Nitric Oxide Increased the Rosmarinic Acid and Essential Oil Production in *In vitro*-cultured *Melissa officinalis*

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Abstract

Background: Nitric oxide is a free radical that recently has emerged as a key signaling molecule in regulating important physiological processes in plants.

Objective: In this work, sodium nitroprusside (SNP) was utilized as the donor of nitric oxide to investigate the effects of exogenous nitric oxide on essential oil, rosmarinic acid and antioxidant activity of *in vitro*-cultured *Melissa officinalis* plants.

Methods: The Plantlets were treated with different concentrations of SNP (0, 5, 10 and 20 mM) at eight leaf stages.

Results: The results showed that SNP decreased trans-caryophyllene and other selected essential oils in *M. officinalis* plants, while increased most of major components of essential oils. The highest content for linalool, neral and thymol was achieved at 5, 5 and 20 mM of SNP respectively. The highest monoterpene (56.17 % v/w) and sesquiterpene content (76.01 % v/w) were achieved by 5 and 20 mM SNP, respectively. In addition, the class of essential oil compounds varied depending on the SNP concentration. Application of SNP on culture media increased rosmarinic acid production and phenolic levels, which in turn improved the antioxidant properties of the extracts.

Conclusion: It seems that nitric oxide elicited *M. officinalis* culture and increased the secondary metabolite production. This current finding open new opportunities for obtaining valuable natural antioxidants for commercial exploitation by using tissue culture systems.

Keywords: ***Melissa officinalis*, Antioxidant activity, Essential oils, Nitric oxide, Rosmarinic acid, Shoot cultures**

Introduction

Lemon balm (*Melissa officinalis* L.) is an aromatic perennial herb that belongs to the Lamiaceae family. It is native to Southern Europe and the Mediterranean region [1]. It has therapeutic properties, such as sedative, carminative, antispasmodic, antibacterial, antioxidant, antiviral and neuroprotective [2, 3, 4]. It is also used for treatment of headache, rheumatism, inflammation and hypersensitivities [5]. Therapeutic properties of lemon balm have been especially related to rosmarinic acid and other phenolic compounds that are found in its essential oil [6]. Furthermore, monoterpenoids and sesquiterpenes including geranial, neral, 6-methyl-5-hepten-2-one, citronellal, geranyl-acetate, caryophyllene, caryophyllene-oxide and 1,8 cineole are regarded as potentially active components of lemon balm [7].

In vitro culture of plant is a potential renewable source of valuable medicinal compounds [8, 9]. The principle advantages of this technology include reliable, simple and more predictable production; rapid and efficient isolation of the phytochemical; avoidance of interfering compounds that occur infield-grown plant [10]. Plant tissue cultures are also widely employed as a model system to investigate the production of specific secondary metabolites because they offer experimental advantages both to basic and applied research and to the development of models with scale-up potential. Additionally, the commercial cultivation of plant tissues represents an alternative source of secondary metabolites, helping to save the genetic diversity of the wild population of medicinal plants. Rapid clonal propagation of *M. officinalis* for obtaining essential oils and rosmarinic acid is necessary; therefore, traditional methods are not efficient. So, tissue-culture can be an effective alternative for mass production of selected genotypes. However, *M. officinalis* grow easily, but this population is not homozygote, more ever top one-third part of it, is contained essential oils, so cultivated in farm (traditional method) is not economically [11]. Numerous strategies have been developed to improve the productivity of plant culture such as medium optimization, precursor addition, elicitation, genetic transformation and metabolic engineering [12]. Nitric oxide (NO) is a free radical gas formed endogenously in many biological systems, including animals, plants and microbes. NO has a regulatory role in plant growth, development, defense responses and seed dormancy [13, 14]. Previously, our results showed that Sodium nitroprusside (SNP) decreased growth and protein content and caused oxidative stress in *M. officinalis* seedlings [15]. SNP as the donor of NO was usually added into culture medium directly to promote the accumulation of secondary metabolites production [13]. SNP at high concentrations stimulated catharanthine in *Catharantus roseus* cells [16]. Moreover, treatment with higher concentration (500 µM) of NO has been shown to significantly enhance accumulation of total tannins, saponins, phenols and total flavonoids in *Ginkgo biloba* callus culture [17]. However, little is known about the effect of NO on plant essential oil composition. In this study, we focused on the influence of SNP treatment on essential oil, rosmarinic acid and antioxidant acitivity of *M. officinalis.*

Material and methods

Plant material

The sterilized seeds of *Mellisa officinalis* were cultured on solidified basal MS [18] medium. The cultures were maintained under white light illumination (fluorescent tubes) under about 3000 lx and daily photoperiod of 16 hours at 25 ± 5°C. In each culture glass, 8 seedlings were kept. The uniform eight leaf plantlets grown in MS medium were treated with different concentrations (0, 5, 10 and 20 mM) of filter sterilized SNP (as NO donor). The experiment was conducted with 3 replications. The plantlets were collected ten days after treatment and the aerial parts were dried at room temperature for several days until establishment of weight.

Essential oil isolation procedure

The essential oil was prepared by hydrodistillation for 3 h using a micro Clevenger-type apparatus. The oil was dried over anhydrous sodium sulphate and stored in sealed vials at low temperature (2°C) prior to analysis. Thereafter the essential oil yield was measured.

Gas chromatography

GC analysis was performed using a TRACE GC gas chromatograph equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm). Oven temperature was held at 40°C for 5 min and then programmed to 250°C at a rate of 4°C/min. Injector and detector (FID) temperature were 280°C; N2 was used as carrier gas with a linear velocity of 1.1 ml/min.

The essential oil quantity was enough and there was no need to dilute it.

Gas chromatography–mass spectrometry

GC–MS analyses were carried out on a TRACE MS GC–MS system equipped with a DB-5 fused silica column (60 m × 0.25 mm i.d.). Oven temperature was 40–250°C at a rate of 4°C/min, transfer line temperature 260°C, carrier gas helium with a linear velocity of 1.1 ml/min, split ratio 1/100, ionization energy 70 eV, scan time 0.4 s, mass range 40–460 amu.

Identification of components

The components of the oil were identified by comparison of their mass spectra with those of a computer library or with authentic compounds and confirmed by comparison of their retention indices, either with those of authentic compounds or with data published in the literature [19].

Quantification of total phenol and flavonoid content

Total phenol content was determined spectrophotometrically, according to the Folin-Ciocalteu method, using Gallic acid (0–1000 μM) as the standard and expressing the results as Gallic acid equivalents (GAE) per gram fresh weight [20]. Flavonoid content of each extract was determined by following colorimetric method. Briefly, 20 μL of each extract were separately mixed with 20 μL of 10 % aluminum chloride, 20 μL of 1 M potassium acetate and 180 μL of distilled water, and left at room temperature for 30 min. The absorbance of the reaction was recorded at 415 nm. The calibration curve was prepared by using Rutin

methanolic solutions at concentrations of 12.5 to 100 µg mL^{-1}. Flavonoid content was expressed as mg Rutin equivalents per gram of fresh weight [21].

Determination of rosmarinic acid

Dried leaves of lemon balm were extracted by ethanol. For spectrophotometric analysis of rosmarinic acid in the crude extracts, method based on the complex reaction with Zr^{4+} ions were used. 200 µL of filtered sample and 3 mL of ethanol was mixed. The mixture contained $ZrOCl_2$ in the concentration of 0.5 M. The mixture was incubated for 5 min at room temperature in the darkness. The absorbance of the reaction medium was read at 362 nm (PerkinElmer, Lambda 650). Calibration was carried out with standard of rosmarinic acid and control without rosmarinic acid [22].

DPPH radical scavenging assay

The antioxidant activity of the extracts, based on the scavenging activity of 1, 1-diphenyl-2-picrylhrdrazyl (DPPH) free radical was determined by the method described by Brand-Williams et al. [23]. 100 µL of methanolic extracts was added to 900 µL of 0.1mM DPPH and mixed vigorously. Absorbance at 517nm was determined after 30 min of incubation in dark. The percentage scavenging of the DPPH free radical was calculated using the following equation.

The percentage of scavenging = AO-AT/AO*100 (where AO=Absorbance of DPPH Solution, AT= Absorbance of Test or Reference)

The inhibition curve was plotted for the triplicate experiments and represented as percentage of mean inhibition ± standard deviation and the IC_{50} values were obtained.

Data analysis

Data analysis was performed by T-test using the SPSS software.

Results

Effect of NO doses on dry weight of *M. officinalis*

The results showed that NO decreased growth of *M. officinalis* in a dose-dependent manner. Growth decreased with increasing NO concentration, so that 20 mg /L of SNP reduced the dry weight by 60% (Fig. 1).

Content and composition of essential oil

The color of oil extracted from aerial parts of *M. officinalis* was light yellow. Results showed that SNP treatment can affect the essential oil content in *M. officinalis* shoots. (Fig. 2). The highest oil content (0.21 % v/w) was achieved by 10 mM SNP treatment, while no treatment control yielded the lowest level of essential oil content (0.09 % v/w) in our experimental condition.

The chemical constituents of the essential oil identified by GC–MS are presented in Table 1. GC–MS analysis identified 18 components in the essential oil of *in vitro M. officinalis* cultured plants, detecting some major compounds, thymol, carvacrol, linalool, hotrienol, α-terpineol, neral, geranial, trans-caryophyllene, aromadendrene, Caryophyllene oxide and spathulenol. There were differences between treatments in terms of essential oil major components. Different levels of SNP had

significant effects on linalool, hotrienol, α-terpineol, neral, thymol and carvacrol. SNP application remarkably increased most of essential oil major components, while decreased trans-caryophyllene, compared to control culture (Table 1).

Notably, changes in the amount of essential oil components varied among different SNP concentrations. For example, the highest content for linalool, neral and thymol was achieved at 5, 5 and 20 mM of SNP respectively.

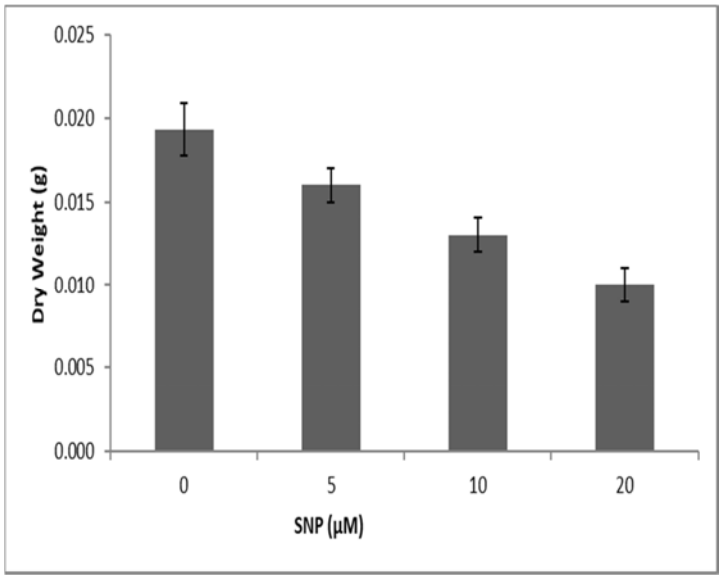

Figure 1- Effect of SNP on dry weight of *M. officinalis*

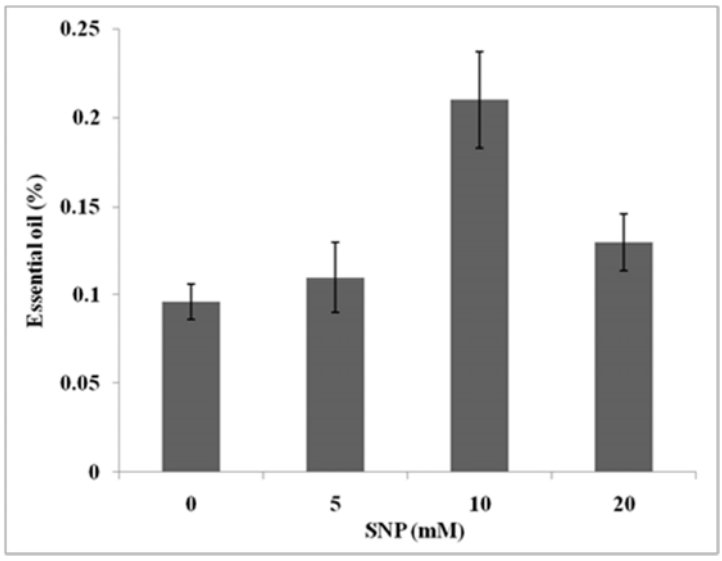

Figure 2- Effect of SNP on essential oil content of *M. officinalis*. Data are expressed as mean ± standard deviations of three determinations

Table 1- Effect of SNP on essential oil chemical compositions of *M. officinalis*

		SNP (mM)			
Compound	**RI**	**0**	**5**	**10**	**20**
unknown		9.01	19.2397	4.5242	1.5078
n-Decane	994.767	1.4096	7.5273	50.8484	0.0558
Linalool	1095.11	0.5249	11.2417	0.203	1.4623
Hotrienol	1100.43	0.3316	29.1694	0.2146	0.3817
α-Terpineol	1109.05	0.4828	2.2393	5.2531	tr
n-Docosane	1199.57	0.2433	6.6368	1.3741	0.5065
Neral	1241.13	0.5657	12.0066	4.0631	0.7559
unknown		2.7835	1.3566	13.1201	0.0687
Geranial	1268.44	0.631	1.1992	1.8096	0.0726
Thymol	1290.78	11.6332	0.319	2.1105	18.1832
Carvacrol	1301.57	1.1705	1.0347	4.6578	72.5191
n-Tetradecane	1388.63	24.4498	3.2497	0.4066	tr
Trans-caryophyllene	1411.79	36.2931	0.1273	1.6478	0.3466
Aromadendrene	1431.3	4.7028	0.2764	5.7471	0.1947
pentadecane	1487.4	2.4037	0.226	0.1262	tr
spathulenol	1571.24	0.8309	0.4339	0.3113	2.0577
Caryophyllene oxide	1575.54	0.5915	2.1021	1.9053	1.0889
n-Hexadecane	1586.7	2.644	0.2835	0.3918	tr
unknown		2.7769	0.2931	0.1864	tr
n-Heptadecane	1685.27	3.2046	0.1339	0.2842	tr
Hexahydrofarnesyl acetone	1842.36	0.6639	0.3221	0.4012	0.351
unknown	1889.7	0.7141	0.5817	0.4136	0.4475
Oxygenated monoterpenes		14.1692	56.1752	13.6539	20.8557
Oxygenated sesquiterpenes		3.2568	3.8928	7.2756	76.0167
Sesquiterpene hydrocarbons		40.9959	0.4037	7.3949	0.5413
Aliphatic hydrocarbons		34.355	18.0572	53.4313	0.5623

The profiles of essential oil compounds were significantly changed when lemon balm shoots were treated with different concentration of SNP (Table 1). The class of sesquiterpene compounds displayed the highest amount followed by monoterpenes, among the above classes. The highest monoterpene (56.17 % v/w) and sesquiterpene content (76.01 % v/w) was achieved by 5 and 20 mM SNP respectively.

Effect of NO doses on total phenol, flavonoid and rosmarinic acid content

The total phenolic and flavonoid content of SNP elicited shoots were compared with unelicited shoots. A remarkable elevation of

total phenolic and flavonoid content was observed at all concentrations of SNP treatments. Among various concentrations of SNP tested, 10 mM treated shoots showed the maximum total phenolic content (7.18 mg gallic acid /g FW) (Fig. 3).

The rosmarinic acid content of elicited plantlets ranged from 3.12 to 4.82 mg/g. NO elicited plantlets exhibited significantly greater capacity to produce rosmarinic acid than control. Among various concentrations of SNP tested, 10 mM treated plantlets showed the maximum rosmarinic acid (4.82 mg gallic acid /g) (Fig. 3).

Effect of NO doses on antioxidant activity

NO elicited plants exhibited significantly greater capacity to scavenge free radicals than control as inferred by DPPH assay. The radical scavenging activity (RSA) of unelicited plants was 43.03% (Fig. 4). By increasing concentration of nitric oxide up to 10mM, radical scavenging capacity of plants increased and then, decreased at upper concentration but still was greater than control. Among various concentrations of NO tested, 10 mM treated plants showed the maximum RSA (78.03%) (Fig. 4), which was 1.8 times more that of the control.

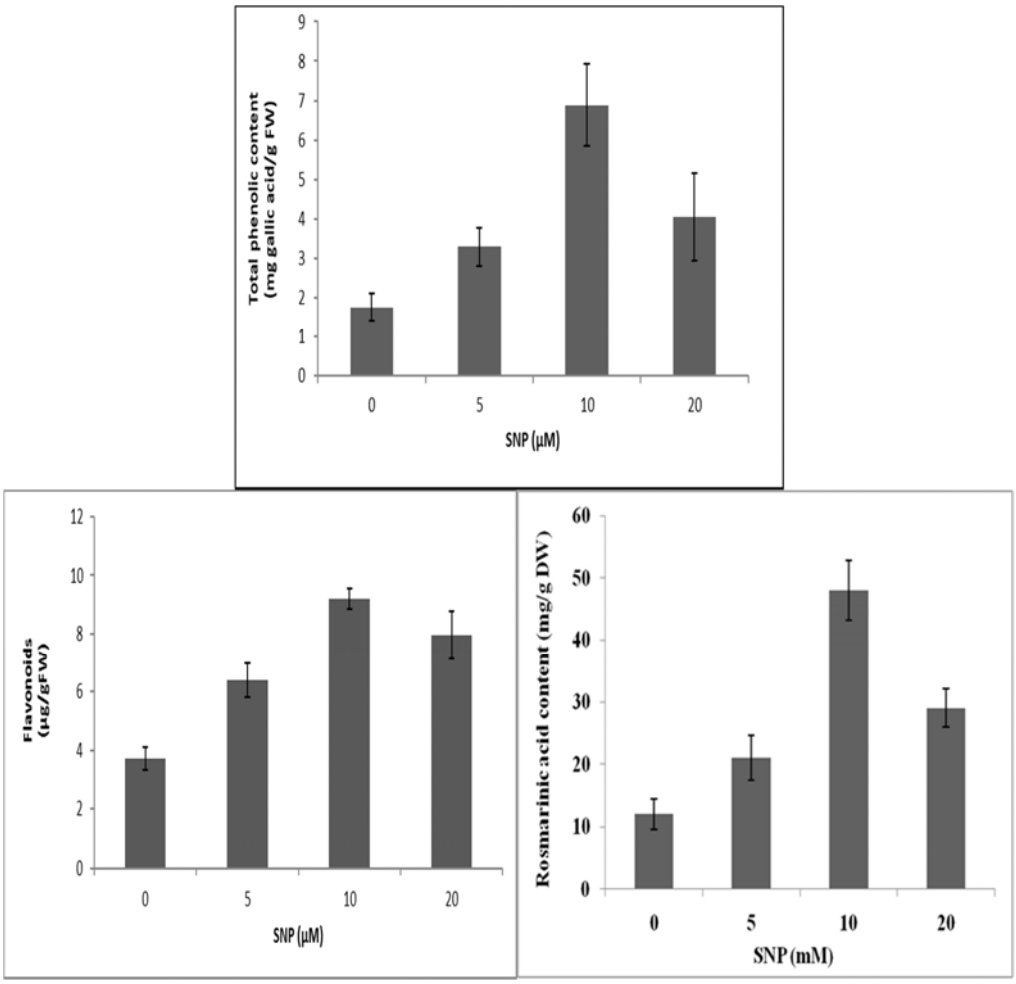

Figure 3- Effect of SNP concentration on total phenol (a) and flavonoid (b) and rosmarinic acid (c) content in *M. officinalis* shoots. Data are expressed as mean ± standard deviations of three determinations

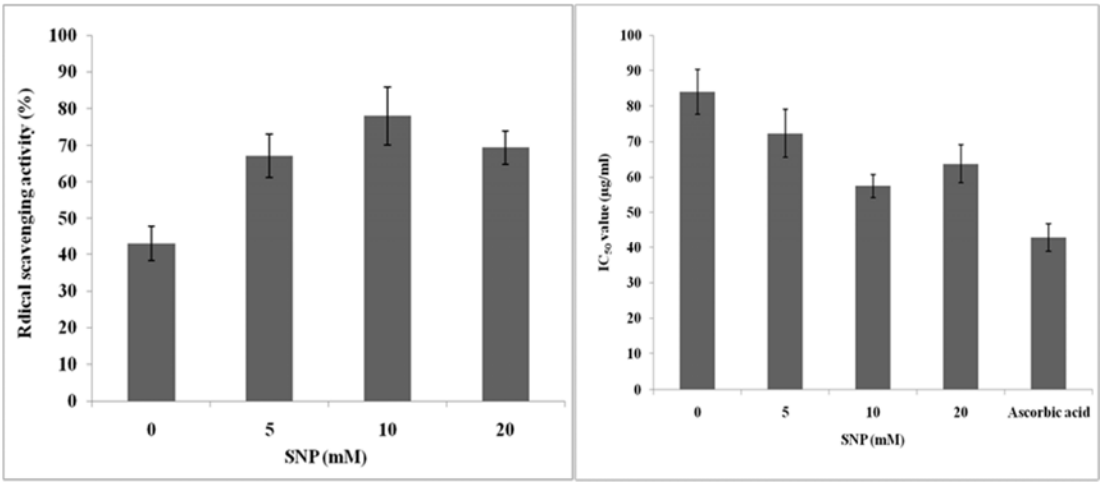

Figure 4- Effect of SNP concentration on radical scavenging activity (a) and IC_{50} value (b) in *M. officinalis* shoots. Data are expressed as mean ± standard deviations of three determinations

Discussion

The chemical composition of the essential oil of *M. officinalis* leaf from different regions has been studied. However, *there are very few reports* available for the *essential oils* of *M. officinalis* in *in vitro* condition. In present study, 18 components were identified in the essential oil of *M. officinalis* cultured plants. The major constituents found in the essential oils were trans-caryophyllene, n-Tetradecane and thymol. *In vitro* cultured plantlets of *M. officinalis* on MS medium presented an increase of 1.4 fold of nerol and 4.1 of geraniol compared to ex vitro cultured plants. The predominant components in the essential oils from kurdestan (Iran) lemon balm were (E)-citral, neral and citronellal [24]. This composition is slightly different to the essential oil of Germany *M. officinalis* in which the major components were acitral, b-caryophyllene, b-citral, citronellal [25] and to the essential oil of Turkey *M. officinalis* in which the major components were citronellal and citral [26]. In India, the major constituents found in the *M. officinalis* essential oil were geranial, neral and trans-caryophyllene [27]. Notable differences in essential oil composition of *M. officinalis* may be due to several factors, such as environment, tested parts, growth season, physiological age of the plant and the method of isolation. The application of elicitors such as NO, which is currently the focus of research, has been considered as one of the most effective methods to improve the production of secondary metabolites in medicinal plants. The current paper represents the assessment of the chemical composition and antioxidant activity of in vitro-cultured *M. officinalis* in response to NO for the first time. Our study showed that essential oil content and composition was significantly affected by the SNP concentrations. This result is supported by the previous reports that the use of NO increased essential oil accumulation [28]. Production of plant secondary metabolites is regulated by multiple endogenous signaling pathways. NO is one of the key signaling molecules in elicitor-induced secondary metabolite biosynthesis such as ginseng, saponin, hypericin, puerarin, catharanthine,

artemisinin and taxanes in plant cell and tissue cultures [14]. Although jasmonic acid (JA) and salicylic acid (SA) may induce secondary metabolite production through distinct signaling pathways [29], they are all interacted with NO in mediating plant secondary metabolite biosynthesis [14]. The role of NO in the biosynthesis of secondary metabolites in plant cells has not been well understood yet. Improvement of essential oil percentage and changes in its composition in response to other elicitors such as SA and methyl jasmonate (MeJA) application have been reported in other plant species [30, 31, 32]. It was suggested improvement in essential oil content by foliar application of SA might be due to the increase in cycle growth, nutrients uptake or changes in leaf oil gland population and monoterpene biosynthesis. The highest amount of methylchavicol (97.6%) was accumulated in the *Agastache foeniculum* plants treated with MeJA [32]. Moreover, MeJA and methyl salicylate (MeSA) treatments showed an increase of 13% and 21.4% of geranial in *M. officinalis* compare to the control [33]. However, little is known on the effect of NO upon essential oil content in plants, thus our data provide additional cue to manipulate essential oil content. All NO treatments significantly increased the total phenolic compounds and RA contents in a dose dependent manner. These data were in compliance with the results of El-Beltagi et al. [17] who reported the accumulation of phenols in inoculated *G. biloba* cells after challenging with different concentrations of SNP. Moreover, RA accumulation in *Thymus membranaceus* in response to SA treatments was dose dependent [34]. Phenolics, flavonoids, and caffeic acid derivatives were significantly enhanced in adventitious roots of *Echinacea purpurea* treated with 100 μM SNP. In present study, the production of some metabolites was initially increased by increasing NO concentration and then a decrease in the metabolite concentration is observed at higher concentration of NO. Although, different response of plants to NO under given set of conditions, like NO concentration and the plant species is studied; However, there are many other aspects in relation to biosynthetic pathways of secondary metabolite production and up-regulation or down-regulation of NO for the production of certain secondary compounds still less known. Results obtained from present study showed that the extract of *M. officinalis* had higher antioxidant activity under elicitation which in turn further indicates different levels of NO have affected antioxidant activities of *M. officinalis* extract (Fig. 3). The results of our study were in line with the results of Shilpha et al. where 4μM MeJA treated *Solanum trilobatum* hairy roots exhibited higher total phenol and antioxidant capacity than control roots [35]. The DPPH is a stable free radical which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants [36]. The lower IC50 value indicates a stronger ability of the extract to act as a DPPH scavenger while the higher IC50 value indicates a lower scavenging activity of the scavengers as more scavengers are required to achieve 50% scavenging reaction. There are various reports [37, 38] on the strong capacity of RA for scavenging DPPH free radicals and

its high reducing properties. Strong correlation between RA level and antioxidant activities have been reported in *Thymus* [34] and in other species belonging to the Lamiaceae [38].

Conclusion

The data presented here constitute the first evaluation of the bioactive compounds and antioxidant activity of *M. officinalis* shoots grown *in vitro* in response to nitric oxide. In summary, our results clearly show that SNP, a donor of NO, can act as an elicitor for increasing essential oil content in *M. officinalis*. Changes in essential oil content and composition as well as significant increase in the total phenolics, RA and radical scavenging activity of elicited shoots were noticed in comparison with control. Thus, this study illustrates the feasibility of the use of *M. officinalis* shoots in bioreactors for a large-scale production of bioactive compounds. Although the effect of NO upon essential oil components in *M. officinalis* was studied only on selected biochemical parameters, future identification of enzymes and corresponding genes that are responsible for the biosynthetic pathways of essential oil components would probably shed more light on the regulatory mechanism of such pathways by NO in *M. officinalis*.

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