



## OPEN Soil-based biochar and foliar-spraying of chitosan enhances the phytochemical traits of yarrow (*Achillea millefolium* L.) under varying moisture levels

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Yarrow (*Achillea millefolium* L.) is an important medicinal and aromatic plant the active biologically compounds in particular essential oil is used as raw material in the pharmaceutical, food, and cosmetics industries. The impacts of the foliar spraying of chitosan (control and 2.5 g/L chitosan), the use of soil-based biochar (control and soil application at 2.0 kg/m<sup>2</sup>) under three moisture levels (irrigation at 80–85%, 60–65%, and 40–45% F.C.) on the agronomic traits and essential oil of yarrow were investigated. Results indicated that the deficit irrigation meaningfully decreased the yields of biological and flower, and essential oil yield, however, the contents of proline and essential oil under reduced irrigation significantly increased. According to essential oils analysis by GC-FID and GC/MS, the major constituents were  $\alpha$ -pinene, 1,8-cineole, borneol,  $\beta$ -bisabolene, and caryophyllene oxide. A considerable increase and decrease were observed respectively in the monoterpenes and sesquiterpenes contents under reduced irrigation. The utilization of biochar along chitosan maintained higher secondary metabolites in particular  $\alpha$ -pinene,  $\beta$ -myrcene, borneol, and 1,8-cineole under deficit irrigation. Generally, it appears that using the foliar spraying of chitosan along the soil application of biochar can be effective in improving the qualitative and quantitative features of the essential oil of *A. millefolium* in arid and semiarid climates.

**Keywords** *Achillea millefolium* L.,  $\alpha$ -pinene, borneol,  $\beta$ -bisabolene, 1,8-cineole, Enzymatic activity, Reduced irrigation

### Abbreviations

F.C.	Field capacity
RCBD	Randomized complete block design
P.W.P.	Permanent wilting point
CAT	Enzymatic activity of catalase
POD	Peroxidase
APX	Ascorbate peroxidase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
GC-FID	Gas chromatography (GC)–Flame ionization detector (FID)
GC/MS	Gas chromatography/mass Spectrometry
GLM	Generalized linear model

Yarrow (*Achillea millefolium* L.) is a perennial herb belonging to the Asteraceae family that grows wildy in Asia, Africa, Europe, and America. Nowadays, the essential oil from the leaves and inflorescences of yarrow as an important therapeutic ingredients is widely used in traditional and modern medicine, food, cosmetic, and pharmaceutical industries<sup>1</sup>. The yarrow essential oil is used as an analgesic, antioxidant, anti-inflammatory, and

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stomach protector, as well as in the production of skincare products and aromatherapy<sup>2,3</sup>. *A. millefolium* has a significant position in the pharmaceutical and cosmetic industries, and conducting agronomic research will greatly contribute to expanding and improving the economic justification for cultivating this herb<sup>1,3</sup>. The major components of essential oil from the yarrow flowers due to the existence of several chemotypes are very complex. The results of previous investigations have indicated that  $\alpha/\beta$ -pinene, borneol, camphor, 1,8-cineol, myrcene,  $\alpha/\beta$ -thujone, bornyl acetate, sabinene, and spathulenol were the main constituents of the yarrow essential oil<sup>1,2,4,5</sup>.

The growth, physiology, and biochemistry responses, as well as the biosynthesis of secondary metabolites in particular essential oils in the medicinal and aromatic plants vary under the influence of genetic<sup>1,3</sup>, environmental factors such as climate, edaphic, and agronomic practices, biotic and abiotic stresses<sup>6–8</sup>, and interaction effects of them. Among environmental stresses, water deficit stress being one of the major abiotic factor affecting the quantity and quality essential oil in the medicinal and aromatic crops<sup>9</sup>. Generally, water deficit stress that requires immediate attention, as the world faces water supply constraints in agricultural areas to achieve higher agricultural productivity. An imbalance between precipitation and evapotranspiration in a specific area leads to drought stress<sup>10</sup>. One of the impacts of drought and water deficit stress is the induction of oxidative damage due to the production of reactive oxygen species, which leads to the oxidation of lipids, proteins, nucleic acids, and carbohydrates, and disrupts physiological processes such as glycolysis and photosynthesis<sup>6,8</sup>. Plants have various defense mechanisms to cope with the detrimental impacts of water deficit stress including antioxidant enzymes (catalase, ascorbate peroxidase, superoxide dismutase, etc.) and non-enzymatic antioxidant compounds (phenols, carotenoids, flavonoids, tocopherols, ascorbate, etc.).

Some biological or organic compounds can act as stimulants for modifying secondary metabolites and, consequently, the therapeutic impacts of the medicinal and aromatic plants. In recent years, the application of signaling compounds as elicitors has evolved as an effective strategy for producing desired secondary metabolites in the medicinal and aromatic plants<sup>11,12</sup>. One of the elicitors is chitosan that a natural polysaccharide obtained through the acetylation reaction of chitin extracted from the exoskeletons of crustaceans such as shrimp and crabs or from the cell wall of certain fungi<sup>13</sup>. Chitosan is a non-toxic, biocompatible, and biodegradable compound, which has led to its widespread application in agriculture. Among the beneficial impacts of chitosan is its ability to improve plant tolerance to biotic and abiotic stresses<sup>11,12,14</sup>. In general, chitosan stands out as a versatile and eco-friendly bio-stimulant with immense potential to revolutionize agriculture. Its ability to enhance disease resistance, improve growth, and mitigate abiotic stress makes it a valuable tool for sustainable crop production. Moreover, the foliar application of chitosan can cause improvement in photosynthesis, respiration and plant metabolisms, plant growth promotion, and nutritional values. These positive effects cause sustainability in the production of biomass and quantity yield, as well as improving the production quality in horticultural and agronomic crops in particular under water deficit stress<sup>10,11,15</sup>.

Another approach to cope with water deficit stress caused by evaporation and moisture depletion is the application of amendments to the soil. The use of natural materials to enhance fertility, improve the physical and chemical structure of the soils, and increase the water retention capacity of the soil has gained attention from researchers, among which biochar can be mentioned<sup>16</sup>. Biochar, or biomass charcoal, is added as a soil amendment for carbon storage in agricultural soils. Biochar is charcoal produced through the pyrolysis process from plant biomass and agricultural waste such as straw, wheat, corn, bran, rice, and sugarcane bagasse. Previous investigations have attempted to convert food waste into biochar and use it in the soil, and positive results have been suggested for soil fertility, crop productivity, and mitigation of environmental stress impacts<sup>17,18</sup>. Biochar is a rich source of many important nutrient elements such as nitrogen, phosphorus, potassium, and micronutrients<sup>16,19</sup>. Notably, biochar has the potential to enhance soil physicochemical properties, improving moisture retention and agricultural productivity. Additionally, biochar can improve plant performance and preserve the environment, with significant implications for the alleviation of drought and sustainable crop production in arid and semiarid climates<sup>19</sup>. Biochar also can alleviate environmental stresses especially salt and drought damage stress to plants through adsorption<sup>20</sup>. Furthermore, investigations have been done on the positive effect of the use of biochar in reducing the negative influence of water deficit stress on some characterization of growth and yield as well as essential oil compounds of the medicinal and aromatic plants such as borage, marjoram, and quinoa<sup>21–23</sup>. Alharbi and Alaklabi<sup>24</sup> reported that the utilization of biochar along exogenous application of jasmonic acid improved salt tolerance in wheat by boosting photosynthetic attributes, mineral uptake from the soil, and enhances in osmolytes and secondary metabolites.

Despite some credible studies on soil amendment of biochar in some herbs and the foliar application of chitosan, the impacts of the biochar utilization along the foliar-spraying of chitosan and their synergist effects on the growth and essential oil of yarrow (*A. millefolium*) have not been well examined yet. Therefore, the aims of this experiment were: (i) to study the impact of the foliar application of chitosan and the soil utilization of biochar on the yield and biochemistry characteristics of yarrow under reduced irrigation; (ii) to assess the chemical constituents, content, and yield of essential oil from yarrow under experimental treatments.

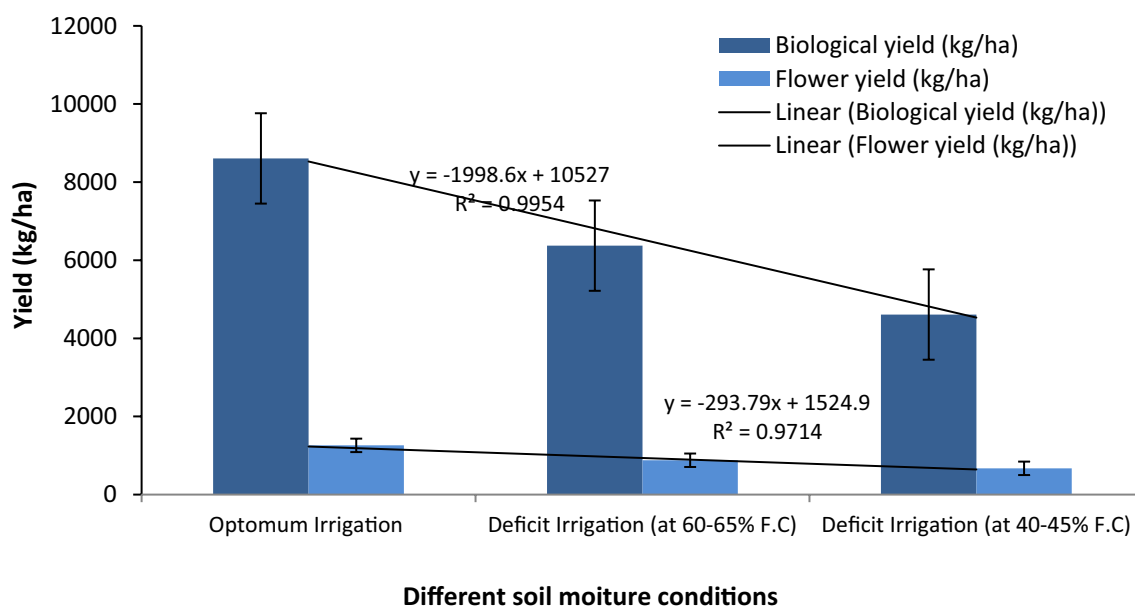
## Results

### Flower and biological yield

According to a combined analysis of variance for two experimental years, the flower and biological yields of yarrow in this study was significantly ( $p \leq 0.05$ ) affected by the interaction of the experimental factors i.e. biochar  $\times$  chitosan  $\times$  soil moisture levels (Table 1). The effect simple of deficit irrigation treatment significantly decreased the flower and biological yields of yarrow (Fig. 1). Indeed, water deficit stress limited the growth and yield parameters of yarrow like other agronomic and horticultural crops. However, the soil amendment of biochar along the foliar-spraying of chitosan had an increasing impact on the yield of this plant under varying soil moisture levels, both with and without water deficit stress. The highest values the flower (1323.3 kg/h) and biological (9197.7 kg/h) yields obtained from the plants treated by biochar and chitosan in optimum irrigation condition

Irrigation	Biochar (kg/m <sup>2</sup> soil)	Chitosan (g/L)	Characteristics								
			Proline (μmol/g wet weight)	Peroxidase (μmol/mg protein/min)	Ascorbate peroxidase (μmol/mg protein/min)	Catalase (μmol/mg protein/min)	DPPH antioxidant capacity (%)	Biological yield (kg/ha)	Dry flower weight (kg/ha)	Essential oil content (% v/w)	Essential oil yield (L/ha)
Irrigation (80–85% F.C.)	0	0	14.4 k <sup>¶</sup>	5.45 g	30.75 h	5.96 h	30.75 h	7714.4c	1161.6c	0.29i	3.52d
		2.5	19.08i	5.93f	32.56 h	7.31f	32.56 h	8615.1b	1259.4b	0.31 h	3.97c
	2	0	16.66j	5.59 g	31.19 h	6.7 g	31.19 h	8899.2b	1296.4ab	0.32 g	4.2b
		2.5	21.45 h	6.3e	35.78 g	7.61ef	35.78 g	9197.7a	1323.3a	0.33 fg	4.39a
Irrigation (60–65% F.C.)	0	0	23.72 g	6.32e	38.59f	7.95e	38.59f	5660.7 g	805.4e	0.34f	2.47 g
		2.5	26.5f	6.87d	44.06e	8.97d	44.06e	6602.7e	921.5d	0.35e	3.25e
	2	0	25.21 fg	6.7d	40.26f	8.67d	40.26f	6050.6f	841e	0.35e	2.99f
		2.5	32.8e	7.15c	46.75d	9.47c	46.75d	7182.6d	948.3d	0.38d	3.64d
Irrigation (40–45% F.C.)	0	0	37.11d	7.45b	53.66c	9.81c	53.66c	4135.9j	609.2 h	0.40c	2.46 h
		2.5	46.92b	7.85a	61.75b	10.71b	61.75b	4652.9i	677.7 g	0.42b	2.79 g
	2	0	40.28d	7.82a	60b	10.65b	60b	4458.2ij	648.6 g	0.43b	2.68 g
		2.5	49.07a	7.72a	65.38a	12.51a	65.38a	5191.6 h	745.9f	0.44a	3.29e
P-value			0.000**	0.01*	0.000**	0.000**	0.01*	0.03*	0.04*	0.00**	0.01*

**Table 1.** The interaction impacts of the soil application of biochar × foliar-spraying of chitosan under varying soil moisture levels on the biochemical, phytochemical, and yield characteristics of yarrow during two years of study. <sup>¶</sup> Different letters within a column indicate significant difference at  $p < 0.05$  according to Duncan's test. \*: significant at the 5% level and \*\*: significant at the 1% level.

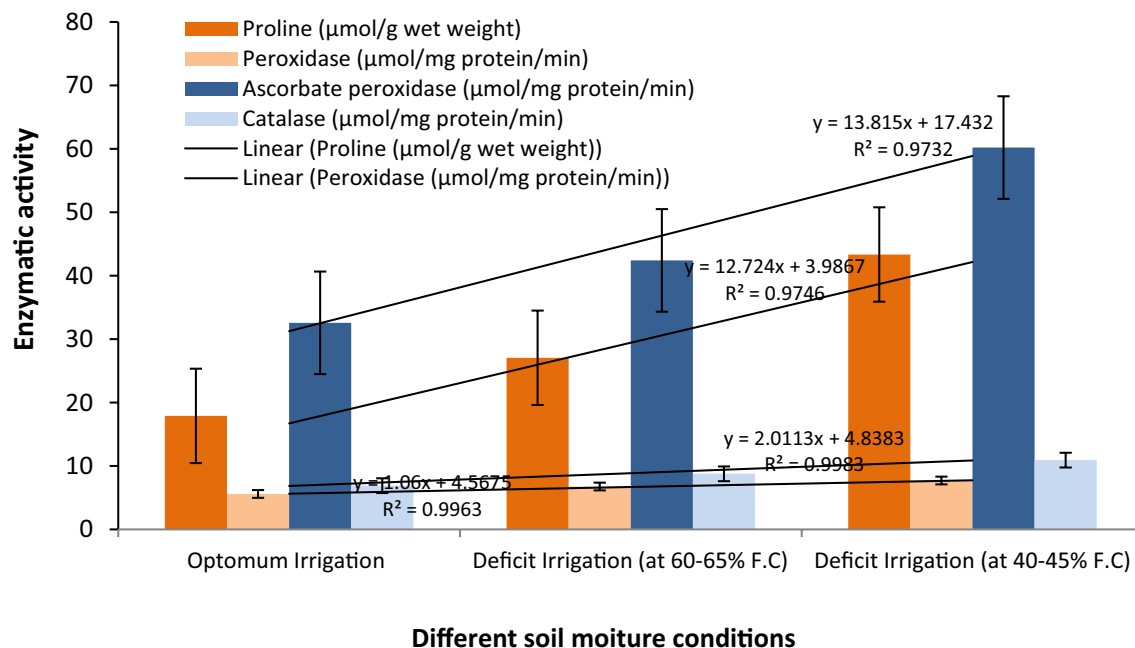


**Fig. 1.** The main effect of irrigation treatment on biological and flower yields (during two experimental years).

(Table 1), whereas, the lowest the flower yield (609.2 kg/h) and biological yield (4135.9 kg/h) were associated with the treatment of the interaction of non-application of biochar and chitosan under severe water deficit stress or deficit irrigation (Table 1).

### Biochemical characteristics

In this study, the interaction impact of triple of the experimental factors ( $p \leq 0.01$ ) on the proline content, as an important physiological trait of the yarrow plants was significant (Table 1). The application of biochar and chitosan, especially under water deficit conditions, increased the proline content (Fig. 2). The highest value of this trait (49.07 μmol/g fresh weight) was associated with the treatment of biochar and chitosan applications under deficit irrigation based on 40% (severe water deficit stress), while the lowest value of the proline content (14.40 μmol/g fresh weight) was related to the treatment without the applications of biochar and chitosan under optimum irrigation (non-stress) (Table 1).



**Fig. 2.** The main effect of irrigation treatment on enzymatic activities (during two experimental years).

For the ascorbate peroxidase and catalase enzymes, there were significant differences ( $p \leq 0.01$ ) between the experimental treatment in particular triple interaction effects of the experimental factors (Table 1). The results indicated that increasing the intensity of drought stress led to an increase in the activity of these enzymes (Fig. 2). The maximum activities of the ascorbate peroxidase and catalase enzymes in the yarrow plants were obtained from the treatment of the interaction of the applications of biochar and chitosan under severe water deficit or irrigation at 40% F.C., while the lowest enzyme activities belonged to the treatment of no biochar application  $\times$  no chitosan application  $\times$  optimum irrigation (Table 1).

Triple effects of the experimental factors had significant impacts ( $p \leq 0.01$ ) on the activity of the peroxidase enzyme in the yarrow plants (Table 1). The maximum activity of this enzyme was observed in the treatment of non-biochar application under severe water deficit stress (Table 1). Meanwhile, the lowest enzyme activity was recorded in the treatment without biochar and chitosan under normal irrigation and non-stress (Table 1).

The interaction effect of the experimental factors on the DPPH antioxidant capacity of the yarrow plants was significant ( $p \leq 0.01$ ) (Table 1). Enhancing the intensity of water deficit stress and the application of biochar and chitosan led to an increase in DPPH antioxidant capacity (Fig. 3). The highest value of this feature (65.38%) was associated with the soil amendment of biochar and the foliar-spraying of chitosan under water deficit stress or irrigation at 40% F.C., whereas, the lowest value was associated with non-applications of chitosan and biochar under normal moisture condition.

Generally, the activity of antioxidant enzymes and the antioxidant capacity of the plant extract measured by the DPPH method increased under deficit water stress conditions compared to the non-stress or full irrigation treatment.

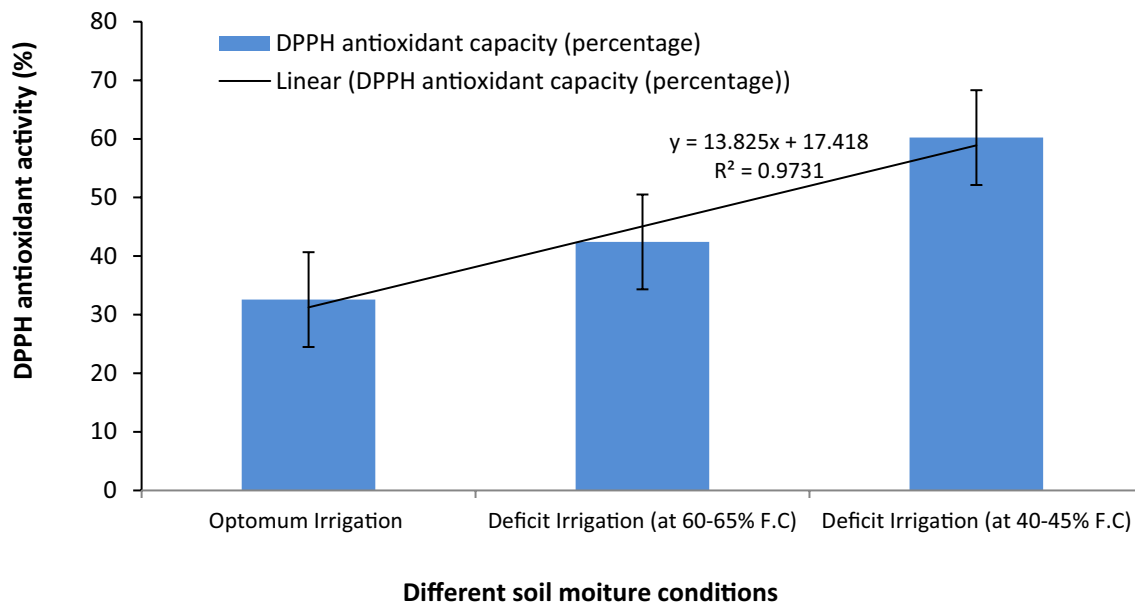
### Essential oil content and yield

The essential oil content of yarrow was significantly affected by the interaction of the soil application of biochar  $\times$  foliar-spraying of chitosan under varying soil moisture levels at the 1% level (Table 1). The results showed that the essential oil content of this medicinal plant experienced a significant increase with the soil application of biochar and the spraying of chitosan under the reduction of available water (Fig. 4a). The lowest essential oil content (0.29%) was recorded in the control (no biochar and no chitosan application) under optimum irrigation, while the highest essential oil content (0.44%) was obtained from the biochar and chitosan applications under severe stress or irrigation at 40% F.C. (Table 1).

As shown in Table 1, triple interactive had significant effects on the essential oil yield ( $p \leq 0.05$ ). The maximum essential oil yield (4.39 L/ha) was obtained from the soil amendment of biochar and the foliar application of chitosan under optimum irrigation, while the minimum essential oil yield (2.46 L/ha) was recorded in the non-applications of biochar and chitosan under water deficit condition (Table 1). In this study, the increased intensity of drought stress due to reduced irrigation water resulted in an increased percent of the essential oil in yarrow (Fig. 4b).

### Essential oil profile

In this study, 26 compounds were detected in the essential oil of the medicinal plant yarrow across the investigated treatments. The major compounds included  $\alpha$ -pinene,  $\beta$ -pinene, and  $\beta$ -myrcene belongs to the



**Fig. 3.** The main effect of irrigation treatment on antioxidant activity (during two experimental years).

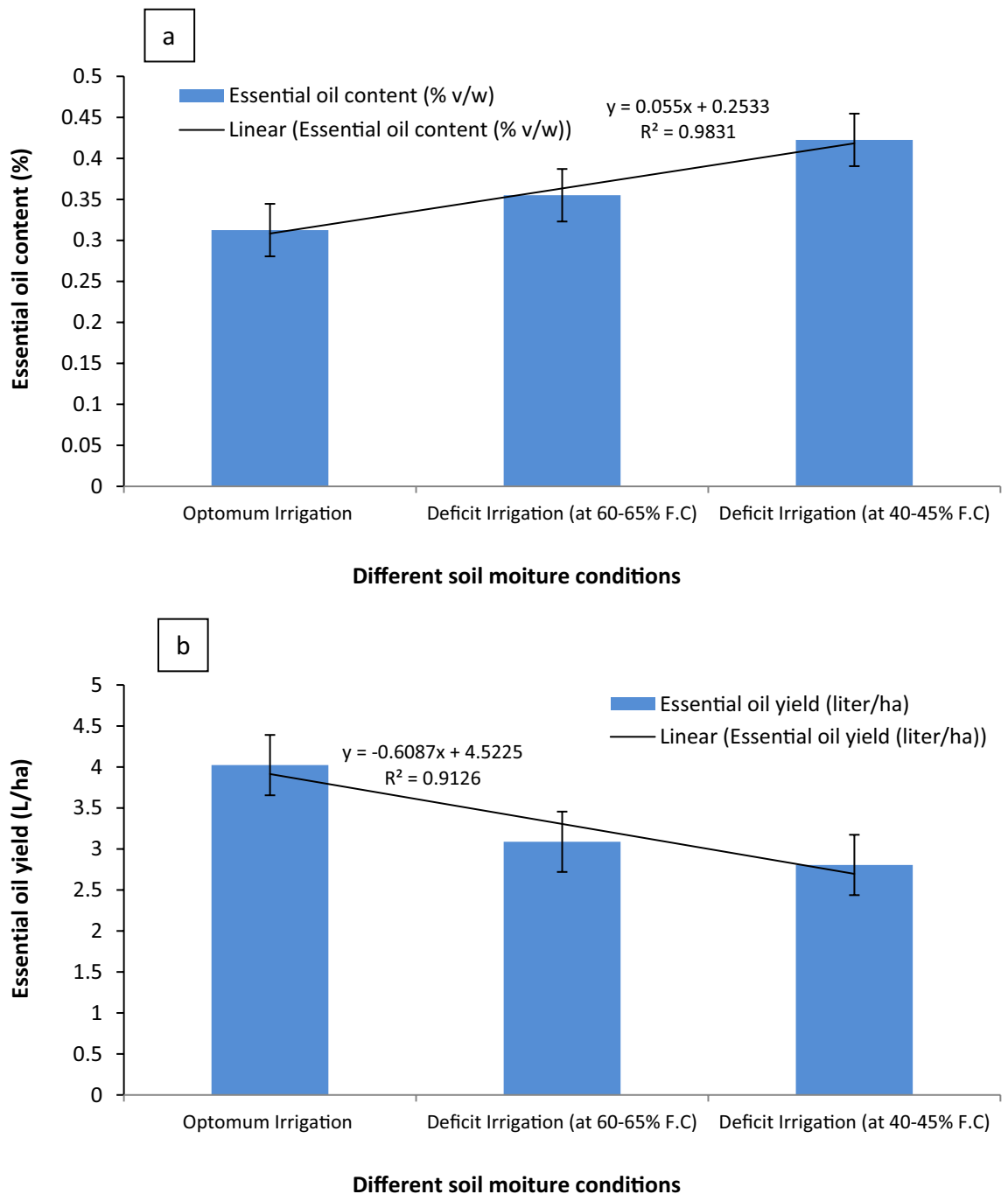
group of hydrocarbon monoterpenes, and 1,8-cineole, camphor, bornyl acetate, borneol, and terpinen-4-ol are part of the oxygenated monoterpenes, along with caryophyllene oxide,  $\beta$ -bisabolene, and nerolidol which belong to the group of sesquiterpenes (Table 2). As shown in Figs. 5, 6 and 7, the moderate and severe water deficit stress induced by irrigation based on 60 and 40% of F.C. in the yarrow plants led to an increase in hydrocarbon and oxygenated monoterpenes and a decrease in hydrocarbon and oxygenated sesquiterpenes.

Based on our finding, the foliar-spraying to the yarrow plants with chitosan and soil-applying of biochar under varying soil moisture levels increased the hydrocarbon and oxygenated monoterpenes and sesquiterpenes in the essential oils of yarrow. In general, the triple interactive impact of biochar  $\times$  chitosan  $\times$  soil moisture levels was significant on the amounts of the major compounds belonging to monoterpenes i.e.  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -myrcene, camphor, bornyl acetate, borneol, 1,8-cineole, and terpinen-4-ol and the main of sesquiterpenes like  $\beta$ -bisabolene, nerolidol, and caryophyllene oxide present in the yarrow essential oil (Table 2). According to our results, with the increase in water deficit stress intensity and the applications of chitosan and biochar, the amounts of these constituents improved (Table 2). The highest percent of monoterpenes e.g.  $\alpha$ -pinene (13.39%),  $\beta$ -pinene (3.60%),  $\beta$ -myrcene (5.73%), camphor (4.51%), bornyl acetate (7.02%), borneol (14.79%), 1,8-cineole (15.34%), and terpinen-4-ol (4.18) were obtained from the soil amendment of biochar and the foliar-spraying of chitosan under deficit irrigation or severe water deficit stress, while the treatment of non-applications of biochar and chitosan under optimum irrigation recorded the lowest values of these compositions (Table 2). However, the maximum concentrations of  $\beta$ -bisabolene (13.12%), nerolidol (8.5%) and caryophyllene oxide (13.11%) were achieved from non-applications of biochar and chitosan under optimum irrigation, whereas, the minimum amounts of  $\beta$ -bisabolene, nerolidol, and caryophyllene oxide were detected in the treatment of non-application of biochar and the foliar application of chitosan under deficit irrigation conditions (1.37, 1.00, and 1.36%, respectively) (Table 2).

## Discussion

In the present work, the reduction in the dry flower and biological yields under water deficit stress condition was likely due to the fact that as drought stress intensity increased, photosynthesis decreased because of reduced chlorophyll, increased protease activity, and stomatal closure. Indeed, water shortage limited morphological features and growth parameters of crops. Similar to the results of the present research, found that the biomass production of yarrow significantly decreased under increasing irrigation intervals. Decrease in the characteristics of the plant growth and its correlation with quantity yield in other medicinal and aromatic species under water deficit conditions has been reported in some investigations<sup>6,8,11,12,14,25,26</sup>. Consequently, the allocation of photosynthetic materials to plant organs especially reproductive organs like flowers, decreased, leading to increased flower drop, abortion, and drying<sup>6,14,26</sup>. In this investigation, the treatments containing biochar, particularly under water deficit stress conditions, likely enhanced plant performance due to the positive impacts of biochar on nutrient retention and mobility and transformation of micronutrients, improvement of soil pH, improvement of soil physical properties including water retention, and increased population and activity of beneficial soil microbes like mycorrhizal fungal<sup>18,21–23,27</sup>. In line with these results, Yang, Akhtar<sup>21</sup> and Seham, El-Din<sup>23</sup> also reported increased yields of quinoa and borage under the biochar application and water deficit condition.

In addition in present research, the foliar-spraying of chitosan was able to improve the growth and development of plants by enhancing the accessibility and absorb of indispensable nutrients and water<sup>28,29</sup>. Moreover, the increase in yarrow yield under the use of chitosan compared to the non-application treatment, particularly



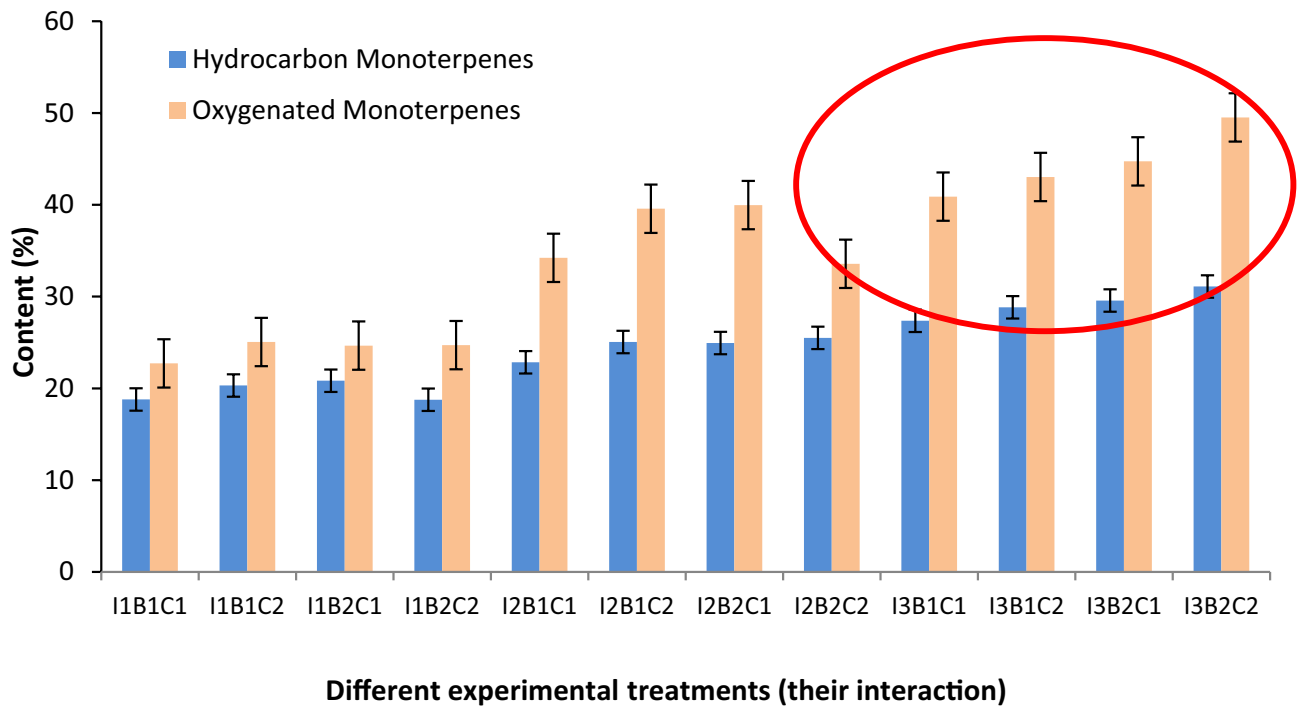
**Fig. 4.** The main effects of irrigation treatment on essential oil content (a) and yield (b) (during two experimental years).

under water deficit stress, was likely due to increased activity of enzymes involved in nitrogen metabolism such as nitrate reductase, glutamine synthetase, and protease, improved photosynthesis rate, increased chlorophyll content, adequate supply of essential nutrients, reduced water evaporation from the plant, which finally they were able to improve plant water status<sup>11,12,25</sup>.

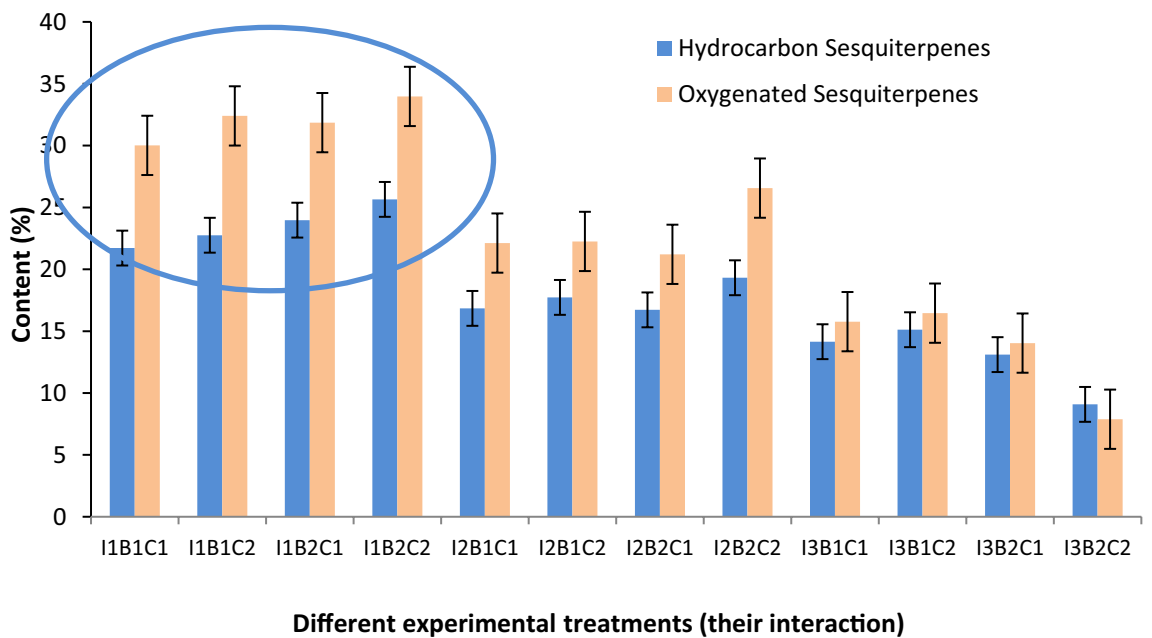
Additionally, deficit irrigation treatment meaningfully decreased the yields of biological, flower, and essential oil, however, enzymatic activity of catalase, peroxidase, and ascorbate peroxidase, and antioxidant activity and as well as the contents of proline and essential oil under reduced irrigation significantly increased. Indeed, the increased synthesis and accumulation of low molecular weight compounds known as osmolytes, such as proline, are also among the defense mechanisms involved in plant cells that reduce osmotic stress<sup>6,30,31</sup>. The reduction of protein content in plants under water deficit stress is associated with increased activity of protein-degrading enzymes and the accumulation of free amino acids such as proline<sup>6,26,32</sup>. According to our finding, the soil application of biochar and the foliar-spraying of chitosan mitigated the negative impacts of water deficit stress

Row	Compositions	RI	Experimental treatments												p-Value								
			Irrigation at 80–85% F.C						Irrigation at 60–65% F.C							Irrigation at 40–45% F.C							
			Non-Biochar		Biochar		Non-Chitosan		Chitosan		Non-Biochar		Biochar			Non-Chitosan		Chitosan		Non-Biochar		Biochar	
1	α-Phene	939	9.07j	9.63hi	9.92gh	9.39ij	10.88e	10.31f	11.29d	12.05c	12.87b	13.13ab	13.39a	0.01*									
2	Camphene	954	0.66h	0.85g	1.00f	1.08ef	1.17e	1.42d	1.55c	1.66bc	1.75b	2.19a	0.01*										
3	Sabinene	975	1.73j	1.84i	1.94h	0.98k	2.42c	2.70a	2.57b	2.21ef	2.16f	2.29de	0.000**										
4	β-Phene	979	1.44j	1.69i	1.63i	1.83h	2.40g	2.51f	2.61e	2.94c	3.22b	3.60a	0.000**										
5	β-Myrcene	985	3.58i	3.82h	3.98gh	3.89h	4.09fg	4.27f	4.60e	5.03c	5.20b	5.73a	0.02*										
6	α-Phellandrene	1003	2.03g	2.15f	2.07g	1.23h	2.38d	2.33d	2.44c	2.61a	2.60ab	2.56ab	0.03*										
7	P-Cymene	1029	0.28g	0.33f	0.29g	0.36e	1.42b	1.40b	0.44d	1.51a	1.51a	1.49a	0.000**										
Sum of hydrocarbon monoterpenes			18.79	20.31	20.83	18.76	22.84	24.94	25.50	27.36	28.83	29.57	31.10										
8	1,8-cineole	1031	9.33h	10.22f	10.40f	9.84g	13.25c	13.40c	11.55e	14.78b	15.12ab	15.34a	0.01*										
9	Pinocarveol < trans- >	1139	0.86h	0.90h	0.87h	0.94g	2.24b	2.43a	1.52f	2.00e	2.15c	2.23b	0.000**										
10	Verbenol < trans- >	1144	0.13i	0.17h	0.20g	0.22g	1.24e	1.29d	0.31f	1.36b	1.34bc	1.45a	0.000**										
11	Camphor	1146	2.90h	3.22g	3.08g	3.55f	5.11c	6.17ab	6.30a	4.38d	4.00e	4.51d	0.000**										
12	Borneol	1169	5.50gh	5.78g	5.57g	5.13h	6.33f	9.38d	6.40f	10.52c	10.88c	14.79a	0.000**										
13	Terpinen-4-ol	1177	1.87j	2.04i	1.95ij	2.19h	2.37g	2.74f	3.15d	3.79c	3.96b	4.18a	0.000**										
14	Bornyl acetate	1285	2.13k	2.72i	2.59j	2.83h	3.74g	4.56e	4.31f	5.22c	5.33b	7.02a	0.000**										
Sum of oxygenated monoterpenes			22.72	25.05	24.66	24.71	34.22	39.57	33.57	40.89	43.03	44.73	49.52										
Sum of hydrocarbon and oxygenated monoterpenes			41.51	45.36	45.49	43.46	58.06	64.62	59.04	68.25	71.86	74.3	80.77										
15	Longifolene	1400	0.73b	0.78ab	0.86a	0.93a	0.59c	0.65bc	0.67bc	0.42d	0.48d	0.53cd	0.04*										
16	Germaacrene-D	1479	1.28a	1.34a	1.33a	1.29a	0.98b	1.06b	1.17ab	0.75c	0.87bc	0.96b	0.02*										
17	α-Curcumene	1481	2.63b	2.82b	2.68b	3.47a	1.20d	1.70d	0.81d	1.94c	1.01d	2.33bc	0.000**										
18	β-Bisabolene	1509	11.68b	12.05ab	12.96a	13.12a	9.98bc	8.80c	10.89b	7.85c	8.19c	1.37e	0.001**										
19	delta-Cadinene	1521	1.68a	0.81bc	1.05b	1.26b	1.20b	1.39ab	1.47ab	0.69c	0.87bc	1.11b	0.02*										
20	α-Calacorene	1546	1.87b	1.99ab	2.09ab	2.56a	1.46bc	0.57d	0.82c	0.92c	0.54d	1.36bc	0.01*										
21	α-Bisabolene < cis- >	1548	1.85a	1.96a	2.00a	2.04a	1.44b	1.56b	1.49b	1.58b	1.69ab	1.75ab	0.02*										
Sum of hydrocarbon sesquiterpenes			21.72	22.75	23.97	25.65	16.84	16.72	19.32	14.15	15.12	13.11	9.08										
22	Nerolidol	1538	7.86b	8.45a	8.21ab	8.44a	6.16d	5.76de	6.9c	4.72f	4.51f	3.62g	0.000**										
23	Caryophyllene oxide	1579	11.68c	12.05b	12.36ab	13.11a	9.97e	8.79g	10.88d	7.85i	8.18h	5.69j	0.000**										
24	Guaiol	1595	5.02a	5.22a	5.35a	5.57a	2.24b	2.71b	3.82b	1.04c	1.25c	2.07b	0.000**										
25	Spathulenol	1635	1.38a	1.52a	1.51a	1.46a	1.14b	1.28b	0.35e	0.85d	1.04c	0.96c	0.02*										
26	Chamazulene	1723	4.08b	5.16a	4.43b	5.39a	2.61d	2.91cd	3.61c	1.58e	1.83e	2.10d	0.000**										
Sum of oxygenated sesquiterpenes			30.02	32.40	31.86	33.98	22.12	21.21	26.56	15.77	16.46	14.04	7.88										
Total			93.24	99.58	99.86	99.95	96.02	99.84	99.86	98.17	99.87	99.48	97.58										

**Table 2.** The interaction impacts of the soil application of biochar × foliar-spraying of chitosan under varying soil moisture levels on the chemical compositions of the yarrow essential oil during two years of study. † Different letters within a row indicate significant difference at  $p < 0.05$  according to Duncan's test. \*: Significant at the five percent level and \*\*: Significant at the one percent level.

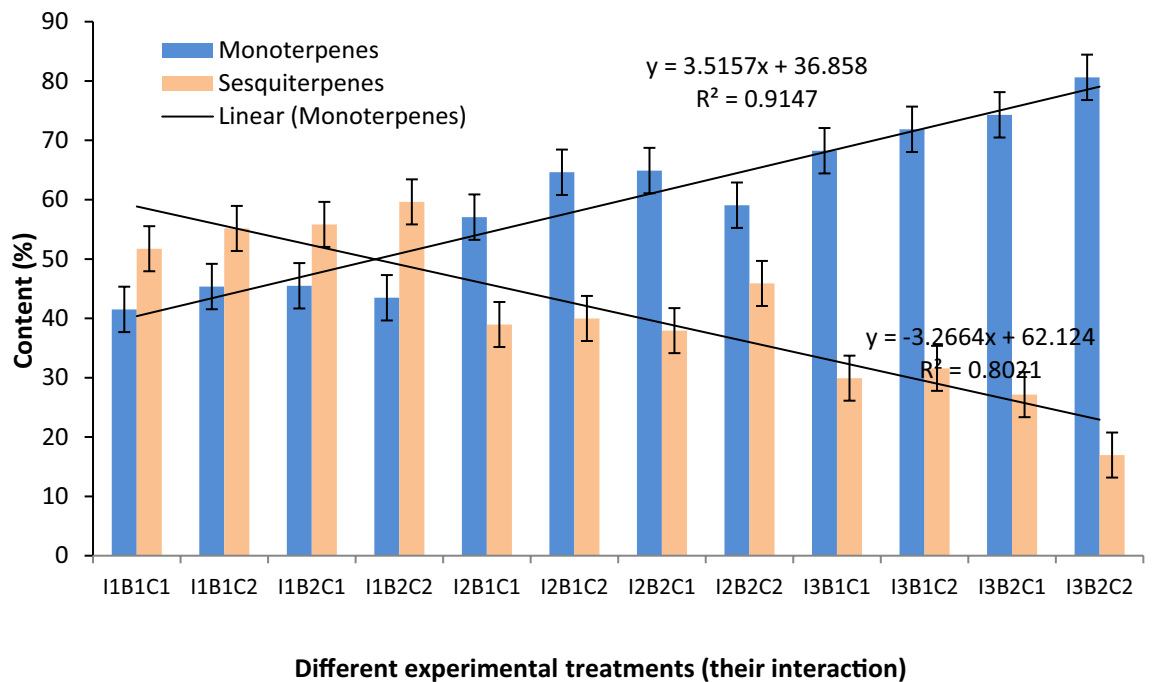


**Fig. 5.** The interaction effects of irrigation × biochar × chitosan on the monoterpenes contents (during two experimental years).



**Fig. 6.** The interaction effects of irrigation × biochar × chitosan on the sesquiterpenes contents (during two experimental years).

caused by irrigation at 60% and 40% of F.C. Also, the increase in proline content in the yarrow plants under reduced irrigation and increased water deficit stress intensity from 60% F.C. (moderate stress) to 40% F.C. (severe stress) was likely due to the increased expression of the genes for the enzymes delta-proline-5-carboxylate synthetase (P5C) and delta-proline-5-carboxylate reductase (P5cR) under water deficit stress conditions, which are involved in proline production from the glutamic acid pathway, as noted by Liang, Zhang<sup>33</sup>. In line with our results, an increase in proline in *Lallemantia royleana* (Benth.) under drought stress was reported<sup>34</sup>. The increase



**Fig. 7.** The interaction effects of irrigation × biochar × chitosan on the monoterpenes and sesquiterpenes contents (during two experimental years).

in proline content in yarrow with the applications of biochar and chitosan under water deficit stress conditions can be related to increasing expression of the delta-1-proline-5-carboxylate synthetase (P5CS) gene influenced by the application of chitosan and biochar, according to the findings of Zhang<sup>35</sup>.

Hafez, Attia<sup>16</sup> and Alavi Samany, Ghasemi Pirbalouti<sup>14</sup> reported an increase in the proline content in barley and hyssop under the foliar-spray application of chitosan and deficit water condition, and Zemanova, Břendová<sup>36</sup> reported an increase in the concentration of proline in spinach with the biochar application. Mehmood, Ahmed<sup>37</sup> also reported that the activity of antioxidant enzymes and gene expression levels in soybean triggered by salinity stress, while the soil utilization of biochar significantly further boosted the expression profile of four genes encoding antioxidant enzyme and two salt-tolerant conferring genes.

Interestingly, the soil application of biochar and the foliar-spraying of chitosan had synergic impacts on the yarrow performances and they were able to alleviate the negative influences of water deficit stress on the dry flower and biological yields of yarrow. Probably, this synergic positive effect of the applications of biochar and chitosan can be related to boosting photosynthetic attributes, mineral uptake, increases in osmolytes and secondary metabolites<sup>24</sup>.

DPPH is a stable free radical that has an unpaired electron on one of its nitrogen bridge atoms. The inhibition of the DPPH radical forms the basis for assessing antioxidant capacity, which is used to measure antioxidant activity in plants and indicates the plants' ability to cope with stress<sup>38</sup>. Water deficit stress leads to increased production of reactive oxygen species in cells, lipid peroxidation of membranes, and increased ion leakage, which enhances the activity of the plant's antioxidant system, thereby controlling the excess production of reactive oxygen species under stress and protecting itself against the harmful impacts of stress<sup>6,31</sup>. In accordance with these results, Arabsalehi, Rahimmalek<sup>39</sup> also reported an increase in antioxidant activity in *Ducrosia anethifolia* Boiss. under drought stress conditions. According to our results, the activities of antioxidant enzymes including catalase, peroxidase, and ascorbate peroxidase and also the DPPH antioxidant capacity in the yarrow plants under the soil application of biochar, especially under water deficit stress, were likely due to increased expression of antioxidant genes in response to stress<sup>40</sup>. Alharbi, Khan<sup>41</sup> reported that the soil application of biochar along with exogenously applied β-sitosterol on the thymus (*Thymus vulgaris* L.) plants significantly decreased the electrolytic leakage of cells in heat stress condition. They claim that the reactive oxygen species was decreased, however, the synthesis of antioxidants enhanced with the treatment of biochar and β-sitosterol. Moreover, they found that the synergistic application of biochar and β-sitosterol led to an upregulation in the synthesis of antioxidant enzymes e.g. 15.29% in SOD, 73.28% in APOX, and 14.67% in POD compared to the control<sup>41</sup>.

In other respects, the increase in the activities of the antioxidant enzymes (catalase, peroxidase, and ascorbate peroxidase) and DPPH antioxidant capacity in yarrow under the foliar-spraying of chitosan can be related to increasing antioxidant activities in stressed plants by chitosan as a strong stimulant, which this increase is induced through the positive signaling regulation of defense-related genes, including antioxidant genes in the plant<sup>11,42</sup>. In addition, the reduction of reactive oxygen species accumulation through increased activity of antioxidant enzymes, stimulation of physiological processes, improved growth, and increased carbon dioxide fixation was influenced by the foliar application of chitosan<sup>11,12,25</sup>.

Complex processes are activated in plants in response to environmental stresses, which include hormonal modulation, measurement and signaling due to transcription factors, and the production of secondary metabolites<sup>6,39</sup>. Among these factors, secondary metabolites like essential oils are significant because these compounds play crucial roles in regulating plant environmental interactions and subsequent adaptive responses<sup>11,32</sup>. Water shortage affects the quantity and quality of essential oils by influencing the expression of genes or the activity of enzymes involved in the biosynthesis pathways of secondary metabolites<sup>8,14</sup>. The increase in the percent of essential oil in yarrow in this study under biochar application, particularly under water deficit stress, was likely due to the fact that essential oils are often terpenoid compounds that require isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) as building blocks, along with a fundamental need for NADPH and ATP.

In addition, according to results of this research, the increased percent of essential oil in yarrow under chitosan elicitor spray, particularly under water deficit stress can be related to chitosan's role in activating new genes and various biosynthetic pathways for essential oil production, as well as increasing the number of essential oil-secreting glands and the biosynthesis of monoterpenes<sup>30</sup>. Previous literature illustrated that the essential oil contents of French lavender (*Lavandula dentata* L.) in the plants treated by the foliar application of chitosan under water deficiency was higher than treatment of water deficiency without chitosan<sup>43</sup>. Consistent with these results, Ghasemi Pirbalouti, Malekpoor<sup>11</sup> also reported increased the content and yield of essential oil of two species of basil under chitosan application and both stress and non-stress conditions. In the present study, the reduction in essential oil yield under moderate and severe water deficit stress compared to optimum irrigation was likely due to the decrease in available soil water and the detrimental impacts of drought stress on the growth and dry matter yield of yarrow. Given that the essential oil yield is a product of the percent of the essential oil and the plant's dry matter yield, the lower essential oil yield in water deficit stress treatments that produced a lower percent of the essential oil is likely due to lower dry matter yield under stress conditions.

In general, the effect interaction of the foliar-spraying of chitosan and the soil application of biochar under deficit water stress on the essential oil content may be due to its impact on enzyme activity and metabolism of essential oil output. The increase in essential oil yield under the biochar and chitosan applications in both stress and non-stress conditions was also due to the increased percent of essential oil and the yield of yarrow influenced by these two stimulants.

In line with our finding, Farhadi, Babaei<sup>1</sup>, 28 compounds were also identified in the essential oil of yarrow, with 1,8-cineole, camphor, chamazulene, and  $\alpha$ -eudesmol being the most significant compounds found. In previous investigation by Alvarenga, Pacheco<sup>2</sup>, 1,8-cineole, borneol,  $\beta$ -caryophyllene, and sabinene accounted for the highest proportion of the essential oil compounds of this species of yarrow. In another study<sup>5</sup>, the major constituents of the essential oil from *A. millefolium* cultivated in Saudi were monoterpenes, e.g., myrcene, 1,8-cineole, camphor,  $\alpha$ -thujone, and  $\beta$ -thujone. Reduction in the primary metabolism of the plants during environmental stresses can result in the accumulating of specific products, which can be shifted toward the biosynthesis and accumulation of secondary metabolites like essential oils<sup>30,44</sup>. The soil amendment of biochar may effectively decrease the harmful impacts of environmental stresses on the crops by improving the soil cation exchange capacity, soluble, and exchangeable  $K^+$ , N use efficiency, and fertilities, decreasing soil electrical conductivity and water evaporation. The results of previous investigation by Eghlima, Mohammadi<sup>44</sup> have indicated that biosynthesis and accumulation of monoterpenes like carvacrol, as the dominate compound in the essential oil of *Satureja* species, in response to environmental stresses like salt and drought. They realized that the percent of carvacrol in salinity stress conditions was higher than control and also stated that the biochar application effectively reduced the damage caused by salinity stress and the utilization of 3% biochar could have the positive impacts on the essential oil content, carvacrol percent, and SPAD index of *Satureja khuzistanica* Jamzad. under salinity stress. Results of a previous research by Abd-Rabhu, Wahba<sup>43</sup> showed that the amounts of hydrocarbons monoterpenes and oxygenated monoterpenes produced from the water deficit stressed plants under 6 m/L chitosan application was higher than the stressed plants without chitosan application. The impact of water deficit stress on the quality and quantity of the essential oil compounds in medicinal plants is likely due to changes in the expression of genes or the activity of enzymes involved in the biosynthesis of monoterpenes and sesquiterpenes or may relate to the way the plant neutralizes water deficit stress<sup>45</sup>. Under such stress conditions, the plant may increase the amount of monoterpenes to counteract oxidative damage caused by the accumulation of reactive oxygen species<sup>2</sup>. Ghassemi and Raei<sup>46</sup> reported that the enhancing all of the essential oil compositions of the garlic bulbs and stimulate the biosynthesis of some constituents by the utilization of biochar and polyamine (putrescine) can be related with up/down regulation of some enzymes and proteins in metabolism of plant carbohydrate, which may affect the balance of the secondary metabolites. The soil application of biochar with high cation exchange capacity and rich in micronutrients and macronutrients (potassium, zinc, iron, calcium, phosphorus, etc.) provided the necessary nutrients as cofactors for the optimal functioning of specific enzymes involved in the synthesis pathways of essential oil compounds, resulting in increased hydrocarbon and oxygenated monoterpenes and sesquiterpenes in yarrow under both stress and non-stress conditions<sup>41,47</sup>. Additionally, in the present study, the chitosan elicitor likely increased these essential oil compounds in yarrow under both stress and non-stress conditions by activating and expressing the genes in the biosynthetic pathways of monoterpenes and sesquiterpenes and also antioxidant enzyme activity, and compatible solutes, sending a series of chemical signals to the plant<sup>48</sup>.

## Conclusions

Based on the results of this study, water deficit meaningfully reduced the biological yield, dry flower weight, and essential oil yield of yarrow (*A. millefolium*), however, enzymatic activity of catalase, peroxidase, and ascorbate peroxidase, and antioxidant activity and as well as the contents of proline, essential oil, and some major constituents of the essential oil under deficit irrigation significantly improved. The soil application of

biochar and foliar spraying of chitosan could be effective on the stability of the growth parameters and dry herbal yield under deficit irrigation condition, improving its tolerance to water deficit stress caused by decreased irrigation water at 60 and 40% F.C. Interestingly, the quality of yarrow essential oil was improved by an enhancement in the biosynthesis and accumulation of monoterpenes e.g.  $\alpha/\beta$ -pinene,  $\beta$ -myrcene, camphor, borneol, 1,8-cineole etc., which the soil amendment of biochar along the foliar-spraying of chitosan under severe water deficit stress had the highest amounts in the yarrow essential oil. However, the highest production of sesquiterpenes in *A. millefolium* e.g. nerolidol and caryophyllene oxide were obtained from the yarrow plants under the non-applications of biochar and chitosan under optimum irrigation. Generally, the interactive of the biochar amendment and the foliar-spraying of chitosan can mitigate the adverse impacts of water deficit stress and improved the active biologically compounds like essential oil in *A. millefolium* under dehydration conditions especially in arid and semiarid climate. According to these findings, it is evident that incorporating biochar and chitosan sustainable agricultural practices could be a viable strategy for farmers to ensure better the quality of yarrow essential oil in the future. The action mechanism of the biochar and chitosan effects on the biosynthesis pathways of secondary metabolites particularly monoterpenes in essential oil of the yarrow plant is not addressed currently. In general, the analysis at molecular levels, identify of secondary metabolites biosynthesis paths need to be studied in *A. millefolium* under soil-based biochar, foliar-spraying of chitosan and water deficit stress conditions.

## Materials and methods

### Experimental treatments and design, and agronomic practices

To investigate the impact of the chitosan and biochar applications on the biochemical and phytochemical properties of yarrow (*Achillea millefolium* L.) under different irrigation regimes, an experiment was conducted during consecutive two years (2020 and 2021) at the Islamic Azad University Experimental Farm located in the Malard region (semiarid climate) of Tehran Province, Iran. The experiment was designed as a split-plot factorial in a completely randomized block design with three replications. The foliar spraying of chitosan (control and 2.5 g/L chitosan) along the use of biochar (control and soil application at 2.0 kg/m<sup>2</sup>) under different irrigation levels (irrigation at 80–85% field capacity or F.C., 60–65% F.C., and 40–45% F.C., respectively) that these treatments were assigned to the main plots. The application of chitosan and biochar treatments were set at two levels including non-application of chitosan and the spraying of chitosan at 2.5 g/L chitosan and the soil application of biochar including non-application of biochar and soil application biochar at a rate of 2 kg per square meter of soil which were factorial placed in subplots (Fig. 8). Biochar was produced by pyrolysis of woody pruning residues of the branches of forest trees (as the major residue in wood factory in the northern Iran).

Before planting, a one-kilogram soil sample was taken from a depth of 0 to 30 cm at the planting site of yarrow to determine the soil's physicochemical properties. This sample was sent to the water and soil laboratory, and nitrogen, phosphorus, and potassium base fertilizers were applied to the experimental field based on the results of the soil test (Table 3) and according to fertilizer recommendations for this plant.

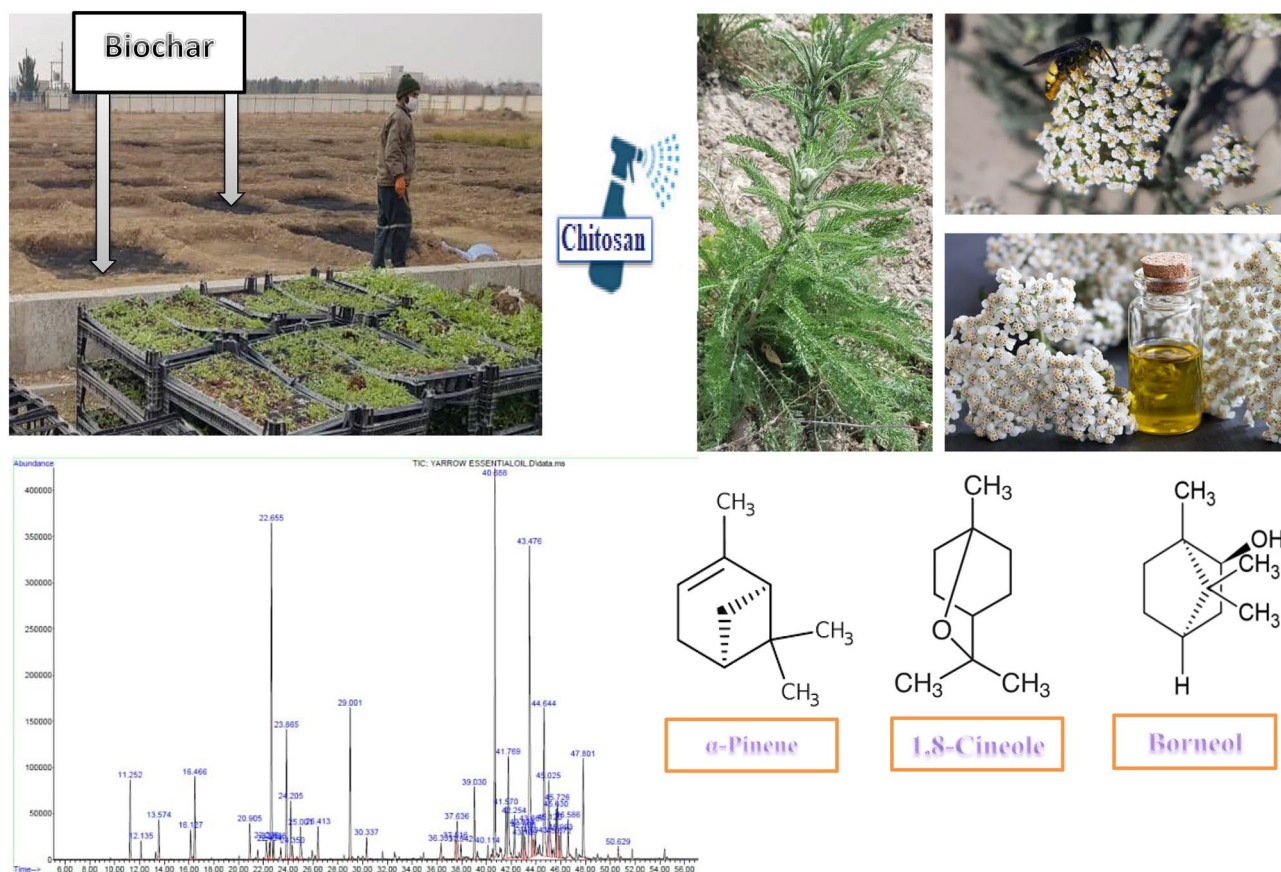
Soil preparation operations included one plowing and two perpendiculars disking before planting. Each experimental plot measured 1.5 m in length and 2 m in width, consisting of three planting rows. The distance between the main plots was 1.5 m, and the distance between blocks was also 1.5 m.

For planting of yarrow, the seeds were purchased from Pakaneh Seed Company, Esfahan, Iran. The collection site access has been approved by A. Modaresi, Ph.D., the local owner. Plant identities were confirmed by H.A. Shirmardi, Ph.D. (Research Center for Agriculture and Natural Resources, Iran). A voucher specimen of the plant was preserved in the TARI herbarium of the Research Institute of Forests and Rangelands (RIFR), Iran (Herbarium specification No. 104120 TARI). We confirm that field studies on cultivated plants in this investigation comply with international, national and/or institutional guidelines. To produce seedlings, the seeds were sown in the greenhouse of Islamic Azad University, Shahriyar branch, in planting trays filled with perlite and peat moss, spaced 5 cm apart and sown to a depth of 0.5 cm, then covered with coco coir. The seedlings were transferred to the main field at the 5-leaf stage. At the time of transplanting, the seedlings in the biochar application treatments, 2 kg of biochar per square meter was mixed with the soil to a depth of 15 cm. The transplanting operations were conducted with planting distances of 20 cm within rows and 50 cm between rows in December 2021 and 2022, according to the planting map. Immediately after planting, the first irrigation was performed. Irrigation of each plot was performed using the soil moisture curve. F.C. and permanent wilting point (P.W.P.) were – 33 and – 1500 kPa, respectively. Soil moisture was measured by a TDR device three times a week following the manufacturer's protocol<sup>49</sup>.

After the plants were established, the irrigation treatments were applied. For the chitosan application treatment, the yarrow plants were foliar sprayed at three stages: establishment, stem elongation, and budding at dew point i.e. early morning (~ 150 mL/plant). Maintenance operations included mechanical control of weeds, pests, and diseases throughout the plant's growth. The flowering tops of yarrow were harvested on July 15th of each growing year. No systemic pesticides and herbicides were used during the experiment, and weeds were controlled manually.

### Measurement of biological and flower yields

To determine the biological and flower yields of yarrow, during the flowering stage (~ 50%), the plants from the middle row of each plot were selected and harvested. After harvesting, the flowering tops were separated and dried in the shade with exposure to free air. The dried samples were weighed and converted to kg/ha. The aerial parts of the plants were placed in an oven at 70 °C for 48 h, then weighed, and combined with the flower yield to record the total biological yield<sup>50</sup>. Subsequently, the essential oil from yarrow flowers was extracted.



**Fig. 8.** Soil application of biochar, foliar-spraying of chitosan on the major compounds in the yarrow essential oil.

Depth (cm)	Soil texture	pH	EC (dS/m)	Organic Carbon (%)	N (%)	P	K	Zn	Mn	Fe	Cu
						(mg/kg)					
0–30	Sandy loam	7.62	3.03	0.25	0.03	5.3	227	0.98	1.03	1.36	1.11

**Table 3.** Some soil physical and chemical traits in the experimental field.

### Measurements of biochemical traits

The enzyme extraction process was carried out at 4 °C using freshly homogenised samples that had been treated with EDTA-Na<sub>2</sub> and ascorbate in potassium phosphate buffer. Centrifugation was used to produce the raw enzyme extract. According to the method of Change and Maehly<sup>51</sup>, 10 mM guaiacol, 70 mM hydrogen peroxide, 250 µL of potassium phosphate buffer, and 467 µL of sterile distilled water were added to a quartz cell and read in a Perkin Elmer UV-Vis spectrophotometer (Perkin Elmer, USA) as a control. Then, the extracted yarrow plant extract was added to the above mixture, and the antioxidant peroxidase enzyme activity was recorded at a wavelength of 470 nm over 180 s. The activity of this enzyme was reported based on the number of micromoles of hydrogen peroxide decomposed by the peroxidase enzyme per milligram of protein in the extracted plant sample.

According to the method by Bergmeyer<sup>52</sup>, 500 µL of sterile distilled water was added to 250 µL of 100 mM potassium phosphate buffer in a quartz cell. Then, 250 µL of 70 mM hydrogen peroxide were added to the mixture and read in a spectrophotometer at a wavelength of 240 nm as a control. After that, 20 µL of the extract from each treatment was added to the above mixture, and the highest absorbance of hydrogen peroxide by the catalase enzyme was reported at a wavelength of 240 nm.

Using the method of Nakano and Asada<sup>53</sup> the antioxidant enzyme activity of ascorbate peroxidase in yarrow was measured. Briefly, 850 µL of 0.5 mM ascorbate (dissolved in 100 mM potassium phosphate buffer) were placed in a quartz cell along with 150 µL of 2 mM hydrogen peroxide (dissolved in double-distilled water) and considered as a control in the spectrophotometer at a wavelength of 290 nm. Then, 20 µL of the extracted enzyme was added to the reaction mixture, and the activity of ascorbate peroxidase was recorded over 180 s. The activity

of ascorbate peroxidase was expressed as the number of micromoles of hydrogen peroxide decomposed per minute per milligram of protein.

To measure the amount of proline in the leaves of the yarrow plant, 0.5 g of plant tissue was ground with 10 mL of 3% sulfosalicylic acid in a porcelain mortar<sup>54</sup>. The resulting solution was transferred to a test tube with a lid, and these tubes were centrifuged for 15 min at 3000 RPM. One milliliter of the centrifuged extract was transferred to another test tube along with 1 mL of ninhydrin and 1 mL of glacial acetic acid, and placed in a 100-degree water bath for one hour. After the extract changed color to a pink hue, to stop the reactions, the extract was placed in ice water. After cooling, 4 mL of toluene solution was added to each of the tubes containing the extract. In each test tube containing the extract, two distinct phases were observable, with the color of the upper phase varying from red to pink based on the concentration of proline in the extract. Therefore, to measure the proline content in the extract, the upper phase was used, and its absorbance was calculated at a wavelength of 541 nm using a spectrophotometer. Standard solutions were prepared to draw the standard curve, and the curve was plotted based on their light absorbance. The concentration of proline in each extract sample was reported in micromoles per gram of fresh weight.

### Essential oil content and yield

To measure the essential oil present in the flowers of yarrow, the water distillation method was used. For this purpose, 40 g of dried flowers from each treatment were weighed and then ground. The ground flower samples were transferred into a flask containing 500 mL of water in a Clevenger apparatus (made by Glass Fabricating of Ashk-e-Shishe Co., Tehran, Iran). The flower and water mixture was boiled for four hours, after which the volume of the extracted essential oil was recorded. By opening the apparatus valve, the essential oil was transferred into dark-colored bottles, and anhydrous sodium sulfate was added to the extracted essential oil. Based on the essential oil obtained per 100 g of dried flowers for each treatment, the essential oil yield of yarrow was calculated in liters per hectare.

### Gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS)

To separate and identify the components of the essential oil, the essential oil obtained from each treatment was injected into GC-FID and GC/MS devices with the following specifications. GC was a Younglin Acme6000 with a column length of 30 m, an internal diameter of 0.25 mm, and a film thickness of 0.25 µm of type BP5. To identify the components of the essential oil, the sample was diluted with *n*-hexane and 1 µL was injected into GC. The temperature program for the column was set as follows:—initial oven temperature of 50 °C, held for 5 min;—temperature gradient of 3 °C/min;—increased to 240 °C, and then increased to 300 °C at a rate of 15 °C/min, with a hold at this temperature for 3 min, and a total response time of 75 min.

GC/MS analyses of the samples were performed on an Agilent Technologies 6890 gas chromatograph coupled to an Agilent 5973 C mass selective detector (MSD) and quadrupole EI mass analyzer (Agilent Technologies, Palo Alto, CA, USA) with a column length of 30 m, an internal diameter of 0.25 mm, and a film thickness of 0.25 µm of type BPX5. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 40–500. For identifying the components of the essential oil, a sample diluted with *n*-hexane was injected at a rate of 1 µL into the GC/MS device. The temperature program for the column was set same GC method.

The injection chamber temperature was set at 290 °C with a split ratio of 1 to 35. The FID detector temperature was set at 300 °C, and helium gas was used as the carrier gas with a flow rate of 1 mL/min. Helium gas was used as the carrier gas with a flow rate of 0.5 mL/min. The software used was Chemstation. The identification of the spectra was performed using their retention indices and compared with indices available in reference books and articles, using mass spectra of standard compounds and information available in the computer library<sup>55–57</sup>.

### Statistical analysis

All data were tested for normality and homogeneity of variance. The observations were statistically analyzed based on the generalized linear model (GLM) procedure of the SAS statistical package (v.9.2.). The Duncan multiple range test at 5% level was employed for comparing means, and Excel software was used for drawing tables and graphs.

### Data availability

Data is provided within the manuscript or supplementary information files.

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## Author contributions

F. Rezaei-Adl carried out the field experiment, collected the plant sample, and essential oil extraction; A. Ghassemi Pirbalouti shared the work idea, revising and writing the draft of the manuscript, supervising, and administering of project; T. Rahimi edited of the manuscript and supervising, analyzing of data; F. Rajabzadeh edited of the manuscript and consulting of project; H. Mozafari edited of the manuscript and consulting of project manuscript. All authors read and approved the final manuscript.

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## Declarations

## Competing interests

The authors declare no competing interests.

## Ethics approval and consent to participate

This study is complied with relevant institutional, national, and international guidelines and legislation. This study does not contain any studies with human participants or animals performed by any of the authors.

## Additional information

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