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SILVER NANOPARTICLES ELICITED *IN VITRO* SHOOTS OF *SALVIA OFFICINALIS* L. FOR ACCUMULATION OF 1, 8- CINEOLE ESSENTIAL OIL

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Abstract

The effects of silver nanoparticles (AgNPs) on the fresh weight, dry weight and content of 1, 8-cineole from *in vitro* shoots of *Salvia officinalis* L. were evaluated. The results showed that the benzyl adenine had the ability to *in vitro* regeneration of shoots from nodes of *S. officinalis* L. Murashige and Skoog (MS) medium supplemented with 1 mg/l BA gave the highest shoots fresh weight (3.552 g) and dry weight (325.5 mg). The application of AgNPs on *in vitro* shoots of *S. officinalis* L. results in the alteration of the shoots growth and some biochemical parameters. MS medium supplemented with 10 mg/l of AgNPs and 1 mg/l of BA gave the highest fresh weight (5.19 g) and dry weight (497.9 mg) of shoots. The defense system of *S. officinalis* L. showed an increase in activity of superoxide dismutase and catalase under the effect of AgNPs. MS medium supplemented with 80 mg/l of AgNPs and 1 mg/l of BA gave the highest superoxide dismutase activity (0.363 unit/ ml). MS medium supplemented with 20 mg/l of AgNPs and 1 mg/l of BA revealed the highest catalase activity (15.763 unit/ ml). Gas chromatography–mass spectrometry (GC-MS) analysis showed the highest content of 1, 8-cineole in medium supplemented with 1 mg/l of BA and 20 mg/l of AgNPs. AgNPs at low concentration enhance the fresh and the dry weight of *S. officinalis* L. *in vitro* shoots and can be used for the enhancement of the production of bioactive compounds and activate the antioxidants system of *S. officinalis* L.

Key words: *Salvia officinalis* L., *in vitro*, 1,8-cineole, silver nanoparticles, benzyl adenine, shoots, regeneration.

Introduction

Plant tissue culture is an important field of plant biology, which deals with the improvement of plant, propagation of callus, production of bioactive compound and genetic manipulation (Kumar *et al.,* 2019). The applications of nanoparticles (NPs) have successfully led to evaluate the positive role of NPs in callus induction, organogenesis, somatic embryogenesis, somaclonal variation, genetic transformation and secondary metabolite production (Kim *et al.,* 2017). The nanoparticles with less than 100 nm in diameter have physiochemical properties (Ali *et al.,* 2019). There are many types of nanoparticles such as gold, titanium, silver, zinc, silicon, copper and magnesium are available commercially and interferes with many applications such as agriculture, biology and medicine (Ruttkay-Nedecky *et al.,* 2019). AgNPs among other types of nanomaterial attract global attraction due to their high physiological properties including enhancement plant cell growth, secondary metabolites production in plant cell cultures, biomass production and higher anti-microbial activity (Elechiguerra *et al.,* 2005). *S. officinalis* L. is a plant in the mint family lamiaceae, subfamily nepetoideae, tribe mentheae and genus Salvia (Dinc *et al.,* 2009), *S. officinalis* is a perennial subshrub up to 60 cm in high. The leaves are opposite and simple with white hairs on the lower leaf surface and greenish or greenish-grey on the upper surface. Stems are erect or procumbent with abundant hairy dark green branches. Leaves are elongated and petiolate with a serrate margin, rugose surface, and sometimes with basal lobes (Devansh, 2012). The essential oil of *S. officinalis* L. is characterized by yellow color with intense odor and the major constituents were camphor (27.59%), camphene (23.70%), α-pinene (13.75%), and β-pinene (6.28%); followed by limonene (5.38%), borneol (3.58%), 1,8-cineole (3.54%), caryophyllene oxide (2.24%) and other compounds were present in amounts less than 2% (Cruz-Silva *et al.,* 2017). The oil and leaves of *S. officinalis* L. is commonly used

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for therapeutic and non-therapeutic purposes such as antioxidant, antimicrobial, anticancer, anti-stress, anti-alzheimer, anti-cardiovascular diseases, memory improving and anti-inflammatory (Miraj and Kiani, 2016). Plant cell cultures offer promising technology for the rapid propagation and secondary metabolites production in plants in short period of time (Rani *et al.,* 2017). The aims of this study were to assess the ability of benzyl adenine to shoots regeneration of *S. officinalis* L. and to investigate the effect of AgNPs on fresh and dry weight and accumulation of essential oil 1,8-cineole from *in vitro* shoots of *S. officinalis* L.

Materials and Methods

Preparation and Culturing of Seeds

Seeds of *S. officinalis* L. were purchased from New Rama Seed Corporation Company in India and diagnosed in the lush of College of Science, University of Babylon. Seeds were sterilized dependent on the method descripted by (Awika and Rooney, 2004). The sterilized seeds were cultured in pots contain sterilized peat moss at three seeds in each pot. Then incubated under light conditions (16 hours day and 8 hours night) and intensity illumination of 1000 lux at temperature of 24±2^{0}C. Three months-old seedlings were used as a source of explants for shoots induction. The research experiments were performed in College of Science labs, University of Babylon, during the period of August-2019 to April-2020.

Shoots Induction

The explants used in this experiment for *in vitro* induction of shoots were nodes from mother plant at three months old. Nods segments were cut off into 2cm and sterilized, then cultured in the test tubes containing MS medium supplemented with different concentrations of BA (1, 2 and 3 mg/l) at a rate of 10 replicates for each concentration. These tubes were incubated under light conditions (16 hours day and 8 hours night) and intensity illumination of 1000 lux at temperature of 24±2^{0}C for 30 days. After one month, the shoots were harvested and determined their fresh weight and dry weight.

Silver Nanoparticles Treatment

Nano Silver Media Preparation

MS medium supplemented with 1mg/l of benzyl adenine was melted by hot plate stirrer for 5 minutes to mix the media and then adjusted the pH to 5.8 using sodium hydroxide or hydrochloric acid (0.1 N) before adding the agar and autoclaved. The medium was left to cool and supplemented with different concentrations of AgNPs (5, 10, 20, 40 and 80 mg/l) before being solidified.

Nodes Explant Cultured on Nano Silver Media

One node explant from *in vitro* shoots induced in MS medium supplemented with BA (1mg/l) was sub-culture on AgNPs media at concentrations of 5, 10, 20, 40 and 80 mg/l supplemented with 1mg/l BA. Ten repeats were cultured for each nano silver concentration. Culture process was made at sterilized conditions and the fresh and dry weight was measured after one month of agriculture.

Estimation of Catalase Enzyme Activity

Catalase enzyme activity was estimated by the method mentioned by (Aebi, 1984), this method depends on the amount of change in absorbance at 240nm of hydrogen peroxide solution (30mM) and phosphate buffer solution (50mM) at pH= 7. The method consist crushing 1g of shoots fresh weight with 10 ml phosphate buffer solution and added 0.3 g polyvinyl pyrophosphate during the crushing using ceramic mortar above amount of ice for 10-15 minutes, then filtered and the filter centrifuged at 10000 rpm for 10 minutes at 4^{0}C. 40 µl of enzymatic extract was added to 2 ml of phosphate buffer solution and 2 ml of hydrogen peroxide solution (H_2O_2, 30 mM) and incubated at 25^{0}C for one minute then read the absorbance at 240 nm was read. The blank solution was used which composed of the same materials with the replacement of the material foundation by phosphate buffer solution. The activity of catalase enzyme was calculated by the following equation:

Catalase activity (unit) =

$$\frac{\Delta^{bs} / \min \times reaction\,volume}{0}.01$$

Δbs = absorbance difference within a minute and the reaction volume= 4.04 ml

Estimation of Superoxide Dismutase Activity

The activity of the SOD enzyme was estimated as mentioned by (Marklund and Marklund, 1974), the interaction mixture of 50 µl of the extraction solution added to 2 ml of tris-buffer solution (pH = 8.2) and 0.5 ml of pyragallol solution (0.2 mM). This solution absorbs the light at a wavelength of 420 nm. The method consists of a mixture of 10 ml of the phosphate buffer (pH = 7.2-7.4) with 1g of shoots and centrifuged at 10,000 rpm for 15 min (50^{0}C). 50 µl of the extract was added to 2 ml of the tris buffer solution and 0.5 ml of pyragallol solution for the test solution, then compare with the change in absorption of the control solution containing distilled water instead of the enzyme. Distilled water was used as a blank solution. The following equation was used to determinate the enzyme efficacy:

SOD Activity (units) =

$$\frac{\%inhibition\ of\ pyragallol\ reduction}{50\%} \times reaction\ volume \Big/ total\ test\ period\ (10\ \text{min})$$

Gas Chromatography–Mass Spectrometry Analysis

The amount of 1, 8-cineole was estimated by the method mentioned by (Basma *et al.,* 2013), this method dependent on the separation of essential oils by Clevenger apparatus. GC–MS analysis was carried out using Japanese instrument (30mm × 0.25 mm × 0.25µm) of capillary column. Injection temperature was maintained at 250^{0}C, Helium flow rate as 1.5 ml/min and ion source temperature at 240^{0}C. Injection was performed using the split less mode and the volume was 1 µL. The instrument was set to an initial temperature of 70^{0}C, and maintained at this temperature for 3 min. At the end of this period the oven temperature was arisen up to 300^{0}C, at the rate of an increase of 10^{0}C/min and maintained for 15 min (Idan *et al.,* 2015). The content of 1, 8- cineole was calculated using the following equation:

Sample concentration (ppm) =

$$\frac{Sample\ area}{S\tan dard\ area} \times \text{Standard conc.} \times \text{dilution factor}$$

Statistical Analysis

The experiments were designed according to the full random design (C.R.D.) depending on the less significant difference (L.S.D.) ($p \leq 0.05$) (Al-Rawi and Khalaf Allaha, 2000).

Results and Discussion

Effects of Benzyl Adenine on Shoots Regeneration of *S. officinalis* L.

Table 1 and Fig. 1 showed the significant effect of BA concentrations which added to MS medium on the fresh and dry weight of regenerated shoots. BA with a concentration of 1 mg/l gave a higher mean of fresh and dry weight reached to 3.552 g and 325.5 mg, respectively, which differed significantly with 2 mg/l and 3 mg/l of BA in MS medium ($p \leq 0.05$). Generally, a cytokinin is required for shoot multiplication (Miri, 2020). The cytokinins such as BA have ability to inter into plants cells and affected on cell metabolisms and stimulate the cell division and shoot formation (Erfani *et al.,* 2017). BA has ability to induce endogenous hormones for morphogenesis induction (Ahmed and Anis, 2014). BA has also an effect on the reduction of multiplied shoot length, shoots fresh and dry weight (Mousavi *et al.,* 2012). The used of high concentration of BA in medium for plant shoots regeneration results in reduction of shoots growth (Arab *et al.,* 2014). Rasool *et al.,* (2009) reported

Table 1: Effect of BA on shoots fresh and dry weight of *S. officinalis* L.

Concentration of BA (mg/l)	Fresh weight (g) (Mean ± SE)	Dry weight (mg) (Mean ± SE)
0	0	0
1	3.552 ± 0.169	325.5 ± 15.696
2	1.669 ± 0.099	155.0 ± 10.028
3	0.934 ± 0.053	83.4 ± 4.975
L.S.D. (0.05)	0.6834	64.6002

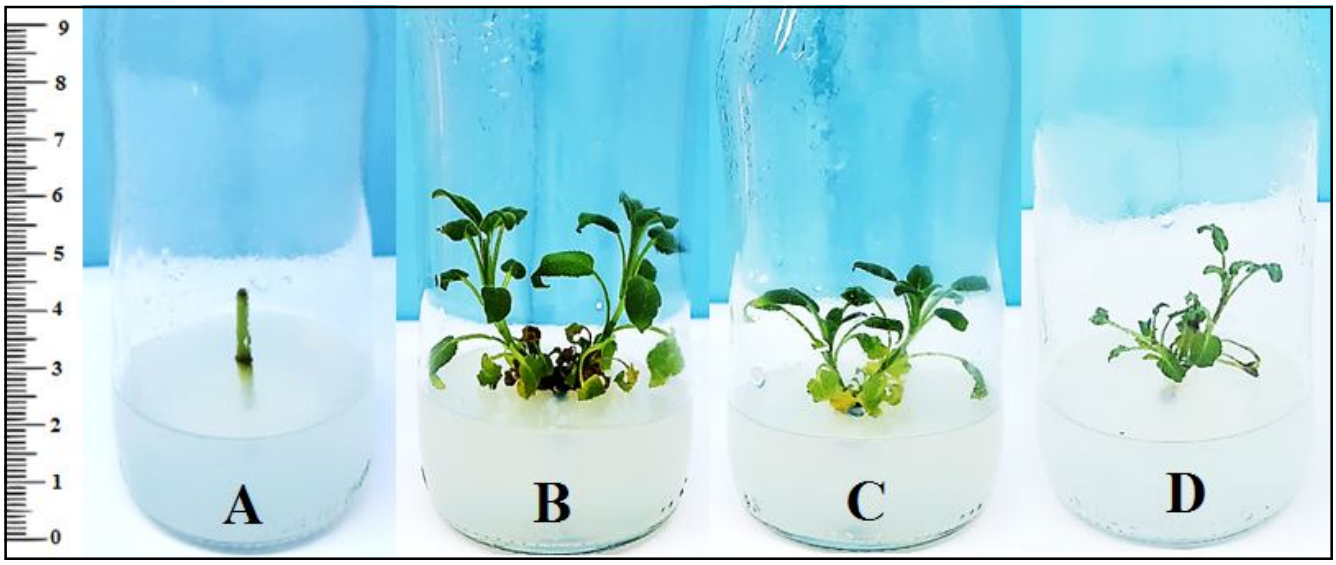

Fig. 1: Morphological features of *S. officinalis* L. shoots grown in 20 X 5 cm test tubes containing MS medium with different concentrations of BA incubated at 25 C° ± 3 C° for 30 days. A. Without BA as a control. B. With 1mg/l of BA. C. With 2mg/l of BA. D. With 3mg/l of BA.

that the used of BA at concentration of 15 µM results in highest number of shoot buds. Addition of different concentrations of BA for shoots regenerations of *Zingiber officinale* was very affected on increasing of the shoots fresh and dry weight (Miri, 2020).

Effect of Silver Nanoparticles on Shoots Fresh and Dry Weight

Shoots fresh and dry weights were determined after 30 days of growing under AgNPs stress. Table 2 and Fig. 2 indicated the presence of a variation in shoots fresh and dry weight mean with different concentrations of AgNPs. It was found that the increase of AgNPs concentrations in medium caused significant increase in shoots fresh and dry weight mean at 10 mg/l, which gave the higher fresh and dry weight mean reached to 5.19 g and 497.9 mg, respectively, compared with the control and other treatments. The other concentrations (20, 40 and 80 mg/l) of AgNPs caused decreasing in shoots fresh and dry weight mean and the lowest shoots fresh and dry weight mean was in 80 mg/l of AgNPs reached to 1.43 g and 126.8 mg, respectively. AgNPs have been used to enhance the plant growth, improve bioactive compound production and plant genetic modification (Kim *et al.,* 2017; Ruttkay-Nedecky *et al.,* 2017). AgNPs can improve the plants growth when the plant cultivated on nutrient medium supplemented with AgNPs due to the improvement efficiency uptake of nutrient from medium by plant (Jhanzab *et al.,* 2015). AgNPs can also induce stress on plant cultured on nutrient medium supplemented with AgNPs, but at mild stress will causes induction of reactive oxygen species (ROS) which leading to the activation of antioxidant defenses (Poschenrieder *et al.,* 2013). AgNP causes increase macronutrient and micronutrient contents in plant, subjected with different concentrations of AgNP such as N, Mg, N, Mg, and Fe. The increase of important nutrients such as N, Mg and Fe lead to increase the plant growth rate because these elements are associated with the biosynthesis of chlorophyll, an essential molecule of photosynthesis during plant growth (Bello *et al.,* 2017). Homaee and Ehsanpour (2015) found that the area of leaf, dry weight and root length were improved at 2 mg/l of AgNPs. The fresh weight, total chlorophyll, chl-a and chl-b were increased at 50 mg/l of AgNPs. The plant-based AgNPs have a role in increasing of dry weight of aerial structures (Ngo *et al.,* 2014). The dry weight of wheat exposed to different concentrations of AgNPs was increased with the increasing of concentrations of AgNPs and decreased at high concentrations. (Iqba *et al.,* 2017).

Effect of Silver Nanoparticles on Catalase Activity

The maximum catalase activity was showed in MS medium supplemented with 20 mg/l of AgNPs which

Table 2: Effect of AgNPs on fresh weight, dry weight, catalase activity, SOD activity and 1,8- cineole content of *in vitro* shoots of *S. officinalis* L.

1,8- cineole content (ppm) (Mean ± SE)	SOD activity (Unit/ml) (Mean ± SE)	Catalase activity (Unit/ml) (Mean ± SE)	Dry weight (mg) (Mean ± SE)	Fresh weight (g) (Mean ± SE)	AgNPs conc. (mg/l)
9.163 ± 0.096	0.113 ± 0.0066	9.243 ± 0.187	325.5 ± 15.696	3.55 ± 0.169	0
18.018 ± 2.520	0.123 ± 0.0176	10.853 ± 0.274	366.5 ± 7.591	3.85 ± 0.075	5
32.957 ± 2.089	0.143 ± 0.0185	13.263 ± 0.159	497.9 ± 9.120	5.19 ± 0.092	10
72.976 ± 5.599	0.223 ± 0.0223	15.763 ± 0.635	342.1 ± 11.378	3.31 ± 0.111	20
50.094 ± 4.301	0.303 ± 0.0212	8.173 ± 0.165	202.8 ± 15.503	2.22 ± 0.150	40
7.936 ± 0.127	0.363 ± 0.0232	6.403 ± 0.142	126.8 ± 7.690	1.43 ± 0.745	80
19.586	0.120	1.929	66.2	0.67	L.S.D. (0.05)

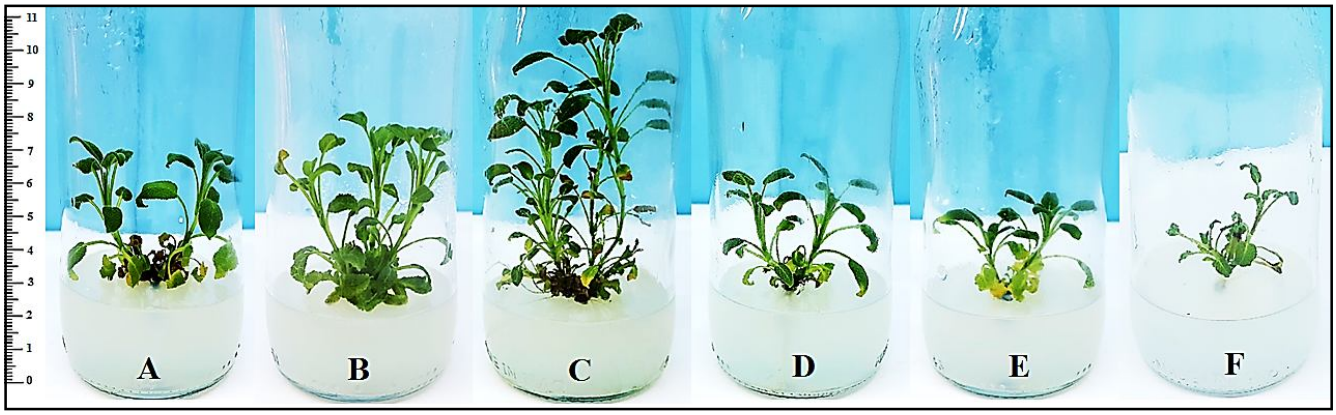

Fig. 2: Morphological features of *S. officinalis* L. shoots grown in 20 X 5 cm test tubes containing MS medium with 1mg/l of BA and different concentrations of AgNPs incubated at 25 C° ± 3 C° for 30 days. A. Without AgNPs as a control. B. With 5 mg/l of AgNPs. C. With 10 mg/l of AgNPs. D. With 20 mg/l of AgNPs. E. With 40 mg/l of AgNPs. F. With 80 mg/l of AgNPs.

reached to 15.763 unit/ml, differed significantly with control treatment (0 mg/l) and all the treatments table 2. While, the lowest catalase activity was found in shoots grown with high AgNPs concentration (80 mg/l) which reached to 6.403 unit/ml, differed significantly compared with all treatments. Antioxidant enzymes such as catalase play an important role in scavenging H_2O_2 and converted it into water and oxygen (Rastogi *et al.,* 2017). The first defense line of plant in responding to stress is converting of O^{2-} to H_2O_2 and then convert H_2O_2 by antioxidant enzyme (catalase and ascorbate peroxidase) into oxygen and water (Lokhande and Suprasanna, 2012). AgNPs have phytotoxic property on plant cells causes increasing the production of antioxidants, the catalase activity increased with the increasing of concentrations of AgNPs in plant which treated with 1- 5 mM of AgNPs. An increase in catalase activity indicates an increased amount of hydrogen peroxide (zivcak *et al.,* 2019). The activity of catalase was decreased at high concentration of AgNP due to ability of AgNP and their precursor to interact with thiol moieties of CAT and altered the CAT structure, which led to inhibition of its functioning and decreased the CAT activity (Khan *et al.,* 2019).

Effect of Silver Nanoparticles on Superoxide Dismutase Activity

The results indicated to presence of significant differences (L.S.D. = 0.120) ($p \leq 0.05$) in superoxide dismutase activity among different concentrations of AgNPs table 2. The increase of AgNPs concentrations in medium caused a significant increase in superoxide dismutase activity. While, the highest superoxide dismutase activity was found with the treatment of 80 mg/l of AgNPs that reached to 0.363 unit/ml which differed significantly with the control. The antioxidant defense system responsible for scavenging of ROS which can be activated to alleviate nanomaterial induced toxicity in plants. Superoxide anion (O_2^-) radical content was regulated by SOD (Mittler *et al.,* 2004), It is converted in to less toxic oxygen (O_2) and hydrogen peroxide (H_2O_2) by SOD which represent the first enzyme defense that catalyze the ROS (Ma *et al.,* 2015). This results agreed with the results found by (Khan *et al.,* 2019), and (Ali *et al.,* 2019), who observed the increased of SOD activity at various doses of AgNPs. Shakeran *et al.,* (2015) demonstrated that the increase of exposure of *Spirodela polyrhiza* to AgNPs cause increasing of the activity of SOD enzyme and the magnification of the scavenging process of ROS.

Quantitative Determination of 1,8- Cineole Using GC-MS under AgNPs Stress

The results of GC-MS indicated the presence of monoterpenoid 1,8-cineole compound in the samples. table 2 showed the concentration of 20 mg/l of AgNPs encouraged the plant to produce of 1, 8-cineole more than of control treatment which reached to 72.976 ppm dry weight and represent the higher amount of 1, 8-cineole which differed significantly with the control and all treatments. While the lowest concentration of 1, 8-cineole was with the concentration of 80 mg/l of AgNPs which reached to 7.936 ppm dry weight. Fig. 3 showed the peak of 1, 8- cineole standard solution and the peak of 1, 8-cineole of shoots which cultured on MS medium with the concentration of 20 mg/l of AgNPs. Nano metal particles have shown high capacity for attaching to plant tissues and activate enzymatic pathways responsible for the production of secondary metabolites (Shakeran *et al,* 2015). They also contribute to the peroxidation of cellular membranes in plant cells and influence the expression of genes responsible for the production of biologically active compounds (Raei *et al.,* 2014). The additions of AgNPs increase the production of secondary metabolites of the cultures of *Artemisia annua* (Zhang *et al.,* 2013), *Brugmensia candida* (Jamshidi *et al.,* 2016) and *Prunella vulgaris* (Fazal *et al.,* 2014). Hatami *et al.*, (2016) and Ghanati and Bakhtarian (2013) showed that

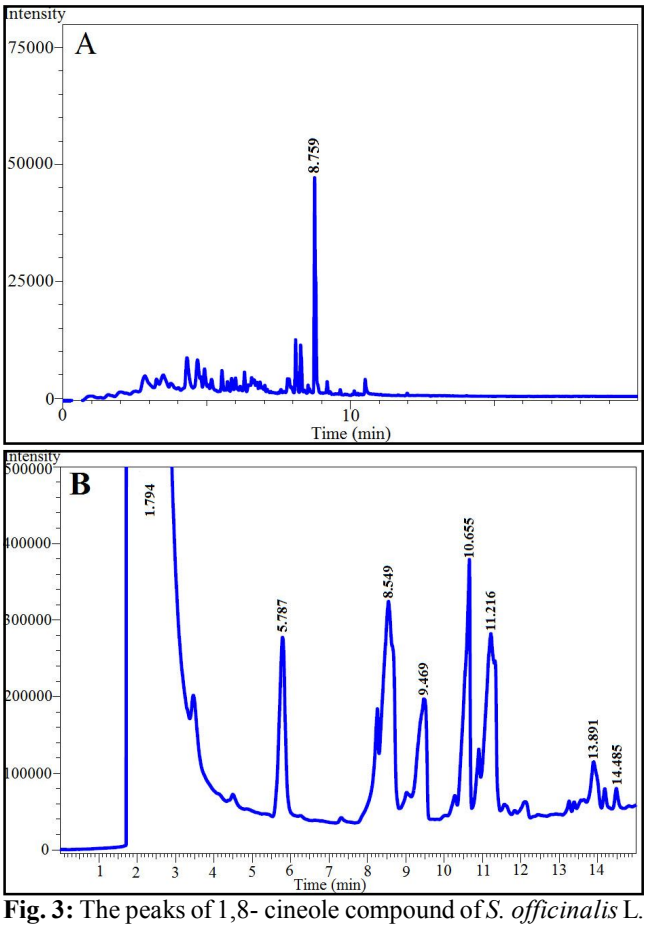

Fig. 3: The peaks of 1,8- cineole compound of *S. officinalis* L. using GC- MS under AgNPs stress. A. The peak of 1,8- cineole standard solution. B. The peaks of 1,8-cineole and other secondary metabolites in shoots cultured on MS medium with 20mg/l of AgNPs.

the application of metal nanoparticles to plants growing under natural conditions cause the change in the essential oil content extracted from their tissues. Ali *et al.,* (2019) Noticed that the callus cultures of *Caralluma tuberculata* grown under different AgNPs levels stress resulted in progressive decrease of total flavonoid contents and phenylalanine ammonia lyase content. The reason for reduction of 1, 8-cineole compound under high level of AgNPs may be due to the influence of some enzyme included in the biosynthesis of essential oils. While the increase production of 1, 8-cineole under low level of AgNPs may be due to that the essential oils that have a potential to remove of free radicals which would preserving the cells from damage, so the plant may resort to increase the production of essential oil under low level of AgNPs (Anjum *et al.,* 2019). These results showed that the benzyl adenine had the ability to formation of shoots *in vitro* from the nodes explant of *S. officinalis* L. The low levels of AgNPs revealed the positive effect on *in vitro* growth of *S. officinalis* L., but it had inhibition effects at high levels. The secondary metabolite contents and antioxidant system of *in vitro* shoots of *S. officinalis* L. were enhanced with the application of AgNPs.

Conclusions

Plant growth regulator (benzyl adenine) causes stimulation *in vitro* shoots formation of *S. officinalis* L. from nodes explant at different concentrations (1, 2 and 3 mg/l). The application of AgNPs on *in vitro* shoots of *S. officinalis* L. proved their effects on growth, antioxidant system and secondary metabolites production. AgNPs at low concentration (10 mg/l) stimulates the growth of *S. officinalis* L. shoots, but at high concentrations (80 mg/l) caused inhibition to the growth of *S. officinalis* L. shoots. The antioxidant system of *in vitro* shoots of *S. officinalis* L. was affected by the addition of AgNPs to the medium. AgNPs enhance the activity of catalase enzyme and superoxide dismutase enzyme. The accumulation of secondary metabolites in shoots of *S. officinalis* L. cultured *in vitro* under AgNPs stress was increased. These findings indicated that there is a role for benzyl adenine in the activation *in vitro* organogenesis of *S. officinalis* L. shoots from nodes explant, in addition to the effect of AgNPs on shoots development, antioxidant system and secondary metabolites production from *in vitro* shoots of *S. officinalis* L. which were increased.

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