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بررسی شاخص های بیوشیمیایی گیاه دارویی بادرشبو تحت اثر نانوذره مس و پلاسمای سرد اتمسفری

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چکیده. هدف این مطالعه، بررسی اثر نانو ذرات مس و پلاسمای سرد اتمسفری بر شاخص های بیوشیمیایی گیاه دارویی بادرشبو (*Dracocephalum moldavica*) است. عوامل دخیل در آزمایش شامل نانوذرات مس و ذرات غیرنانو در چهار سطح صفر (کنترل)، ۲۵، ۵۰، ۷۵ میلی‌گرم بر لیتر (mgl^{-1}) و پلاسمای سرد اتمسفری در سه فاصله زمانی صفر (کنترل)، ۲۰ و ۳۰ ثانیه بود. نتایج نشان داد که پلاسمای سرد اتمسفری باعث افزایش درصد اسانس می شود، در حالی که فلانوئید و فعالیت آنزیم‌های کاتالاز و پراکسیداز را کاهش می دهد. بیشترین تاثیر پلاسمای سرد اتمسفری بر درصد اسانس در تیمار ۲۰ ثانیه مشاهده شد و بین تیمارهای ۲۰ و ۳۰ ثانیه با توجه به تأثیر پلاسمای سرد اتمسفری بر صفات دیگر، تفاوت معناداری مشاهده نشد. همچنین، مشاهده گردید که مس غیر نانو موجب تنش فلزی سنگین در گیاه بادرشبو می‌شود. سطوح ۲۵ و ۵۰ میلی‌گرم بر لیتر نانوذرات مس موجب ارتقا صفات شد، اما سطح سوم آن (۷۵ میلی‌گرم بر لیتر) به‌عنوان فلز سنگین عمل کرد، بنابراین استفاده از نانوذرات مس ۲۵ و ۵۰ میلی‌گرم بر لیتر توصیه می‌شود. برهم‌کنش پلاسمای سرد اتمسفری و نانو ذرات مس نشان داد که پلاسمای سرد اتمسفری اثر فزاینده‌ای بر بهبود صفات مورد اندازه‌گیری داشته و اثر نانوذرات مس را افزایش داده است. می‌توان نتیجه گرفت که استفاده از نانوذرات مس با غلظت ۲۵ میلی‌گرم بر لیتر و پلاسمای ۲۰ ثانیه در مقایسه با مس غیرنانو، تأثیر معنادارتری بر بهبود شاخص‌های بیوشیمیایی گیاه بادرشبو دارد.

واژه های کلیدی. پراکسیداز، درصد اسانس، فلانوئید، کاتالاز، نعناییان

The effects of copper nanoparticles and cold atmospheric plasma on biochemical indices of *Dracocephalum moldavica*

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Abstract. The Moldavian dragonhead (*Dracocephalum moldavica* L., Lamiaceae) is an annual medicinal plant with beneficial nutritional sources that plays important roles in human and animal feed. Nanoparticles and cold atmospheric plasma increase biochemical compounds in plants. In this study, the effects of copper nanoparticles and cold atmospheric plasma on biochemical indices of the medicinal plant *Dracocephalum moldavica* were investigated. Moldavian dragonhead plants were subjected to four doses of copper nanoparticles (0, 25, 50 and 75 mgl^{-1}) and cold atmospheric plasma at three durations (zero, 20 and 30 s). The results showed that cold atmospheric plasma significantly increases the essential oil percentage, while it decreases the amount of flavonoid content and activity of catalase and peroxidase enzymes. Cold atmospheric plasma (20 s) showed significant positive impact on essential oil content, while different time duration (20 and 30 s) did not show a significant impact on other traits. Lower doses of copper nanoparticles (25 and 50 mgl^{-1}) showed positive impacts on measured traits, while 75 mgl^{-1} dose negatively affected the measured traits and functioned as a heavy metal. The cold atmospheric plasma and copper nanoparticles interactions indicated that cold atmospheric plasma had an incremental effect on the improvement of measured traits and increased the effect

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of copper nanoparticles. In conclusion, the results showed that copper nanoparticles with 25 mgl^{-1} dose along with cold atmospheric plasma with 20 s duration had significant positive effects on the improvement of biochemical indices of *Dracocephalum moldavica.*

Keywords. catalase, essential oil percentage, flavonoids, Lamiaceae, peroxidase

INTRODUCTION

Dracocephalum moldavica L. is an annual herbaceous plant, belongs to Lamiaceae family, and capable of growing almost in any climate. The distillate of *D. moldavica*is used as an anti-epileptic drug, stomach enhancer, digestive facilitator, and for treating cardiac arrhythmias (Abd El-Baky&El-Baroty, 2008). This plant has many applications in traditional and modern medicine and is used to treat dyspepsia, abdominal bloating and stomach upset. In addition, it is used in the food industries such as beverage industry, sanitary and cosmetic industries (Hussein et al., 2006).

The absorption of carbon and metal nanomaterials by plants is a new field of research. Absorption, displacement and accumulation of nanoparticles vary depending on species, the type and size of chemical composition, the structure and strength of nanoparticles (Rico et al., 2011). Recently, many studies have been conducted on the severe toxicity of nanoparticles and their positive and negative effects on plants (Menard et al., 2011).

Nanoparticles are atoms or molecules with dimensions ranging from 1 to 100 nm, and can change their physical and chemical properties in comparison to coarse particles. Nanoparticles can be made of a wide variety of coarse particles, and their performance depends on their chemical composition, and their size or shape (Monica & Cremonini, 2009). In a study in the context of Canada, it was found that using nano-fertilizers could prevent a 2000-million-dollar loss, indicating higher utilization efficiency of nano-fertilizers compared to non-nanoscale fertilizers (Monreal, 2010). The nanoparticles mainly exist in soil and find a way to water. Information about the fate of nanoparticles in soil is limited, but it may enter the food chain and ultimately accumulate in the body of organisms. Plants are an important part of the environment and are considered as a potential route for transferring nanoparticles to food chain (Zhu et al., 2008; Asgari et al., 2018).

Nanotechnology is gradually transitioning from the experimental stage to practical and operational stage, and this will lead to a more tangible presence of this technology in agriculture (Baruah & Dutta, 2009). The effect of nanoparticles on plants largely depends on their composition, concentration, size, and physical and chemical properties (Shalaby et al., 2016). Copper is an important element for the growth and development of plants; and plays a major role in the processes of protein and carbohydrate synthesis (Olszewska et al., 2008). Important characteristics of copper are high catalysis and electrical conductivity (Chandra et al., 2014). Application of CN10 to 30 mgl^{-1} in soil has increased the growth and yield of wheat (Hafeez et al., 2015). Heavy metal stress causes lipid peroxidation, damage to the membrane, excessive production of hydrogen peroxide, and protein degradation (Manikandan et al., 2016). However, plants activate a series of antioxidant enzymes such as superoxide dismutase, catalase, peroxidase and ascorbate peroxidase to overcome the active oxygen species (Manikandan et al., 2015).

Catalase and peroxidase are also metalloenzymes, which repel the superoxide ion destruction effect. These enzymes provide a defensive system for the survival of aerobic organisms (Pandey *et al*., 2009; Posmyk *et al*., 2009). It has been found that copper oxide nanoparticles (CN) in *Lemna minor* reduced the growth while increased the activity of superoxide dismutase enzymes, catalase, peroxidase, and malondialdehyde (Song et al., 2016).

Plasma is a radiation flash which is very reactive. Chemical species such as charged particles, free radicals and some rays occur with electrical discharge while in contact with gas are called ‘clod atmospheric plasma’ and are produced under mild conditions (Fernández & Thompson, 2012). Plasma-activated water (PAW) increases the germination rate of radish seeds by up to 80% and improves bud burst in comparison with normal water. Plasma treatment improved seed germination and seedling growth. Although the effect of plasma discharge on the surface of wet seeds is rarely studied, the water molecules absorbed by the seed skin can react with plasma discharge and produce active species, consequently, the germination and bud burst can be highly influenced (Sivachandiran & Khacef, 2017).

The positive effects of osmotic plasma on disinfection, breaking seed dormancy, seed germination, and root and stem growth have been reported in the bean, as it accelerates germination and breaking seed dormancy and increases the length of the roots and stems (Bormashenko et al., 2015). Cold atmospheric plasma (CAP hereafter) treatment exerts a positive effect on seed germination, growth, and yield of radish, accelerating these processes (Mihai et al., 2014).

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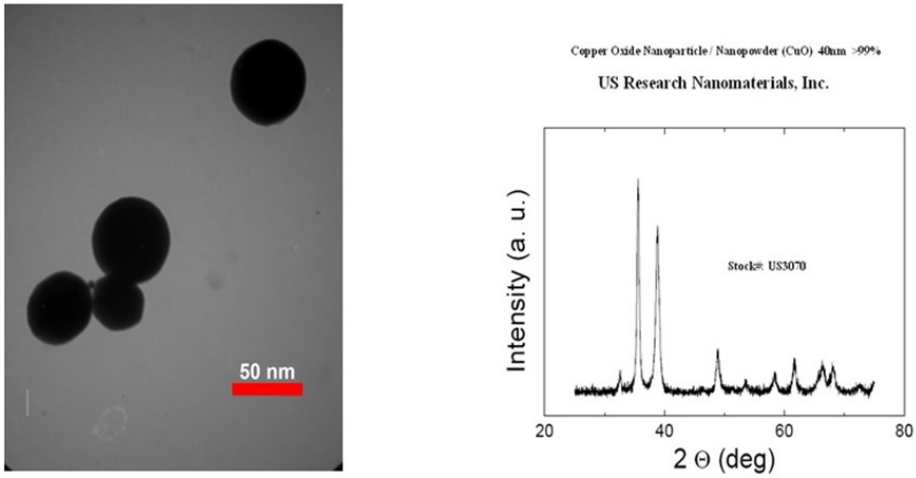

Figure 1. Transmission electron microscope photograph of copper nanoparticle and Zeta potential of CuO nanoparticles.

The application of CAPcan improve the metabolism and physiological properties of the plant, such as dehydrogenase activity, superoxide dismutase (Meiqiang et al., 2005), peroxidase (Jiang et al., 2014), photosynthetic pigments, photosynthesis efficiency, and nitrate reductase activity (Wu et al., 2007).

In our previous study, the effects of copper oxide nanoparticles and cold atmospheric plasma on *D. moldavica* were investigated and an increase in the height of aerial parts, the dry weight of aerial parts, protein percentage and a decrease in proline levels were observed (Haddadian et al., 2017). aim of the present study was to investigate the effects of CN and CAP on biochemical indices of *D. moldavica* L. and providing practical solutions for the development of medicinal plants cultivation, especially *D. moldavica* L.

MATERIALS AND METHODS

The CuO NPs were purchased from NANOSANY Company, Mashhad, IRAN with a purity of 99%, particle size of > 40 nm. The morphology of the CuO nanoparticles was examined using transmission electron microscopy (TEM, JEOL, 100 CX, Japan) (Fig. 1).

This study was a randomized full-factorial design with four replications. The factors included the copper nanoparticles (CN hereafter) and the non-nanoscale particles (NNC hereafter) at four levels of zero (control), 25, 50 and 75 mgl^{-1} and cold atmospheric plasma (CAP hereafter) at three durations of zero (control), 20 and 30 s. The required seeds were provided from Ahvaz Seed and Plant Research Center. The seeds were disinfected in 5% sodium hypochlorite solution for 5 m. The culture medium was 1-liter pots filled with 55% Cocopeat and 45% perlite. The pots were irrigated daily with 100 ml of Hoagland solution. Using the CN and NNC oxide took 90 days, i.e. 1 time per 72 h. The temperature of greenhouse was about 29-32 °C during the day and about 20-25 °C at night. Then the plants were harvested after three month from cultivation-time. The CN was provided from the Iranian Nanomaterials Pioneers Company.Two groups of seeds were selected and exposed to Argon for 20 and 30 s in the form of radiation. The Baker and Nogues method was adopted to determine the total content of flavonoids. To measure the activity of catalase after extracting the protein, 2.5 ml of 50 mM phosphate buffer (pH=7) and .3 ml of 3% hydrogen peroxide were mixed with .2 ml of the enzyme extracts in an ice bath, and the absorbance changes were read at the wavelength of 530 nm after 2 m (Aebi, 1984). Peroxidase (POD) activity was determined using the method of Kar and Mishra (1976), briefly, protein extract, 2 ml of 100 mMTris buffer (pH=7), .3 ml of 5 mM hydrogen peroxide, and .2 ml of 10 mM Pyrogallol were mixed with 50 µl of the enzyme extract in an ice bath, and the absorbance changes were measured at the wavelength of 450 nm after 2 m (Kar & Mishra 1976). To calculate the percentage of essential oil, a 5-g specimen was prepared from young twigs of each experimental unit and was extracted with a Clevenger-based distillation method. Analysis of Variance (ANOVA) was conducted with SAS, and mean comparison was run by Duncan at the probability level of 1%.

RESULTS

Flavonoid

The results of ANOVA (Table 1) showed that CAP, CN and their interaction had significant effects on flavonoid ($p < 0.01$).

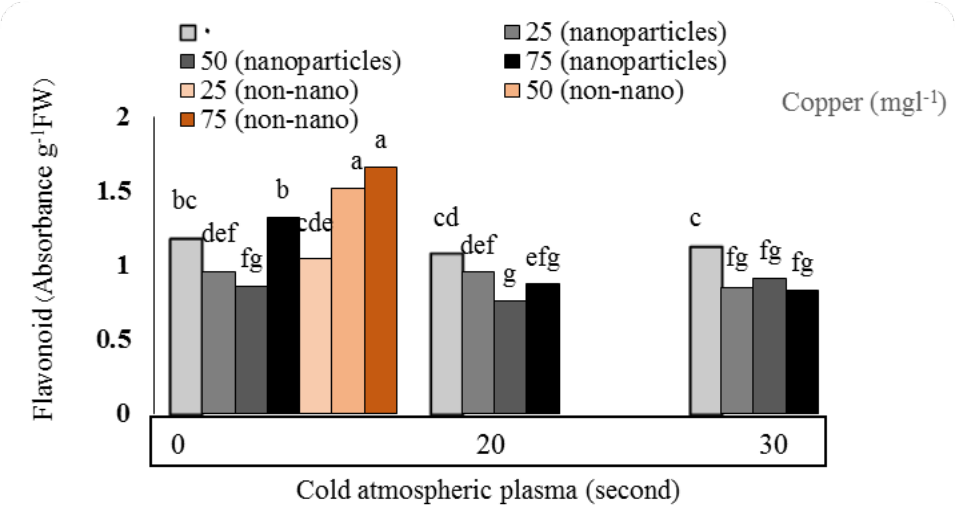

Figure 2. Investigating flavonoids under the influence of various levels of CAP and copper.

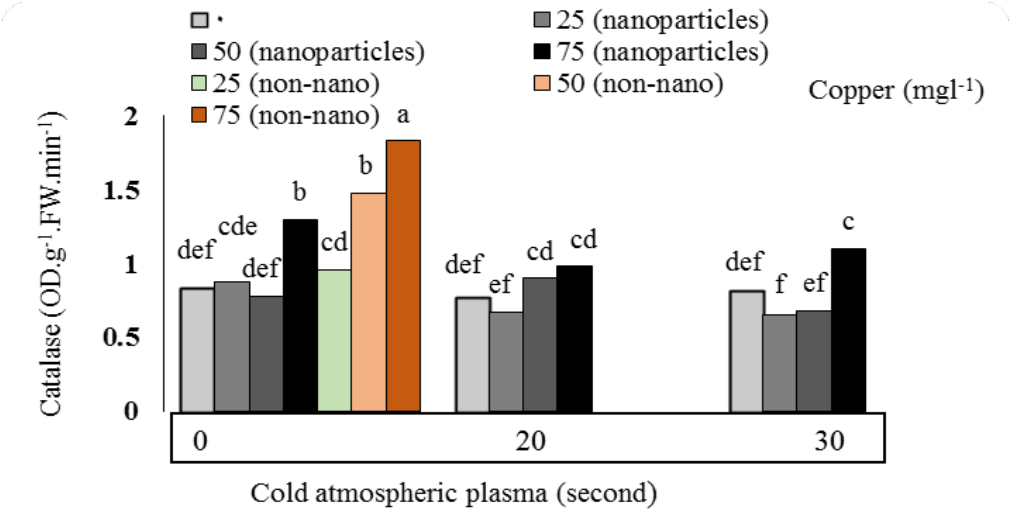

Figure 3. Investigating the activity of catalase enzyme under various levels of CAP and copper

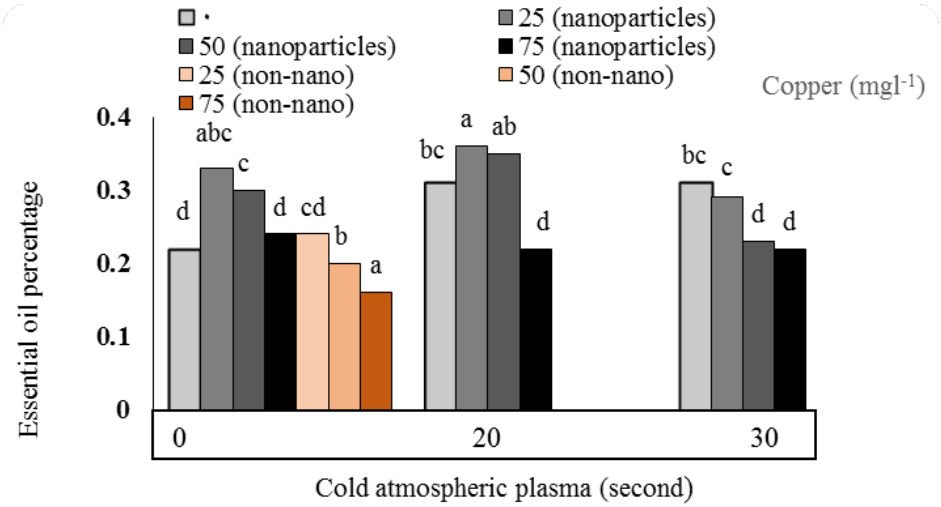

Figure 4. Investigating essential oil percentage under the influence of various levels of CAP and copper

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Table 1. Analysis of variance of the effects of CN and CAP on vegetative and biochemical indices of *Dracocephalum moldavica* L.

Sources of changes	df	Flavonoid	P-Value	Catalase	P-Value	Peroxidase	P-Value	Essential oil percentage	P-Value
Cold atmospheric plasma (a)	2	0.13**	0.002	0.09*	0.01	0.008*	0.02	0.01**	0.006
Copper nanoparticles (b)	6	0.35**	0.001	0.7**	0.002	0.06**	0.003	0.02**	0.001
a*b	6	0.07**	0.001	0.05*	0.04	0.003ns	0.1	0.006**	0.001
Error	45	0.01	–	0.02	–	0.002		0.001	
%CV		9.9	–	13.77		16.24		12.03	

* and ** mean significant effect at the probability level of 5% and 1% respectively; ns means non-significant effect.

Table 2. Comparison of the means, the effect of CAP on vegetative and biochemical indices of *Dracocephalum moldavica* L.

Cold atmospheric plasma (s)	Flavonoid (Absorbance g-1FW)	P-Value	Catalase (OD.g-1.FW.min-1)	P-Value	Peroxidase (OD.g-1.FW/min-1)	P-Value	Essential oil percentage	P-Value
0	1.22a	0.7	1.15a	0.3	0.33a	0.4	0.24c	0.07
20	0.91b	0.4	0.83b	0.2	0.24b	0.2	0.31a	0.1
30	0.93b	0.5	0.81b	0.2	0.22b	0.2	0.26b	0.09

In each column, the means with common letters have no statistically significant difference at the probability level of 5% in the Duncan test.

Table 3. Comparison of the mean effect of CNon vegetative and biochemical indices of *Dracocephalum moldavica* L.

Copper (mgl-1)	Flavonoid (Absorbance g-1FW)	P-value	Catalase (OD.g-1.FW.min-1)	P-value	Peroxidase (OD.g-1.FW/min-1)	P-value	Essential oil percentage	P-value
0	1.13c	0.2	0.84de	0.3	0.22de	0.1	0.28b	0.1
25 (non-nano)	1.04cd	0.2	0.96d	0.2	0.27d	0.1	0.24c	0.1
50 (non-nano)	1.52b	0.4	1.48b	0.1	0.43b	0.08	0.2c	0.1
75 (non-nano)	1.66a	0.5	1.83a	0.08	0.52a	0.06	0.16d	0.08
25 (nano)	0.91ef	0.1	0.73e	0.4	0.2e	0.1	0.33a	0.2
50 (nano)	0.84f	0.1	0.78e	0.4	0.22de	0.1	0.29ab	0.2
75 (nano)	1.01de	0.2	1.12c	0.1	0.33c	0.09	0.22c	0.1

In each column, the means with common letters have no statistically significant difference at the probability level of 5% in the Duncan test.

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- CAP was associated with a decrease in flavonoid. The CAP treatments of 20 and 30 s decreased flavonoid by 25.41% and 23.77%, respectively (Table 2).
- CN was also associated with a reduction in flavonoid, and the most significant reduction was observed to be 25.66% in the 50 mgl^{-1} CN treatment.
- The 25 mgl^{-1} NNC treatment decreased flavonoid by 7.96%. But as its concentration increased to 50 and 75 mgl^{-1}, flavonoid significantly was increased by 34.51 and 46.9%, respectively (Table 3).
- The combined use of CAP and CN also significantly decreased total flavonoid content. The highest flavonoid level was observed in the absence of CAP and presence of the 50 and 75 mgl^{-1} NNC with the amounts of 1.52 and 1.66 absorbed in the wet weight.
- The lowest flavonoid level was in CAP of 30 s combined with CN of 50 mgl^{-1} with the amount of .76 absorbed in the wet weight (Fig. 2).

Activity of catalase and peroxidase enzymes

The effects of CAP and CN and their interaction on the catalase enzyme activity were significant ($p < 0.05$) (Table 1). Besides, CAP ($p < 0.05$) and CN ($p < 0.01$) exerted a significant effect on the peroxidase enzyme activity, but their interaction effect was not statistically significant ($p > 0.05$).

1. The application of CAP caused a decrease in the activity of catalase and peroxidase enzymes. That is, the CAP treatments 20 and 30 s lessened the activity of catalase enzyme by 27.83 and 29.57% and also decreased the activity of peroxidase enzyme by 27.27 and 33.33%, respectively (Table 2), although there was no significant difference between CAP treatments of 20 and 30 s.
2. The 25 and 50 mgl^{-1} CN treatments respectively reduced the activity of catalase enzyme by 9.88% and 3.7%, while the 75 mgl^{-1} CN treatment increased the activity of catalase enzyme by 38.27%.
3. The 25, 50 and 75 mgl^{-1} NNC treatments showed an increase in the activity of catalase enzyme by 18.52%, 82.72% and 125.93%, respectively. The 75 mgl^{-1} CN treatment increased the activity of peroxidase enzyme by 50%, but the presence of 25 and 50 mgl^{-1} CN made no significant difference.
4. The NNC treatments also increased the activity of the peroxidase, with the highest increase to be 136.36% by 75 mgl^{-1} NNC (Table 3). The highest activity of the catalase with the amount of 1.83 absorbed in the wet weight per minute was associated to the absence of CPA and presence of 75 mgl^{-1} NNC, and the lowest activity of the catalase with the amount of 0.65 absorbed in the wet weight per minute was connected with the application of CPA for 30 s and the presence of 25 mgl^{-1} CN (Fig. 3).

Essential Oil Percentage

The results of ANOVA, as indicated in Table 1, showed that the effects of CAP, CN and their interaction on the essential oil percentage were significant ($p < 0.01$).

1. The CAP treatment of 20 s increased the essential oil percentage by 29.17% (Table 2).
2. The 25 mgl^{-1} CN enhanced the essential oil percentage by 17.86%, the 75 mgl^{-1} CN indicated a reduction of 21.43%, and 50 mgl^{-1} CN made no significant difference.
3. All NNC treatments were associated with a decrease in the essential oil percentage. The NNC treatments of 25, 50 and 75 mgl^{-1} lessened the essential oil percentage by 14.29%, 28.57% and 42.86%, respectively (Table 3).
4. The maximum essential oil percentage was observed to be .36% in the CAP treatment of 20 s combined with 25 mgl^{-1} CN, while its minimum was 0.16% in the absence of the CAP and presence of 75 mgl^{-1} NNC (Fig. 4).

DISCUSSION

Plants have important roles in ecological stability of ecosystems, while the impact of nanoparticles upon them is not well known, especially for their effects of biochemical compounds in economical aromatic plants (Zuverza-Mena et al., 2017). Several studies have shown that the nanoparticles may have both negative and positive impacts on different plant species, depending on size, concentration and shape of nanoparticles (Costa & Sharma, 2016; Wang et al., 2016; Tripathi et al., 2017). In this study, for the first time we investigated the effects of different doses of copper nanoparticles along with cold plasma atmospheric treatment on the biochemical compounds of *D. moldavica* L.

Our results showed that copper nanoparticles with 25, 50 and 75 mgl^{-1} doses significantly increased the catalase and peroxidase activity and led to increase the essential oil contents.

The copper increases the activity of some enzymes, the photosynthesis concentration, the durability of the leaf surface, and thus the photosynthesis amount of plant, resulting in plant growth (Bauder & Waskom, 2003). Flavonoids and phenolic compounds are secondary metabolites and have protective and antioxidant roles (Posmyk et al., 2007). In this regard has been proven that exposure of plants to high level of heavy metals can highly active phenolic compounds and flavonoids and protect the plants against toxicity (Dai et al., 2006; Posmyk et al., 2009).

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The toxic effects of heavy metals are due to the production of free radicals. In high plants, heavy metals, such as copper, induce the production of superoxide radical, hydrogen peroxide, hydroxyl and oxygen alone (Tiwari et al., 2008). ROS can quickly target all types of biological molecules, such as nucleic acids, proteins, amino acids and lipids, which leads to unrecoverable metabolic deficiency and ultimately cell death (Pandey et al., 2009; Wang et al., 2009). By increasing the level of ROS in the cells, the activities of the antioxidant enzymes increases (Pandey et al., 2009; Posmyk et al., 2009). In many plant species, the increase in the absorption of heavy metals, such as copper, induces a very high increase in the peroxidase activity and causes changes in the quality of its isoenzymes (Posmyk et al., 2009). On the other hand, the increase in the activity of peroxidase, as the main and key enzyme, in heavy metal stresses has been proven in such a way that this enzyme is known as stress markers in heavy metal stress (Choudhary et al., 2007).

In general, the production of essential oil is due to a set of physiological and morphological processes. In this regard, environmental factors, including nutrients such as copper have a significant effect on the production of essential oil.

Copper as a low-consumption element plays a very important role in the growth and development of essential oil from the medicinal plants. This element at low levels increases metabolic and physiological activities, photosynthesis and respiration, the energy required for biosynthesis of terpene compounds, leaf area and the number of essential oil oil-secreting glands. Furthermore, high levels of this element reduce essential oil percentage due to the negative effects on metabolic and physiological activities (Pande et al., 2007; Street, 2012).

In conclusion, our study showed positive impacts of copper nanoparticles in interaction with cold plasma atmospheric on *D. moldavica* biochemical compounds and enzymatic activity. The literature reviews in this area few and it is necessary to conduct more detailed researches to understand molecular mechanism of plant nanoparticles interaction. Our results showed beneficial impacts on copper nanoparticles, but the mechanisms at large extent are not understood, and our results are in primitive stages. Therefore, we recommend for future studies on the effects on nanoparticles before bringing the nanoparticles to the field.

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