreover, medicinal plants with strong antioxidant capacities protect their cells against oxidative damages caused by free radicals. Among the populations, Yazd (MS), Yazd (Ls), Yazd (SS) were found to have the strongest antioxidant activities. Finally, future studies may be directed toward investigating the enzymes or genes following biosynthetic pathways in different ajowan species under different growing conditions. Such studies will certainly provide novel insights into the possibility of increasing the quantities of the main components extracted from this genus.

Materials And Methods

Plant Material and field experimental design. The seeds of twenty-five populations of ajowan originated from Iran were obtained from Research Institute of Forests and Rangeland gene bank, Tehran, Iran. The species identification was performed by Dr. Valiollah Mozzafarian using Flora Iranica and three populations also obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany were also included in this study (Table 9). The study was in compliance with relevant institutional, national and international guidelines and conservation policy of endangered plants.

The seeds were sown in a randomized complete block design (RCBD) in under field conditions at Lavark Research Farm of Isfahan University of Technology. The soil texture of the field was a clay loam with a bulk density of 1.4 g cm^{-3} and a pH of 7.8. The plot size was 1×2 m^2 and the individual plants were spaced 30 cm apart.

Salt stress experiment. In this experiment, four selected ajowan populations (namely, Yazd, Esfahfo, Qazvin, and Arak) were used. The populations were chosen based on their geographical origin, oil yield and seed weight. Fifteen seeds from each population were sown under controlled greenhouse conditions including a temperature of 25 °C and an average humidity of 50%. Each pot contained nine kilograms of soil at a soil to sand ratio of 3:1. Salt treatments were performed at early flowering stage. The experiment was conducted in a factorial design with a randomized complete block design layout replicated three times. Four treatments of 0 (control), 60 (Low stress=LS), 90 (Moderate stress=MS), and 120 mM NaCl (Severe stress=SS) were applied. Prior to the flowering stage, the plants were exposed to different levels of salinity supplied in normal irrigation water. For the control treatment, the pots were irrigated 2 days in the week. In each irrigation applied two liters normal water to each pot. The salt solution was gradually applied in the following steps over a period of three weeks: Initially, two liters of the saline water was applied at 60 dSm^{-1} to all the experimental pots during the first week followed by a treatment of 90 dSm^{-1} in the second week, and one of 120 dSm^{-1} used as severe stress treatment during the third week. The pot soils were leached before each treatment to avoid salt aggregation. At the end of the salt treatment period, total soil electrical conductivities of all the pots, including the control, were determined using an EC meter and the values were recorded (3.1, 4.8, 6, and 8.5 dSm^{-1}).

Essential oil extraction. A Clevenger type device was used to extract the essential oil from the seeds. Briefly, 10-20 g of seed was in used in every hydro-distillation to which 400 mL of distilled water was added and boiled for five hours. Subsequently, the essential oil was collected in a glass container and the yield was reported based on dry matter.

GC/MS analysis. Essential oil compositions were determined by gas chromatography using the Agilent 7890 mass selective detector (Agilent Technologies, Palo Alto, CA, USA) with a HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness). Oven temperature was set to 60 °C and held constant for 4 min before it was increased to 260 °C at a rate of 4 °C/min. The temperature of the GC injector port was kept at 290 °C and that of the detector at 300 °C. Helium was used as the carrier gas at a flow rate of 2 mL/min. The mass unit was operated at an ion source temperature of 240 °C and an ionization voltage of 70 eV. The RI_s of the EO components were calculated experimentally using the retention time of the homologous n-alkane series (C_5-C_{24})[49]. The percentages of EO components were computed from the GC/MS peak areas without any correction factors.

Phenolic and flavonoid evaluation. To prepare a sample extract, 100 mL of 80% methanol was added to 6 g of the seed powder samples and shaken slowly for 24 h. Then, the solution was filtered to remove the solid residues and collected for further experiments. Phenolic compounds were determined according to the Folin-Ciocalteu method[50]. Briefly, 2.5 mL of the Foline-Ciocalteu's reagent (1:10 diluted with distilled water) was mixed with 0.5 mL of the methanolic extract. The samples were incubated for 5 min at room temperature, then 2 mL of 7.5% sodium carbonate solution was added in a tube test and shaken well. The mixture was maintained at 45 °C in a hot water bath for 15 min. Then, the absorbance of the mixture was measured at 765 nm using a spectrophotometer. Tannic acid equivalents (TAE) were used as the reference standard and the total phenolic content (TPC) was expressed as mg of TAE per gram of each extract on a dry basis.

The aluminum chloride colorimetric method was used to determine total flavonoid content (TFC) as described by Tohidi et al.[33]. In initial, a volume of 125 µL of the extract was added to 75 µL of a 5% $NaNO_2$ solution. The studied samples were kept in the dark for 6 minutes before a solution of 10% $Alcl_3$ (150 µL) was added to each and maintained in the dark for an additional five minutes to complete the reaction before a solution of 5% NaOH (750 µL) and water (2500 µL) were added. The absorbance of the samples was determined at 510 nm. The TFC was presented in mg of quercetin equivalents (QE) per gram of the extract.

Antioxidant capacity

DPPH assay. Antioxidant activity performed using the DPPH scavenging method as described in Baharfar et al.[51] with some modifications. Briefly, the ajowan extracts were prepared in the different concentrations of 50, 300, and 500 mg/l in methanol. At the first, 5 mL of 0.1 mM DPPH (2, 2-diphenylpicrylhydrazyl) methanol solution as the free radical source and kept for 30 min at 25 °C. The absorbance was reported at 517 nm against a blank (plant extract without DPPH) and BHT was used as standard controls for comparison. After that, by plotting the graph of extract concentrations against the scavenging activity, a specific concentration of the sample that needed to provide 50% inhibition (IC_{50}) was calculated.

Reducing power. The reducing power of each extract was assessed using the method described in Gharibi et al.[52]. Briefly, 2.5 mL of the methanol extracts (with the different concentrations of 50, 300, and 500 mg/l) plus BHT were mixed in a solution of phosphate buffer (2.5 mL, 0.2 M, pH = 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was initially incubated at 50º C for 20 min before 2.5 mL of trichloroacetic acid (10%) was added and the reaction mixture was centrifuged at 3000 rpm for 10 min. Finally, the supernatant obtained after centrifugation (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of ferric chloride (0.5 mL, 0.1%). The absorbance of the resulting solution was read at 700 nm versus a blank. The increased absorbance of the reaction mixture signified a greater reducing power.

Malondialdehyde (MDA) content. In the present study, malondialdehyde content was measured as an indicator for fatty acid peroxidation. For this purpose, at the end of the third week of salinity stress, 1 g of fresh leaf sample was powdered with liquid nitrogen and then 5 ml of 0.1% TCA was gradually added and completely homogenized. In the next step, the homogenized material was centrifuged at 10,000 rpm for 5 minutes. Then, 500 µl of the supernatant was removed and 2 ml of 20% TBA + 0.5% TCA solution was added. The samples were heated at 95 ° C in Ben Marie for 30 minutes and then rapidly placed in ice. After cooling, they were again centrifuged at 10,000 rpm for 15 minutes. The absorbance of the supernatant was read at 532 nm.

Hydrogen peroxide (H_2O_2) content. For this purpose, the samples were powdered with liquid nitrogen and then 5 ml of 0.1% TCA was gradually added to it and completely homogenized. Then, the homogenized material was centrifuged. Then 500 µl of potassium phosphate buffer (prepared from KH_2PO_4 and K_2HPO_4) was added. Finally, the absorbance at 390 nm was read by a spectrophotometer. The amount of H_2O_2 was determined using a standard line drawn with specified amounts of H_2O_2.

Assessment of ascorbate peroxidase activity (APX). To measure the activity of ascorbate peroxidase, 250 mM phosphate buffer, 1.2 mM hydrogen peroxide, 0.5 mM ascorbic acid and 0.1 mM EDTA were mixed. Enzyme activity was initiated by adding hydrogen peroxide to the mixture. Reduction of absorption due to ascorbic acid peroxidation at 290 nm for two minutes was read. Absorption changes per minute were used to calculate enzyme activity.

Assay of guaiacol peroxidase activity (GPX). Guaiacol peroxidase activity was evaluated. The reaction medium consisted of 25 mM potassium phosphate buffer, 40 mM hydrogen peroxide and 20 mM guaiacol. The reaction was started by adding 100 µl of enzyme extract to a final volume of 3 ml. Increased adsorption was recorded by tetragyakol formation at 470 nm for 3 minutes. The enzyme activity was then expressed as changes in absorption per minute per gram of fresh weight per minute.

Protein assay. The fresh shoots were weighed and homogenized with 2 ml of 0.1 M phosphate buffer. After homogenization, each sample was transferred to 2 ml vials. The samples were then centrifuged at 15000 rpm for 12 minutes at 4 °C. Bradford (1976)[53] method was used to measure the protein concentration of total plant extracts.

Measurement of chlorophyll content. After applying salinity stress, the amount of chlorophyll in the leaves was evaluated by Arnon (1949)[54] method. The chlorophyll content was then calculated using the following equation:

(1) Chl a (mg/g.f.w) =[12.7(abs 663)-2.69(645)]*v/1000*w

(2) Chl b (mg/g.f.w) =[22.9(abs 645)-4.69(663)]*v/1000*w

(3) Chl a+b (mg/g.f.w) =[20.2 (abs 645)+8.02(663)]*v/1000*w

(4) Carotenoid (mg/g.f.w) =1000(abs 470)-1.8(chlo a)-85.02(chlo)/198

Statistical analysis. The data analysis was accomplished using SAS (version 9.1). Cluster and PCA analyses were performed using Stat graphics ver. XVI.I.

Declarations

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Author contributions

Each of authors contributed to this study as following: G.M. performed experiment and contributed to analysis and interpretation of data, and writing the manuscript. M.R. and A.A. contributed to study conception and project design and critically revised the manuscript for important intellectual content. P.Y. was responsible for data analysis. M.H.E. was responsible for a part of project design.

Competing interests

The authors declare no competing interests.

Additional information

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Tables

Means of Square											
Source of variation	df	Height (cm)	N. of flowering branches	Inflorescence diameter	N. of umbel	N. of umbellule per inflorescence	N. of flowers per umbel	N. of seeds per umbel	Crown cover diameter (cm2)	One thousand seed weight (gr)	Seed yi plant
Year	1	24396.**	2648.14.**	60.84.**	785321.**	1618.82.**	840793.**	3363172.**	357643.**	0.037.**	386539
Rep(year)	2	195.21**	23.70*	0.40**	9048.53**	5.86**	3063.36ns	12253.47ns	228663**	0.0006ns	4356.9
Genotypes	27	381.72**	110.35**	0.80**	25217.35**	16.58**	21926.23**	87704.9**	596830**	0.05**	10394.
Year* Genotypes	2	323.7**	130.9**	0.92**	24389.7**	23.23**	23900.9**	95603.8**	4386.02**	0.0001**	10673.
Error	144	99.51	8.62	0.31	2260.86	4.39	3868.95	15475	36513	0.0003	1517.5

Table 1. Analysis of variance for morphological traits and essential oil content of 28 ajowan (*Trachyspermum ammi* L.) populations. ns: non-significant. *: Significant at 5 % level of probability. **: Significant at 1 % level of probability.

Essential oil yield		Seed yield per plant		One thousand seed weight (gr)		Crown cover diameter (cm2)		N. of seeds per umbel		N. of flowers per umbel		N. of umbellule per inflorescence		N. of umbel		Infl dia
2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	201
4.51	4.35	80.31	31.93	0.75	0.72	1896.9	1462.7	675	439.67	337.5	219.83	18.5	13	105.2	58	5.5
4.47	4.35	86.37	52.48	0.92	0.87	569.8	351.5	329	345.33	164.5	172.67	17.1	11.83	262.7	76	3.7
4.13	3.63	286.80	27.81	0.79	0.76	1056	741.2	943	279	471.5	139.50	21	10.833	389.3	78.67	4.2
3.16	3.01	61.94	21.43	0.61	0.58	697.1	449.1	465.7	361	232.8	180.50	20	13.16	199	102.83	4.5
4.43	4.36	58.44	41.31	0.82	0.80	489.1	278.6	410.7	396.67	205.3	198.33	15	13.16	167	82.50	4.5
4.16	4.09	71.39	72.45	0.75	0.72	987.9	688	698.3	395.67	349.1	197.83	19.5	13	135	273.39	4.1
2.90	2.79	67.61	32.09	0.73	0.70	1097.4	778.7	560	385.33	280	192.67	15.8	13.16	162.8	140	4.4
5.20	5.23	63.60	43.38	0.96	0.92	1906.2	1548.7	528	439.67	264	219.83	20.6	13.66	127.6	57.50	4.1
5.13	5.08	71.90	15.53	0.86	0.83	833.2	563.9	551.3	275	275.6	137.50	18.8	12	147.3	65.50	4.1
4.66	4.61	187.27	34.70	0.80	0.77	755.7	491.9	648	408.67	324	204.33	18.5	12.83	342	88.33	4
2.98	2.78	228.05	58.53	0.75	0.73	705.6	458.3	724.3	546.33	362.1	273.17	18.5	16	413.3	143	4.3
4.69	4.61	91.90	39.11	0.94	0.91	969.5	669.6	550.3	361.33	275.1	180.67	20.3	12	173.3	132.50	3.8
4.15	4.03	128.71	32.19	0.87	0.85	905.1	626.4	721	345.67	360.5	172.83	22.6	12	204.1	110.83	5.2
4.85	4.72	106.39	20.46	0.98	0.96	1059.9	771.1	546.7	387.33	273.3	193.67	15.6	15.16	194.5	81.33	4.8
1.74	1.62	51.56	22.19	0.66	0.63	886.2	606.7	472	565.33	236	282.67	16.3	13.83	154.8	61.50	3.8
3.53	3.43	127.19	23.46	0.61	0.58	832.8	537	940	342.33	470	171.17	23.8	11.83	250.5	130.25	4.5
5.58	5.51	177.60	14.16	0.96	0.92	584.9	354.4	809.7	507.33	404.8	253.67	20.6	15.83	235.8	29.83	4.8
4.37	4.23	114.77	69.33	0.72	0.70	585.5	357.9	422.7	526.67	211.3	263.33	16.6	13.50	372.1	145.65	3.7
5.52	5.32	315.02	24.11	0.77	0.74	1021.4	694.9	1228	435	614	217.50	29.6	13.83	326.8	86	4.1
4.94	4.73	159.55	25.71	0.80	0.78	672	417.5	1020.3	384	510.1	192	22.3	14.33	188.1	102.33	5.1
2.28	2.07	53.59	9.85	0.74	0.71	954.6	644.8	888.7	251	444.3	125.50	20.5	11.50	80.6	56.33	5.5
3.91	3.80	171.82	12.13	0.70	0.67	896.1	625.3	477.7	411.33	238.8	205.67	20.1	13.83	550.3	43.50	4.1
4.40	4.34	74.06	20.09	0.78	0.75	612.5	358.7	795.3	477.67	397.6	238.83	16.5	14	134.5	44.09	3.8
2	1.20	290.74	10.61	0.73	0.70	848.8	432.9	986.7	228	493.3	114	24.6	9.33	399.8	61	3.9
3.67	3.53	118.15	40.72	0.75	0.72	1098.7	755.2	605.7	351.33	302.8	175.67	17	12	344	163.67	3.3
4.18	4.04	19.30	11.60	0.76	0.71	733.9	434.8	329.3	273	164.6	136.50	15.5	14.16	76	60.67	3.3
4.44	4.33	46.90	23.89	0.76	0.73	839.4	560.4	582.3	374	291.1	187	16.1	13.16	111.6	103.25	4.6
4.39	4.17	247.64	41.16	0.75	0.74	1289.8	932.1	994.7	487.33	497.3	243.67	20.8	16.16	258.1	99.75	4.6
0.36	0.38	83.64	33.72	0.03	0.03	335.5	288.2	219	186.94	109.5	93.47	3.1	3.72	107.7	22.38	0.6

Table 2. Means of morphological characters and essential oil content of 28 ajowan (*Trachyspermum ammi* L.) populations in two years of 2017 and 2018.

Species	TFC [a] (mg QE g^{-1} DW)	TPC [b] (mg TAE g^{-1} DW)	ESO (%)	N. of seeds per plant	MAD	H_2O_2	GPX	APX	Pro	Chla	Chlb	Car
Qazvin (C)	3.50^{k}	124.32^{h}	2.16^{o}	48.98^{g}	5.36^{a}	2.40^{a}	0.22^{a}	0.007^{d}	0.23^{ab}	0.07^{c}	0.031^{c}	0.041^{d}
Qazvin (LS)	4.22^{g}	157.32^{b}	3.11^{l}	52.55^{e}	0.86^{d}	1.94^{b}	0.067^{b}	0.02^{b}	0.23^{a}	0.18^{a}	0.06^{a}	0.08^{b}
Qazvin (MS)	4.37^{f}	135.15^{d}	3.53^{j}	29.22^{o}	1.81^{d}	1.59^{c}	0.075^{b}	0.006^{c}	0.23^{a}	0.22^{b}	0.063^{b}	0.104^{b}
Qazvin (SS)	3.49^{k}	119.26^{i}	4.26^{d}	28.59^{p}	4.35^{a}	1.95^{a}	0.277^{a}	0.014^{a}	0.238^{a}	0.136^{b}	0.040^{a}	0.076^{b}
Esfahfo (C)	3.99^{h}	64.85^{m}	3.22^{k}	63.79^{c}	1.46^{d}	2.20^{b}	0.11^{c}	0.016^{b}	0.24^{a}	0.17^{b}	0.037^{bc}	0.077^{c}
Esfahfo (LS)	3.76^{j}	77.32^{l}	3.74^{i}	67.34^{b}	3.62^{b}	2.12^{a}	0.261^{a}	0.01^{c}	0.24^{a}	0.13^{b}	0.04^{a}	0.07^{c}
Esfahfo (MS)	5.35^{b}	127.44^{f}	2.64^{n}	49.50^{f}	3.10^{c}	1.97^{a}	0.014^{c}	0.011^{a}	0.24^{a}	0.36^{a}	0.089^{a}	0.155^{a}
Esfahfo (SS)	5.00^{c}	133.58^{e}	3.77^{h}	38.01^{l}	2.28^{d}	1.52^{d}	0.012^{d}	0.010^{b}	0.243^{a}	0.179^{a}	0.040^{a}	0.092^{a}
Arak (C)	4.26^{g}	81.86^{k}	4.77^{a}	47.66^{h}	2.51^{b}	0.91^{d}	0.18^{b}	0.025^{a}	0.23^{b}	0.19^{b}	0.065^{ab}	0.099^{b}
Arak (LS)	4.42^{f}	183.83^{a}	3.01^{m}	40.46^{j}	3.70^{a}	0.45^{d}	0.022^{c}	0.025^{a}	0.23^{a}	0.23^{a}	0.05^{a}	0.10^{a}
Arak (MS)	4.71^{d}	125.99^{g}	4.22^{e}	39.94^{k}	3.84^{a}	1.54^{c}	0.089^{a}	0.010^{b}	0.24^{a}	0.14^{c}	0.070^{ab}	0.083^{b}
Arak (SS)	4.40^{f}	99.13^{j}	4.10^{f}	34.10^{m}	3.30^{c}	1.73^{b}	0.208^{b}	0.014^{a}	0.240^{a}	0.177^{a}	0.048^{a}	0.078^{b}
Yazd (C)	3.48^{k}	61.76^{n}	4.61^{b}	68.75^{a}	1.69^{c}	1.66^{c}	0.17^{b}	0.014^{c}	0.23^{b}	0.34^{a}	0.080^{a}	0.154^{a}
Yazd (LS)	4.50^{e}	147.00^{c}	4.39^{c}	59.13^{d}	2.81^{c}	1.41^{c}	0.078^{b}	0.002^{d}	0.23^{a}	0.05^{c}	0.03^{a}	0.03^{d}
Yazd (MS)	5.94^{a}	133.58^{e}	4.10^{f}	45.00^{i}	3.13^{b}	1.80^{b}	0.077^{ab}	0.011^{a}	0.24^{a}	0.11^{c}	0.023^{c}	0.044^{c}
Yazd (SS)	3.92^{i}	77.32^{l}	3.97^{g}	30.74^{n}	3.50^{b}	1.60^{c}	0.125^{c}	0.008^{c}	0.240^{a}	0.137^{b}	0.025^{b}	0.072^{b}

Table 3. Contents of total phenolics, total flavonoids, essential oil yield of four selected *Trachyspermum ammi* populations. Means with different letter are statistically significant at 5% level probability. C: Control; LS: Low stress; MS: Moderate stress; SS: Severe stress; TAE: Tannic acid equivalents; QE: Quercetin equivalent; TFC: Total flavonoid content; TPC: Total phenolic content; ESO: Essential oil yield; MAD: Malondialdehyde content; H_2O_2: Hydrogen peroxide content; GPX: Guaiacol peroxidase activity; APX: Ascorbate peroxidase activity; Pro: Protein; Chla: Chlorophylla content; Chlb: Chlorophyllb content, Car: Carotenoid; [a] Total flavonoid content; [b] Total phenolic content.

Populations		Arak				Esfahfo				Qazvin				Yazd
Compounds (%)	RI[a]	C	LS	MS	SS	C	LS	MS	SS	C				
α-Thujene	927	0.36	0.93	0.59	0.57	0.31	0.9	1.15	0.72	0.42				
α-Pinene	941	0.15	0.27	0.16	0.17	0.11	0.23	0.30	0.22	0.13				
Sabinene	972	0.18	0.38	0.24	0.22	0.21	0.35	0.48	0.31	0.25				
β-Pinene	979	1.01	1.00	0.35	0.46	0.66	0.42	0.46	0.78	0.43				
Myrcene	996	0.35	1.02	0.72	0.63	0.47	1.16	1.43	1.00	0.48				
α-Terpinene	1017	0.38	0.75	0.53	0.66	0.19	0.74	0.85	0.67	0.34				
p-Cymene	1025	18.74± 0.01[a]	22.18±0.02[j]	25.42±0.01[d]	22.34±0.02[i]	20.32±0.01[m]	26.16±0.02[a]	25.71±0.02[b]	19.21±0.01[n]	18.8(				
β-Thujone	1110	0.81	1.11	0.79	0.95	0.74	0.18	0.24	0.34	0.77				
γ-Terpinene	1057	26.89±0.04[d]	21.94±0.01[o]	26.71±0.02[e]	24.27±0.03[i]	23.1±0.0.04[j]	22.75±0.04[l]	25.51±0.01[g]	21.71±0.01[p]	22.8				
Pulegone	1246	0	0	0	0	0.71	0.08	0.03	0.02	0				
Terpinene-4-ol	1181	0.21	0.23	0.31	0.27	0.29	0.35	0.12	0.12	0.31				
Thymol	1290	50.16±0.04[e]	50.5±0.05[d]	40.08±0.02[l]	47.92±0.02[h]	52.2±0.02[b]	37.63±0.05[m]	42.11±0.05[k]	51.58±0.01[c]	54.2				
Carvacrol	1315	0.65	0.85	0.63	0.62	0.63	0.45	0.82	0.98	0.66				
Total	-	99.80	98.75	98.94	99.08	99.99	98.19	92.42	97.66	99.4(				

Table 4. Volatile compounds (%) of essential oils in studied ajowan populations under different salt concentration and control condition. Means with differer components. C: Control; LS: Low stress; MS: Moderate stress; SS: Severe stress.

	PC1	PC2	PC3
p-Cymene	0.465	0.091	-0.383
y-Terpinene	0.537	-0.016	-0.006
Thymol	-0.568	-0.087	0.229
Essential oil yield	0.204	0.451	0.429
Total phenolic content	-0.147	-0.004	-0.673
Total Flavonoid content	-0.295	0.368	-0.395
DPPH	-0.0001	-0.640	-0.050
Reducing power (500mg/l)	-0.139	0.483	-0.058
Eigenvalue	2.696	1.956	1.500
percent of variance	33.706	24.455	18.754
Cumulative percentage	33.706	58.161	76.915

Table 5. Principal component loadings for the measured traits on studied ajowan populations.

Traits								
0.007 $^{n.s}$	0.947*	-0869 $^{n.s}$	-0.643 $^{n.s}$	-0.281 $^{n.s}$	-0.870 $^{n.s}$	0.371 $^{n.s}$	1	*p*-Cymene
0.003 $^{n.s}$	0.390 $^{n.s}$	0.114 $^{n.s}$	0.210 $^{n.s}$	-0.188 $^{n.s}$	-0.733 $^{n.s}$	1	0.796 $^{n.s}$	γ-Terpinene
-0.225 $^{n.s}$	-0.773 $^{n.s}$	0.580 $^{n.s}$	0.475 $^{n.s}$	0.076 $^{n.s}$	1	-0.961*	-0.931$^{n.s}$	Thymol
0.953 $^{n.s}$	-0.573 $^{n.s}$	0.062 $^{n.s}$	-0.429 $^{n.s}$	1	-0.669 $^{n.s}$	0.804 $^{n.s}$	0.430 $^{n.s}$	Essential oil yield
-0.597 $^{n.s}$	-0.401 $^{n.s}$	0.874 $^{n.s}$	1	-0.707 $^{n.s}$	0.614 $^{n.s}$	-0.561 $^{n.s}$	-0.642$^{n.s}$	Total phenolic content
-0.152 $^{n.s}$	-0.758 $^{n.s}$	1	-0.289 $^{n.s}$	0.403 $^{n.s}$	0.383 $^{n.s}$	-0.219 $^{n.s}$	-0.524$^{n.s}$	Total flavonoid content
-0.309 $^{n.s}$	1	0.093 $^{n.s}$	0.574 $^{n.s}$	-0.872 $^{n.s}$	0.919 $^{n.s}$	-0.990**	-0.780$^{n.s}$	DPPH^{a}
1	0.799 $^{n.s}$	-0.407 $^{n.s}$	0.361 $^{n.s}$	-0.914 $^{n.s}$	0.499 $^{n.s}$	-0.712 $^{n.s}$	-0.165$^{n.s}$	Reducing power

Table 6. Correlation coefficients between bioactive components on studied ajowan populations under control and low salt condition. (below diagonal) Control and Low Salt stress condition (on diagonal). **Significant at 1% level of probability. *Significant at 5% level of probability. ^{a} 1,1-Diphenyl-2-picrylhydrasyl.

Traits								
-0.270 $^{n.s}$	-0.088 $^{n.s}$	-0.949 $^{n.s}$	-0.268 $^{n.s}$	0.969 $^{n.s}$	-0.927 $^{n.s}$	0.944*	1	*p*-Cymene
-0.388 $^{n.s}$	0.221 $^{n.s}$	-0.888 $^{n.s}$	0.032 $^{n.s}$	0.905 $^{n.s}$	-0.998 $^{n.s}$	1	0.931 $^{n.s}$	γ-Terpinene
0.405 $^{n.s}$	-0.267 $^{n.s}$	0.869 $^{n.s}$	-0.079 $^{n.s}$	-0.887 $^{n.s}$	1	-0.994**	-0.916 $^{n.s}$	Thymol
-0.463 $^{n.s}$	-0.028 $^{n.s}$	-0.843 $^{n.s}$	-0.187 $^{n.s}$	1	0.179 $^{n.s}$	-0.154 $^{n.s}$	-0.461 $^{n.s}$	Essential oil yield
-0.637 $^{n.s}$	0.980 $^{n.s}$	0.404 $^{n.s}$	1	0.138 $^{n.s}$	0.970*	-0.939 $^{n.s}$	-0.815 $^{n.s}$	Total phenolic content
-0.026 $^{n.s}$	0.219 $^{n.s}$	1	0.019 $^{n.s}$	-0.006 $^{n.s}$	0.257 $^{n.s}$	-0.359 $^{n.s}$	-0.472 $^{n.s}$	Total flavonoid content
-0.663 $^{n.s}$	1	-0.668 $^{n.s}$	-0.017 $^{n.s}$	-0.732 $^{n.s}$	-0.211 $^{n.s}$	0.265 $^{n.s}$	0.584 $^{n.s}$	DPPH^{a}
1	-0.527 $^{n.s}$	0.969*	0.204 $^{n.s}$	-0.142 $^{n.s}$	0.419 $^{n.s}$	-0.516 $^{n.s}$	-0.549 $^{n.s}$	Reducing power

Table 7. Correlation coefficients between bioactive components on studied ajowan populations under moderate and serve salt conditions. (below diagonal) Moderate salt stress and serve stress condition (on diagonal). **Significant at 1% level of probability. *Significant at 5% level of probability. [a] 1,1-Diphenyl-2-picrylhydrasyl.

Sources of variation	df	DPPH	RP	TFC	TPC	NCP	ESO	Thymo	γ - Terpinene	p-Cymene	MAD	H_2O_2	GPX	APX
Gen	3	3499789**	0.115**	1.03**	2882.8**	663.74**	3.27**	39.73**	40.08**	3.05**	1.24**	1.73**	0.008**	0.000
Salt	3	27167123**	0.323**	3.51**	8003.4**	919.11**	0.44**	97.89**	10.20**	30.41**	0.97**	0.22**	0.031**	0.000
Gen*Salt	9	66623**	0.015**	0.82**	2543.8**	344.43**	1.27**	181.4**	47.95**	22.06**	5.99**	0.49**	0.024**	0.000
Error	32	1875.01	0.001	0.002	0.018	128.42	0.033	0.081	0.181	0.006	0.056	0.029	0.0002	0.000
Coeff Var	-	1.16	13.37	1.21	0.11	24.37	4.91	0.63	1.68	0.35	8.05	10.26	11.81	5.19

Table 8. Analysis of variance for the measured traits on studied ajowan populations. *, ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively. TFC: Total flavonoid content; TPC: Total phenolic content; ESO: Essential oil yield; MAD: Malondialdehyde content; H_2O_2: Hydrogen peroxide content; GPX: Guaiacol peroxidase activity; APX: Ascorbate peroxidase activity; Pro: Protein; Chla: Chlorophylla content; Chlb: Chlorophyllb content, Car: Carotenoid; [a] Total flavonoid content; [b] Total phenolic content.

No	Accession Number	Location	Accession code	Geographical region	latitude	longitude	Altitude(m)
1	37477	Nahadjan,Khorasan,Iran	khorsar	East	32˚32′N	59˚47′E	1816
2	38913	Boshruieh,Khorasan,Iran	khorbos	East	33˚53′ N	57˚27′ E	880
3	38924	Birjand,Khorasan,Iran	khorbir	East	32˚53′ N	59˚13′ E	1461
4	38929	Sarbisheh,Khorasan,Iran	khorsa	East	32˚34′ N	59˚48′ E	1827
5	37492	Boztanj,Khorasan,Iran	khorbi	East	32˚51′ N	59˚12′ E	1458
6	37483	Mohammadieh,Khorasan,Iran	khormo	East	32˚55′ N	59˚13′ E	1460
7	37529	Ghayen,Khorasan,Iran	khorgh	East	33˚43′ N	59˚10′ E	1455
8	15226	Khomein,Markazi,Iran	arakkho	West	33˚38′ N	50˚4′ E	1811
9	14743	Arak,Markazi,Iran	arak	West	34˚5′ N	49˚42′ E	1735
10	14492	Shazand,Markazi,Iran	araksha	West	33˚56′ N	49˚24′ E	1914
11	14322	Hamedan,Hamedan,Iran	hamdan	West	34˚47′ N	48˚30′ E	1818
12	37251	Mollasadra,Yazd,Iran	yazmol	Center	31˚50′ N	54˚22′ E	1242
13	31831	Markaz tahghighat,Yazd,Iran	yaztah	Center	31˚54′ N	54˚16′ E	1213
14	33683	Saduq,Yazd,Iran	yazsad	Center	32˚1′ N	53˚28′ E	2091
15	15484	Shahedieh,Yazd,Iran	yazshah	Center	31˚56′ N	54˚16′ E	1193
16	15864	Sadooqi,Yazd,Iran	yazist	Center	31˚52′ N	54˚20′ E	1228
17	1085	Yazd,Yazd,Iran	yazd	Center	31˚53′ N	54˚21′ E	1215
18	4077	Ghahderijan,Isfahan,Iran	esfahgh	Center	32˚34′ N	51˚26′ E	1615
19	943	Fozveh,Isfahan,Iran	esfahfo	Center	32˚36′ N	51˚26′ E	1615
20	20055	Qazvin,Qazvin,Iran	qazvin	North	36˚16′ N	49˚59′ E	1305
21	906	Tehran,Tehran,Iran	tehran	North	35˚41′ N	51˚23′ E	1168
22	10569	Ardabil,Ardabil,Iran	ardebil	Northwest	38˚16′ N	48˚18′ E	1332
23	17902	Marvdasht,Fars,Iran	farsmar	South	29˚52′ N	52˚49′ E	1600
24	17861	Shiraz,Fars,Iran	farsfars	South	29˚35′ N	52˚35′ E	1508
25	23011	Rafsanjan,Kerman,Iran	rafsanj	South	30˚21′ N	56˚0′ E	1545
26	TRACH 1	Genebank IPK, Germany	IPK1	Unknown	-	-	-
27	TRACH 5	Genebank IPK, Germany	IPK2	Unknown	-	-	-
28	TRACH 6	Genebank IPK, Germany	IPK3	Unknown	-	-	-

Table 9. Geographical location of 28 ajowan populations.

Figures

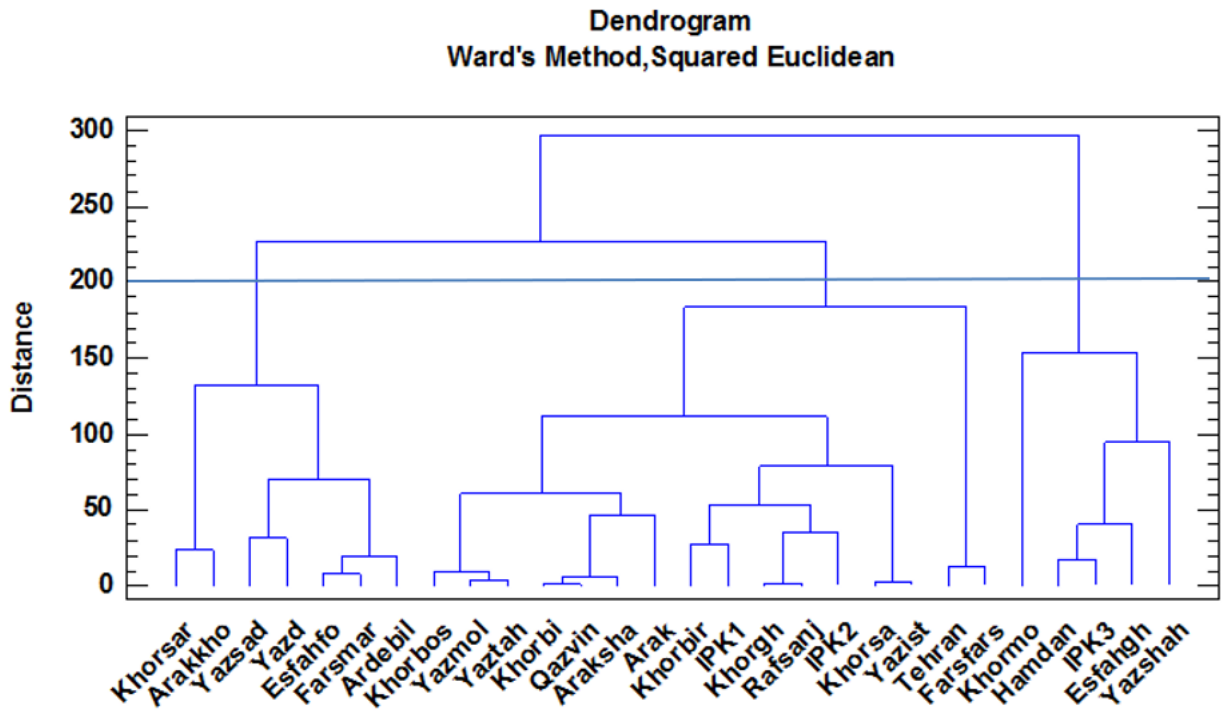

Figure 1

Dendrogram generated from cluster analysis of 28 ajowan (Trachyspermum ammi L.) populations based on agro-morphological characters and essential oil content using WARD based on the Squared Euclidean dissimilarity calculated of 2017.

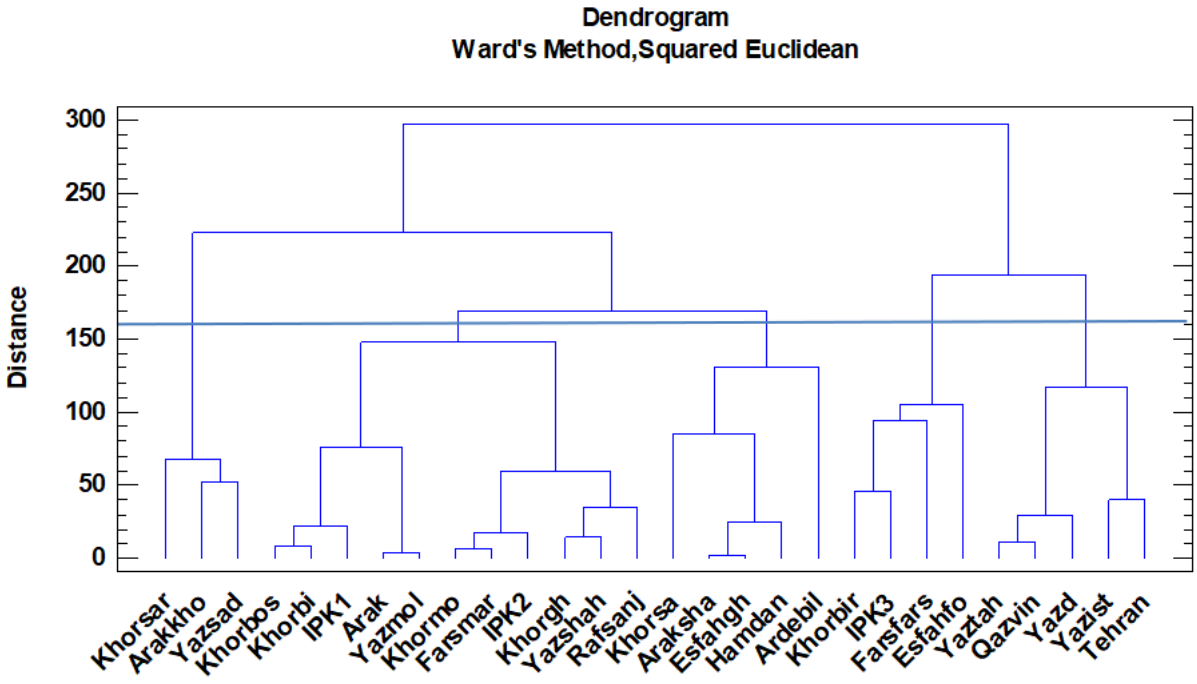

Figure 2

Dendrogram generated from cluster analysis of 28 ajowan (Trachyspermum ammi L.) populations based on agro-morphological characters and essential oil content using WARD based on the Squared Euclidean dissimilarity calculated of 2018.

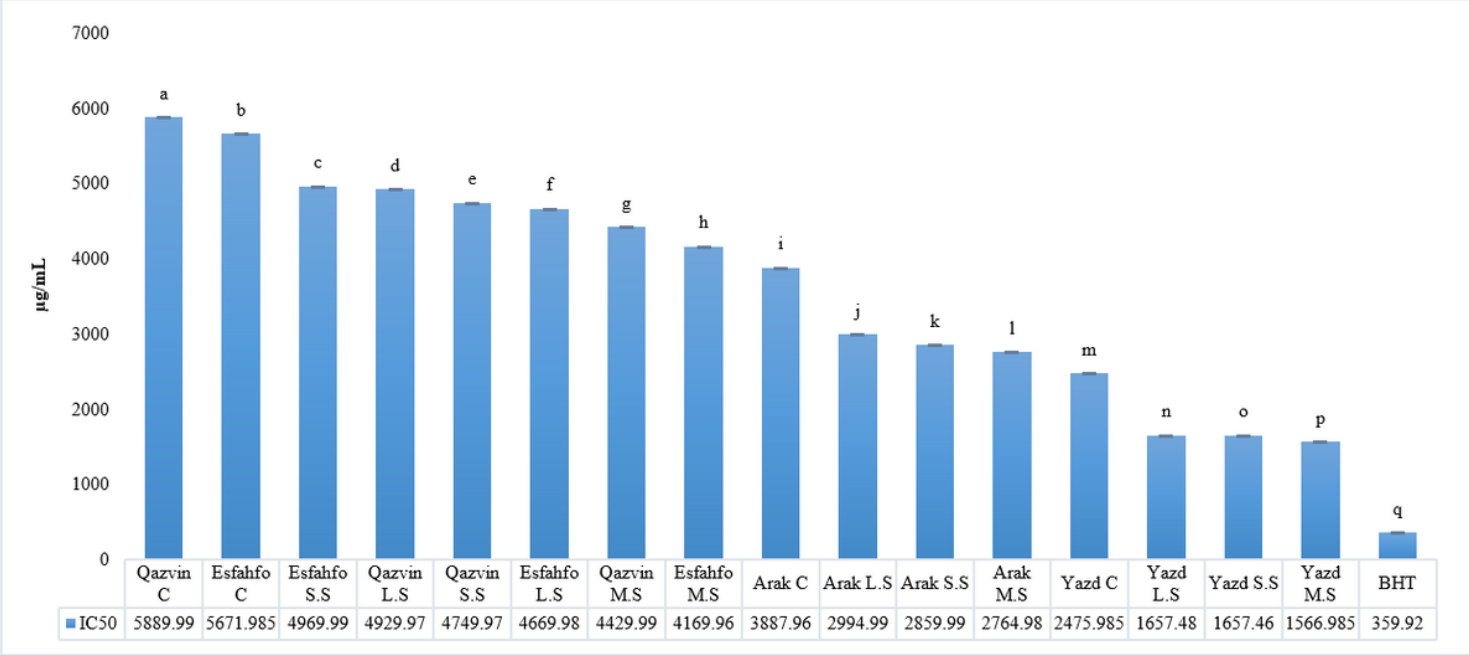

Figure 3

IC50 (µg/mL) of ajowan populations extracts as compared to BHT.

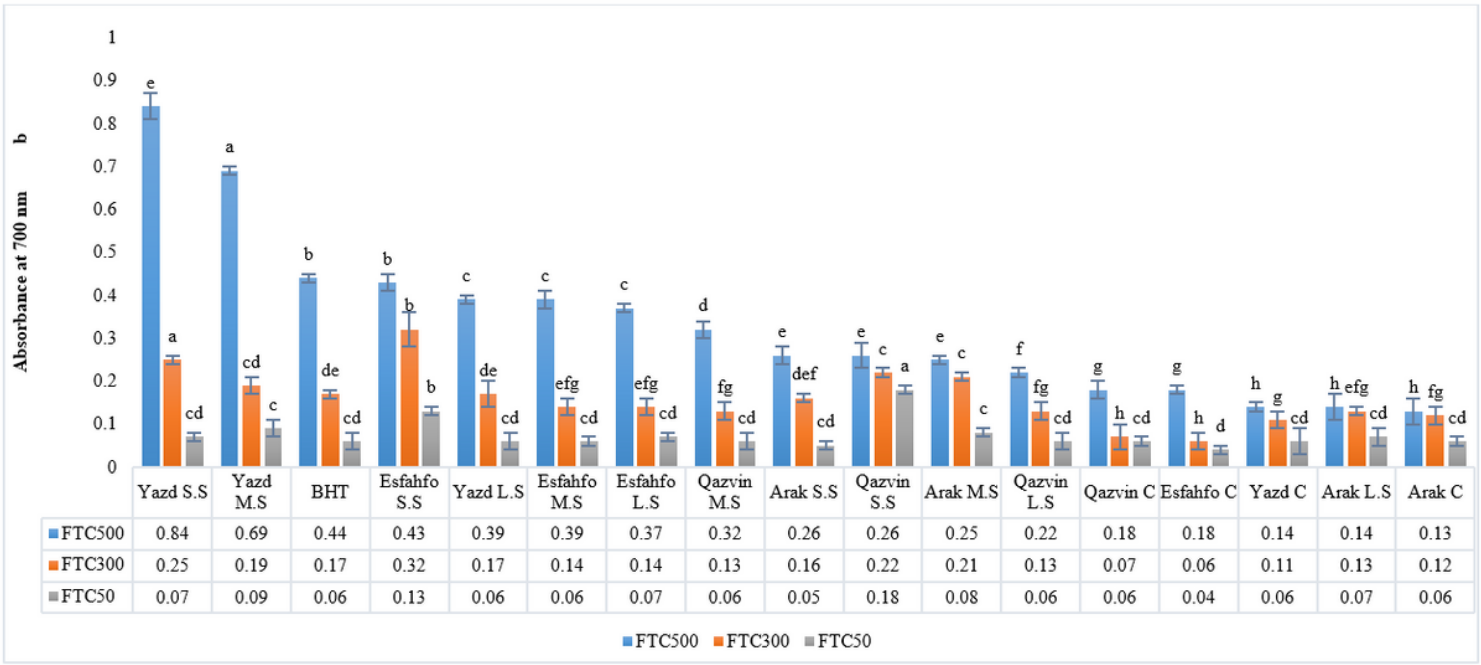

Figure 4

Evaluation of antioxidant capacity based on reducing power in ajowan extracts as compared to BHT. Control (C), 60 (Low stress=LS), 90 (Moderate stress=MS) and 120 dSm-1 (Severe stress=SS). BHT: butylated hydroxytoluene.

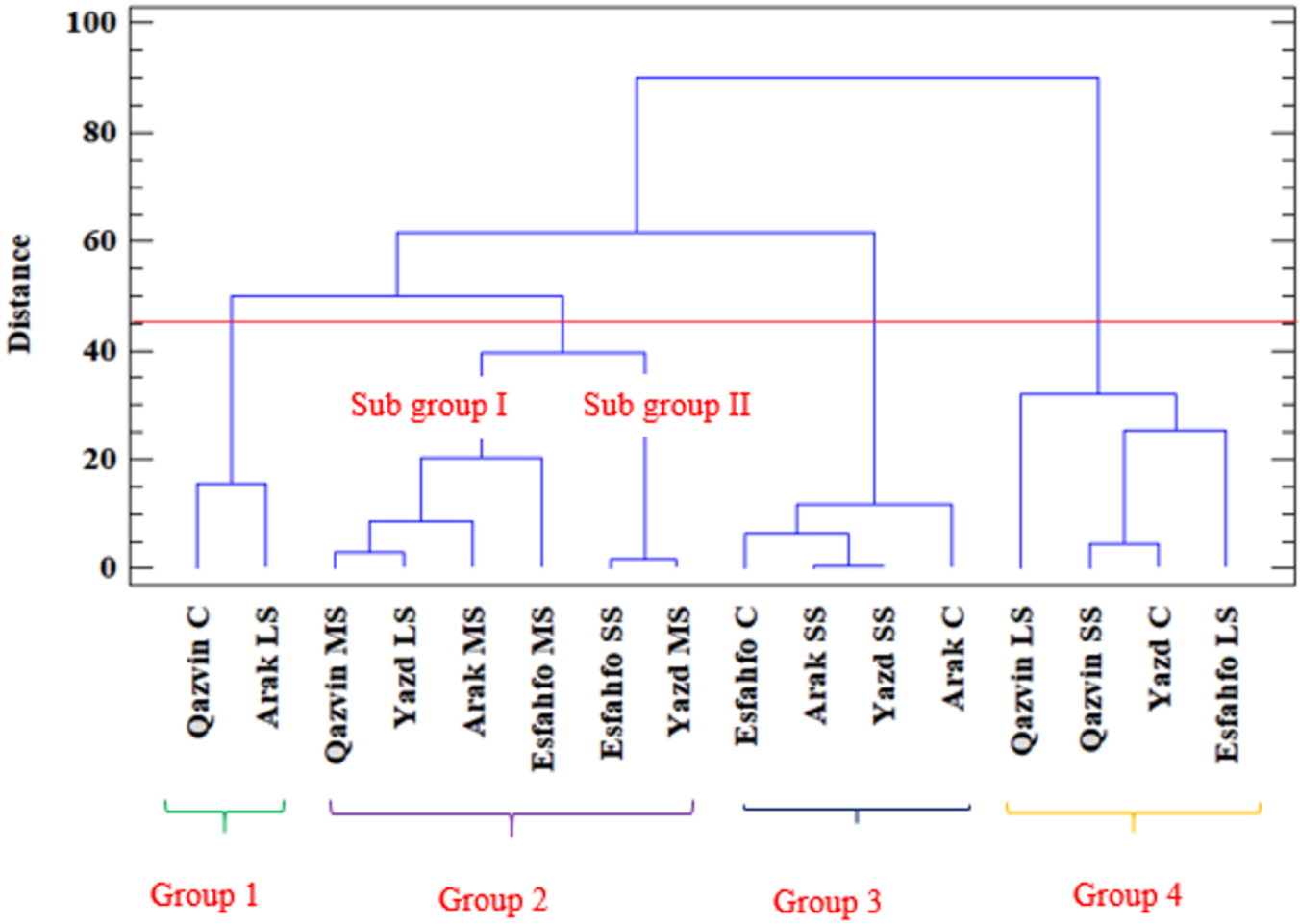

Figure 5

Dendrogram of four ajowan populations under different salinity levels using Ward clustering method. C: Control; LS: Low stress; MS: Moderate stress; SS: Severe stress.

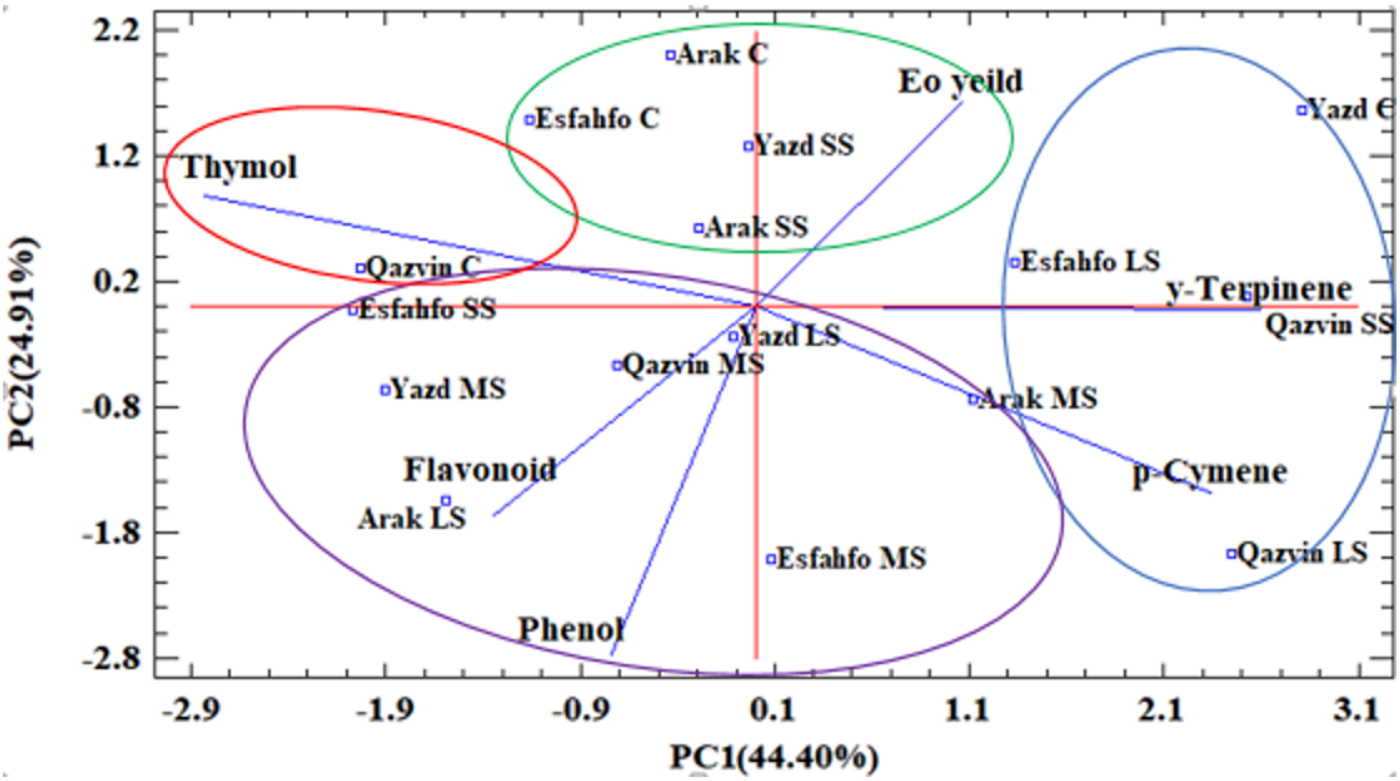

Figure 6

Principle component analysis of ajowan populations based on major essential oil components and other phytochemical traits. C: Control; LS: Low stress; MS: Moderate stress; SS: Severe stress.