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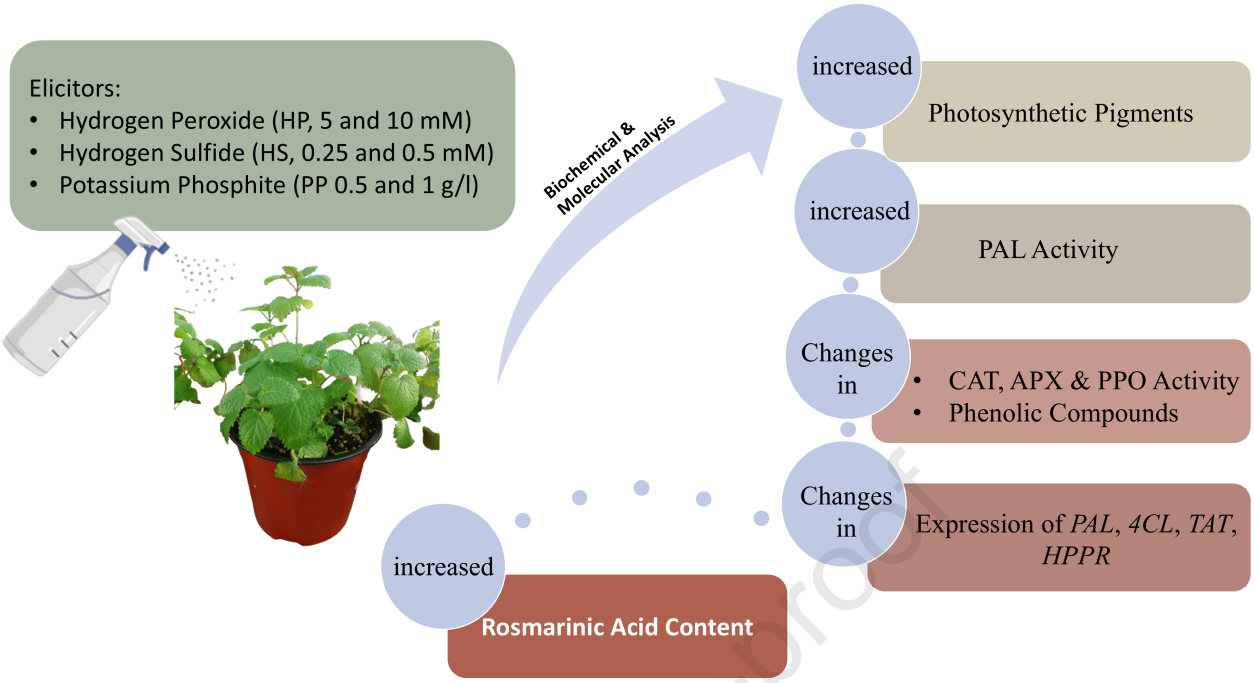

Exogenous Application of Hydrogen Peroxide, Hydrogen Sulfide, and Potassium Phosphite Enhances Rosmarinic Acid Accumulation and Modulates Key Biosynthetic Gene Expression in *Melissa officinalis* L.

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Abstract

Melissa officinalis L. (lemon balm) is a well-known medicinal plant traditionally valued for its pharmacological properties. Its aerial parts are rich in bioactive compounds such as rosmarinic acid (RA), which play key roles in the plant defense system and contribute to its antispasmodic, sedative, and memory-enhancing activities. However, the natural concentrations of these secondary metabolites are typically low in medicinal plants. This study investigated the influence of exogenous elicitors, hydrogen peroxide (HP, 5 and 10 mM), hydrogen sulfide (HS, 0.25 and 0.5 mM), and potassium phosphite (PP, 0.5 and 1 g/L) on the accumulation of photosynthetic pigments, antioxidant enzyme activity, phenolic content, RA production, and the expression of RA biosynthesis-related genes. All elicitors significantly increased chlorophyll *a*, *b*, and carotenoid levels. Treatment with HP and HS notably elevated total phenolic and flavonoid contents. The activity of CAT and APX enzymes varied depending on the elicitor type and concentration applied. Elicitation enhanced RA accumulation by enhancing PAL activity and modulating the expression of key biosynthetic genes (*PAL*, *4CL*, *TAT*, and *HPPR*). HS treatment upregulated genes in both phenylalanine and tyrosine pathways, whereas HP was more effective in stimulating the tyrosine pathway. PP elicitation led to differential gene expression depending on its concentration. These results demonstrate that HP, HS, and PP can modulate key metabolic and signaling pathways, thereby boosting RA production and potentially enhancing the medicinal value of *M. officinalis* in a safe and environmentally friendly manner.

Keywords: *Lemon balm, Secondary metabolites, Elicitor, Foliar application*.

1. Introduction

Melissa officinalis L., a perennial herb belonging to the Lamiaceae family, is native to Western Asia and the Mediterranean region (Shakeri et al., 2016). Traditionally used for various medicinal purposes, it serves as a tonic, antispasmodic, carminative, and sedative, while also being recognized for enhancing memory and alleviating stress-induced headaches (Blumenthal et al., 2000). The leaves of *M. officinalis* contain numerous bioactive compounds, including flavonoids, phenolic acids such as rosmarinic acid (RA), monoterpene glycosides, triterpenes, sesquiterpenes, tannins, and essential oils (Petrisor et al., 2022). RA, a water-soluble phenolic compound, is synthesized through two parallel biosynthetic pathways, the phenylalanine and tyrosine pathways (Fig. 1). In plants, RA acts as a secondary metabolite involved in defense mechanisms, while in humans, it demonstrates antiviral, antibacterial, antidiabetic, antioxidant, antimutagenic, and anti-inflammatory properties (Elufioye and Habtemariam, 2019; Ngo et al., 2018; Trócsányi et al., 2020).

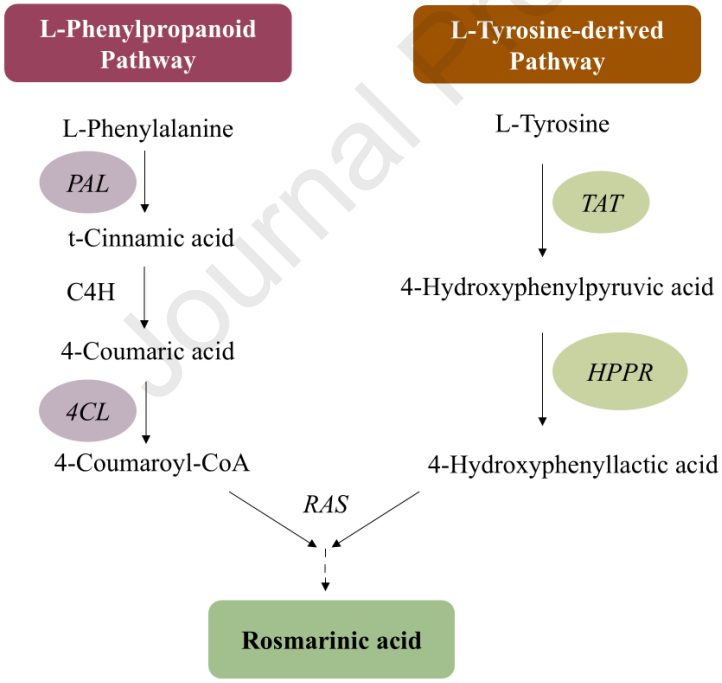

Fig. 1. Rosmarinic acid biosynthesis pathways: L-Phenylalanine and L-Tyrosine. *PAL*: phenylalanine ammonialyase, *C4H*: cinnamic acid 4-hydroxylase, *4CL*: 4-coumarate-CoA ligase, *TAT*: tyrosine aminotransferase, *HPPR*: 4-hydroxyphenylpyruvic acid, *RAS*: rosmarinic acid synthase (hydroxycinnamoyl-*CoA*: hydroxyphenyllactate hydroxycinnamoyl transferase) (Trócsányi et al., 2020).

The accumulation of secondary metabolites in medicinal plants is generally low, often constituting less than 1% of dry weight, and is influenced by physiological and developmental conditions (Oksman-Caldentey and Inzé, 2004). Environmental stresses or elicitor application can stimulate phenolic compounds production in Lamiaceae (Carović-Stanko et al., 2016). Studies indicated that using the elicitor influenced the rosmarinic acid. Application of methyl jasmonate significantly influenced the accumulation of RA, phenolic compounds, and flavonoids in *M. officinalis*, likely due to gene activation from the phenylpropanoid pathway (Kianersi et al., 2022). Chitosan application led to the production of RA and triggered the defense-related enzymes, up-regulating the expression of *TAT* and *RAS* genes in lemon balm (Fooladi Vanda et al., 2019). Therefore, applying external stimuli is an effective strategy to enhance bioactive compound synthesis and improve the medicinal and nutritional value of these plants (Trivellini et al., 2016). Elicitors initiate a series of responses, including the production of reactive oxygen species (ROS), the activation of defense genes, and the accumulation of secondary metabolites. ROS are byproducts of plant metabolism that serve as signaling molecules within plants. In plants, enzymatic compounds like ascorbate peroxidase (APX) and catalase (CAT), as well as non-enzymatic compounds such as phenolics, flavonoids, and carotenoids, protect cells from the harmful effects of ROS (Ali et al., 2023; Nyanasaigran et al., 2024; Woch et al., 2023). It is important to choose appropriate elicitors with consideration for safety, cost-effectiveness, and environmental sustainability.

Hydrogen peroxide (HP), a natural and safe substance, is a signaling molecule involved in plant defense responses under stress conditions. It can diffuse freely across membranes and exhibits a relatively long lifespan, making it crucial for stress signal transduction (Belchí-Navarro et al., 2019; Qaiser et al., 2024). For example, foliar application of HP to *Amaranthus hypochondriacus* increased antioxidant capacity, total phenolic compounds, condensed tannins, and anthocyanins (Ferriz-Martínez et al., 2023). Hydrogen sulfide (HS), a natural substance that is generated internally within cells, is another emerging signaling molecule in higher plants. It regulates photosynthesis, stomatal closure, seed germination, root development, and abiotic stress tolerance (Corpas and Palma, 2020; Fotopoulos et al., 2015). Exogenous HS application in grape (*Vitis vinifera* L.) activates secondary metabolite biosynthesis genes, enhancing plant development and abiotic stress resistance (Ma and Yang, 2018). Phosphite, as a bio-stimulant, triggers hormesis in plants, a phenomenon where low doses of an environmental agent stimulate plant growth (Mattson, 2008). Potassium phosphite and chitosan have been shown to enhance Cucurbitacin E production and antibacterial properties in cucumber plants (Ramezani et al., 2017).

Applying hydrogen peroxide, hydrogen sulfide, and potassium phosphite as elicitors is expected to enhance specialized metabolite production in *Melissa officinalis* by acting as signaling stimulants and affecting different biochemical pathways. These treatments may

increase phenolic compounds, especially rosmarinic acid, and alter the expression of key genes in the biosynthesis pathway. This study aimed to investigate and compare the effects of three elicitors, including hydrogen peroxide, hydrogen sulfide, and potassium phosphite, on both primary and secondary metabolic processes in *M. officinalis* L. Their effects on photosynthetic pigments, antioxidant enzymes, and phenolic compounds, which may serve as a shared biological framework for activating RA biosynthetic pathways, were analyzed. Additionally, the expression patterns of key RA biosynthesis-related genes, *PAL*, *4CL*, *TAT*, and *HPPR*, under different elicitor treatments to identify transcriptional regulatory mechanisms driving RA accumulation were assessed.

2. Materials and Methods

2.1. Plant materials

The experiment was conducted in a research glass greenhouse at the Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari, Iran (36°33′47″ N and 53°03′36″ E). The temperature ranged from ±19 °C at night to ±25 °C during the day, with an average humidity of 56%. In the greenhouse, the photoperiod was set to 16 hours of light and 8 hours of darkness. Seeds of *M. officinalis* were soaked in water for 24 h, kept in moist wipes for 72 h, and then planted in pots (12.5 * 15.5 mm) containing an equal substrate mixture of cocopeat, peat moss, and perlite. After three weeks, the plantlets were thinned, leaving five morphologically similar plants per pot. Hoagland's nutrient solution was used weekly to meet the plants' nutritional needs.

2.2. Elicitor treatment

Hydrogen peroxide (HP) (Sigma-Aldrich, USA) at concentrations of 5 and 10 mM, sodium hydrosulfide (NaHS) (Sigma-Aldrich, USA) as an HS donor at 0.25 and 0.5 mM, and potassium phosphite (PP) at 0.5 and 1 g/L were applied. To prepare PP stocks, phosphorous acid (AppliChem, Germany) was partially neutralized with KOH by gradually mixing and pH adjusted to 6.3 (Mofidnakhaei et al., 2016). Foliar treatments were applied to plants in the 6-8 leaf stage using a hand-held sprayer equipped with a fine mist nozzle. Freshly prepared treatment solutions were applied twice weekly, at a distance of approximately 25- 30 cm from the plant, ensuring even wetting of both sides of the leaf surfaces. Plants were sprayed until dripping (~20 ml/plant), and the soil surface was covered with a plastic sheet to avoid unintended uptake by roots. Plants were sampled seven days post-treatment for phytochemical analysis and stored at -80 °C (Hawrylak-Nowak et al., 2021).

2.3. Photosynthetic pigments

Briefly, 200 mg of fresh leaves was combined with 1800 µl of pure methanol. The samples were stored in darkness for 24 h before centrifugation. The absorbance of the

supernatant was recorded at 665, 652, and 470 nm using a WPA Biowave II UV-Vis spectrophotometer (Cambridge, UK). Chlorophyll (Chl) *a*, *b*, and carotenoid concentrations were determined using the formulas provided by Lichtenthaler and Buschmann (2001).

Chlorophyll $a = 16.72*A_{665} - 9.16*A_{652}$

Chlorophyll $b = 34.09*A_{652} - 15.28*A_{665}$

Carotenoid = $(1000* A_{470} - 1.63*\text{Chlorophyll } a - 104.96*\text{Chlorophyll } b)/221$

2.4. Total phenolic compounds

For the extract preparation, 100 mg of fresh leaf was mixed with 1800 µl of pure ethanol and centrifuged at 10,000× g for 10 min. The upper phase (ethanol extract) was placed on a shaker for 24 h to estimate the total phenolic compounds and flavonoids. Total phenolic content was determined using the Folin-Ciocalteu reagent following the method described by Meda et al. (2005). A mixture of 2.8 ml deionized water and 2 ml of 2% sodium carbonate was added to the extract, followed by 50% Folin-Ciocalteu reagent. After 40 min incubation at RT, absorbance was measured at 760 nm, and the amount of phenolic content was expressed in terms of gallic acid equivalent (mg Eq of GAE g^{-1} FW extract). The flavonoid content in the extracts was determined using the aluminum chloride colorimetric method described by Chang et al. (2020). A mixture of 100 µl of 10% aluminum chloride and 1.5 ml of 70% ethanol was added to extract and incubated for 1 h at RT. The absorbance was measured at 415 nm, and the concentration was expressed in terms of quercetin equivalent (mg Eq of QE g^{-1} FW extract).

2.5. Measurement of total protein and preparation of enzyme extract

Fresh leaves (100 mg) were mixed with 1800 µL of cold potassium phosphate buffer (pH 7.5) and centrifuged at 12,000×g at 4°C. The supernatant was collected and stored at -20 °C for subsequent analysis. Total protein content was measured at 595 nm with Bradford's reagent, and bovine serum albumin (BSA) was used as the standard (Bradford, 1976).

2.6. Determination of enzyme activities

Catalase (CAT, EC 1.11.1.6) enzyme activity was determined using the method of Aebi (1984) by monitoring the decrease in absorbance at 240 nm. The reaction mixture contained 2.45 mM phosphate buffer (pH 7), 7.5 mM H_2O_2, and 50 µL of enzyme extract. CAT enzyme activity was calculated using the extinction coefficient of 36.6 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX, EC 1.11.1.11) enzyme activity was measured according to Nakano and Asada (1987) by monitoring the absorbance change at 290 nm. The reaction mixture consisted of potassium phosphate buffer (pH 7), 1 mM ascorbate, 0.1 mM EDTA,

1 mM hydrogen peroxide, and 50 µl of enzyme extract. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was applied for quantification.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) enzyme activity was determined as described by Goldson et al. (2007) through the measurement of cinnamic acid production. The reaction comprised 100 µL of enzyme extract, 500 mM Tris-HCl buffer (pH 7), and 10 mM phenylalanine, incubated at 37 °C for 1 h. The reaction was terminated with 4N HCl and toluene. Enzyme activity was determined at 290 nm using a spectrophotometer and calculated using a standard trans-cinnamic acid curve.

Polyphenol oxidase (PPO, EC 1.10.3.1) enzyme activity was assayed using the method of Mayer et al. (1966). The reaction mixture, totaling 1.5 ml, consisted of 100 mM sodium phosphate buffer (pH 6.5), 200 µl of enzyme extract, and 100 mM catechol. Absorbance was measured at 495 nm, with activity calculated using an extinction coefficient of 35.7 mM^{-1} cm^{-1}.

2.7. HPLC analysis

HPLC was used to analyze rosmarinic acid content in dried ground *M. officinalis* samples harvested on days 1 and 7 following treatment. The quantity of RA in the methanolic extracts was quantified using a high-performance liquid chromatography (HPLC) system, model 1260 Infinity II LC System (Agilent, US), featuring a Zorbax Eclipse XDB-C18 column (150×4.6 mm, Netherlands). The mobile phase comprised two solutions: (A) 0.1% phosphoric acid in 10% methanol and (B) 0.1% formic acid in methanol. Separation was performed using a gradient of 67% phase A and 33% phase B over 40 min. Detection occurred at 330 nm, as Koca and Karaman (Koca and Karaman, 2015) outlined. The standard curve for rosmarinic acid was generated using concentrations of 0.5, 0.3, 0.2, 0.1, 0.01, and 0.001 mg/mL. The linear calibration model was applied, resulting in R^2>0.99. Fig. 2 shows the HPLC diagram of the rosmarinic acid standard.

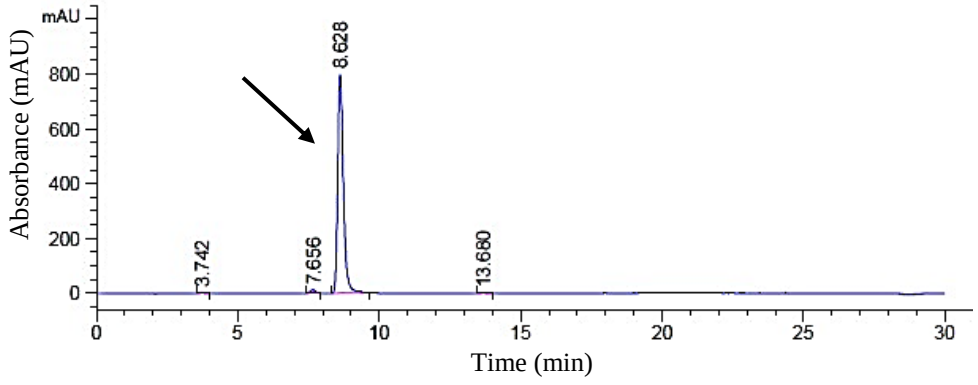

Fig. 2. Chromatogram of 0.2 mg/ml standard rosmarinic acid injection. The arrow points to this peak.

2.8. qRT-PCR assay

Leaf samples were harvested 24, 48, and 72 h post-treatment for gene expression analysis. Total RNA was isolated using TRIzol reagent (Riragene, Iran), followed by DNaseI (Thermo Fisher Scientific, US) treatment to eliminate gDNA contamination, according to the manufacturer's instructions for both steps. The cDNA was synthesized using the SinaClon First Strand cDNA Synthesis Kit (SinaClon, Iran). Gene-specific *PAL, 4CL, TAT, and HPPR* primers were designed using Primer3 software. Primer specificity was validated with the Primer-BLAST tool (Coulouris et al., 2012). The *ß-Actin* gene was used as the internal reference. The primer sequences are shown in Table 1.

Reactions were performed in a 12 µL volume containing 6 µL SYBR Green Master Mix (Ampliqon, Denmark), 15 µM of each primer, 2 µL diluted cDNA template, and nuclease-free water. Amplification was carried out on an ABI StepOne Plus Real-Time PCR system. The thermal cycle program was performed under the following conditions: pre-denaturation at 95 °C for 8 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1. Sequences of primers used in this study

Gene	Accession number	Primer pair	(5′–3′)
PAL	FN665700.1	Forward	CGCTCAATTCTCCGAACTCGTC
		Reverse	AGTAGGAAGCCATGGCGATTTC
4CL	FN665699.1	Forward	AGACGATCATGCTCTTGCTCCC
		Reverse	GGCCTTGGCTTGCTTGATTACC
TAT	JN863949.1	Forward	GATCTCCCCTACAAGCTACCAGC
		Reverse	CCTTCTCGGGATGAAGATCGAAG
HPPR	MW118284.1	Forward	GCGGATTTGTGAGTGCGATAAG
		Reverse	CGATTCTGCCCAATCCTATGATGC
ß-Actin	DQ423374.1	Forward	TGTATGTTGCCATCCAGGCCG
		Reverse	AGCATGGGGAAGCGCATAACC

2.9. Statistical analysis

The experiment was structured in a factorial design arranged as a completely randomized design (CRD). Factors were elicitors and the concentrations used. Each treatment included three replicates, with each replication consisting of a single pot containing five plants. All data were subjected to two-way analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC). Before ANOVA, data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests,

respectively. Means were compared using the least significant differences (LSD) test (a=0.05) to determine significant differences between treatments.

3. Results

3.1. Elicitor efficacy and plant safety

All elicitor doses were specifically selected to function as signaling stimulants, which ensured that no phytotoxic effects occurred. No signs of phytotoxicity, such as leaf necrosis, chlorosis, or stunted growth, were noted during the study (data not shown). This indicates that the chosen concentrations successfully achieved a balance between elicitation effectiveness and the safety of plant health.

3.2. The content of Photosynthetic pigments

Exogenous elicitor treatments significantly increased photosynthetic pigment levels in *M. officinalis* leaves (Fig. 3). No significant difference between PP concentrations in the alteration of Chl *a* or Chl *b* levels of treated plants was observed. In contrast, the effect of HS treatment was pigment-specific; 0.5 mM HS significantly increased Chl *b* content, whereas no statistically significant difference was observed in the Chl *a* concentration between the two HS concentrations. HP at 10 mM induced a significant decline in both Chl *a* and Chl *b* content compared to the 5 mM dose, likely attributable to oxidative stress-driven chlorophyll degradation. All treatments significantly enhanced the accumulation of carotenoids in *M. officinalis*; however, no significant differences were observed between the concentrations of elicitors used.

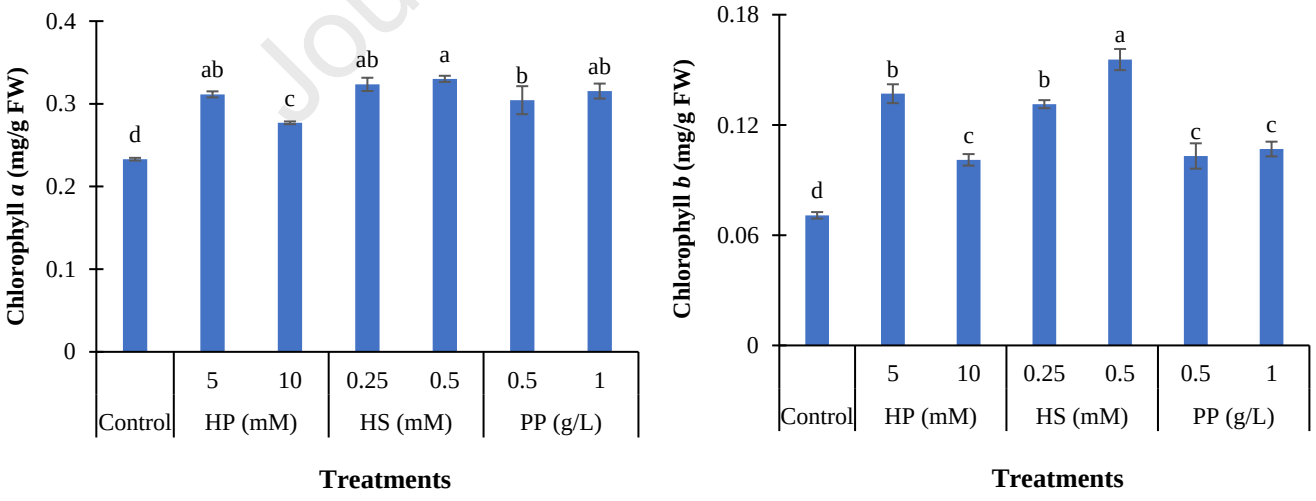

Fig. 3. Effect of different treatments and concentrations (HP: 5 and 10 mM, HS: 0.25 and 0.5 mM, PP: 0.5 and 1 g/L) on *M. officinalis* photosynthetic pigments, chlorophyll *a* and chlorophyll *b*. Data are shown as the mean of three replicates with error bars indicating ±SD (n = 3). Different letters indicate significant differences at $P \leq 0.05$ based on the LSD test.

3.3. Total phenolic and flavonoid content

The total phenolic content in *M. officinalis* leaves varied depending on the elicitor type and concentration (Fig. 4). Exogenous HP (5 and 10 mM) elicited a robust increase of phenolic compounds in treated plants compared to the control. Notably, the 5 mM HP treatment showed the highest phenolic levels among all elicitor treatments. Similarly, HS-treated plants exhibited significantly elevated phenolic content relative to the control. In contrast, the effect of PP on the content of phenolic compounds was different. At 0.5 g/L PP, the treatment effect was not significant, while at 1 g/L, a significant decrease in phenolic compounds was measured compared to the control.

A comparable trend was observed for flavonoid content. The 5 mM HP treatment yielded the highest flavonoid levels, significantly exceeding those of the control and other treatments. HS treatment at all concentrations also enhanced flavonoid content compared to untreated plants. However, at 0.5 g/L PP impact on flavonoid levels was not significant, while the 1 g/L PP led to a notable decrease in flavonoid content relative to the control (Fig. 4).

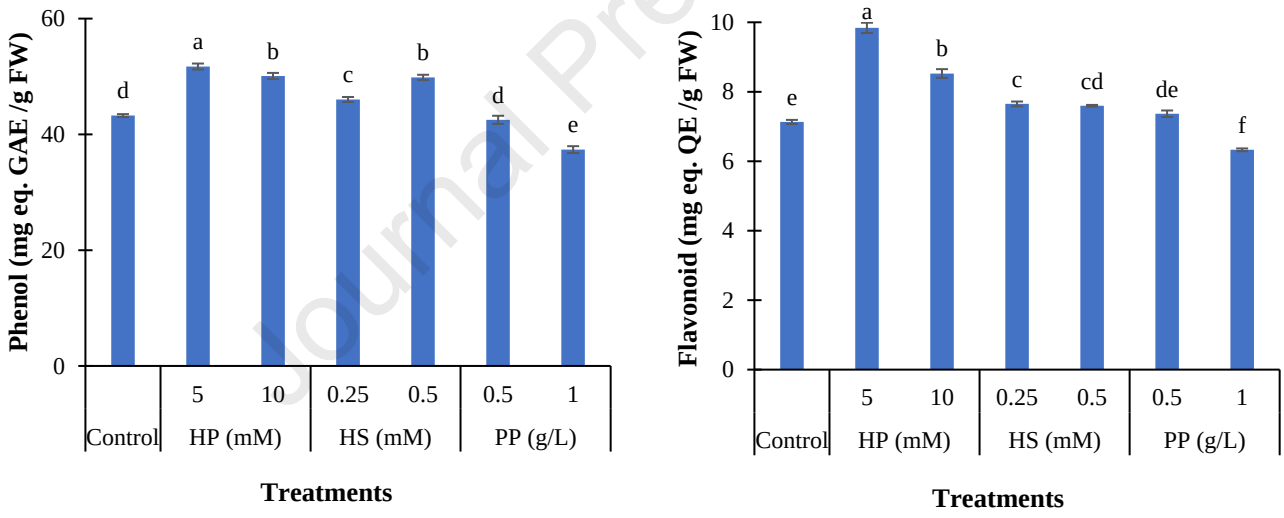

Fig. 4. Effect of different treatments and concentrations (HP: 5 and 10 mM, HS: 0.25 and 0.5 mM, PP: 0.5 and 1 g/L) on total phenolic and flavonoid contents of *M. officinalis*. Data are shown as the mean of three replicates with error bars indicating ± SD (n = 3). Different letters indicate significant differences at $P \leq 0.05$ based on the LSD test.

3.4. Enzyme activities

Elicitor treatments differentially modulated antioxidant enzyme activity in *M. officinalis* (Fig. 5). Both HP and HS significantly elevated APX activity at all tested concentrations relative to the control, with the highest increases observed at 5 mM HP and 0.25 mM HS. Notably, lower concentrations of these elicitors consistently induced higher APX activity than their higher-dose counterparts. On the other hand, PP treatments

exhibited no significant effects on APX activity compared to control (Fig. 5A). For catalase (CAT), only 0.5 mM HS elicited a significant activity boost, whereas PP treatments (0.5 and 1 g/L) decreased CAT enzyme activity below the control (Fig. 5B). PAL activity generally increased across all elicitor types and concentrations in comparison with control plants. The most marked enhancement occurred at 0.25 mM HS; the difference among other treatments was not significant (Fig. 5C). PPO responses varied by treatment; 1 g/L PP triggered significant PPO enzyme activity, while 0.5 g/L PP showed no effect. HP treatments moderately increased PPO activity at both concentrations, whereas 0.25 mM HS decreased PPO below the control (Fig. 5D).

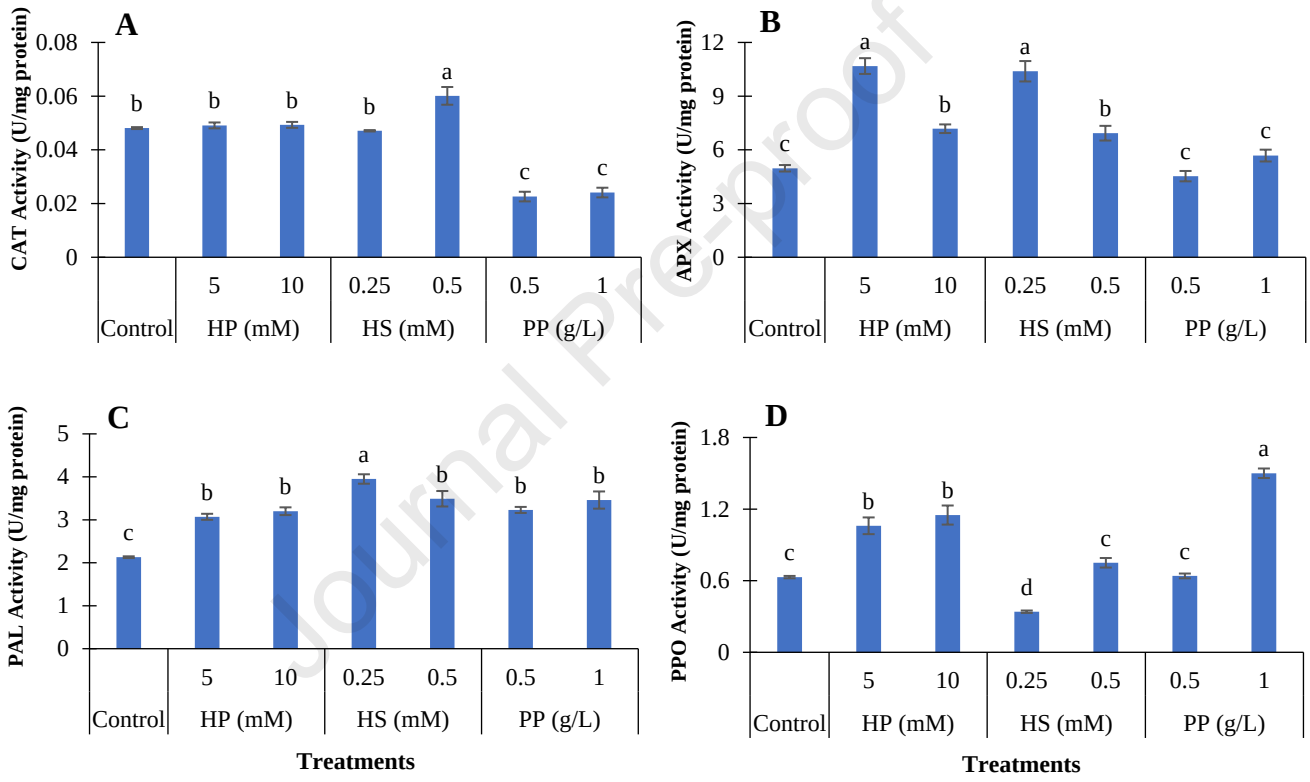

Fig. 5. Effect of different treatments and concentrations (HP: 5 and 10 mM, HS: 0.25 and 0.5 mM, PP: 0.5 and 1 g/L) on *M. officinalis* antioxidant enzyme activity: APX (A), CAT (B), PAL (C), and PPO (D). Data are shown as the mean of three replicates with error bars indicating ± SD (n = 3). Different letters indicate significant differences at $P \leq 0.05$ based on the LSD test.

3.5. Rosmarinic acid content

Fig. 6 displays the chromatogram of the sample. HPLC analysis revealed that elicitation significantly influenced RA accumulation in *M. officinalis*, with variations depending on the treatment and sampling time (Fig. 7). At 24 hours post-elicitation, RA levels in plants treated with HP (5 and 10 mM) and HS (0.25 and 0.5 mM) showed no significant differences between applied elicitor concentrations. However, the HS-treated plants

exhibited higher RA content than the HP-treated counterparts. In contrast, PP treatment at 1g/L induced a significant elevation in RA concentration relative to the 0.5 g/L PP treatment. By the One-week post-treatment, RA levels in HP-treated plants remained unchanged. However, HS treatments elicited time-dependent fluctuations, at 0.25mM a significant decline and at 0.5 mM a marked increase (2.77-fold higher than control) in RA content compared to initial sampling (1st day) was measured. Similarly, PP treatments demonstrated concentration-specific effects; 0.5 g/L PP significantly enhanced RA accumulation, while 1 g/L reduced it relative to the first sampling time point (1st day).

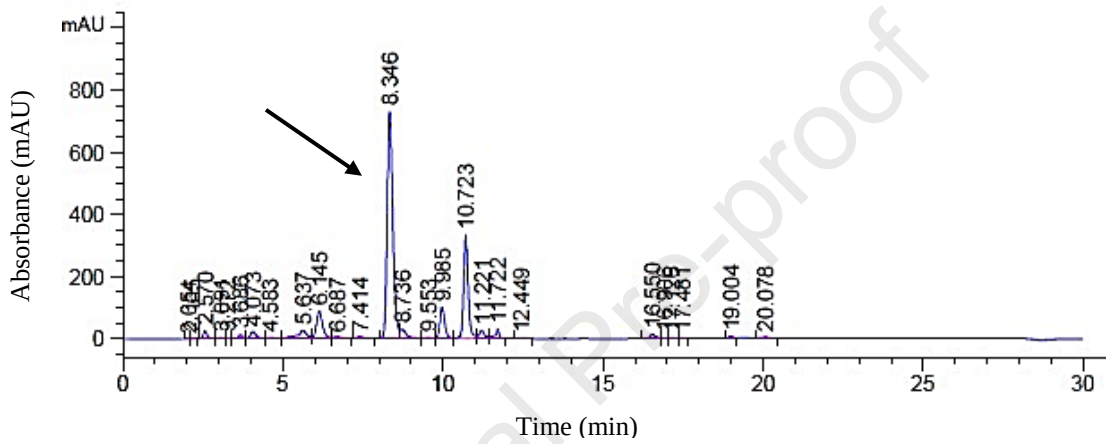

Fig. 6. Chromatographic diagram of sample injection. The arrow symbol shows the presence of rosmarinic acid.

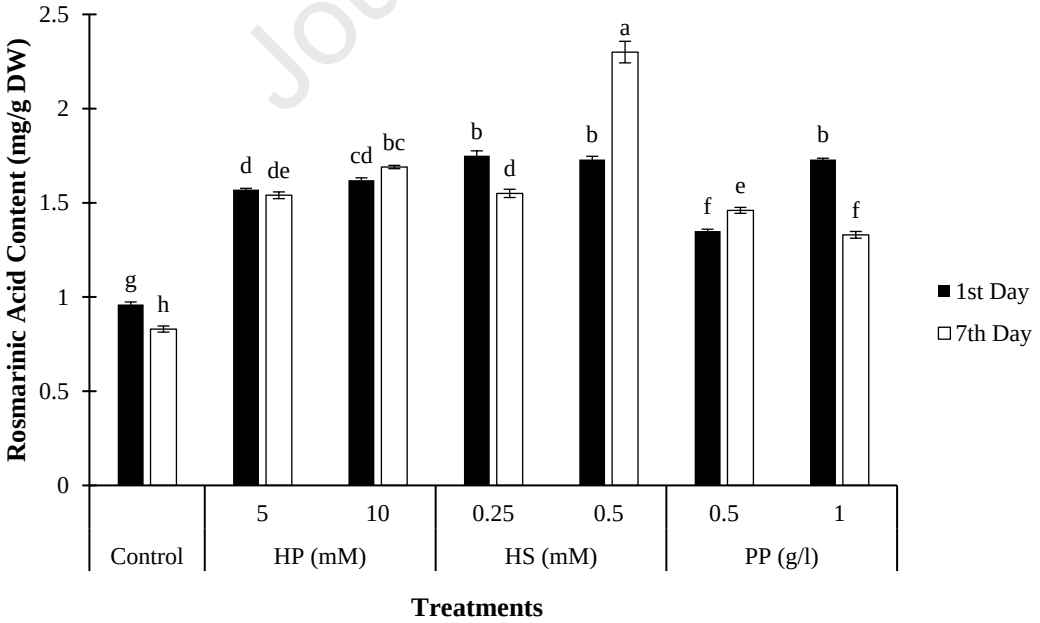

Fig. 7. Effect of different treatments, concentrations (HP: 5 and 10 mM, HS: 0.25 and 0.5 mM, PP: 0.5 and 1 g/L), and days after treatment (1st and 7th) on rosmarinic acid content. Data are shown the mean of three replicates with error bars indicating ± SD (n = 3). Different letters indicate significant differences at $P \leq 0.05$ based on LSD test.

3.6. Expression of genes involved in RA biosynthesis pathway

The results indicated that hydrogen peroxide, hydrogen sulfide, and potassium phosphite elicitors had different effects on the expression of genes in the phenylalanine and tyrosine biosynthetic pathways examined in the *M. officinalis* plants (Fig. 8). In phenylpropanoid pathway, by application of HP in both concentrations, *PAL* and *4CL* showed a significant reduction in expression at 24 and 48 h, while at 72 h, *PAL* returned to control expression in contrast to *4CL*. HS at 72 h showed a notable increase in *PAL* by 6.9 and 5.8 fold at 0.25 and 0.5 mM, respectively. In addition, HS-treated plants showed a significant increase in *4CL* by 32.7 and 7.5 fold at 0.25 and 0.5 mM, respectively. Significant reduction in *PAL* was shown in PP treatments in all time courses of 0.5 g/L, 24 and 72 h of 1 g/L. There was a notable increase of 0.5 g/L PP application in *4CL* by about 3 and 2 fold at 24 and 48 h, as well as 1.97 and 7.1 fold at 48 and 72 h in 1g/L of PP.

TAT and *HPPR* have the same trend in HP- and HS-treated plants, after a significant reduction in 24 and 48 h, a notable increase was observed in 72 h. Among these, 19.5 and 10.7 fold were notable, which were observed in HS 0.25 and 0.5, respectively. PP-treated Plants showed a different trend in *TAT* and *HPPR*. There was a significant increase in *TAT* 24 h of PP at 0.5 g/L, followed by a decrease at 48 and 72 h. However, at 1 g/L of PP, after reduction in 24 and 48 h, a significant increase was found in 72 h.

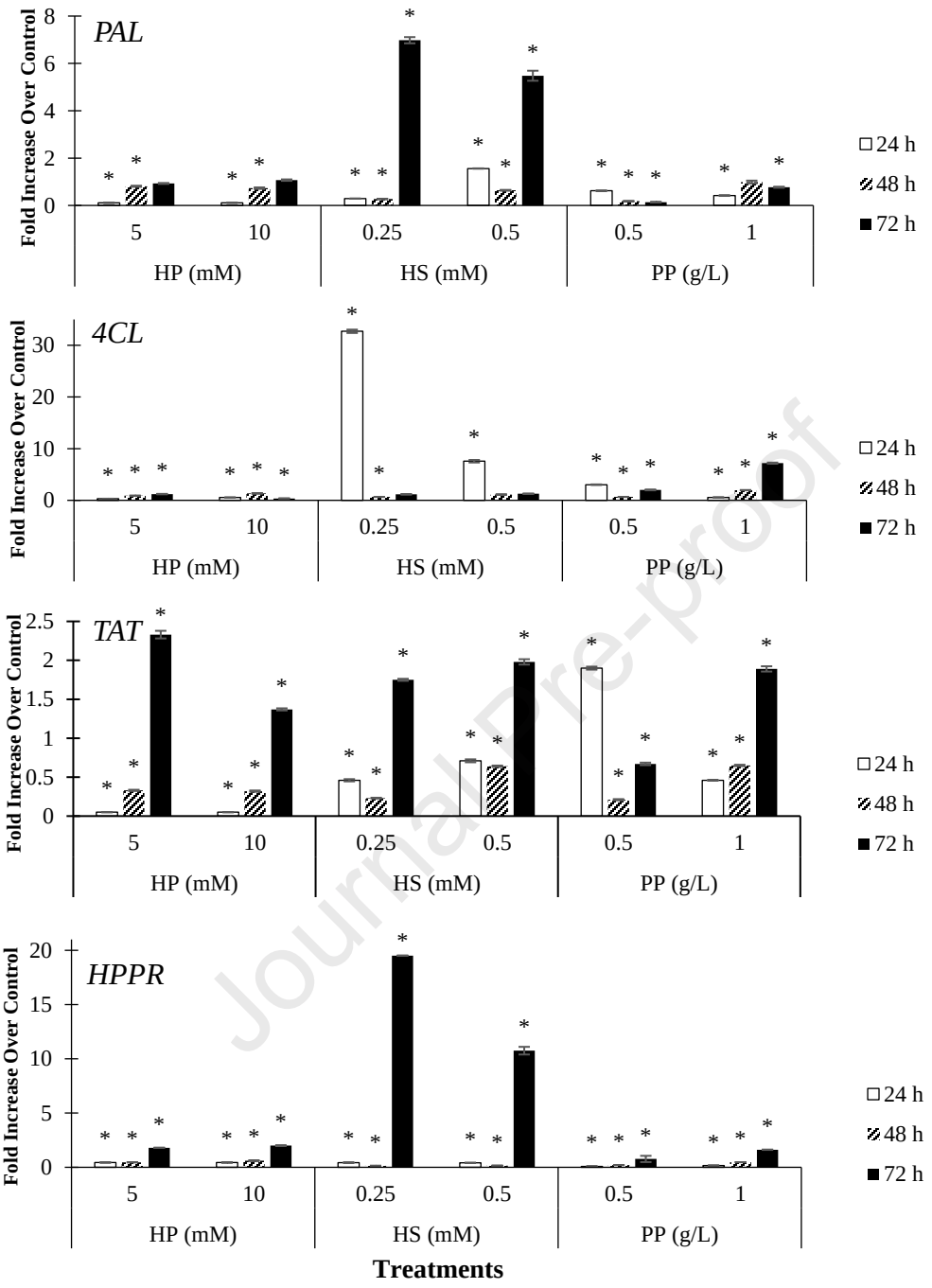

Fig. 8. Effect of different treatments and concentrations (HP: 5 and 10 mM, HS: 0.25 and 0.5 mM, PP: 0.5 and 1 g/L) on genes expression of *PAL* and *4CL* from phenylpropanoid, *TAT* and *HPPR* from tyrosine-derived pathway at 24 h, 48 h, and 72 h after treatments; the star (*) at the top of the graphs indicates the significance of the results at the 5% level, data represent mean ± SD (n=3).

4. Discussion

In this study, different concentrations of hydrogen peroxide (HP), hydrogen sulfide (HS), and potassium phosphite (PP) were applied by foliar spraying to evaluate their effects

on the biochemical properties of *M. officinalis*, including rosmarinic acid (RA) content and the expression of genes involved in the RA biosynthesis pathway.

The results indicate that photosynthetic pigments significantly increased in elicitor-treated plants, as HP and HS showed the highest effects on chlorophyll content (Fig. 3). Jamaludin et al. (Jamaludin et al., 2020) highlighted HP's role as a signaling molecule that regulates photosynthesis, stomatal movement, and biochemical processes during plant growth. Other studies have also demonstrated that HP improves stomatal size and conductance, gas exchange, as well as net photosynthetic rates (Gondim et al., 2013; Konstantinos et al., 2010). Fariduddin et al. (2014) found that HP treatment improves reactive oxygen species (ROS) scavenging activity and the recovery of photosynthetic efficiency in *Vigna radiata* under copper stress. It appears that HP treatment plays a role in protecting the photosynthetic apparatus from ROS activity. Carotenoids are known to protect cells from ROS (Mittler et al., 2004), and HP-induced oxidative stress can stimulate carotenoid biosynthesis, as observed in *Haloferax mediterranei* (Giani and Martínez-Espinos, 2020). Studies showed that sodium hydrosulfide (NaHS), as an HS donor, enhances endogenous HS signaling and photosynthetic pigment content in tobacco (Dai et al., 2024). HS has been shown to enhance photosynthesis by increasing chlorophyll content in both higher and lower plants, including algae (Chen et al., 2011; Joshi et al., 2020; Liu et al., 2020; Parveen et al., 2017). Phosphite is recognized as a biostimulant that enhances growth, yield, and stress tolerance in crops (Gómez-Merino et al., 2022). Studies have shown that PP application increases chlorophyll content and photosynthesis under UV-B and infection stress (Mohammadi et al., 2020; Oyarburo et al., 2015).

Elicitors trigger responses like generating reactive oxygen species (ROS), turning on defense genes, and building up secondary metabolites. Plants regulate ROS levels by activating antioxidant enzymes like APX and CAT, and increasing non-enzymatic antioxidants such as carotenoids and phenolic compounds (Hossain et al., 2015). In this study, CAT activity in HP-treated plants remained unchanged compared to the control. In contrast, APX activity increased significantly, particularly at lower HP concentrations (Fig. 5A). Exogenous HP significantly increased H_2O_2 generation in a dose-dependent manner (Mejía-Teniente et al., 2013). It is suggested that CAT is more active at high H_2O_2 concentrations than APX (Palma et al., 2020). There are also other reports that CAT typically responds to HP in advance in shorter times, whereas APX responds later, with notable activity at 240 h after elicitor application (Gondim et al., 2012). HP-induced APX activity is often linked to chloroplasts, where APX isoforms are located (Barzotto et al., 2024). In tomato plants, HP increased APX activity in roots under normal and cold stress conditions, while CAT levels remained stable (İşeri et al., 2013). Similarly, HP pre-treatment enhanced APX and POD activities in *B. napus* L. under Cr stress, without affecting CAT or SOD activity (Yıldız et al., 2013). APX activity showed a similar trend with the accumulation patterns of phenolic compounds and flavonoids.

HS treatment also significantly increased APX activity, with higher HS concentrations boosting CAT activity. NaHS application stimulates endogenous HS production, reducing ROS levels by enhancing antioxidant enzyme activities (Giani and Martínez-Espinos, 2020). The HS pathway interacts with H_2O_2 and nitric oxide signaling, further activating antioxidant enzymes like CAT, SOD, and APX (Aroca et al., 2018; Lisjak et al., 2011; Zhang et al., 2010). Previous studies showed that HS-treated plants exhibit increased APX, CAT, and other antioxidant enzyme activity under different stress conditions (Valivand and Amooaghaie, 2021; Wang et al., 2011; Zhang et al., 2009). Also, Exogenous HP significantly increased antioxidant enzyme activities in Ginkgo leaves during the ROS burst (Fan et al., 2024). Based on the concentration of HS used, the increases in PAL and APX exhibited a similar trend, as did the increases in CAT, phenolic compounds, and RA content.

PP treatment had different behaviors based on the concentration used. At higher PP concentrations, increased PPO activity (Fig. 5D) led to a decrease in phenol and flavonoid levels (Fig. 4), since these compounds are known substrates for PPO, although enzymes display specific substrate selectivity during catalysis (Boeckx et al., 2015; Zou et al., 2025). CAT activity is also decreased, but the RA content increased (Fig. 7), which is linked to high PAL activity. APX also remained unchanged. At lower PP concentration, significant differences were observed not in phenol and flavonoid, nor in PPO and APX activity. However, increasing PAL activity and RA content suggested that PP promoted phenolic acid synthesis in *M. officinalis*. It was found that PP treatment of soybean plants in water deficit stress did not significantly change CAT and APX activity (Batista et al., 2023).

HS, HP, and PP treatments showed a positive correlation between PAL activity and RA accumulation observed in *Melissa officinalis* leaves. In HP and HS treatment, this correlation is associated with phenolic compounds and flavonoids. Although PAL enzyme is not part of the plant's antioxidant system, its produced compounds greatly enhance the plant’s overall antioxidant capacity. PAL is a crucial biosynthetic enzyme that initiates the production of phenolics, which are primarily involved in defense mechanisms. (Jakovljević et al., 2025; Paul et al., 2023). In similar studies, induction of PAL activation and RA biosynthesis in *M. officinalis* treated plants has been reported (Fooladi Vanda et al., 2019; Mousavi and Shabani, 2019; Ulgen et al., 2021). Other studies in Lamiaceae, such as *Dracocephalum kotschyi* and *Salvia leriifolia,* showed increased PAL activity, leading to higher production of secondary metabolites, especially RA content (Rastgoo et al., 2025; Vafadar et al, 2020). So, it is suggested that PAL activity directly influences RA synthesis.

Gene expression analysis revealed that HP treatment induced the expression of *TAT* and *HPPR* genes at 72 h post-treatment, while *4CL* expression varied with concentration and timing (Fig. 8). According to these results, by increasing RA content (Fig. 7), it is suggested that RA production is more closely linked to the tyrosine pathway than to the phenylalanine

pathway in HP-treated plants. The observations align with the results of Kwon et al. (2021), indicating that tyrosine aminotransferase showed a higher transcript level compared to the phenylpropanoid pathway for producing RA in *Ocimum basilicum*, regardless of whether the plants were under dark or light conditions. HP signaling regulates plant physiological processes and interacts with other signaling molecules to co-regulate antioxidant systems and defense-related gene expression (Niu and Liao, 2016). HS treatment triggered *PAL*, *TAT*, and *HPPR* expression at 72 h, along with *4CL* at 24 h. The notable increases in *PAL* at 72 h and *4CL* at 24 h (Fig. 8) were associated with higher RA levels suggesting that activating the phenylalanine biosynthesis pathway could boost RA production (Fig. 7). Moreover, in the tyrosine biosynthesis pathway, the upregulation of *TAT* and *HPPR* at 72 h after treatment was linked to RA content production. Additionally, these findings are associated with the total phenol and flavonoid content. It is consistent with previous research that indicated chitosan treatment promoted the expression of *PAL* and *TAT* genes, resulting in the accumulation of RA and phenolic compounds in lemon balm shoots (Fooladi Vanda et al., 2019). The positive correlation between *4CL* and *PAL*, as well as *TAT* and *HPPR* expression, with RA accumulation under elicitor treatment, also aligns with findings in other Lamiaceae species (Kim et al., 2025), confirming the crucial role of these genes in RA biosynthesis. HS is a critical signaling molecule in higher plants, influencing various metabolic pathways and interacting with other signaling molecules and hormones (Chen et al., 2011). PP treatment increased *4CL* expression at lower concentrations (24 and 48 h) and higher concentrations (24 and 72 h), while *TAT* and *HPPR* expression peaked at 72 h, which is correlated with RA production. According to this result, it is suggested that in PP-treated plants, the production of RA is influenced by both pathways. Another study showed that elicitation induced both the PAL- and TAT-derived pathways in *M. officinalis* (Mousavi and Shabani, 2019). *PAL* expression decreased or remained unchanged under different concentrations of PP, while RA content increased at all applied concentrations. Previous studies showed that the effect of elicitors on *PAL* isn't always linked to phenolic compound accumulation (Salar et al., 2021). The positive effects of PP treatment on plant growth are linked to its role in activating plant defense responses rather than serving as a direct phosphorus source (Vinas et al., 2020). Plants' elicitation triggers the accumulation of various defensive secondary metabolites. The perception of the elicitor's signal triggers the initiation of a signal transduction network, which subsequently activates the biosynthesis of transcription factors that regulate plant secondary metabolism (Zhao et al., 2005). No data has been published regarding the effects of these elicitors on RA biosynthesis-related genes. As the use of elicitors influenced the genes related to the phenylalanine and tyrosine pathways in RA production, it is suggested that both pathways are activated in *M. officinalis*. It is important to highlight that the effects observed on RA biosynthesis depend on the dose and conditions, and results may differ in field settings due to natural biological and environmental differences. Several study limitations should be noted, such as the focus solely on RA without a full profile of related

metabolites and the absence of characterization for possible RA derivatives. Overcoming these limitations could enhance the practical relevance of this research.

5. Conclusion

Treatment of *M. officinalis* with HP, HS, and PP enhanced RA accumulation by boosting PAL activity and modulating the expression of *PAL*, *4CL*, *TAT*, and *HPPR* genes, thereby activating the phenylpropanoid and tyrosine-derived biosynthetic pathways. All elicitors increased photosynthetic pigment levels. HP and elevated HS concentrations notably increased CAT and APX activities, along with total phenolic and flavonoid contents. Notably, PP's effects were concentration-dependent, while higher doses increased PPO activity at the expense of phenolic compounds. These findings demonstrate that application of these safe and accessible elicitors represents an effective and cost-effective practical strategy to enhance the production of bioactive specialized metabolites in *M. officinalis*. However, the responses observed depended on the dose and pathway, highlighting the importance of precise optimization in practical use. Future research should expand metabolite profiling to include RA derivatives and validate these findings in field settings to link controlled experiments with agricultural scalability.

CRediT authorship contribution statement

Nahid Abdi: conceptualization, writing- original draft, experimental investigation, formal analysis and data curation. **Nima Ahmadi:** review and editing, methodology, validation of results, supervision and resources provision. **Ali Pakdin-Parizi:** review and editing, data validation, supervision, resource provision, conceptualization, methodology and formal analysis. **Marie-Laure Fauconnier:** supervision, resource provision, and methodology development.

Ethical approval

Not applicable.

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Declaration of competing interest

All authors state that there are no conflicts of interest.

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- Hydrogen peroxide (HP), hydrogen sulfide (HS), and potassium phosphite (PP) foliar application increased photosynthesis pigment content
- Phenolic compound enhanced by HP and HS treatment.
- Rosmarinic acid content promoted by HP, HS, and PP.
- HP, HS, and PP affected *PAL*, *4CL*, *TAT*, and *HPPR* gene expression.

Declaration of 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: