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COPPER ION INDUCED PRODUCTION OF ROSMARINIC ACID IN LEMON BALM (*Melissa officinalis* L.) SEEDLINGS

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Abstract

Rosmarinic acid (RA), one of the most important active ingredients of lemon balm (*Melissa officinalis* L.), exhibits antiviral, antibacterial, antioxidative and anticancer properties. Furthermore, it can improve functionality in baking process. Cu acts as a cofactor of several proteins and plays a key role in photosynthesis, respiration, lignin synthesis, response to oxidative stress and cell wall metabolism, but can be toxic to plants in high concentrations. We hypothesized that abiotic stresses, as one of the external factors inducing the defense mechanism of plants, may contribute to the production of secondary metabolites, especially RA, in representatives of the *Lamiaceae* family. In current study, RA accumulation, expression of tyrosine aminotransferase gene (TAT), contents of flavonoid and anthocyanin as well as antioxidant enzymes activities were investigated in 45-day-old *M. officinalis* seedlings after treatment with different concentrations of Cu^{2+} (0, 5, 10, 20, and 30 μM). Samples were collected and analyzed after 8 and 16 hours of treatment. Lower concentrations of Cu^{2+} positively affected RA accumulation at both aforementioned treatment times, which is consistent with the increase in TAT gene expression profile. Flavonoid, anthocyanin and soluble protein contents of the seedlings significantly decreased (except at 20 and 30 μM Cu^{2+}-treated seedlings after 8 hours). RA content and expression of TAT gene decreased significantly at the highest concentration of Cu^{2+} for 16 hours. Concurrently, elevated levels of superoxide dismutase and peroxidase activities were measured in these seedlings. Latter can indicate that lower concentrations of Cu^{2+} cause oxidative stress. Reactive oxygen species (ROS), which act as signal molecules, are accumulated and due to their positive effects on the expression of TAT gene more RA is produced. In contrast, at the highest concentration of copper ions, ROS suppressed TAT gene expression and prevented the degradation of the gene product.

Keywords: antioxidant enzyme, superoxide dismutase, catalase, peroxidase, *Melissa officinalis*, rosmarinic acid, flavonoids, anthocyanins, tyrosine aminotransferase.

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, and it has been identified from lemon balm (*Melissa officinalis* L.) as one of the major active phenolic compounds [1, 2]. It has shown various biological activities such as anti-inflammatory[3], antiviral [4], antibacterial, antioxidative [5, 6], anticancer [7] and antiallergic activities [8]. Furthermore, RA as a potential

enhancer can provide functional properties to bakery products [9]. RA is biosynthesized through two different pathways in plants. Phenylpropanoid pathway, which is considered as the main biosynthesis pathway, starts with activation of phenylalanine ammonia-lyase (EC 4.3.1.24) (PAL) enzyme and uses phenylalanine amino acid as its substrate. The second pathway is initiated with tyrosine aminotransferase (TAT) reaction that uses tyrosine as its substrate [1]. Previous reports have shown that RA production can be changed in presence of carbohydrates [10] as well as by yeast extract, Ag^+ and methyl jasmonates [11-13]. Studies in *Salvia miltiorrhiza* hairy root have shown that treatment with Ag+/yeast increases TAT activity and decreases PAL activity [13]. It was demonstrated exposure of *Phyllanthus tenellus* with Cu^{2+} increased PAL activity in the leaves [14]. Similarly, a rise in PAL activity was observed in of *Camellia sinensis* leaves in presence of copper, mercury and nickel [15].

Copper, essential microelements for plant growth and development, acts as a cofactor of several proteins such as plastocyanin (PC), Cu/Zn superoxide dismutase (Cu/Zn SOD) [16], cytochrome c oxidase [17] and plays key roles in photosynthesis, respiration, lignin synthesis, responding to oxidative stresses and cell wall metabolism [18]. This element, along with other microelements, is responsible for plant resistance to diseases [19]. Although copper is highly present in nature and needed for plant growth, its high concentrations can lead to toxicity in plants [20]. Plants exposed to heavy metal stress often encounter oxidative stress and consequently a high production of reactive oxygen species ROS [15]. Plants prevent damages caused by ROS through antioxidant systems consisting of both non-enzymatic (ascorbic acid, glutathione, tocopherol, flavonoids, etc) and enzymatic reactions (catalase, peroxidase, superoxide dismutase, glutathione, and ascorbate peroxidase and reductase) [21]. Phenolic compounds play important roles in color-stabilizing mechanism [22] and defending against pathogens attacks [23] and ultraviolet ray [24] in plants. Furthermore, they are used as indicators to investigate stresses and scavenger of free radicals [25, 26].

We hypothesized abiotic stresses as one of external factors inducing plant defense mechanism can promote the production of secondary metabolites and particularly RA in *Lamiaceae* family. The aim was to treat lemon balm seedlings with different copper concentrations and to measure the amount of RA and to analyze related plant defense mechanisms such as flavonoid and anthocyanin content, and antioxidant enzymes activities. We also aimed to measure TAT gene expression as one of important genes in RA biosynthesis pathway in *M. officinalis* seedlings.

Materials and methods. F_1 seeds of lemon balm (*M. officinalis*) were obtained from Pakanbazr (Esfahan, Iran). Methanol (HPLC grade) and orthophosphoric acid were obtained from Merck (Germany). Water (HPLC grade) was provided by membrane purification system. External standard of RA ($C_{18}H_{16}O_8$, MW = 360 $g \cdot mol^{-1}$) and other analytical reagents materials were purchased from Sigma (United States).

The seeds were treated with 2% sodium hypochlorite solution and rinsed three times with sterile distilled water. These seeds were planted in MS medium (Murashige and Skoog, 1962) with 0.8% (w/v) agar, and transferred to dark incubator (relative humidity 55±5%; 28±2 °C) for two weeks. Then, plants were transferred to 16:8 hour (Light:Dark) at 30±2 °C. The 45-day-old seedlings were collected from the medium and washed carefully with sterile distilled water and transferred to liquid MS medium. The medium was supplemented with the final Cu^{2+} concentrations of 0 (control), 5, 10, 20 and 30 μM by using $CuSO_4 \cdot 5H_2O$ salt. The treatment was applied for 8 and 16 hours as recently reported [27]. Treated

seedlings were harvested and rinsed with sterile distilled water for several times to remove the surface ions. Treated seedlings were divided into two groups: one was dried in the shade (for three days) and used to measure the RA concentration; the other group was rinsed, dehydrated and frozen in liquid nitrogen, and kept at –80 °C until measuring flavonoid and anthocyanin contents, antioxidant enzymes activity and analyzing TAT gene expression level.

RA identification and quantification was performed as recently reported [27, 28]. In brief, 0.1 g of dried sample was ground into powder and mixed with 25 mL ethanol/water (30:70 v/v) solution, then it was sonicated for 10 min and centrifuged at 4500 rpm for 5 min at 4 °C. The supernatant was transferred to new collection tube and its volume was increased to 50 ml by adding sterile distilled water. The extract solution was filtered with 0.2 μM single-use syringe filter, before injecting into column of High Performance Liquid Chromatography (HPLC, ZORBAX SB-C18 column, Agilent 1100 series, USA). The mobile phase containing 40% solvent A (orthophosphoric acid in water, 1.0% v/v) and 60% solvent B (orthophosphoric acid in methanol, 1.0% v/v), was run at 1.0 ml/min at room temperature. Identification of RA was achieved by comparing retention time of each sample with the standard, which was acquired at 330 nm.

RNX plus™ kit (Cinnagen, Iran) was used to extract total RNA as stated by manufacturer's protocol. The quality and quantity of extracted RNA were assessed by 1% agarose gel and spectrophotometer apparatus (Varian cary 50, Australia), respectively. Finally, 200 $U \cdot \mu l^{-1}$ M-MuLV Reverse Transcriptase (Fermentas, EP0441, USA), Oligo(dT)20 Primer (Fermentas, SO131, USA), dNTP (1 mM of ecah) and 20 U RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA) were used to synthesize cDNA at 42°C for 120 min.

TAT gene primers (Forward primer: 5′-CCG CTA CTT CGA TCT TCA TCC-3′ and reverse primer: 5′-CCA TTG GAA CAA AAG GGT TCG-3′) were designed by Oligo Primer Analysis Software v. 7 (https://www.oligo.net/downloads.html) in reference to available mRNA sequence of Tyrosine aminotransferase gene in *Melissa officinalis* (Gene Bank Accession No. JN863949). TAT gene was amplified by *Taq* DNA polymerase (Qiagen, Germany) with an initial denaturation for 4 min at 94 °C, followed by 30 cycles amplification (1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C) and a final extension at 72 °C for 10 min using thermocycler (Eppendorf 5331 Mastercycler Gradient PCR, Germany).

To assess TAT gene expression levels in the presence of different Cu^{2+} concentrations, semi-quantitative PCR method was used and comparisons were done by using Gene Tools software (Syngene, Cambridge, UK), employing intensities of the amplified bands separated on 1% agarose gel. This software can quantify the fluorescence intensity from electrophoresis bands and enables the investigation of gene expression levels [29].

To determine the flavonoid content, 0.1 g of fresh sample was ground into powder in 10 ml ethyl alcohol: acetic acid (1:99, v/v) and centrifuged for 10 min at 4000 rpm. The supernatant was slowly heated over a hot water bath for 10 min at 80 °C. Absorbance measurement was done at wavelengths of 270, 300 and 330 nm, considering the extinction coefficient of 33,000 $M^{-1} \cdot cm^{-1}$. Flavonoid contents were calculated cumulatively and reported in $\mu M \cdot g^{-1}$ fresh weight (fw) [30].

To measure anthocyanin content, method of Krizek et al. (1993) was applied. Briefly, 0.2 g of fresh sample was ground into powder in 3 mL acidified (0.1% HCl) methanol and then centrifuged at 12000 rpm for 20 min at 4 °C. Supernatant as a pigment container was stored in darkness for 24 hours. Absorbance was measured at 550 nm and the extinction coefficient of 33,000 $M^{-1} \cdot cm^{-1}$

was used to calculate anthocyanin content. Finally, anthocyanin content was reported in $\mu M \cdot g^{-1}$ fw [31].

Protein was extracted through grounding 0.5 g of fresh sample into powder in 50 mM potassium phosphate buffer (pH 7.5) comprising 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The mixture was centrifuged at 11000 rpm for 20 min at 4 °C. The supernatant was used for measuring protein content and investigating enzyme activities [32]. Total content of protein was determined from plant extractions according to the Bradford's (1976) method by using Bovine serum albumin (BSA) as the standard and the absorbance was measured at 595 nm [33].

To measure superoxide dismutase (SOD; EC 1.15. 1.1) activity, method of Giannopolitis and Reis (1977) was used, which emphasizes on preventing photochemical reduction of nitro blue tetrazolium (NBT) through the enzyme's capability. One unit of the enzyme activity is defined as the amount of enzyme required to 50% inhibition of NBT reduction into blue formazan under light conditions. Reaction mixture included 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 50 mM potassium phosphate buffer (pH 7.8) and 0.1 mM EDTA. Finally, absorbance was measured at 650 nm [34].

To measure catalase (CAT: EC 1.11. 1.6) activity, method of Dhinsda et al. (1981) was used, which relies on enzyme's capability to degrade H_2O_2 in 1 min. One unit of catalase enzyme is defined as the amount of enzyme, which degrades 1 ml of H_2O_2 per minute. The reaction mixture included 15 mM H_2O_2 and 50 mM potassium phosphate buffer (pH 7). Amount of H_2O_2 in reaction mixture after 1 min and absorption differences were measured at 240 nm and then the activity was defined in $U \cdot mg^{-1}$ protein [35].

To assay peroxidase (POD; EC 1.11. 1.7) activity, method of Plewa et al. (1991) was applied, which concentrates on absorbance of tetraguaiacol formed by oxidation of guaiacol which, was catalyzed by peroxidase at 470 nm in 3 min. Reaction mixture contained 4% guaiacol, 1% H_2O_2 and 50 mM potassium phosphate buffer (pH 7). POD activity was measured using an extinction coefficient of tetraguaiacol, $\varepsilon = 26.6\ mM^{-1} \cdot cm^{-1}$ [36].

All experiments were done through completely random designs with 3 independent replicates. Data means were used for Duncan's multiple range test. Afterwards, one-way analysis of variance (ANOVA) with a significance level of 0.05 was used for analyses of data with SAS 9.1.3 (SAS Institute, Cary, NC).

Results. Treatment of lemon balm seedlings with different Cu^{2+} concentrations led to different responses of RA production. RA content of experimental samples, compared to the standard sample, was measured after 16 minutes retention time (data not shown). The maximum amount of RA reached about 36 mg/g dry weight, in treatment with 5 μM Cu^{2+} after 8 hours, which was 3.5 fold more than the control (8.5 mg/g) (Fig. 1, A). Treatment with 10 μM Cu^{2+} resulted in 12 mg/g RA, being significantly bigger than the control. RA content of samples treated with higher Cu^{2+} concentration was similar to the control. The RA concentration was reduced after 16 hours of treatment and only 10 μM Cu^{2+} treatment was comparable to the results at 8 hour timepoint.

TAT gene expression was studied in a semi-quantitative method. Extracted RNA quality was determined by 18S and 28S bands related to ribosomal RNA on 1% agarose gel, which were clearly observable for all samples, indicating high quality of extracted RNA (Fig. 2, A). After cDNA library construction and TAT gene amplification, the gene expression level was calculated through loading of 4 μl of each PCR product (242 bp in length) on 1% agarose gel by using Gene Tools software (Fig. 2, B).

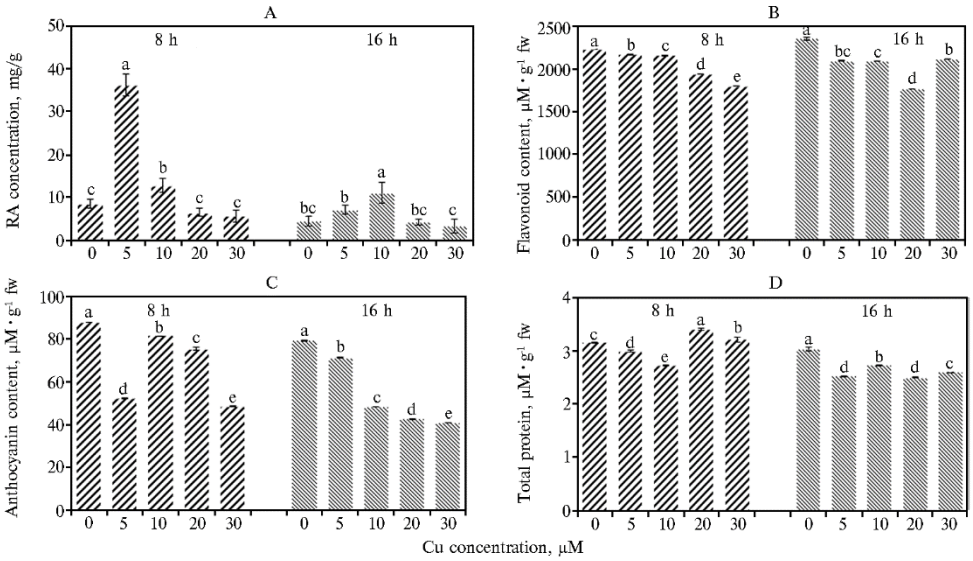

Fig. 1. Rosmarinic acid (RA) (A)**, flavonoids** (B)**, anthocyanin** (C)**, and protein** (D) **content of lemon balm treated seedlings with different Cu^{2+} concentrations after 8 and 16 hours (***n* = 3, *M*±SD, lab tests). Different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Test.

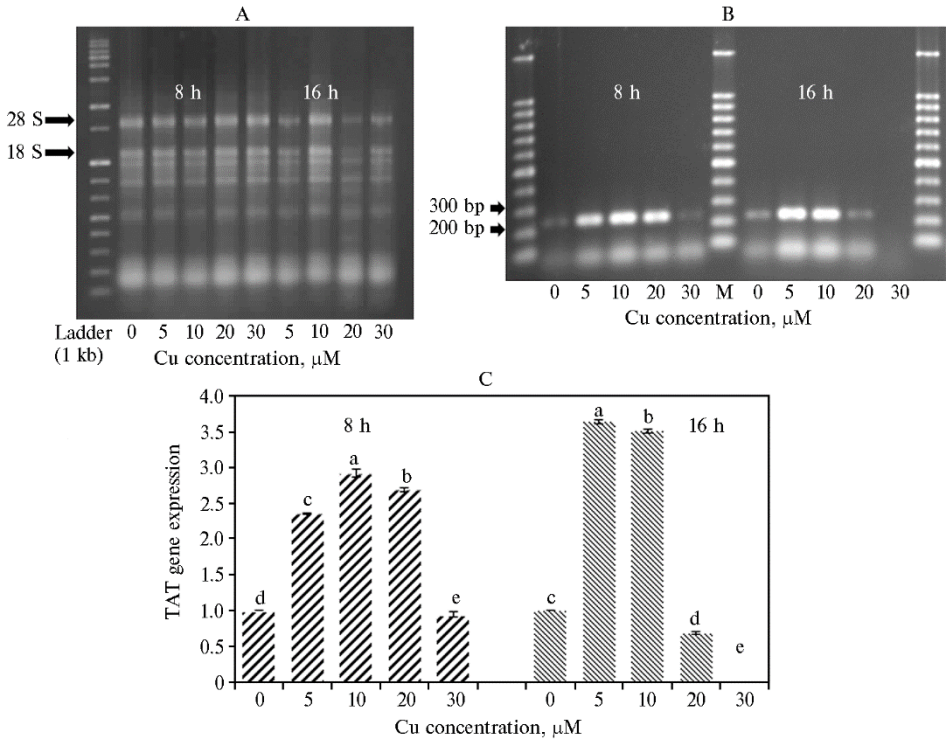

Fig. 2. RNA extracted from Cu^{2+}-treated lemon balm seedlings (A)**, amplified TAT gene on agarose gel** (B)**, and comparing of TAT gene expression** (C) **in treated seedlings with different concentrations of Cu^{2+} after 8 and 16 hours** (*n* = 3, *M*±SD, lab tests). GelPilot 1 kb Ladder (Qiagen, Germany)**.** Different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Test.

Treatment of seedlings with 5, 10 and 20 μM concentrations of Cu^{2+} for 8 hours led to an increase in TAT gene expression level compered to control (Fig. 2, C). Similarly, the seedlings treated with 5 and 10 μM of Cu^{2+} for 16 hours showed a significant rise in the level of the expression. On the other hand, by

increasing Cu^{2+} concentration in media, expression of this gene was decreased which was more considerable in 16 hours timepoint, while no band was observed on the gel in 30 μM concentration of Cu^{2+}.

Flavonoid content of the Cu^{2+}-treated seedlings tended to decrease with the increase of ions concentration in both treatment times (Fig. 1, B). Decrease in the flavonoid content was comparatively bigger at 16 hours than 8 hours although 30 μM Cu^{2+} concentration treatment recovered flavonoid content approximately up to level of 5 μM Cu^{2+}.

Treatment of lemon balm seedlings with different Cu^{2+} concentrations at the both treatment times led to a tendency to decrease in anthocyanin content compared with control (Fig. 1, C). Decrease in anthocyanin content was more considerable at 16 hours than 8 hours as well as in higher Cu^{2+} concentrations.

Treatment of lemon balm seedlings with different Cu^{2+} concentrations at the both treatment times led to significant increase in the SOD activity and to significant decrease in CAT activity compared to control (Table).

SOD, CAT and POD activity of treated lemon balm seedlings with various concentrations of Cu^{2+} at different treatment times (n = 3, M±SD, lab tests)

Cu^{2+} concentration, μM	SOD, U/mg protein		CAT, U/mg protein		POD, U/mg protein	
	8 h	16 h	8 h	16 h	8 h	16 h
0	38.4±7.7[c]	51.6±17.2[e]	179.7±2.3[a]	161.9±1.8[a]	3.61±0.4[b]	3.51±0.3[d]
5	395.6±15.6[a]	142.8±10.8[d]	89.3±1.1[d]	156.7±0.9[b]	6.29±0.5[a]	7.65±0.4[b]
10	277.0±19.7[b]	232.4±21.7[c]	157.0±1.1[b]	105.7±1.3[c]	5.87±0.1[a]	6.15±0.2[c]
20	376.8±16.3[a]	382.9±25.5[a]	86.6±1.1[e]	93.7±1.8[d]	5.58±0.4[a]	8.86±0.4[a]
30	397.9±14.5[a]	301.5±17.1[b]	107.3±0.7[c]	65.9±1.4[e]	5.81±0.1[a]	6.99±0.4[b]

N o t e. Different letters in each group indicate significance at $p \leq 0.05$ according to Duncan's Multiple Range Test.

POD activity was significantly increased in Cu^{2+}-treated seedlings proportionally to Cu^{2+} concentration increase in medium, except for 30 μM Cu^{2+} for 16-hour treatment, where the enzyme activity was the same as the level of 5 μM Cu^{2+} (see Table).

Soluble protein content of the Cu^{2+}-treated seedlings significantly decreased in 5 and 10 μM treatments at both timepoints. At 8-hour treatment in presence of 20 and 30 μM Cu the protein content increased instead (Fig. 1, D). However, longer treatment still significantly decreased the protein content compared to the control.

In the present study, effects of different Cu^{2+} concentrations were analyzed on RA accumulation, TAT gene expression as well as antioxidant system in 45-day-old *M. officinalis* seedlings at different timepoints. According to the results, copper had positive effect on the RA content especially at lower doses (5 and 10 μM) whereas at higher concentrations RA content was similar to the level of control. The maximum amount of RA was obtained in the presence of 5 μM Cu^{2+} after 8 hours of treatment, when RA concentration increased 3.5 times more than the control. These observations were consistent with the TAT gene expression profiles. On the other hand, although the maximum gene expression was seen in the presence of 5 and 10 μM Cu^{2+} after 16 hours of treatment, it was completely suppressed at 30 μM Cu^{2+}. Interestingly, in comparison with control, flavonoid and anthocyanin contents significantly decreased when the seedlings were treated with the different concentrations of this ion at the treatment timepoints, particularly at 16-hour treatment. Since flavonoid, anthocyanin and RA are phenolic compounds, it may be suggested that RA is produced more through tyrosine derived pathway [37]. Furthermore, as recently reported by Nasiri-Bezenjani et al. (2014), RA and TAT gene expression of lemon balm seedlings were induced by yeast extract after 17 hours and the data revealed these two parameters had a

similar pattern. Decrease in flavonoid and anthocyanin content was also consistent with our recent report, which showed the decrease of these metabolites when lemon balm seedlings were treated with Fe^{2+} concentrations [27]. Treatment of *Pinus sylvestris* L. seedlings with high levels of copper or nickel showed a decline in their phenolic compounds contents [38]. Based on this report, the decrease of phenolic compounds content possibly is related to suppression of other genes such as PAL, which are involved in their biosynthesis pathway or degradation of the enzymes involved in this pathway due to high toxicity of these ions in the medium. PAL, one of the most important enzymes in phenylpropanoids pathway [1], can be activated under stress conditions in plants to protect the plant during the synthesis of secondary metabolites such as simple phenols, anthocyanin, flavonoids, and lignins [39]. Previous studies have shown an increase of PAL activity in the *Phyllanthus tenellus* and *Camellia sinensis* leaves treated with copper, mercury and nickel [14, 15].

In comparison with other abiotic stresses, heavy metals induce the synthesis of heat shock proteins (HSPs), messenger molecules such as salicylic acid, abscisic acid and jasmonates and ethylene [40]. It has been proposed that free radicals have reciprocal roles in cells, while at higher concentrations they may damage cell membrane, nucleic acids and proteins [41], at lower concentrations they can act as signalling molecules [40]. Jasmonate, as a key signalling molecule, plays an important role in signalling network adjustment, which leads to biosynthesis of plant secondary metabolites [42]. The effects of these compounds on biosynthesis of different types of secondary metabolites such as alkaloids, terpenoids, glycosinolates and phenylpropanoids have been clarified [43]. Rapid accumulation of jasmonic acid in plant species such as *Phaseolus coccineus* and *Arabidopsis thaliana* after treatment with copper was proved [44]. Increase in RA production in exposure to some elicitors (yeast extract and methyl jasmonate) in several plant species including *Orthosiphon aristatus* [45], *Coleus blumei* [46] and *M. officinalis* [28] were reported. Yan et al. (2006) declared that increase of RA content in *Salvia miltiorrhiza* after treatment with yeast extract and silver ion is due to an accretion in activity of TAT gene. By considering the antioxidant properties of RA [47], it can be proposed that increase in RA content in Cu^{2+}-treated seedlings may be attributed to the induction of ROS and activation of a signaling pathway such as internal jasmonate [48], and consequently, activation of the genes involved in defensive system (e.g. TAT). Decrease in the level of these compounds due to antioxidant effects is related to the activation of other antioxidant pathways such as enzymatic pathway or degradation and inactivation of the enzymes, which are involved in biosynthetic pathways of these compounds [49]. Interestingly, SOD, which is the first enzyme in detoxification of ROS [50] and plays an important role in conversion of superoxide radicals to H_2O_2 and O_2 [51], increased in Cu^{2+}-treated seedlings compared with control after 8-hours exposure. In other words, in Cu^{2+}-treated seedlings, concentration of POD, which participates in removal of hydrogen peroxide inside the cell [52], was significantly increased in this research. However, the activity of CAT, another H_2O_2 scavenging enzyme in peroxisomes [53], was decreased significantly in Cu^{2+}-treated seedlings. Thus, it seems that lemon balm seedlings remove free radicals using expression of antioxidant enzymes such as SOD and POD.

Many studies showed that SOD, CAT and POD are necessary for protection of cells against side effects of ROS [50]. Miteva et al. (2005) declared that addition of arsenic acid concentration to culture medium led to significant increase in POD activity in tomato *(Lycopersicon esculentum* Mill.), which indicates

confrontation of POD with harmful effects of oxidative stress [54]. Moreover, significant increases in activities of some enzymes such as peroxidase and superoxide dismutase were reported in red cabbage (*Brassica oleracea*). Aforementioned plant used enzymatic and non-enzymatic antioxidants during heavy metal stress [55]. Wang et al. (2004) demonstrated that treatment of *Brassica Junica* seedlings with copper led to increase in the activities of SOD, POD, and decrease in CAT activity [56] which is consistent to our current study. Decrease in CAT activity may be due to lower affinity to combine with H_2O_2 or inhibition due to presence of high concentrations of heavy metals such as copper [57, 58]. It is pertinent to mention that cadmium caused CAT oxidation in pea (*Pisum sativum* L.) and as a result leading to a decrease in its activity [44]. Additionally, under toxic conditions of heavy metals, lack of a system to neutralize the by-products of oxygen radicals resulted in production of hydroxyl radicals through Fenton and Haber-Weiss reactions [59]. These radicals can change enzymatic activities, gene expression, protein content and soluble sugar content as well as release calcium from cell spaces, and also they can cause irreversible damages to plasma membrane and nucleic acid as were reported earlier [60]. Hence, decrease in protein content in Cu^{2+}-treated seedlings can be attributed to accumulation of these radicals. Decrease in protein content in wheat seedlings in presence of Cu^{2+} was shown by Singh et al. (2007) [61]. Furthermore, Singh et al. (2006) reported that protein content of *Pteris vittata* was decreased due to oxidative stress resulted from arsenic acid and degradation of some proteins [62]. On the other hand, elevated levels of soluble proteins at 20 and 30 μM after 8 hours of treatment may be attributed to the synthesis of heat shock proteins. Thereby, decrease in total flavonoid content, anthocyanin and RA in Cu^{2+}-treated seedlings may be attributed to inhibition of the enzymes, which are involved in RA biosynthesis pathway or may be attributed to degradation of these enzymes due to oxidative stress.

It can be concluded that the Cu^{2+} ion at investigated concentrations in this study leads to oxidative stress due to inducing the production of free radicals. Thus, production of these radicals, specially hydrogen peroxide, directly activate signalling pathways or induce expression of other genes involved in the pathway through biosynthesis of certain compounds such as plant hormones [48]. Increase in TAT gene expression level can be due to signalling roles of these radicals, which in turn leads to synthesis of RA when the seedling are treated with low concentrations of Cu^{2+}. Although the production of free radicals usually coincides with increase of SOD and POD activities, higher concentration of free radicals leads to degradation of some proteins or their functions or decrease in gene expression of these proteins. This assumption is consistent with the observed decrease in total protein content as well as activities of antioxidant enzymes and severe decrease in TAT gene expression in the treatment with highest copper concentration. This issue can also be due to decrease in flavonoid and anthocyanin content along with decrease of RA production in Cu^{2+}-treated seedlings at higher ion concentrations. Future experiments could test the effect of copper on RA in soil systems. If copper can increase RA content, it can be used in crop production to enhance the valorization properties of lemon balm and to help bioeconomy.

To summarize, lower concentrations of Cu^{2+} caused oxidative stress, followed by accumulation of ROS as signals molecules, which induced RA accumulation by an increase of TAT gene expression level. On contrary, at the highest applied Cu^{2+} concentration for 16 hours, ROS suppressed TAT gene expression and decreased RA production, where elevated levels of superoxide dismutase and peroxidase activities were measured in these seedlings.

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