

Enhancing Thymol and Carvacrol Biosynthesis in *Thymus vulgaris* L. using *Laurencia caspica* Seaweed Extract: Biostimulant Potential and Gene Expression Insights

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Abstract

This study investigates the biostimulant potential of *Laurencia caspica* seaweed liquid extract (SLE) on *Thymus vulgaris* L., focusing on its impact on various biological parameters and the biosynthesis pathway of thymol and carvacrol. Results demonstrate significant enhancements in vegetative and reproductive traits, including leaf area, root and shoot length, and biomass, with inoculated plants showing notable increases in fresh and dry weights compared to controls. Moreover, SLE application led to elevated levels of total phenolic and flavonoid content, as well as increased activity of key enzymes such as phenylalanine ammonia lyase (PAL) and cell wall peroxidase (POD), along with enhanced lignin accumulation in leaves. Gene expression analysis revealed upregulation of *DXR* and *TPS2* genes, resulting in higher accumulation of thymol and carvacrol in plant tissues compared to controls. This pioneering study underscores the practical utility of *L. caspica* seaweed as a biostimulant in agriculture, offering insights for further advancements in harnessing *Laurencia* species for sustainable agricultural practices.

Introduction

Thymus vulgaris L., one of the most valuable member of the Lamiaceae family, holds significant value in various industries including food, cosmetics, and medicine. Characterized by its perennial nature, shrubby stature, and aromatic gray-green leaves adorned with pink or purple flowers, this plant thrives in native habitats spanning the Mediterranean, North Africa, and diverse regions of Asia (Kuate 2017). Notably, the essential oil derived from thyme boasts a plethora of medicinal applications attributed to its robust antiseptic, antibiotic, and antifungal properties (Ekoh et al. 2014). Central to its medicinal prowess are phenolic compounds, primarily thymol (10–64%) and carvacrol (0.4–20.6%), which confer the distinct spicy flavor and potent therapeutic benefits (Li et al. 2019). Recent elucidation of the biosynthetic pathway governing carvacrol and thymol monoterpenes in thyme's aerial parts has shed light on their intricate molecular synthesis (Crocchi, 2011; Turner et al. 2000). This biosynthetic journey unfolds within plastids through the methylerythritol phosphate (MEP) pathway, which is regulated by enzymes catalyzing the conversion of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) (Dudareva et al. 2005). Beginning with the synthesis of DXP (1-deoxy-D-xylulose-5-phosphate) via the DXS enzyme, the pathway progresses with DXP reductase isomerase (DXR) converting DXP to MEP (Takahashi et al. 1998). Geranyl diphosphate synthase (GDS) then facilitates the condensation of IDP and DMADP, yielding geranyl diphosphate (GDP), a pivotal precursor in monoterpene production. Further enzymatic conversions lead to the formation of key intermediates like γ -terpinene under the influence of γ -terpinene synthase (TPS) (Davis and Croteau 2000; Lichtenthaler 1999). Beyond intrinsic biochemical pathways, the chemical composition of essential oils is intricately influenced by various environmental factors like heat, UV-B radiation, heavy metals, salt, drought and different kinds of elicitors, with nutrient levels playing a crucial role in optimizing plant growth and bioactivity (Mihajilov-Krstev et al. 2010; Arvin 2019; Aqeel et al. 2023). Seaweeds are integral components of marine biosystems and serve as sources for numerous valuable bioactive compounds, such as polysaccharides (alginate, agar and carrageenan), lipids, flavonoids, polyphenols, alkaloids, glycosides, and steroids. Consequently, they find applications across

diverse industries including cosmetics, pharmaceuticals, food, fertilizers, and as dietary supplements (Aziz et al. 2020). The growth stimulating effect of seaweed liquid extracts and their application on several plant species have been demonstrated, which have been successfully used for enhanced plant growth and improved seed germination (Kumar and Sahoo 2011; Hong et al. 2007; Ramarajan et al. 2012), increased vegetative growth (Khan et al. 2009; Shukla et al. 2019), and improved biochemical characteristics (Hassan et al. 2021; González-González et al. 2020) in various agricultural products. Among the diverse seaweed species, *Laurencia caspica*, a red macro algae inhabiting the southern coast of the Caspian Sea, stands out for its rich reservoir of halogenated secondary metabolites (Karimzadeh et al. 2020). Notably, recent studies underscore the pivotal role of red algae metabolites in augmenting the growth and biochemical profile of various plant species, including Faba Bean (*Vicia faba* L.) (Ragaa and Safinaz 2013).

Harnessing the growth-promoting potential of seaweed organic fertilizers has yielded promising results, as evidenced by notable improvements in the growth parameters and essential oil yield of medicinal plants like hyssop, mint, and basil (Elansary et al. 2016b; Pirani et al. 2020). Intriguingly, the application of methyl jasmonate (MeJA) has been shown to modulate the metabolic profile and expression of key genes governing the thymol/carvacrol biosynthetic pathway in Iranian thyme species, leading to enhanced essential oil accumulation (Kianersi et al. 2021).

Building upon these insights, the present study endeavors to explore the potential of *Laurencia caspica* seaweed extract as a biostimulant in enhancing the seed germination, vegetative growth, essential oil yield, and physiological attributes of *Thymus vulgaris* L. plants. Furthermore, a comprehensive investigation into the molecular mechanisms underpinning carvacrol and thymol biosynthesis post-seaweed extract application promises to unveil novel strategies for bolstering productivity in medicinal plants. Through a multidimensional approach encompassing molecular, biochemical, and metabolic analyses, this study aims to elucidate the intricate interplay between seaweed-derived biostimulants and the biosynthesis of key phytochemicals in thyme plants.

Materials and methods

Collection and preparation of seaweed liquid extract

Samples of the marine red seaweed, *L. caspica*, were collected from Ramsar coasts, Caspian Sea, Mazandaran, Iran, in April 2021, and transported in plastic bags to the Phycology Laboratory of Shahid Beheshti University. The identification of samples followed the method introduced by Sterrer (1986). Upon collection, the seaweeds were washed thoroughly with fresh seawater to remove epiphytes, sand, and other extraneous matter, and then shade dried for 10 days followed by oven drying at 60°C for 48 hours. The oven-dried algae were then ground into a fine powder using a mechanical grinder. The powdered samples were stored in airtight vessels for future use. To prepare the algal extract, 50 g of powdered samples were boiled with 500 ml of distilled water for 60 minutes. After filtration, the obtained extract was considered as 100% concentration. Subsequently, different concentrations of seaweed liquid

fertilizer (1%, 1.5%, 2%, 2.5%, and 5%) were prepared from the 100% extract using double distilled water (Ramarajan et al. 2012).

Seedling growth test

Seeds of *T. vulgaris* L. were sourced from the Institute of Medicinal Plants, ACECR, Karaj, Iran. The seeds underwent surface sterilization with 1% mercuric chloride for 2 minutes, followed by rinsing with distilled water. Subsequently, they were treated with various concentrations (1%, 1.5%, 2%, 2.5%, 5%) of *L. capsica* extract. Each treatment and control group consisted of 20 seeds placed on filter paper in Petri dishes, with three replicates, and submerged in 5.0 mL of seaweed extract or distilled water. The Petri dishes were then incubated in a culture chamber at 25°C under a 12/12-hour light-dark cycle. After 10 days, seedling length (SL cm), fresh weight (FW g), saturated weight (SW g) (after 24 hours in distilled water), and dry weight (DW g) were measured. Relative water content (RWC %), vigor index (VI), and germination percentage (GP %) were calculated using the following formulas:

$$\text{RWC\%} = [(FW - DW) / (SW - DW)] \times 100$$

$$\text{GP} = (n/N) \times 100$$

(n = number of germinated seeds on the 10th day, N = total number of seeds)

$$\text{VI} = \text{Average SL (cm)} \times \text{Standard GP (\%)}$$

Pot culture

Homogenous seedlings of *T. vulgaris* L. were obtained from the Institute of Medicinal Plants, ACECR, Karaj, Iran. These seedlings were planted in 3-L pots with dimensions of 18 cm in diameter. The potting mix consisted of 31% silt, 40% sand, and 29% clay. The cultivation period lasted for 4 months. All experiments were conducted with five replicates, and the arrangement of the pots was completely randomized. The cultivation took place in a greenhouse under standard conditions: a photoperiod of 8/16 (Dark/light), a temperature maintained at 25.0 ± 2 °C, and a relative humidity of $55 \pm 5\%$. The soil in the pots was inoculated with SLE (2.5%) one week before planting and then repeated every 20 days after planting for the duration of 4 months. Each inoculation involved the application of approximately 100 ml of the extract. Control plants were solely watered, receiving 100 ml of water per irrigation session.

Measurement of Growth Parameters

Thyme plants were harvested four months after planting. Upon harvesting, the roots and shoots were separated, and the roots were thoroughly washed to remove soil particles. Subsequently, various growth parameters were measured, including plant height (root and shoot length), fresh and dry weights of both roots and shoots, leaf surface area, and the number of flowers per stem.

Extraction and quantification of essential oil and analysis of its compounds

Air-dried aerial parts of the samples (30 g) were subjected to hydro-distillation for 3 hours using a Clevenger-type apparatus to extract the essential oil. The distilled oils were then dried with waterless sodium sulfate and stored in a refrigerator (4 °C) prior to weighing. The extraction efficiency for each sample was calculated based on the weight fraction of dried oils and the dry weight of the whole plant used (w/w %). Gas chromatography-mass spectrometry (GC/MS) analysis was conducted to identify and analyze the components of the essential oil. The analysis was performed on a ThermoQuest-Finnigan Trace GC-MS system equipped with a DB-5 fused silica column (30 m × 0.25 mm × 0.25 µm), following the method described by Zarezadeh et al. (Zarezadeh et al. 2020). The components in the essential oil were identified by comparing their mass spectrum with the internal reference mass spectrum library and confirming their retention indices with indices reported in the literature (Adams 2007).

Variability of photosynthetic pigments

To measure the changes in the content of photosynthetic pigments, 0.2 g of plant tissue from each treatment were powdered in liquid nitrogen and mixed with 1800 microliters of pure methanol. The samples were centrifuged after 24 hours incubation in dark. Then, the absorbance of the liquid phase was recorded at wavelengths of 665, 652, 470 nm using a WPA biowave II UV-Vis spectrophotometer (Cambridge, UK). The concentrations of chlorophyll a, b and carotenoids were calculated from the following equations. (Lichtenthaler and Buschmann 2001).

$$\text{Chl}_a (\mu\text{g. ml}^{-1}) = 16.72 A_{665.2} - 9.16 A_{652.4}$$

$$\text{Chl}_b (\mu\text{g. ml}^{-1}) = 34.09 A_{652.4} - 15.28 A_{665.2}$$

$$\text{Carotenoid } (\mu\text{g. ml}^{-1}) = (1000 A_{470} - 1.63 \text{ Chl}_a - 104.96 \text{ Chl}_b)/221$$

Measurement of phenol, flavonoid and lignin content

"To measure phenol, flavonoid, and lignin content, 0.2 g of powdered plant tissue was mixed with 1800 µL of pure methanol and placed in an ultrasonic bath for 30 minutes. After centrifugation (10-1000 g), the liquid phase (methanolic extract) was used. Total phenolic compounds were determined by adding 200 µL of 10% Folin reagent and 800 µL of sodium carbonate to 100 µL of methanolic extract, with absorbance read at 765 nm after 3 hours of incubation at ambient temperature. Total phenol content was expressed as Gallic Acid equivalent (mg GA gFW⁻¹ of extract) (Singleton et al. 1999). For total flavonoids, 1 ml of methanolic extract was mixed with 1 ml of 2% aluminum chloride, with absorbance recorded at 420 nm after one hour of incubation at room temperature. Flavonoid content was presented as mg GA gFW⁻¹ of the extract (Kamtekar et al. 2014). Lignin was measured using phloroglucinol reagent (Zimmer 1999). Leaf powder was treated with methanol, agitated for 24 hours, and then transferred to a 45-degree oven for 48 hours. After reweighing the sediments, ethanolic acid was used to extract lignin. The resulting extract was mixed with 1 mM Phloroglucinol and absorbance read at 488 nm to estimate lignin content based on the standard curve (Y= 0.0002X-0.0093, R2=0.9894)."

Determination of PAL and POD activity

The determination of PAL (phenylalanine ammonia-lyase) and POD (peroxidase) enzyme activity was conducted using a standardized procedure. Powdered leaves were homogenized with extraction buffer, and the supernatant obtained after centrifugation was used for enzyme assays. PAL activity was measured by monitoring the production of cinnamic acid from phenylalanine, while POD activity was determined by observing the increase in absorbance at 470 nm due to the oxidation of guaiacol by hydrogen peroxide. Protein concentration was measured using the Bradford method. Detailed protocols for both assays, including reaction conditions and spectrophotometric readings, were followed as described in the references (Rodriguez-Rosales et al., 1999; Goldson et al., 2008; Tang and Newton, 2005).

GAPDH, *DXR* and *TPS2* expression analysis

The genes selected for expression analysis, including *GAPDH*, *DXR*, and *TPS2*, were chosen based on their relevance to the biosynthetic pathways of thymol and carvacrol, as well as previous research findings. The primers for these genes were designed using the oligo7 program and following qPCR prerequisites (Table 3). RNA extraction from plant tissue was performed using the Chomczynski and Sacchi method (Chomczynski and Sacchi 1987) with RNX-Plus solution (Cinnagen Co., Iran), followed by assessment of quantity and quality using UV spectroscopy and agarose gel electrophoresis. To eliminate genomic DNA contamination, RNA was treated with DNase I (Fermentas, Cinagen Co., Iran), and the absence of DNA was confirmed by PCR. First-strand cDNA synthesis was carried out using 1 microgram of RNA with Oligo (dT) and random primers in the presence of Reverse Transcriptase enzyme. qRT-PCR analysis was conducted using the StepOne Real-Time PCR system (ABI, USA), with a reaction volume of 12 microliters containing RealQ Plus 2x Master Mix (Ampliqon, Copenhagen, Denmark), 15 micromoles of each primer, 2 microliters of cDNA, and ddH₂O. The primer binding temperature was optimized using the gradient PCR method, and the integrity of cDNA was confirmed by PCR of the control gene, *GAPDH*, in all samples. Gene expression changes were determined using the 2- $\Delta\Delta$ CT method (Schmittgen and Livak 2008), where Δ CT was calculated as the difference between the CT value of each reaction and the CT value of *GAPDH*, and $\Delta\Delta$ CT was calculated as the difference between the Δ CT of the treatment group and the Δ CT of the control group. Table 3 provides a list of primers used for the qRT-PCR reactions.

Statistical analysis

Statistical analysis was conducted using the SAS statistical package (2009). One-way ANOVA was employed to analyze all the data, followed by Duncan's multiple range test for means comparison. Data analysis charts were created using Excel 2016 software (Microsoft Corporation).

Results

Inoculation of *Thymus vulgaris* L. with seaweed extract

Results from the inoculation of *T. vulgaris* L. with seaweed extract indicate that among various concentrations of *L. caspica* seaweed extract (1%, 1.5%, 2%, 2.5%, 5%), the most notable increase in *T. vulgaris* L. seedling growth indices occurred with a 2.5% seaweed extract treatment compared to the control. Specifically, thyme seeds treated with this seaweed extract exhibited significant improvements in germination percentage (90%), seedling dry weight (0.006 ± 0.0005), and vigor index (5.19 ± 0.57) compared to untreated seeds (Table 1, Fig. 1). Consequently, this species was chosen for further investigation. In subsequent pot culture experiments, treated plants displayed considerable enhancements in growth indices compared to control plants. Notably, those treated with *L. caspica* exhibited significant differences in various vegetative and reproductive parameters, including leaf area, root and shoot length, fresh and dry weight, and number of flowers, leading to increases in plant growth (33.84%), fresh weight (49.49%), dry weight (41.21%), and number of flowers (31.57%) (Fig. 2,3). Additionally, the application of seaweed extract positively and significantly affected the weight of essential oil, resulting in an approximately 30% increase in treated plants compared to controls (Fig. 3). Analysis of the essential oil composition revealed the presence of carvacrol methyl ether metabolite in treated samples, which was absent in controls. Furthermore, concentrations of thymol and carvacrol, key components of thyme essential oil, were notably higher in treated plants by 6.15% and 18.60%, respectively. Moreover, linalool (19.35%), para-cymene (12.52%), and caryophyllene oxide (71.43%) exhibited significant increases in treated plants. Conversely, the tissue content of gamma terpinene and camphor was lower in treated plants compared to controls, while the concentration of other compounds remained relatively unchanged (Table 2).

Table 1				
The effect of <i>Laurencia caspica</i> extract on the growth parameters of <i>Thymus vulgaris</i> L. seedlings. GP: Germination Percentage, VI: Vigor Index, DW: Dry Weight (Means $\pm$ SE, n = 3). significance at P < 0.05 level.				
Treatment	VI	DW	RWC	GP
Control	2.57 ± 0.13	0.004 ± 0.0005	0.69 ± 0.22	85%
<i>L. caspica</i>	$5.19 \pm 0.57^*$	$0.006 \pm 0.0005^*$	0.66 ± 0.03	90%*
*Significance at P < 0.05 level.				

Table 2

Effect of 0.2% seaweed extract on changes in compound and profile of *Thymus vulgaris* L. essential oil (%) based on GC MS analysis (Mean $\pm$ SE).

NO	RI	Compound	Type	Control	<i>Laurencia caspica</i>
1	10.88	α - Thujene	MH	0.49 $\pm$ 0.02	0.45 $\pm$ 0.06
2	11.26	α - pinene	MH	0.57 $\pm$ 0.11	0.68 $\pm$ 0.07
3	13.58	β - pinene	MH	0.19 $\pm$ 0.01	0.17 $\pm$ 0.04
4	26.91	Carvacrol, methyl ether	MO	-	0.70 $\pm$ 0.11
5	14.79	Octanol < 3>	MH	0.11 $\pm$ 0.03	0.13 $\pm$ 0.02
6	15.64	α - Terpinene	MH	1.38 $\pm$ 0.15*	1.09 $\pm$ 0.09
7	16.16	Para Cymene	MH	17.01 $\pm$ 0.51	19.14 $\pm$ 0.51*
8	16.30	Limonene	MH	0.33 $\pm$ 0.06	0.34 $\pm$ 0.04
9	17.83	Gamma Terpinene	MH	8.61 $\pm$ 0.04*	5.46 $\pm$ 0.10
10	18.57	Cis Sabinene hydrate	MO	1.53 $\pm$ 0.11	1.41 $\pm$ 0.09
11	20.05	Linalool	MO	2.48 $\pm$ 0.06	2.96 $\pm$ 0.06*
12	20.19	Trans Sabinene hydrate	MO	0.42 $\pm$ 0.04	0.43 $\pm$ 0.06
13	22.67	Camphor	MO	1.41 $\pm$ 0.06*	0.84 $\pm$ 0.08
14	23.89	Borneol	MO	2.81 $\pm$ 0.11*	2.36 $\pm$ 0.06
15	24.23	Terpinene-4-ol	MO	0.40 $\pm$ 0.04	0.41 $\pm$ 0.04
16	27.78	Thymoquinone	MO	0.26 $\pm$ 0.03	0.25 $\pm$ 0.03
17	29.73	Thymol	MO	48.32 $\pm$ 0.04	51.29 $\pm$ 0.19*
18	30.04	Carvacrol	MO	3.01 $\pm$ 0.06	3.57 $\pm$ 0.09*
19	34.95	caryophyllene	SH	3.43 $\pm$ 0.08	3.21 $\pm$ 0.14
20	41.79	Caryophyllene oxide	SO	0.35 $\pm$ 0.02	0.60 $\pm$ 0.08*
MH: Monoterpene Hydrocarbons, MO: Oxygenated Monoterpenes, SH: Sesquiterpene Hydrocarbons, SO: Oxygenated Sesquiterpenes, * Significant at the 0.05 level.					

Table 3. The primer sequences designed for expression analysis of the GAPDH, DXR, and TPS2 genes in *Thymus vulgaris* L.

Gene name	Accession number	Primer	Primer sequences	PCR product	length	Tm	GC%
GAPDH	MF373628	Forward	5' GTGGATGGTGGACATGAG 3'	141	18	54	55
		Reverse	5' GTAGGAGTGAACGAGAAGG 3'		19	54	52
DXR	KY621335	Forward	5' CTGACTCGGAACATTCTGC 3'	105	19	56	52
		Reverse	5' GCAAATCCCTGAAAGCACC 3'		19	57	52
TPS2	JQ957866.1	Forward	5' GCTAGAGGCGAGATGGTTC 3'	112	19	59	57
		Reverse	5' GAATTAGTCCACCACCTCG 3'		19	57	52

Variability of biochemical characteristics

Table 4 illustrates the changes in photosynthetic pigments, indicating notable enhancements in chlorophyll a (by 50.19%), chlorophyll b (by 52.68%), and carotenoid (by 49.50%) compared to the control group (Table 4). Moreover, this study investigated the total phenol and flavonoid content, crucial for the medicinal properties of thyme (Fig. 5a). The treated plants exhibited a 15.25% increase in total phenol content and a significant 76.19% increase in total flavonoid content compared to the control group. Regarding lignin content, an essential defense barrier against stressors, seaweed treatment substantially augmented lignin levels in thyme leaves. Specifically, the treated plants demonstrated a 100% increase, rising from $0.15 \mu\text{g. g FW}^{-1}$ in control plants to $0.31 \mu\text{g. g FW}^{-1}$ (Fig. 5a). Furthermore, the activity of key enzymes involved in secondary compound biosynthesis was evaluated. The application of seaweed extract notably enhanced PAL enzyme activity by 12.5% compared to control plants. Additionally, seaweed treatment had a significant impact on the activity of the POD enzyme (Fig. 5b).

Table 4

Effects of 2.5% seaweed extract on the concentration of photosynthetic pigments in *Thymus vulgaris* L. leaves. Chl a: chlorophyll a, Chl b: chlorophyll b, Carot: carotenoids. (Means $\pm$ SE, n = 3).

	Chl a ($\mu\text{g. ml}^{-1}$)	Chl b ($\mu\text{g. ml}^{-1}$)	Carot ($\mu\text{g. ml}^{-1}$)
Control	2.61 ± 1.60^b	2.24 ± 1.18^b	1.01 ± 0.24^b
<i>Laurencia caspica</i>	3.92 ± 1.18^a	3.42 ± 0.90^a	1.51 ± 0.57^a
^a Significant at the 0.05 level.			

Changes in gene expression

In order to explore the impact of seaweed extract on gene expression related to thymol and carvacrol biosynthesis pathways, this study investigated the expression of *DXR* and *TPS2* genes in *T. vulgaris* leaves using qPCR. The analysis revealed a significant difference in plants treated with SLE. The relative expression levels of *DXR* and *TPS2* genes in treated plants were 4.19 and 3.49 times higher than the control, respectively (Fig. 4). These findings are groundbreaking, shedding light on the potential of SLE as a potent inducer of gene expression related to the biosynthesis of phytochemical compounds. By enhancing the expression of genes involved in thymol and carvacrol production, SLE emerges as a promising candidate for optimizing the yield of these valuable bioactive compounds in thyme plants. This study not only underscores the significance of seaweed extract in agricultural practices but also unveils new avenues for enhancing the production of phytochemicals through targeted gene expression modulation.

Discussion

The current study aimed to explore the impact of the red algae *L. caspica* as a biofertilizer on thyme plants. Results revealed that a 2.5% *L. caspica* seaweed extract significantly enhanced the growth indices of *T. vulgaris* L. plants such as seedling dry weight, germination percentage and vigor index compared to the control group. Increasing the rate of germination is crucial for optimizing agricultural productivity, as successful germination directly correlates with crop yield and seedling establishment (Panahabadi et al. 2022). An increase in seed germination and vigor index was reported with seaweed extract treatment for *Oryza sativa* L. (Layek et al. 2018b), *Solanum lycopersicum* L. (Hernandez-Herrera et al. 2014) and *Oryza sativa* L. (Layek et al. 2018a). The efficacy of seaweed extract in promoting plant growth is attributed to its content of growth regulators such as auxins, gibberellins, and phenylacetic acid, which enhance germination and vigor index (Sivasankari et al. 2006). For instance, the brown seaweed *Ascophyllum nodosum* extract induces Gibberellic Acid (GA3)-independent amylase activity, thereby increasing seed germination rate in barley (Rayorath et al. 2008). Pot culture experiments demonstrated significant improvements in vegetative and reproductive parameters, as well as in the weight of essential oil in treated plants. Key metabolites such as thymol, carvacrol, linalool, para cymene, and caryophyllene oxide increased in plants treated with seaweed extract compared to controls. Seaweed extract acts as a

biostimulant, enhancing plant metabolism by releasing soil nutrients and increasing their absorption by plants (Hussain et al. 2021; Shukla and Prithiviraj 2021). This effect is attributed to the presence of polysaccharides, glycerol, and plant growth hormones like auxins, cytokinins, and gibberellins in seaweeds, which promote plant growth and productivity (Hamed et al. 2018). However, excessive use of seaweed extract may negatively impact essential oil production in thyme leaves due to accelerated vegetative growth outpacing the synthesis and accumulation of essential oil. Nevertheless, optimal consumption of seaweed extract can maximize dry matter yield, essential oil yield, and the main constituents of essential oil using the response surface method (Muetasam Jafr et al. 2022). Previous studies have extensively documented the positive effects of seaweed on various aspects of plant growth and development, including increased yield, improved quality, enhanced resistance to environmental stress, and protection against diseases (Hassan et al. 2021; Mahmoud et al. 2019). Additionally, seaweed extract has been found to stimulate flower and fruit production in tomato plants by elevating hormone levels, promoting nutrient accumulation, enhancing photosynthetic efficiency, and delaying senescence (Dookie et al. 2021). Importantly, the influence of seaweed extract on the production of plant secondary compounds, such as essential oils, is largely attributed to the presence of complex carbohydrates in seaweed tissues (Elansary et al. 2016b). Seaweed extract comprises a diverse array of beneficial constituents, including polysaccharides, nutrients, hormones, sterols, and betaines, which have been demonstrated to positively impact the essential oil content of aromatic plants like thyme (Boutekrabt et al. 2022; Muetasam Jafr et al. 2022; Waly et al. 2020), as well as medicinal plants in general (Ghatas et al. 2021; Tawfeeq et al. 2016). For instance, Waly et al. (2020) conducted a study showing that treatment of *Thymus vulgaris* L. with a blend of seaweed extracts (including *Ascomyllum* spp., *Laminaria* spp., *Sargassum* spp., and *Fucus* spp.) significantly enhanced the essential oil content.

The quality of aromatic plant species is often determined by their essential oil content and valuable ingredients (Riahi et al. 2020), influenced by various factors such as climate, genotype, growth conditions, and harvesting time (Preedy 2015). Additionally, the application of seaweed extract, such as *Sargassum latifolium*, either alone or under salt stress, has been found to increase the essential oil content of Rosemary plants (Gharib et al. 2014). In another study, treatment of *Salvia officinalis* L. with *Enteromorpha intestinalis*, *Fucus spiralis*, and *Ulva rigida* extracts as foliar spray showed considerable effects on the levels of certain essential oil compounds (α -pinene, 1,8-cineole, and camphene). Environmental factors such as temperature, day length, light intensity, planting date, and soil conditions can also affect essential oil compounds (Mansori et al. 2022). In the current study, the main components of the essential oil were identified as thymol, p-cymene, γ -terpinene, caryophyllene, and carvacrol, consistent with previous research (Muetasam Jafr et al. 2022; Nhu-Trang et al. 2006; Pavela et al. 2018). Different factors such as fertilizers and irrigation levels have shown inverse changes on the amount of thymol and carvacrol. For instance, in a study by Karamanos and Sotiropoulou (2013), the application of nitrogen on *Origanum vulgare* had differing effects on the amounts of thymol and carvacrol.

Furthermore, research has demonstrated the beneficial effects of seaweed extract treatment on the physiological responses of plants, such as alterations in photosynthetic pigments, PAL enzyme activity, and total phenol and flavonoid content. For example, the application of seaweed fertilizer has been

shown to enhance the synthesis of photosynthetic pigments in treated plants, likely attributable to its rich organic and mineral composition (Hussain et al. 2021; Manaf 2016). Similarly, studies have indicated that seaweed liquid fertilizer inoculation can elevate chlorophyll levels in various plants, including soybeans (Hashem et al. 2019; Ramarajan et al. 2012). Moreover, carotenoids present in seaweed extracts play a crucial role in mitigating oxidative stress in plants by scavenging reactive oxygen species (ROS) (Christaki et al. 2013).

According to our report, the carotenoid content experienced a notable increase of approximately 49% following seaweed treatment. Similarly, a study by Muetasam Jafr et al. (2022) found that the application of the highest levels of nitrogen combined with seaweed extract on *T. vulgaris* L. plants resulted in the highest carotenoid levels. The observed enhancement in leaf pigment concentration due to seaweed extract can be attributed to the presence of natural phytohormones, particularly cytokinins, which have been shown to delay leaf aging, reduce leaf pigment degradation, or enhance leaf pigment biosynthesis (Jannin et al. 2013).

The comparison of total phenolic and flavonoid content observed in treated plants compared to the control demonstrated a significant difference. These findings align with previous studies where seaweed extract, specifically *Ascophyllum nodosum*, was utilized as a biostimulant to enhance total phenol and flavonoid content as well as antioxidant activity in various plants such as *Brassica oleraceae* L., *Achillea millefolium* L., and *Calibrachoa x hybrida* (Lola-Luz et al. 2013; Shafie et al. 2021; Elansary et al. 2016a). For instance, the assessment of phenol and flavonoid content in red radish (*Raphanus sativus* L.) treated with seaweed extract revealed that a concentration of 3 ml/l resulted in the highest accumulation of leaf flavonoid and root phenolic and anthocyanin, while the control treatment exhibited the lowest levels of these phytochemicals (Mahmoud et al. 2019). Similarly, Kasim et al. (2016) and Lola-Luz et al. (2014) reported increased concentrations of phytochemical compounds and antioxidant activity in *Raphanus sativus* L. and *Brassica oleraceae* var. *italica* plants treated with seaweed extract, consequently enhancing the nutritional value of the plants. Phenolic compounds possess strong antioxidant properties and are crucial for plant growth, development, and defense mechanisms. Canola (*Brassica napus* L.) plants exhibit elevated accumulation of phenols and flavonoids under salt stress conditions, contributing to their ability to tolerate high salt levels (Kubalt 2016). According to Mzibra et al. (2021), the induction of phytochemical biosynthesis, such as polyphenols, may be attributed to the hydrolysis of seaweed extract polysaccharides, which are recognized as signaling molecules by the cell walls of *Solanum lycopersicum* L. Additionally, Gonzalez-Gonzalez et al. (2020) reported a twofold increase in polyphenol content in tomato (*Solanum lycopersicum* L.) plants inoculated with seaweed extract compared to those inoculated with nutrient solution, attributing this increase to plant growth under stress conditions.

Our study highlights the regulatory effect of seaweed extract (SLE) treatment on lignin, PAL, and cell wall peroxidase (POD) activity. In a study conducted by Samadi et al. (2015), treatments involving methyl jasmonate and salicylic acid resulted in heightened PAL enzyme activity, along with increased levels of total phenolic and flavonoid content. These results underscored the significant correlation between phenol content and PAL enzyme activity, further emphasizing the influence of biostimulant concentration.

Moreover, Mehrabanjobani et al. (2019) demonstrated that rice plants supplemented with extra iron exhibited elevated wall POD and PAL activity, leading to enhanced lignin accumulation in roots. These findings suggest that optimal biocatalyst utilization can facilitate the production of secondary compounds in appropriate quantities.

While numerous reports have documented the efficacy of seaweed extract in enhancing plant growth and performance, there remains a lack of information regarding the mechanisms underlying these biostimulants' action on plant growth processes. In our study, we measured the expression levels of *DXR* and *TPS2* genes in response to SLE treatment compared to control plants, revealing a significant increase. This suggests that SLE may serve as a suitable treatment for inducing the expression of genes involved in the biosynthesis of phytochemical compounds. The expression patterns of thymol and carvacrol biosynthesis genes are influenced by seaweed treatment, resulting in increased thymol and carvacrol content in *T. vulgaris* L. This indicates a direct relationship between the production of secondary metabolites and the expression of corresponding genes at the transcriptional level. The *DXR* gene plays a pivotal role in controlling the MEP pathway, directing mediators for the production of IPP and DMAPP, thereby influencing terpene biosynthesis in the plastid (Carretero-Paulet et al. 2002). Its role in controlling the accumulation of isoprenoids in plants has been confirmed, as evidenced by the high expression of *DXR* in transgenic *Mentha piperita* L., leading to increased monoterpene content in essential oil (Mahmoud and Croteau 2001). Another key gene in the thymol biosynthesis pathway is terpinene synthase, with changes in its expression affecting thymol synthesis in plants (Crocchi 2011). Rasuli et al. (2024) investigated the effect of cyanobacterial treatment as biofertilizer on the expression of key genes of thymol and carvacrol biosynthesis pathway (*DXR* and *TPS2*) of thyme plant and reported an increase in the expression of these genes in treated plants (Rasuli et al. 2024). Studies on the effect of salicylic acid on gene expression patterns in black cumin (*Nigella sativa* L.) revealed increased expression levels of monoterpene synthase, geranyl diphosphate synthase, beta-amyrin synthase, and squalene epoxidase genes upon treatment (Elyasi et al. 2015). Salinity stress was also found to enhance thymol and carvacrol biosynthesis in *T. vulgaris* L. by affecting genes involved in the MEP biosynthetic pathway (Hosseini et al. 2018). Furthermore, *DXR* gene expression patterns in *T. vulgaris* L. showed higher expression in flower tissue, with various treatments such as methyl jasmonate, salicylic acid, trans-cinnamic acid, and UV increasing its expression after 24 hours (Malek Zadeh et al. 2015). Similarly, methyl jasmonate treatment in *Salvia miltiorrhiza* Bunge plants and *Withania somnifera* L. led to significant increases in *DXR* gene expression (Yang et al. 2012; Gupta et al. 2013). Cold stress was also found to significantly affect the expression of thymol biosynthesis pathway genes in *T. vulgaris* L., with *DXR* and *TPS1* genes potentially playing a role in defense function and message transmission of terpenes (Habibi et al. 2017). In summary, the tissue accumulation of terpenoids, as the most prevalent and diverse plant secondary metabolites, influenced by various environmental variables (Huang et al. 2019).

Conclusion

In summary, the study revealed that the application of *L. caspica* seaweed extract could significantly enhance the growth, yield, and biochemical composition of *T. vulgaris* L. plants. Moreover, it was

observed that SLE increased the expression of key genes involved in the biosynthesis of thymol and carvacrol, potentially due to the presence of active compounds like flavonoids and polyphenols. These findings highlight the promising role of SLE in regulating the biosynthesis of important secondary metabolites in medicinal plants. The successful use of *L. caspica* seaweed extract underscores its potential as a natural and eco-friendly substrate for improving plant performance and metabolic profiles. This study marks a significant step in harnessing the benefits of seaweed extracts for agricultural applications, offering insights into the development of more sustainable strategies for enhancing secondary metabolite production. Moving forward, further research on the utilization of seaweed biostimulants, particularly from *Laurencia* species, holds promise for advancing modern agricultural practices in an environmentally conscious manner.

Declarations

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Author Contributions N. Rasuli collected data, wrote and prepared the manuscript. H. Riahi and Z. Shariatmadari supervised the project, interpreted data and critically read the manuscript. P. MehrabanJoubani assisted in enzyme assays and critically reviewed the manuscript. M. Ghorbani Nohooji and P. MehrabanJoubani advised the project. A. Dehestani assisted in parts of molecular analysis. All authors approved the final version of the manuscript.

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Data availability The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declarations of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures



Figure 1

Effect of red algae extract on *Thymus vulgaris* L. seedling growth rate, 0. Control, 1. *Laurencia caspica*. (bar 0.5 cm).



Figure 2

Comparison between the control and treated plants with 2.5% *Laurencia caspica* extract on growth parameters of *Thymus vulgaris* L. 0. control. 1. *Laurencia caspica* (bar 5 cm).

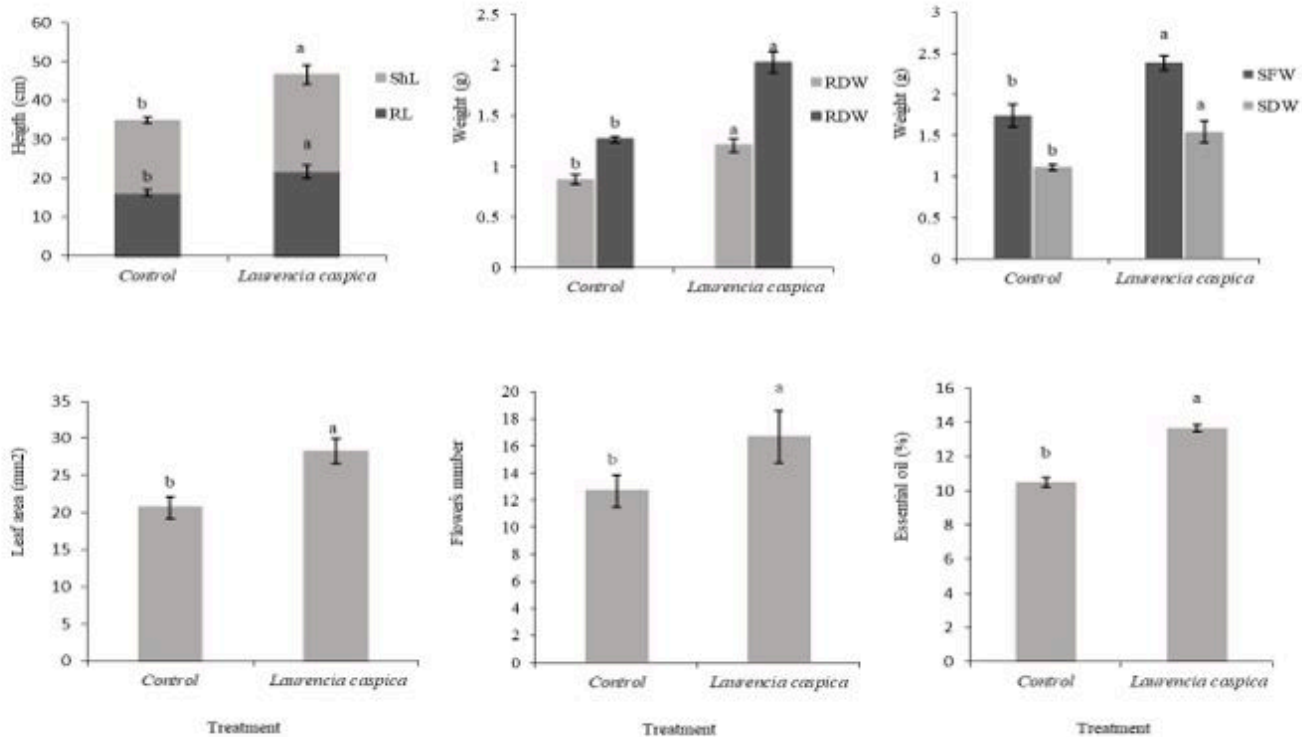


Figure 3

Changing of quantitative plant growth parameters of *Thymus vulgaris* L. with the treatment of 2.5% *Laurencia caspica*. ShL: Shoot length, RL: Root length, Rfw: Root fresh weight, Rdw: Root dry weight, Sfw: Shoot fresh weight, Sdw: Shoot dry weight. (Mean ± SE, n=3). ^a Significant at the 0.05 level.

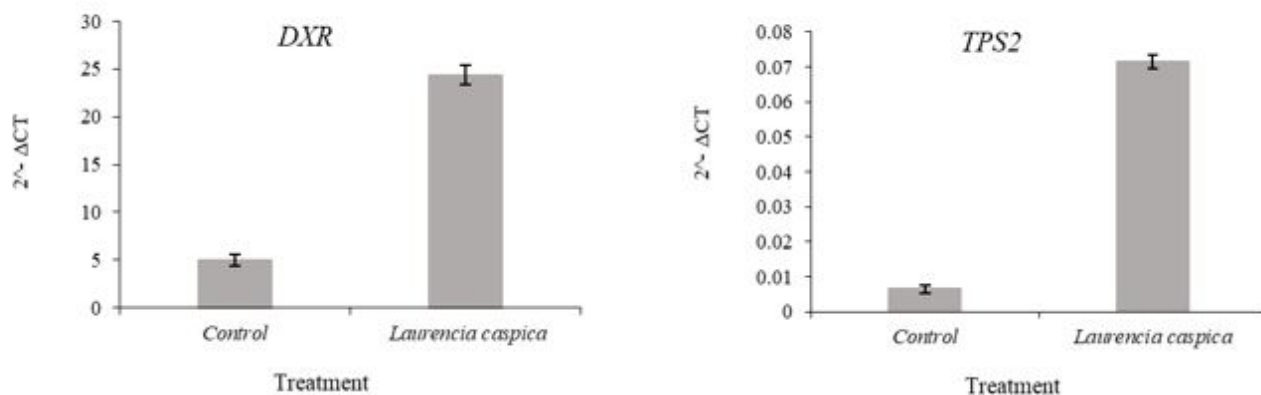


Figure 4

The effect of *Laurencia caspica* treatments on the expression of *DXR* and *TPS2* genes in *Thymus vulgaris* L. (Means ± SE, n = 3).

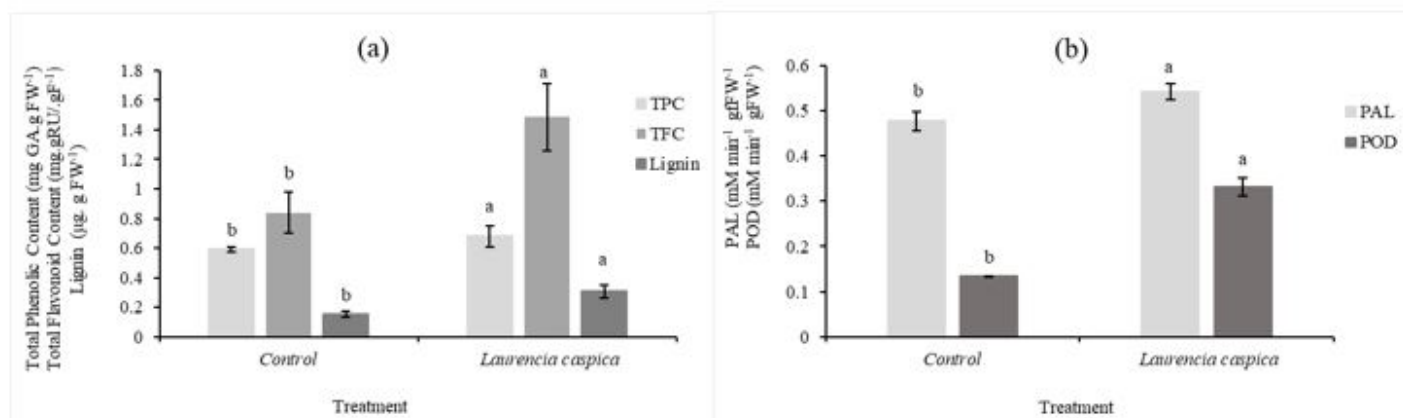


Figure 5

Variation of the PAL and POD enzymes activity (b), total phenolic (TPC), flavonoid and lignin contents (TFC) (a) under SLE treatment with 2.5% concentration in thyme plants. (Means $\pm$ SE, n = 3). ^a Significant at the 0.05 level.