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Enhancing biosynthesis and bioactivity of *Trachyspermum ammi* seed essential oil in response to drought and *Azotobacter chroococcum* stimulation

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Abstract

Background: Plant growth-promoting bacteria have fundamental role in enhancing natural bioactive compounds and proved to increase the plant growth and mineral availability in soil. These phytochemicals, like phenolic and essential oils, illustrated wide range of biological properties. This study was designed to evaluate the effect of *Azotobacter chroococcum* (*A. chroococcum*) alone or in combination with slight (irrigation at 80% filed capacity) or moderate (irrigation at 60% filed capacity) drought stresses on the yield, phytochemicals, antioxidant, and the toxicity of *Trachyspermum ammi* (*T.* ammi) seeds essential oil.

Results: Overall, the application of *A. chroococcum* as plant growth-promoting agent together with slight drought stress significantly ($p < 0.05$) resulted in higher essential oil yield, total phenolic, total flavonoid, and higher antioxidant activity. The gene expression analysis in the developing seeds confirmed the up-regulation in the expression of antioxidant-related gene (SOD) and thymol synthesis gene (TSG) upon *A. chroococcum* bacteria treatment in combination with slight drought stress. The toxicity study showed no prominent signs of toxicity in mice upon oral administration of essential oil up to 100 mg/kg body weight for 28 days.

Conclusion: The slight drought stress (irrigation at 80% filed capacity) together with treatment of *T. ammi* plant with *A. chroococcum* bacteria as plant growth-promoting agent could be promising approach in improving the yield and medicinal value of the *T. ammi* seeds essential oil.

Keywords: *Trachyspermum ammi*, Essential oil, Gene expression, Abiotic stress, Biological value, Pharmaceutical properties

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Graphical Abstract

Background

In recent years, there has been renewed interest in the treatment of different diseases using herbal medicine as they are generally considered to have lesser side effects in human application [1]. There have been several studies on cells, animals, and human clinical trials which are able to provide substantial proof that bioactive components found in the medicinal plant exhibited wide range of biological potential, including anti-inflammatory and antioxidant activities [2, 3].

Trachyspermum ammi L. is a spice and aromatic herb that widely distributed throughout the world and it is famous as ajwain. The seed parts of this medicinal plant are commonly used traditionally for curing different types of illness in animal and human. The bioactive constitute and volatile compounds from *Trachyspermum ammi* seeds exhibited the substantial role as therapeutic agents in drug discovery. Apiaceae is a family of plants that contain valuable phytochemicals and essential oils, such as thymol, g-Terpinene, isobornyl isobutyrate, o-Cymene, *p*-Cymene, a-Pinene, silphine, verbenene, ionone myrcene, and thymyl acetate [4, 5].

The application of natural constituents from herbal plants as the therapeutic agents has been great attention in biomedical, food industries, and natural product research recently [6, 7]. The potential of this bioactive compounds may be an excellent alternative strategy for developing future effective, safe anti-inflammatory, and anticancer drugs [8, 9]. A variety of natural chemical compounds, including essential oils, phenolic, and flavonoid components, illustrated outstanding efficacy as an anti-tumor, anticancer, antioxidant, and various pharmacological properties because they may prevent ROS generation and DNA damage, inhibit the lipid peroxidation, and induce apoptosis through the caspase, P53, and other involved genes [10, 11]. Whereas the role and importance of these natural compounds have been well known and fully documented, there is rising interest in improving strategies by elevating and increasing these secondary metabolites [12, 13]. Several studies have been proved that different biotic and abiotic factors, such as UV light, acute gamma irradiation, carbon dioxide, nutrient, drought stress, and water availabilities, can significantly increase the concentration of phenolic and flavonoid contents [14–17].

Among the plant growth-promoting rhizobacteria, *Azotobacter chroococcum* as one of the eco-friendly management practice, safe, and sufficient techniques has been applied to enhance the natural bioactive compounds. They behave as the promising resource of

biotechnologically valuable phytochemicals with high pharmaceutical potential besides promoting growth of the plants. Numerous researches were carried out to confirm the notable role of bacteria and endophytic fungi in the production of natural phytochemicals, like phenolic, flavonoid, alkaloids, and quinols [13, 18–20]. The application of N2 fixation bacteria biofertilizer on Glycyrrhiza uralensis Fisch, *Juglans regia* L., and *T. foenumgraecum* L seedlings displays the significant development of bioactive compounds yields [21–23].

Drought stress as one of the important environmental stresses causes the oxidative damage and is the main restrictive factor in plant growth and development [24, 25]. Under these stresses the synthesis of secondary metabolite, including phenolic, flavonoids, and essential oils, was enhancing to overcome the photoinhibition by contributing to the antioxidant potential and detoxify reactive oxygen species [26, 27]. Previous studies have been manifested that the bioactive compounds, including betacarotene constitute in *Choy sum* varieties [28] and in perennial herbaceous [29], phenolic and flavonoid compounds in buckwheat [30], and polyphenolic and flavonoid content among with antioxidant potential in *Achillea* species [31], have been significantly developed under drought stress.

The biological potential of *Trachyspermum ammi* seed in response to *A. chroococcum* bacteria under drought stress was not demonstrated earlier. Therefore, this study was designed to evaluate the effect of *A. chroococcum* and drought stress individually or in combination on biochemical profiling and biological potential of *T. ammi* seeds. Furthermore, the plausible mechanisms of action were further investigated through the SOD and TSG genes expression analysis.

Results and discussion

Total phenolic and flavonoid analysis

The plant secondary metabolites are responsible in adaptation of plants to their environment and stressors. The plant under drought stress generally produces higher concentrations of bioactive metabolites to protect them against free radicals and reactive oxygen species and to maintain the photosynthesis. The plant metabolites, particularly phenolic compounds in addition to the plant protection, have great applicability in human health, playing critical roles as antioxidant agents. Thus, applying any approach to enhance the production and biosynthesis of plant bioactive metabolites could be very helpful to the pharmaceutical industry.

The results of essential oil content and bioactive compounds, including total phenolic and flavonoids present in essential oil, are shown in Table 1. Findings illustrated that the application of *A. chroococcum* as plant growth-promoting agent significantly ($p < 0.05$) improved the yield, total phenolic, and flavonoid content of the essential oil. The results indicated that the slight drought stress could significantly ($p < 0.05$) improve the essential oil content of the seed, while the increase in the drought stress level up to moderate stress significantly ($p < 0.05$) limited the seeds essential oil production. In addition, the increase in the drought stress significantly ($p < 0.05$) enhanced the concentration of total phenolic and flavonoid compounds in the essential oil. Incorporation of *A. chroococcum* as a growth-promoting agent significantly ($p < 0.05$) improved the phenolic and flavonoid content of the essential oil either in slight or moderate drought stress conditions.

Table 1 Total phenolic and flavonoid contents in the essential oil of *T. ammi* seed under different treatments

Samples	Seeds essential oil (%)	Phenolic Content 1 (mg/g)	Flavonoid Content 2 (mg/g)
T1	$1.9^{e} \pm 0.14$	$14.6^{e} \pm 0.85$	$2.6^{e} \pm 0.54$
T2	$2.2^{d} \pm 0.08$	$15.3^{d} \pm 0.92$	$3.7^{d} \pm 0.68$
T3	$2.8^{b} \pm 0.05$	$16.4^{c} \pm 0.51$	$4.3^{c} \pm 0.75$
T4	$2.5^{c} \pm 0.07$	$18.5^{b} \pm 0.24$	$5.1^{b} \pm 0.54$
T5	$3.1^{a} \pm 0.11$	$20.1^{a} \pm 0.28$	$6.4^{a} \pm 1.02$
T6	$2.6^{c} \pm 0.05$	$18.8^{b} \pm 0.71$	$5.3^{b} \pm 0.12$
SEM	0.09	0.16	0.11

1 mg gallic acid equivalent/g essential oil; 2 mg rutin equivalent/g essential oil

Means with different letters in the same columns show significant differences at $p < 0.05$

T1: control (Irrigation at 100% filed capacity), T2: *T. ammi* treated by *A. chroococcum* bacteria (Irrigation at 100% filed capacity), T3: *T. ammi* under slight drought stress (Irrigation at 80% filed capacity), T4: *T. ammi* under moderate drought stress (Irrigation at 60% filed capacity), T5: *T. ammi* treated by *A. chroococcum* bacteria under slight drought stress (Irrigation at 80% field capacity), T6: *T. ammi* treated by *A. chroococcum* bacteria under moderate drought stress (irrigation at 60% field capacity)

The common physiological response in plants challenged by drought stress is stomata closure. As a result, the uptake of CO_2 notably decreases and the consumption of reduction equivalents (NADPH + H+) required during CO_2-fixation via Calvin cycle declines considerably, producing a massive oversupply of NADPH + H+. Consequently, all metabolic processes are pushed toward the synthesis of highly reduced compounds, such as phenolics, flavonoids, isoprenoids, and alkaloids [32]. The results obtained in the current study were in agreement with the earlier studies reported the positive role of slight drought stress (~ irrigation up to 80% filed capacity) in enhancing the essential oil production and bioactive phenolic compounds [33, 34]. In line with the current study, in several experiments moderate drought stress (~ irrigation up to 60% filed capacity) limited the biosynthesis of essential oil and bioactive compounds [35, 36].

In addition, the drought stress resulted in accumulation of significant content of proline, glycine betaine, sugar, inositol, and phenolic compounds in the leaves of Mentha piperita and Catharanthus roseus, implying osmotic adjustment as stress resistance mechanism in these plants [37].

Antioxidant activity

The antioxidant activity of the seeds essential oil is shown in Table 2. The results revealed that treatment of *T. ammi* plant by *A. chroococcum* bacteria as a plant growth-promoting agent could enhance the antioxidant activity of essential oil. Apart from that, the antioxidant activity of essential oil significantly ($p < 0.05$) increased when *T. ammi* plant challenged by slight and moderate drought stress. The treatment of plant by *A. chroococcum* bacteria could significantly ($p < 0.05$) increase the antioxidant activity of essential oil even in the slight and moderate drought stress conditions. These results augur well with the results of total phenolic and flavonoid compounds present in the essential oil upon different treatments. From these results, the T5 was selected as promising treatment in enhancing the essential oil production with the highest concentrations of bioactive phenolic and flavonoids. Hence, this treatment was selected for further evaluations.

In line with the present study, the early experiment conducted by Ref. [38] indicated that slight drought stress (irrigation at 75% filed capacity) in the *Rosmarinus officinalis* L. enhanced the essential oil yield and production of phenolic compounds, and increased the antioxidant activity of essential oil. The severe drought stress (irrigation at 55% filed capacity) decreased the essential oil yield, phenolic compounds, and antioxidant activity of essential oil.

Table 2 The antioxidant activity of seeds essential oil upon different treatments and vitamin c as reference antioxidant in FRAP and ABTS assays

Treatments	Ferric reducing antioxidant power	ABTS scavenging activity
	IC50 (µg/mL)	
T1	$217.1^{a} \pm 1.15$	$113.5^{a} \pm 2.02$
T2	$205.6^{b} \pm 2.29$	$100.8^{b} \pm 1.19$
T3	$169.7^{c} \pm 1.07$	$62.0^{c} \pm 0.65$
T4	$158.3^{d} \pm 2.81$	$58.4^{d} \pm 2.07$
T5	$149.2^{f} \pm 2.36$	$53.8^{f} \pm 2.85$
T6	$152.6^{e} \pm 1.95$	$56.3^{e} \pm 1.96$
Vitamin C	$22.7^{g} \pm 1.85$	$14.5^{g} \pm 2.15$
SEM	2.23	2.18

Means with different letters in the same columns show significant differences at $p < 0.05$

T1: control (Irrigation at 100% filed capacity), T2: *T. ammi* treated by *A. chroococcum* bacteria (Irrigation at 100% filed capacity), T3: *T. ammi* under slight drought stress (Irrigation at 80% filed capacity), T4: *T. ammi* under moderate drought stress (Irrigation at 60% filed capacity), T5: *T. ammi* treated by *A. chroococcum* bacteria under slight drought stress (Irrigation at 80% field capacity), T6: *T. ammi* treated by *A. chroococcum* bacteria under moderate drought stress (irrigation at 60% field capacity)

Gene expression analysis

The gene expression study was performed in the developing seeds to confirm the molecular mechanism involved in the increase in the total phenolic content and subsequently the antioxidant activity of the essential oil. The results of SOD and TSG genes expression are shown in Figs. 1 and 2, respectively. Based on these results, it is postulated that treatment of *T. ammi* plant with *A. chroococcum* under slight drought stress (irrigation at 80% field capacity) significantly ($p < 0.05$) up-regulated the expression of antioxidant-related gene (SOD) and thymol synthesis gene (TSG) in the developing seeds as compared to the control group. In fact, the drought stress and *A.*

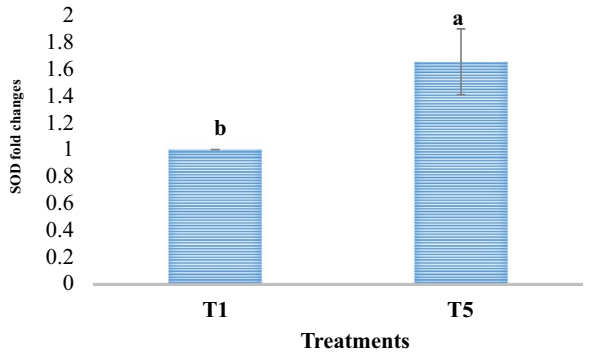

Fig. 1 Super oxide dismutase (SOD) gene expression in the developing seeds. Charts with different bars are significantly different ($p < 0.05$). T1: control, T5: *T. ammi* treated by *A. chroococcum* bacteria under slight drought stress (irrigation at 80% field capacity)

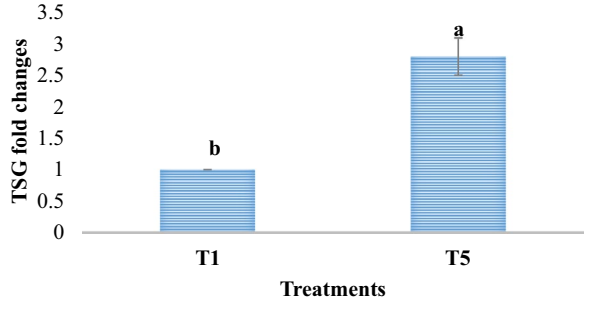

Fig. 2 Thymol synthesis gene (TSG) expression in the developing seeds. Charts with different bars are significantly different ($p < 0.05$). T1: control, T5: *T. ammi* treated by *A. chroococcum* bacteria under slight drought stress (irrigation at 80% field capacity)

Table 3 The final weight and total feed intake of mice receiving different treatments

Average	T1	T2	T3	T4	SEM
Average daily weight gain (mg)	$221.3^{d} \pm 2.31$	$263.5^{c} \pm 2.97$	$297.2^{a} \pm 2.15$	$280.8^{ab} \pm 2.08$	6.45
Average daily feed intake (mg)	$4.3^{c} \pm 0.85$	$4.7^{b} \pm 0.19$	$5.8^{a} \pm 1.16$	$4.8^{ab} \pm 1.65$	0.51

T1: 0 mg/Kg BW, T2: 50 mg/Kg BW; T3: 100 mg/Kg BW; T4: 200 mg/Kg BW

Different letters in the same raw indicate significant difference ($p < 0.05$)

Table 4 The liver enzymes biochemical assay

	ALP(U/L)	ALT(U/L)	AST(U/L)	MDA (%)*
T1	$602.5^{a} \pm 1.64$	$188.1^{a} \pm 3.44$	$46.4^{bc} \pm 3.85$	$100^{a} \pm 1.66$
T2	$535.2^{b} \pm 2.02$	$162.8^{c} \pm 1.55$	$58.2^{b} \pm 2.76$	$92.5^{ab} \pm 1.21$
T3	$461.7^{c} \pm 3.37$	$145.5^{d} \pm 1.92$	$55.1^{b} \pm 2.18$	$85.1^{b} \pm 1.57$
T4	$485.3^{c} \pm 2.51$	$171.9^{b} \pm 1.88$	$61.3^{a} \pm 1.57$	$97.4^{a} \pm 2.34$
SEM	8.3	7.5	1.5	2.5

T1: 0 mg/Kg BW, T2: 50 mg/Kg BW; T3: 100 µg/Kg BW; T4: 200 mg/Kg BW

Different letters in the same column indicate significant difference ($p < 0.05$)

The analysis was performed in triplicates

*Relative to T1 as a control

chroococcum bacterial activity regulated the net photosynthesis and transpiration rate and as a consequence, the expression of some genes involved in biogenic volatile organic compounds and essential oil biosynthesis is altered [34, 39, 40].

Toxicity evaluation

The essential oil obtained from the seeds of *T. ammi* treated by *A. chroococcum* bacteria under slight drought stress (Irrigation at 80% field capacity) was evaluated for the toxicity at different concentrations (0, 50, 100, and 200 mg/Kg BW). No signs of toxicity, including diarrhea, abdominal contortions, sedation, alterations in locomotor activity, or deaths, were recorded during the 28 consecutive days of treatments by oral administration of essential oil. The results of food intake and body weight changes are presented in Table 3. The results demonstrated that mice administrated with the essential oil concentrations of 50 and 100 mg/kg BW showed enhancement in the final weight and feed intake significantly ($p < 0.05$) as compared to control group (T1). The increase in the concentration of essential oil up to 200 mg/kg BW significantly ($p < 0.05$) suppressed the food intake and body weight changes.

The liver enzymes (ALP, ALT, and AST) and MDA as lipid peroxidation value are considered as biomarkers of hepatocyte damage and hepatotoxicity (Table 4). The oral administration of essential oil up to the concentration of 100 mg/kg BW alleviated the liver enzymes and lipid peroxidation in the liver. The alleviation of liver enzymes production and lipid peroxidation is reflecting the improvement in the health status. The increase in the concentration of essential oil up to 200 mg/kg BW increased the liver enzymes production and lipid peroxidation which confirmed the liver damage.

The results of blood parameters, including RBC, WBC, lymph, monocyte, and neutrophils, are presented in Table 5. The oral administration of essential oil during 28 days of treatment did not significantly ($p > 0.05$) affect the blood parameters, including RBC, WBC, lymph, monocyte, and neutrophils.

Histopathological examination

The histopathological images of liver, kidney, jejunum, and spleen section obtained from different treatments are illustrated in Fig. 3. The results manifested that administration of essential oil through oral gavage at the concentrations of 0, 50, 100, and 200 mg/Kg BW for 28 days exhibited no histological alteration.

The morphometric analysis, including villus height, villus width, crypt depth, and the number of goblet

Table 5 The blood analysis of mice receiving different treatments

Treatment	RBC	WBC	Lymph (%)	Monocyte (%)	Neutrophils
T1	7.36 ± 0.45	5.4 ± 0.52	63 ± 0.27	2 ± 0.76	33 ± 0.69
T2	7.41 ± 1.04	5.5 ± 0.47	53 ± 0.36	4 ± 1.29	39 ± 0.42
T3	7.25 ± 0.31	5.1 ± 0.93	56 ± 0.33	3 ± 0.97	38 ± 1.15
T4	7.22 ± 0.64	5.7 ± 0.85	60 ± 1.05	4 ± 0.82	32 ± 1.48
SEM	1.2	1.8	1.5	0.9	1.7

T1: 0 mg/Kg BW, T2: 50 mg/Kg BW; T3: 100 µg/Kg BW; T4: 200 mg/Kg BW

Different letters in the same raw indicate significant difference ($p < 0.05$)

The analysis was performed in triplicates

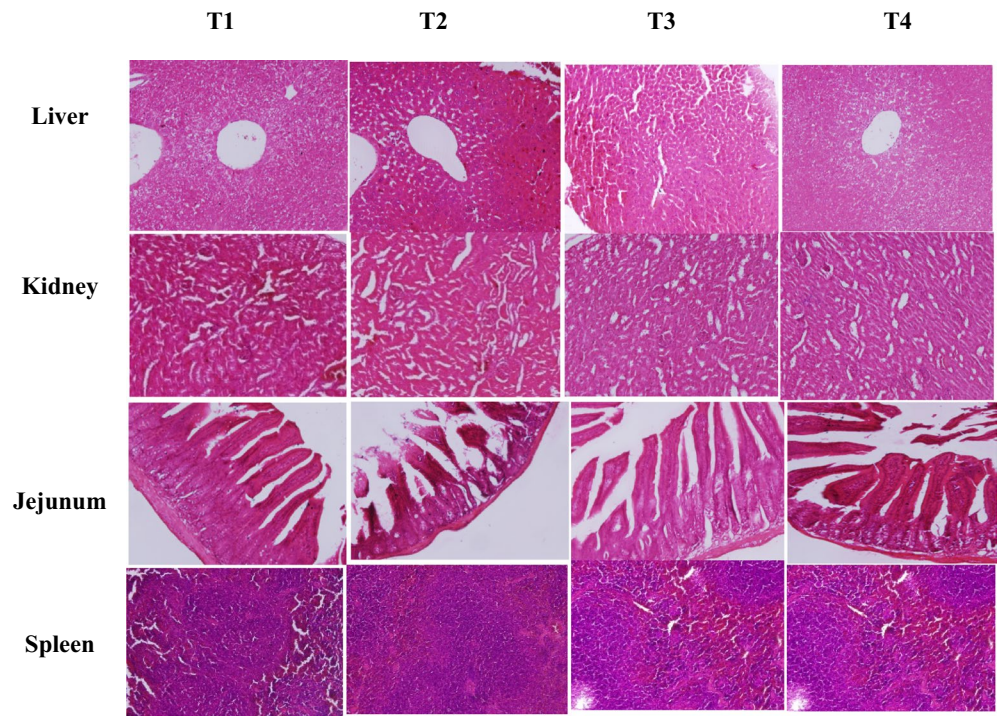

Fig. 3 Histopathological analysis of liver, kidney, jejunum, and spleen of the mice under different treatments (T1: 0 mg/Kg BW, T2: 50 mg/Kg BW; T3: 150 mg/Kg BW; T4: 200 mg/Kg BW)

Table 6 Morphometric analysis of ileum upon different treatments

Parameters	T1	T2	T3	T4	SEM
Villus height (μm)	$342^{d} \pm 1.72$	$352.2^{c} \pm 1.55$	$378^{a} \pm 0.81$	$361^{b} \pm 1.19$	6.32
Villus width (μm)	$88^{c} \pm 1.24$	$118^{b} \pm 2.11$	$128^{a} \pm 1.07$	$117^{b} \pm 1.15$	7.16
Crypt depth (μm)	$156^{a} \pm 0.98$	$120^{c} \pm 1.32$	$110^{d} \pm 1.62$	$145^{b} \pm 1.64$	4.92
Mean number of Goblet cells	$3.9^{b} \pm 1.66$	$3.9^{b} \pm 1.06$	$4.4^{a} \pm 1.32$	$3.3^{c} \pm 1.47$	0.46

T1: 0 mg/Kg BW, T2: 50 mg/Kg BW; T3: 100 mg/Kg BW; T4: 200 mg/Kg BW

Different letters in the same raw indicate significant difference ($p < 0.05$)

The analysis was performed in triplicates

cells in the jejunum of mice receiving different treatments, are illustrated in Table 6. These findings indicated that the oral administration of essential oil at the concentrations of 50 and 100 mg/kg BW for 28 days significantly ($p < 0.05$) increased the villus height, villus width, and number of goblet cells and deceased the crypt depth. These results were in accordance with the results of earlier study [41] who reported that inclusion of phenolic compounds in the animal diet improved the morphometric parameters of the small intestine and subsequently the intestinal absorption of nutrients is increased. Hence, the significant increase in the feed intake and average daily weight gain of mice upon oral administration of essential oil could be associated to the improvement in the morphology of the jejunum and increase in the absorption of nutrients.

Conclusion

The results revealed that *T. ammi* plant upon treatment by *A. chroococcum* bacteria as plant growth-promoting agent under slight drought stress (irrigation at 80% field capacity) indicated the highest biosynthesis of essential oil and total phenolic and flavonoid contents. The essential oil obtained upon this treatment possessed higher antioxidant potential. The gene expression analysis confirmed the up-regulation in the expression of

Table 7 The treatments applied in this study

Treatments	*T. ammi* treatment by *A. chroococcum*	Drought stress level
T1	–	Irrigation at 100% filed capacity
T2	Treated	Irrigation at 100% filed capacity
T3	–	Irrigation at 80% filed capacity (slight stress)
T4	–	Irrigation at 60% filed capacity (moderate stress)
T5	Treated	Irrigation at 80% filed capacity (slight stress)
T6	Treated	Irrigation at 60% filed capacity (moderate stress)

antioxidant-related gene (SOD) and thymol synthesis gene (TSG) in developing seeds upon slight drought stress in combination with *A. chroococcum* bacteria treatment. The toxicity study showed no prominent sign of toxicity in mice upon oral administration of 100 mg essential oil/kg body weight for 28 days. The slight drought stress (irrigation at 80% filed capacity) together with treatment of *T. ammi* plant with *A. chroococcum* bacteria as plant growth-promoting agent could improve the biological value of the essential oil. As a consequence, the slight drought stress together with microbial stimulants could be a feasible strategy to improve the production of bioactive compounds concentrations in medicinal plants for future cultivation.

Materials and method

Chemicals and plant material

In the present research the seeds of *Trachyspermum ammi* were purchased from the Pakan Bazr Esfahan Company**.** Folin-Ciocalteu reagent, gallic acid, rutin, and ascorbic acid were purchased from Fisher Scientific, USA. All the other solvents and chemical for this study were purchased from Merck, Germany. The *A. chroococcum* (10^9 Cfu/ml) as liquid biofertilizer was kindly provided by the Dayan Agricultural Company, Mashhad, Iran.

Seedling and treatments

This research was conducted in the greenhouse of Islamic Azad University of Mashhad during 2018–2019. The seeds were cultivated in pots filled with sandy soil, arable soil, and leaf soil with respective values of 1:1:1. The experiment was carried out in a complete randomized design and each treatment consisted of five replicate plants. The treatments were performed by irrigation at 100% (no drought stress), irrigation at 80% (slight drought stress), and irrigation at 60% (moderate drought stress) of field capacity. The *A. chroococcum* was applied through inoculation of seeds with 10^8 cfu/g of seed and then irrigation through water at the population of 10^5 cfu/L. Microorganisms for re-treatment through irrigation water were added in the first irrigation after planting and on the 31st day after planting. The average daily maximum and minimum temperatures in the greenhouse during the growing period were 25 and 18 °C, respectively, and relative humidity was between 60 and 78%. The seeds were harvested after 100 days and used for further analysis [42]. The treatment of *T. ammi* seeds was applied as described in Table 7.

Extraction and determination of bioactive compounds

The *T. ammi* seeds were ground, using a mechanical grinder, and the essential oil was extracted by using a Clevenger apparatus [43]. The total phenolic (TP) and total flavonoid (TF) contents were assessed using colorimetric assay with detection at the wave length of 765 nm and 510 nm, respectively. The gallic acid (phenolic) and rutin (flavonoid) were used as standards and data were reported as milligrams equivalent per gram of essential oil (mg/g) [17].

Antioxidant properties

The antioxidant activities of essential oil were evaluated using ferric reducing antioxidant power (FRAP) and ABTS free radical cation-scavenging assay. Briefly, the FRAP potential of the essential oil was calculated using a method as described by Taheri et al. [15]. The ABTS was determined by the method of Giao et al. [44]. ABTS radical cation (ABTS*+) was produced by reacting ABTS stock solution with 2.45 mM $K_2S_2O_8$ and allowing the mixture to stand at room temperature (dark place) overnight before utilization. For both assay the vitamin C was used as a reference standard.

Gene expression

The expressions of super oxide dismutase as biomarker gene responsible for oxidative stress and defense mechanism against free radicals in the plant were determined [45]. Moreover, the expression of cyp71d180 gene involved in thymol biosynthesis [46] and in seed development in response to abiotic and biotic elicitors was analyzed. At the end of each experiment, the plant samples were frozen in liquid nitrogen and stored at − 80 °C. Then, the tissues were ground totally and the RNA was

extracted by RNeasy Mini kit (Qiagen, Hilden, Germany). The cDNA synthesis was performed by a Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). The SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was used in a comparative Real-time PCR (Stratagene Mx-3000P). The targeted genes were amplified as follows: 95 °C for 5 min (1X), 95 °C for 20 s, then 60 °C for 20 s, and 72 °C for 30 s (35X). The expressions of genes were normalized to β-actin as a reference gene and then normalized to the expression of respective genes in the control group. The characteristics of the primer used in this study are presented in Table 8.

In vivo study

Based on the results of total phenolic, flavonoid, and antioxidant potential, the best treatment was selected for the cytotoxicity study. Briefly, the 24 Balb/c male mice (28–30 g, 8 to 10 weeks) were acclimatized in animal house for 10 days in the room temperature under 12 h light/dark cycle. Animals were randomly assigned into four treatments and each treatment possessed six mice as a replicate. Different concentrations of essential oil (0, 50, 100, 200 mg/Kg BW) were administered by oral gavage for 28 days. Finally, they were euthanized with pentobarbital HCL (50 mg/kg, i.p.) and sacrificed. The blood, liver, kidney, jejunum, and spleen tissues were collected immediately for further investigation. The mice trial in this research was approved by the ethical committee of Islamic Azad University of Mashhad and the laws, norms, and regulations dealing with international animal ethics (IR.IAU.S.REC.1399.001). The toxicity study test was conducted in compliance with the OECD guideline No. 407 [47].

Liver enzyme and hematological assays

The liver enzymes, including alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), were carried out on an automated chemistry analyzer (Hitachi 902 analyzer, Japan). The blood parameters, including red blood cell (RBC), white blood cell (WBC), lymphocytes, monocyte, and neutrophil, were evaluated using an automated hematology analyzer (2800 Hematology Auto Analyzer).

Table 8 The list of the primers used for gene expression analysis

Genes	5′→ 3′	Primer list
SOD	F	TGCTGGTGATCTCGGGAATG
	R	GTCAGCATGGACAACAACCG
*CYP71D180	F	CGAGAGGGGACAATCCGAAA
	R	ACTCTGCAGCTAGCTTCTCC

*CYP71D180 is a gene involved in biosynthesis of thymol

Histopathological examination

To investigate the toxicity of essential oil on different organs, including liver, kidney, jejunum, and spleen, each tissue was sampled and stored at buffered formalin (10% formalin in 0.1 M sodium phosphate buffer, pH7) immediately. The paraffin section was made from the paraffin embedded using microtome, then sliced and stained based on the protocol. Finally, the slides were observed under Olympus light microscope (X 400) [48].

Statistical analysis

The data were subjected to one-way analysis of variance using SAS software [17]. The mean comparisons were performed by Duncan's New Multiple Range Test [49] and the difference were considered as significant at $p < 0.05$.

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Authors' contributions

MH contributed to study design, experimental work, formal analysis, and writing original draft; BB was involved in analysis and methodology. EK and EO performed project administration, supervision, review, and editing of the original draft. All the authors read and approved the final manuscript.

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Availability of data and materials

The datasets applied during the current study are available on reasonable request.

Declarations

Ethics approval and consent to participate

The mice trial in this research was approved by the ethical committee of Islamic Azad University of Mashhad and the laws, norms, and regulations dealing with international animal ethics (IR.IAU.S.REC.1399.001).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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