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Influence of Ag nanoparticles on physiological and biochemical aspects of callus of *Thymus* species and *Zataria multiflora* Boiss.

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Influence of Ag nanoparticles on physiological and biochemical aspects of callus of *Thymus* species and *Zataria multiflora* Boiss.

Abstract: *Thymus* species have found remarkable importance in food and medicine industries. The present study investigates the potential effect of Ag nanoparticle elicitors on proliferation of callus, and production of carvacrol and thymol in *Zataria multiflora* and three *Thymus* species. Firstly, callus was induced on Murashige and Skoog (MS) medium containing 2 mg l^{-1} of 2, 4-dichlorophenoxy acetic acid (2,4-D) and 1 mg l^{-1} of kinetin (Kin)). Secondly, the effects of two different concentrations of Ag nanoparticles (4 and 8 mg l^{-1}) were studied on callus growth and its secondary metabolites production. Results elucidated that after elicitation by 8 mg l^{-1} of Ag NPs, significantly the highest callus growth rate (CGR) (0.02 mm day^{-1}), callus fresh mass (CFM) (0.99 g), and carvacrol (0.68 mg l^{-1}) and thymol (11.09 mg l^{-1}) content was achieved. Comparing different *Thymus* species, notably the greatest carvacrol and thymol amount was obtained in *.kotschyanus* Boiss. & Hohen. and *T. Daenesis Čelak.* at 8 mg l^{-1} concentration of Ag NPs. Hence, it is evident that the stimulation by NPs is dose-dependent. This study has potential to be commercially applied for the enhancement of pharmaceutical compounds in different species of *Thymus*.

Key words: Ag nanoparticles; *Thymus* species; *Zataria multiflora*; callus; carvacrol thymol

Abbreviations: 2,4-D, 2, 4-dichlorophenoxyacetic acid; Kin, kinetin; PGRs, plant growth regulators; HPLC, high performance liquid chromatography; NPs, nanoparticles

Vpliv nanodelcev srebra (Ag) na fiziološke in biokemične lastnosti kalusa dveh vrst materine dušice (*Thymus* sp.) in vrste *Zataria multiflora* Boiss.

Izvleček: Vrste iz rodu materine dušice (*Thymus* sp.) imajo velik pomen v prozivodnji hrane in zdravil. V raziskavi je bil preučevan potencialni učinek nanodelcev srebra kot eliciatorja na rast kalusa, tvorbo karvakrola in timola pri vrsti *Zataria multiflora* in treh vrstah materine dušice. Najprej je bil na Murashige in Skoog (MS) gojišču, ki je vsebovalo 2 mg l^{-1} 2,4-D in 1 mg l^{-1} kinetina, vzgojen kalus. Potem je bil preučevan učinek dveh različnih koncentracij srebrovih nanodelcev (4 in 8 mg l^{-1}) na rast kalusa in in tvorbo sekundarnih metabolitov. Rezultati, pridobljeni z visokotlačno tekočinsko kromatografijo (HPLC) so pokazali, da je bila po uporabi 8 mg l^{-1} srebrovih nanodelcev kot iliciatorjev dosežena značilno največja rast kalusa (CGR) (0,02 mm dan^{-1}), največja sveža masa kalusa (CM) (0,99 g) in največja vsebnost karvakrola (0,68 mg l^{-1}) in timola (11,09 mg l^{-1}). V primerjavi različnih vrst materine dušice je bila dosežena največja vsebnost karvakrola in timola pri vrstah *T. kotschyanus* Boiss. & Hohen in *T. daenesis* Čelak pri koncentraciji srebrovih nanodelcev 8 mg l^{-1}. Očitno je, da je stimulacijski učinek nanodelcev odvisen od doze. Izsledke raziskave bi lahko komercialno uporabili za povečanje tvorbe zdravilnih spojin pri različnih vrstah materine dušice.

Ključne besede: Ag nanodelci; vrste iz rodu *Thymus*; *Zataria multiflora*; kalus; karvakrol; timol

Okrajšave: 2,4-D: 2, 4-diklorfenoksi ocetna kislina; Kin: kinetin; PGRs: rastlinski rastni regulatorji; HPLC: visokotlačna tekočinska kromatografija; NPs: nanodelci

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1 INTRODUCTION

Nanobiotechnology has enormous applications in diverse fields including agriculture, cosmetics, pharmaceutics, and food industry) Kim et al., 2017; Rastogi et al., 2017). Nanoparticles (NPs) are employed as elicitors of various cell signaling pathways in metabolism of plants (Kim et al., 2017; Marslin, et al., 2017). NPs have been found to play crucial role in enhancement of plant secondary metabolites (SMs) by imposing oxidative stress and increasing cell membrane permeability (Jasim et al., 2017; Marslin et al., 2017; Ahmad et al., 2020). The effects of NPs on growth rate of plants (Sharma et al., 2012), germination of seeds (Zaka et al., 2016), production of SMs (Marslin et al., 2017; Mosavat et al., 2019; Zaka et al., 2016; Golkar et al., 2021) and plant physiology (Jasim et al., 2017; Sharma et al., 2012) have been studied recently. Although there are few studies related to effect of NPs on callus culture development, physiology and secondary metabolism (Dykman & Shchyogolev, 2017; Kokina et al., 2013; Marslin et al., 2017; Sanzari et al., 2019; Zuverza-Mena et al., 2017), still this research domain needs to be explored further. Silver (Ag) NPs possess unique properties in terms of toxicity and alteration of yield, development, antioxidant activities, and SMs production of plants due to their high catalysis and reactivity (Rastogi et al., 2017; Sadak 2019). Furthermore, the influence of Ag NPs on the callus cultures of *Solanum nigrum* L. (Ewais et al., 2015), *Prunella vulgaris* L. (Fazal et al., 2019) and *Caralluma tuberculata* N.E. Brown (Ali et al., 2019) has recently been studied.

Thymus L., belonging to Lamiaceae family, has world-wide distribution (Sajed et al., 2013). However, it is dominantly present in Asia, Europe and North Africa (Zarshenas & Krenn, 2015). The essential oils and SMs enhance the commercial value of flowers and leaves of *Thymus* making it a valuable crop in cosmetics, pharmaceutics, and food and agriculture industry (Miraj & Kiani, 2016). A thyme-like plant, *Zataria multiflora* Boiss., belonging to Lamiaceae family, is wild plant found in only southern and central Pakistan, Afghanistan, and Iran (Sajed et al., 2013). Tissue culture propagation of thyme plant is well-known because it is a wide source of ingredients of pharmacology. Both *Thymus* sp. and *Z. multiflora* possess anti-cancerous, anti-inflammatory, anti-oxidant, anti-bacterial, anti-fungal, and anti-spasmodic properties (Mathela et al., 2010; Sajed et al., 2013). Naturally occurring terpenoid thymol (2-isopropyl-5-methyl phenol) and its phenol isomer, carvacrol/cymophenol are the phenolic compounds that play an important role in inducing these properties to *Thymus* sp. and *Z. multiflora* (Kianersi et al., 2021; Mathela et al., 2010). Some other properties like their use as additive in perfumes, deodorant, toothpastes, soaps, etc. and as important flavoring agent in foods are also attributed to these compounds (Sajed et al., 2013).

The defense system of plants is activated by chemical, biological or physical elicitors (Asadollahei et al., 2022; Zhao et al., 2005). The gene expression is then modulated that transcribes the formation of SMs (Ajungla et al., 2009). A more efficient method could be the use of callus cultures for extracting SMs naturally from *Thymus* sp. and *Z. multiflora* in a sustained manner (Ramakrishna & Ravishankar, 2011). Previously, there is no report concerning the carvacrol and thymol production by imposing Ag NPs in cell cultures of *Thymus* sp. and *Z. multiflora.* Hence, the current study investigates proliferation of callus and SMs production in three *Thymus* species, i.e., *T. vulgaris* L., *T. daenensis Čelak,* and *T. kotschyanus* Boiss. & Hohen as well as *Z. multiflora* Boiss. after Ag NPs exposure.

2 MATERIALS AND METHODS

The seeds of *Thymus* species, i.e., *T. vulgaris, T. daenensis, T. Kotschyanus* and *Zataria multiflora* (two accessions) (Table 1) were deposited at Botanic Herbarium of Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology (IUT), Isfahan, Iran, after collection from different geographical regions and identification by using Flora Iranica (Rechinger, 1982). Their characteristics is shown in Table 1. The seeds of four different species were surface sterilized with 70 % (v/v) ethanol for 1 min, followed by the addition of 3 % (v/v) sodium hypochlorite for 20 min, and then rinsing in sterile distilled water thrice. After surface sterilization, the seeds were grown in Murashige and Skoog (MS) (1962) medium (Duchefa, Netherland). These were incubated for germination and growth of plantlets. The leaflet explants from about 1-month old plantlets were cultured in MS medium containing 2,4-D (2 mg l^{-1}) and Kin (1 mg l^{-1}) supplemented with 3 % (w/v) sucrose (Sigma-Aldrich, USA), 0.8 % (w/v) agar (Sigma-Aldrich, USA) and 0.1 mg l^{-1} myoinositol for callus induction. The pH was adjusted at 5.7. The samples were exposed to 16h/8h (light/dark) photoperiod for a period of 2 months at 23 ± 2 °C.

Silver (Ag) nano-powder was purchased from US Research Nanomaterials Inc., Houston, TX, USA having an average size of 30–50 nm and purity of 99.99 %. The nanoparticles were characterized by x-ray diffraction (XRD) and scanning electron microscopy (SEM) techniques by following the protocols of Javed et al. (2016). XRD was performed using Carlo ERBA Model EA 1108 analyzer and the instrument for getting SEM image was

Table 1: The geographical origins of *Thymus* sp. and *Z. multiflora* with their geographical traits collected from Iran

Species	Abbreviation	Origin	Genotype code	Latitude (m)	Longitude (m)	Altitude (m)
Zataria multiflora (1)	*Zm* (1)	Dehbala, Yazd, Iran	RIBB/ZM01/2016	31°59' N	54°11' E	2600
Zataria multiflora (2)	*Zm* (2)	Abadeh, Fars, Iran	RIBB/ZM02/2016	31°45' N	51°21' E	2030
Thymus vulgaris	Tv	Marvdasht, Fars, Iran	RIBB/TV01/2016	35°56' N	52°10' E	1620
Thymus daenensis	Td	Aligoodarz, Lorestan, Iran	RIBB/TD01/2016	33°24' E	49°41' E	2022
Thymus kotschyanus	Tk	Lahijan, Gilan, Iran	RIBB/TK01/2016	37°12' N	50°14' E	396

Hitachi S4800 (Japan). These NPs were added to MS medium after filter sterilization. The 2-months old friable callus (0.25 g) was transferred to MS containing 2,4-D (2 mg l^{-1}) and Kin (1 mg l^{-1}) under Ag NPs stress of 4 and 8 mg l^{-1}. This callus material was placed at 23 ± 2 °C under a photoperiod of 16h/8h. After incubation for 21 days of callus with Ag NPs, the callus growth rate (CGR) and callus fresh mass (CFM) was calculated. CGR was measured according to Afshar et al. (2016) every 7 days in 21 days period.

Later on, the quantity of carvacrol and thymol was obtained by high performance liquid chromatography (HPLC). The method of Castro et al. (2016) was utilized for preparing callus extracts. The process involved drying of 200 mg of callus from each treatment in an oven at 50 °C for 24 h, and then soaking it in 5 ml of diethyl ether for a period of 24 h. In order to prevent the evaporation of diethyl ether, the vials were kept closed and extraction was performed in a cold room. After adding the 80 % of methanol (1 ml) to remaining solid material, the extracts were filtered (0.22 µm pore size) into clean vials and prepared for injection to HPLC instrument. The HPLC (SY-8100 series, Beijing Beifan-Ruili Analytical Instrument, China) was performed by UV-VIS detector, a flow rate of 0.9 ml min-1, injection volume of 20 µl at 28 °C, C18 column (25 cm × 4.6 mm, partial size 5 µm), mobile phase methanol-water (80:20; v/v), and flow rate of 0.9 ml min-1. The detection was carried out at 280 nm of wavelength and a pressure of 12 atm. The UV spectra of phenolic compounds were recorded at 280 and 320 nm. The content of carvacrol and thymol were determined based on the calibration curve of standard compounds, including carvacrol (Sigma-Aldrich, USA) and thymol (Sigma-Aldrich, USA). For this purpose, 4 concentrations (10, 25, 50, and 100 mg l^{-1}) of carvacrol and 3 concentrations (25, 100, and 400 mg l^{-1}) of thymol were examined by HPLC. The retention time for carvacrol and thymol were appeared at 3-4 min and 14-15 min, respectively. After calibration of the standards with HPLC, the quantities of carvacrol and thymol in different samples were calculated.

2.1 STATISTICAL ANALYSIS

The experimentation was conducted with three replications in completely randomized design and the statistics was determined using two-way analysis of variance (ANOVA). LSD test ($p \leq 0.05$) in SAS software (SAS 9.1 Inc. USA) was applied to determine significant difference among the treatments.

3 RESULTS AND DISCUSSION

Callus growth and development as well as formation of secondary metabolic compounds is positively or negatively influenced by supplementing the growth medium with abiotic or biotic stress elicitors (Ajungla et al., 2009; Zaka et al., 2016). The contents of SMs show significant changes under elicitation of callus by different stresses (Fazal et al., 2016; Mosavat et al., 2019; Sanzari et al., 2019). NPs, specifically the metallic oxide NPs like ZnO, CuO, TiO_2 act as oxidative abiotic stress elicitors (Lala 2021). According to Al-jibouri et al. (2012), thymol amount was increased by proline in *Origanum vulgare* L. Similarly, the production of significantly the highest content of hyperforin in *Hypericum perforatum* L. (Sharafi et al., 2013), rebaudioside A and stevioside in *Stevia rebaudiana* Bertoni (Javed, et al., 2018), and proline in *Triticum aestivum* L. (Barbasz, et al., 2016) under ZnO NPs elicitation has been previously documented.

Ag NPs show significant effect on the production of SMs in callus culture, resulting in their ultimate increase (Fazal et al., 2016). For instance, Ali et al. (2019) reported that various concentrations of Ag NPs significantly affected the callus proliferation and substantially increased the callus biomass and SMs in *Caralluma tuberculata*. In another study, Fazal et al. (2019) reported the positive effects of Ag NPs and Au NPs on the production of biomass and SMs in the cell culture of *Prunella vulgaris* L.

XRD of Ag NPs is given in Figure 1 which shows 100 % phase purity by the sharpness of peaks. Similar XRD pattern was obtained by Kim et al. (2006). The

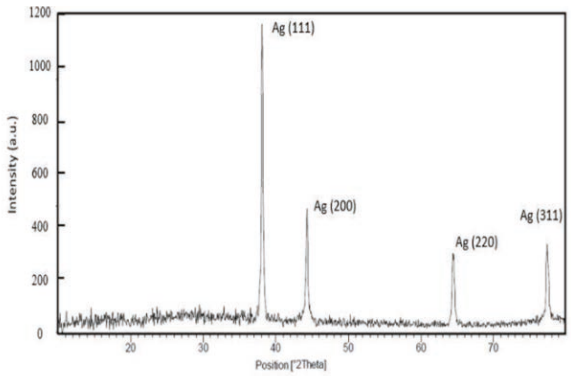


Figure 1: X-ray diffractogram (XRD) of Ag nanoparticles

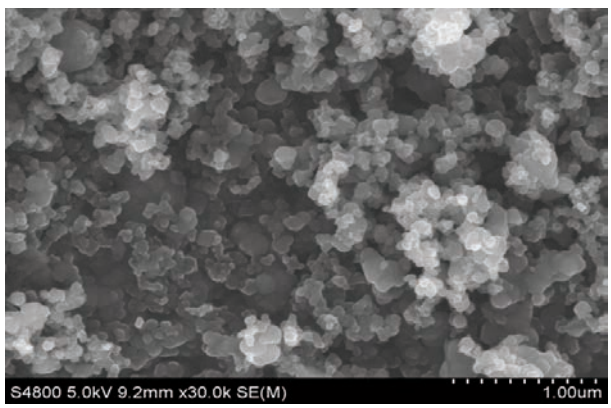
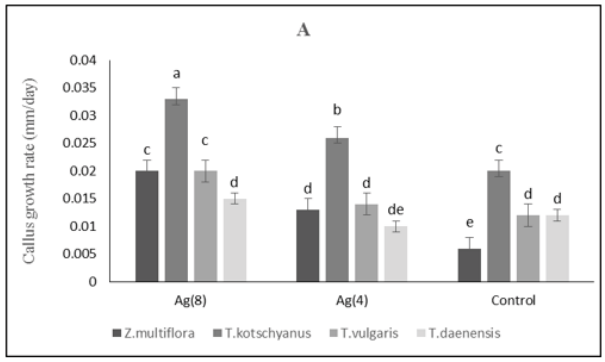


Figure 2: Scanning electron micrograph (SEM) of Ag nanoparticles



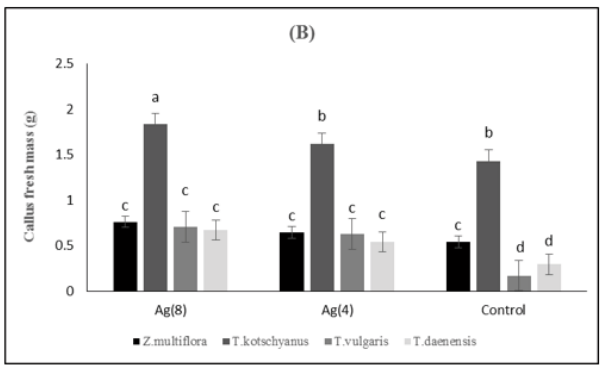


Fig 3: Effect of Ag nanoparticles on callus growth rate (A) and callus fresh mass (B) content of different *Thymus* species and *Z. multiflora* under callus culture

spherical shape of Ag NPs was illustrated by SEM image given in Figure 2 which is coinciding with the results of Elumalai et al. (2010).

The size, shape, surface, concentration and chemical composition of NPs cause stimulatory or inhibitory effects on the growth of callus cells (Al-Jibouri et al., 2012). The synthesis and accumulation of SMs in cells is enhanced by an increased surface area of NPs as a result of reduced size and transport of NPs in to the cells apoplastically which increases electrostatic interactions between living cell membranes (Javed et al., 2017). Table 2 and Figure 3 shows the effect of Ag NPs on callus growth of *Thymus* sp. and *Zataria multiflora*.

Compact calli with white and greenish colour were obtained after 10 days upon control culture (no AgNPs), whereas friable watery calli with white, greenish or yellowish colour were observed after 10-13 days upon culture supplemented with AgNPs (Figure 4.)

A significant effect is produced on callus traits by different concentrations of Ag NPs in this study that is coherent with the reports about effects of Ag NPs on

Table 2: Effect of different concentrations of Ag NPs on color, texture, growth rate, and fresh mass of callus cultures of *Thymus* sp. and *Zataria multiflora*

Nanoparticle	Concentration (mg l^{-1})	Color and Texture	CGR[I] (mm day^{-1})				CFM [II] (g)
			7	14	21	Mean	
Ag	8	Green, friable	0.045	0.06	0.015	0.022 [a]	0.99 [a]
Ag	4	Green, friable	0.07	0.06	0.032	0.015 [b]	0.85 [ab]
Control	-	White to yellow, soft	0.02	0.01	0.006	0.012 [c]	0.61 [b]

Mean values followed by the same letter in each column are not significantly different at $p<0.05$ (Least Significant Difference Test). I: CGR: Callus growth rate, II: CFM: Callus fresh mass

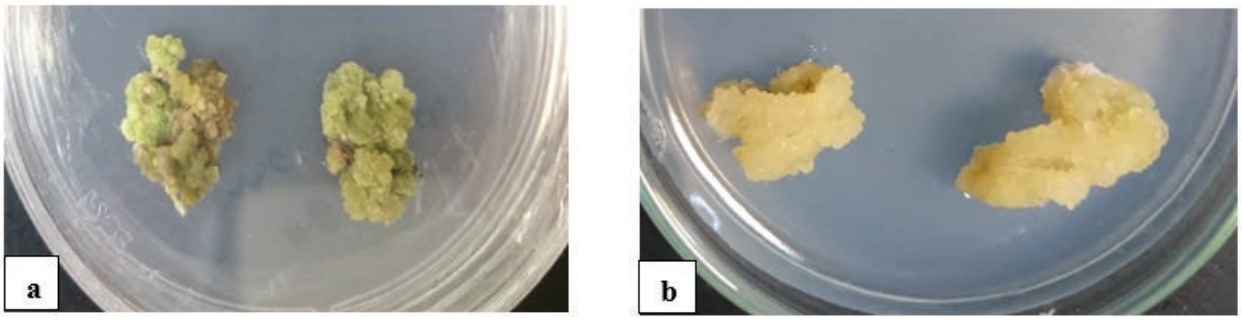


Fig 4: Friable calli with greenish or yellowish colour of *Thymus* species and *Z. multiflora* after culture treatment with $AgNP_s$ (a) and under control culture (b)

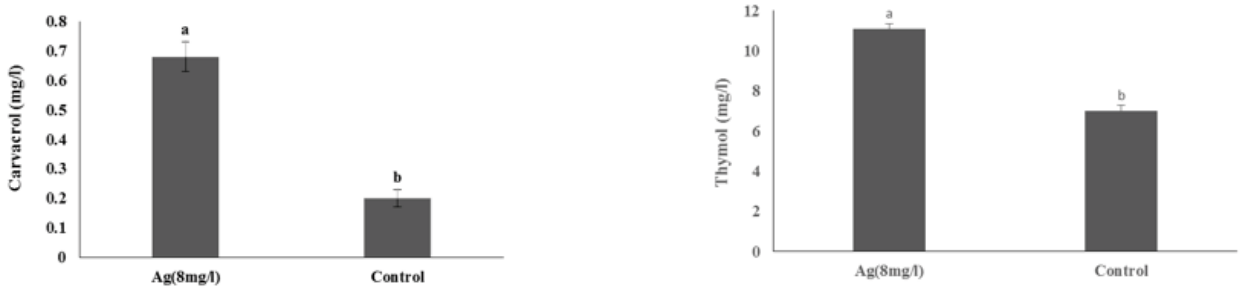


Fig 5: Effect of 8 mg l^{-1} Ag NPs on *in vitro* production of carvacrol and thymol in *Thymus* sp. and *Zataria multiflora*

callus culture of *Solanum nigrum* L. (Ewais et al., 2015) which gain friable watery calli with greenish or yellowish colour were observed after 10-13 days upon culture supplemented with AgNPs and effects of TiO_2 NPs on the callus of *Hordeum vulgare* L. (Mandeh et al., 2012). The higher TiO_2 NPs concentration influenced callogenesis of *Hordeum vulgare* explants in this study. Also according to Kokina et al. (2013) elicitation with Ag and Au NPs shows positive effects on callus width and length in *Linum usitatissimum* L. The positive effects of different NPs on callus growth of *Prunella vulgaris* L. is reported by Fazal et al. (2019). Callus growth traits affected by ZnO NPs in *Solanum lycopersicum* Mill. have also been reported (Alharby et al., 2016).

The effects of elicitation by Ag NPs on production of carvacrol and thymol under *in vitro* conditions are presented in Figure 5. The production of thymol and carvacrol was determined at 8 mg l^{-1} concentration of Ag NPs and control treatment. The carvacrol (0.68 mg l^{-1}) and thymol (11.09 mg l^{-1}) quantity was enhanced under 8 mg l^{-1} of Ag NPs. The chromatographic separation of the methanolic extracts in *Zataria multiflora* for carvacrol and thymol by HPLC is given (Figure 6). Asadollahei et al. (2022) employed different concentrations of CuNPs in *in vitro* culture medium and observed significant rise in thymol and carvacrol content compared to control in *Zataria multiflora*. This study elucidated that the selection of appropriate plant species and suitable elicitor is crucial for increasing the production of bioactive compounds as well as antioxidants of *Zataria multiflora*. This can be done by inducing expression changes in the biosynthetic pathways of thymol and carvacrol. In fact, the gene expression patterns of the pathways of formation of thymol and carvacrol were greatly influenced by the Ag NPs in our study which is the phenomenon well explained by the studies of Kianersi et al. (2021).

The interactive effects of NPs and genotypes/species of *Thymus* and *Zataria multiflora* for production of SMs

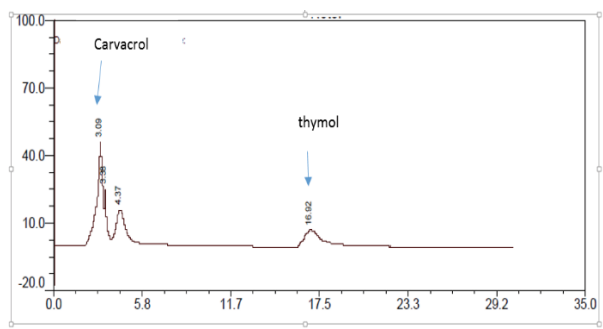


Fig 6: Representative HPLC chromatograms of thymol and carcacrol of *Z. multiflora*. Peak identifications were performed by matching retention time and UV spectra against commercially available reference compounds

have been presented in Table 3. The exposure of Ag NPs has significantly increased the content of two SMs of callus compared to control. Moreover, *in vitro* synthesis of thymol was notably greater than that of carvacrol which is also evident from the molecular studies of Kianersi et al. (2021). The highest content of carvacrol (1.06 mg l^{-1}) was observed at 8 mg l^{-1} concentration of Ag NPs in *T. kotschyanus*, whereas the least amount (0.10 mg l^{-1}) was observed in control treatment of *T. daenensis*. Furthermore, the highest concentration of thymol was obtained at 8 mg l^{-1} of Ag NPs in callus of *T. daenesis* (19.75 mg l^{-1}), while the least thymol content (3.95 mg l^{-1}) was achieved in *T. daenesis* under control condition.

This result can be well supported by the phenomenon that NPs trigger thymol synthetic pathways and/or transcription factors more than the carvacrol pathways (Mosavat et al., 2019). Taking into account of concurrent studies, the phenolics and flavonoids production is activated by ZnO NPs in seedlings of *Brassica nigra* L. (Zafar et al., 2016) Additionally, the significant rise in hyperforin content in cell suspension culture of *Hypericum perfolatum* L. under ZnO NPs stress is reported (Sharafi et al., 2013). A complex variety of elicitation effects on *in vitro* synthesis of SMs is obtained using different types of elicitors (Goswami et al., 2017; Marslin et al., 2017; Syu et al., 2014), plant tissues (Ajungla et al., 2009), and physiochemical environment of various species (Shakya et al., 2019).

4 CONCLUSION

The formation of callus from *Thymus* species and *Zataria multiflora* was performed in the presence of Ag NPs elicitors. Addition of abiotic elicitors, i.e., Ag NPs (8 mg l^{-1}) to the MS medium played a vital role in enhancing the thymol and carvacrol content in the callus cultures of different *Thymus* species and *Zataria multiflora*. In other words, Ag nano-elicitors applied to the *in vitro* callus cultures of *Thymus* species and *Zataria multiflora* in our study resulted in increase in SMs production at a concentration of 8 mg l^{-1}. Our finding opens the way for studies involving relationship between chemical elicitors and formation & accumulation of thymol/carvacrol. In future, transcriptomic and metabolomics studies should be performed to elucidate the regulation of SMs production under an elicitation of Ag NPs in these medicinal plants.

Table 3: The effect of species × NPs interaction on *in vitro* production of thymol and carvacrol in callus culture of *Thymus* species and *Z. multiflora*

Species	Nanoparticles (mg l^{-1})	Thymol (mg l^{-1})	Carvacrol (mg l^{-1})
Z. multiflora	Ag (8)	$12.76^{b} \pm 0.02$	$1.05^{a} \pm 0.03$
	Control	$7.06^{g} \pm 0.01$	$0.20^{bc} \pm 0.01$
T.kotschyanus	Ag (8)	$7.90^{e} \pm 0.01$	$1.06^{a} \pm 0.04$
	Control	$7.39^{f} \pm 0.01$	$0.26^{b} \pm 0.01$
T. vulgaris	Ag (8)	$11.06^{c} \pm 0.04$	$0.29^{b} \pm 0.01$
	Control	$9.64^{d} \pm 0.04$	$0.10^{c} \pm 0.002$
T. daenesis	Ag (8)	$19.75^{a} \pm 0.02$	$0.31^{b} \pm 0.01$
	Control	$3.95^{h} \pm 0.03$	$0.23^{bc} \pm 0.02$

Each value represents Mean ± SE. Mean values followed by the same letter are not significantly different at $p < 0.05$ (Least Significant Difference Test)

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6 DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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