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The effect of activated charcoal and multi-walled carbon nanotubes on the growth, rosmarinic and caffeic acid content, total phenol, total flavonoid and antioxidant activity of *Satureja rechingeri* calluses

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Abstract

Satureja rechingeri belongs to the Lamiaceae family and is native to Iran. The plant produces high amounts of phenolic compounds, especially rosmarinic acid. The effects of multi-walled carbon nanotubes with a carboxyl functional group (MWCNT-COOH) and activated charcoal (AC) on the growth, rosmarinic and caffeic acid content, total phenol, total flavonoid and antioxidant activity of *S. rechingeri* calluses were evaluated. MWCNT-COOH and AC were able to increase the amount of fresh and dry weight compared to control in a wide range (25-500 µg/ml). The maximum amount of rosmarinic acid was observed in concentrations of 100 µg/ml MWCNT-COOH and 250 µg/ml AC. Positive correlations between antioxidant activity and total phenol content and rosmarinic acid content were observed. These data underline the role of rosmarinic acid as a potent antioxidant in plants.

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Introduction

Satureja rechingeri Jamzad belongs to the Lamiaceae family and is native to Iran. This plant is distinguished with yellow flowers, dense white villous hairs and a dense covering of punctate glands on both leaf surfaces (Jamzad, 1996). The plant produce large amounts of phenols mainly rosmarinic acid (Hadian *et al*., 2014). It has been considered for domestication to supply the demand of industry.

Nanotechnology may be able to create many new materials and devices with a vast range of applications in biology, medicine (Bruches *et al.,* 1998; Wang *et al.,* 2002; Mah *et al.,* 2000; Panatarotto *et al.,* 2003; Nam *et al.,* 2003; Ma *et al.,* 2003), electronics (Wang *et al.,* 2009), biomaterials (Balasundaram *et al.,* 2006), energy production, and agriculture (Bouwmeester *et al.,* 2009; Nair *et al.,* 2010; Sharon *et al.,* 2010; Emamifar *et al.,* 2010). Research on the effect of nanoparticles on plants is at a primary stage and whether they are harmful or helpful to plants is questionable. It has been confirmed in some plant species that nanoparticles are safe at low concentrations and are capable of encouraging photosynthesis and nitrogen metabolism and can thus ameliorate the growth of the plant (Zheng *et al.,* 2005; Klaine *et al.,* 2008). Carbon nanotubes (CNTs) may have possibly single or multiple layers of carbons established in a cylinder (Li *et al.,* 1996; Philip *et al.,* 2000). CNTs behave as fibers, with its properties very different from bulk carbon or graphite (Philip *et al.,* 2000). Multi-walled carbon nanotubes (MWCNTs) which consist of multiple rolled layers (concentric tubes) of grapheme. Several experiments have recently been performed to understand the interaction between carbon nanotubes and biological systems. For example, water soluble carbon nanotubes (wsCNTs) increased growth rate in every part of gram (*Cicer arietinum*) plant including the roots, shoots and also branches. Khodakovskaya *et al.* (2012) proved that multi-walled carbon nanotubes (MWCNTs) have the ability to enhance the growth of tobacco cells in a wide range of concentrations (5-500 µg/mL). They also showed that activated charcoal (AC) increases cell growth only in low concentrations but its influence on cell growth was lower than that of MWCNT.

The positive effects of low doses of MWCNTs on germination, dehydrogenase activity, cell elongation, root growth and biomass production has been confirmed (Khodakovskaya *et al.,* 2009; Wang *et al.,* 2012).

In this study, for the first time, the effect of carbon nanotubes and activated charcoal on the growth rate of *S. rechingeri* calluses, and the amount of rosmarinic acid and caffeic acid in the calluses were evaluated. Due to low solubility of carbon nanotubes in water, it used carbon nanotubes with carboxyl functional group.

Materials and methods

Materials

MWCNTs-COOH were purchased from Neutrino company in Iran (purity:> 95 %, OD 10-20nm, Length ~30µm and COOH content 2wt%). Also activated carbon was purchased from Merck company with absorption >30% (nitric acid <10% , Fe <1000ppm and zn <200ppm).

Preparation of calluses

For callus induction of *S. rechingeri,* homogeneous explants were placed in B5 culture medium supplemented with 0.1 mg/l IBA. For maintenance of the calluses and further tests, B5 medium was supplemented with 5 mg/l BA and 1mg/l IBA. An equal amount of initial callus (inoculum), 3g, was placed in each experimental glass.

Experiments with the established calluses

The established calluses were treated with the media supplemented with MWCNTs or activated carbon in the concentrations of 25, 50, 100, 250 and 500 µg/mL in 4 replications (glasses). The experimental glasses were kept in the dark condition at 22-24 °C in a growth chamber for 1 month.

Methanolic extract preparation

After 1 month, Calluses were dried in the freeze dryer. Extraction procedure was performed according to Baskan *et al.* (2007). 500 mg of powder of each individual was suspend in 10 mL of MeOH and mixed vigorously. Extraction was performed by sonic and then the extracts were filtered and stored at 4 ◦ C until analysis.

Determination of caffeic acid and rosmarinic acid by HPLC

The separation of phenolic acids was performed on a Knauer liquid chromatography apparatus consisting of a 1000 smartline pump, a 5000 smartline manager solvent organizer and a 2800 smartline photo-diode array detector. Injection was performed through a 3900 smartline autosampler injector equipped with a 100 µl loop. The temperature control of the column was made with a jet stream 2 plus oven (Knauer, advanced scientific instrument, Berlin, Germany). The column, a Eurospher 100-5 C18, (250 × 4.6 mm) was maintained at 25 C. The solvents used for separation were 0.02% TFA in water (v/v) (eluent A) and 0.02% TFA in methanol (v/v) (eluent B). The gradient used was: 0–5 min, linear gradient from 0% to 20%, 5-10min, 20% to 25% B; 10–20 min, linear gradient from 25% to 30% B; 20-35 min, linear gradient from 30% to 45%, maintained at 90% B until 60 min. The flow rate was 0.5 ml min^{-1}. Detection wavelength was 330 nm. The sample injection volume was 20 µl. The chromatographic peaks of rosmarinic acid and caffeic acid were confirmed by comparing their retention times and UV spectra with those of their reference standards. Working standard solutions were injected into the HPLC and peak area responses were obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

Determination of total phenolic compound

The total phenols of these extracts were determined with folin-ciocalteu reagent at 760 nm (Lister and Wilson, 2001). Totally, 250 µl of each methanolic extract (50µg/ µl) was introduced into test tubes and 1.25 ml of diluted folin-ciocalteu reagent (1:10 with distilled water) and 1 ml of 7.5% sodium carbonate were added to the extracts. All the tubes were allowed to stand for 30 minutes in a dark place at room temperature. Gallic acid was used as standard. The absorbance was read at 760 nm and all the measurements were performed three times. Total phenol values are expressed in terms of gallic acid equivalents (mg/kg or µg/mg of dry mass of extract).

Determination of total flavonoids

Flavonoid contents of the extracts were determined by a colorimetric method described by Zhishen *et al.* (1999) with some modifications. 250 µl of the extracts (50µg/ µl) were mixed with 2ml distilled water and 0.15 ml of a 15% $NaNO_2$ solution. After 6 minutes, 0.15 ml of a 10% $AlCl_3$ solution was added. After 6 minutes, 2 ml of a 4 % NaOH solution was added to the mixture, which was adjusted to 5 ml with distilled water. The solution was mixed well and the absorbances were measured at 510 nm 15 minutes later. Rutin was used as standard and the results were expressed as mg of rutin equivalents per gram of the extract.

Measurement of free radical-scavenging activity (DPPHassay)

The free radical-scavenging activity was measured by the DPPH assay described by Blois (1958). Different volumes of the extracts (2, 10, 40, 60, 80) were added to 96-well plates and allowed to be dried at room temperature. Then 187 µl of MeOH and 63 µl of DPPH solution in MeOH (final concentration of 0.2 mM) were added. The mixtures were shaken and allowed to stand for 45 minutes in a dark place at room temperature. The absorbance was measured at 517 nm. The ability of the extracts to scavenge the DPPH radical was calculated using the following equation: inhibition% = (Ac-As)/Ac*100 where As is the absorbance of the sample, and Ac is the absorbance of the mixture of 187 µl MeOH and 63 µl DPPH solution as control. BHT was used as standard

and the results were expressed in BHT equivalent capacity.

Results and discussion

One month after callus incubation with MWCNT-COOH and AC, fresh and dry weight of callus, the amount of caffeic acid and rosmarinic acid, total phenolics, total flavonoids and antioxidant activity in methanolic extract of callus were measured. Changes in fresh and dry weight of the callus are showed in Fig. 1. As can be seen, MWCNT-COOH and AC were able to increase the amount of fresh weight and dry weight compared with control. This suggests that MWCNT-COOH and AC not only increase the absorption of water by the callus but also increase cell division. It has been proven that expression of genes related to cell division were in the highest level when the cells were treated with MWCNTs (Khodakovskaya *et al.,* 2012). The effect of AC on callus growth may be attributed to the adsorption of inhibitory materials in the culture medium (Horner *et al.,* 1977; Fridborg *et al.,* 1978; Theander *et al.,* 1988; Weatherhead *et al.,* 1978), decrease in the phenolic oxidation or brown exudate accumulation (Carlberg *et al.,* 1983; Liu *et al.,* 1993; Teixeria *et al.,* 1994), regulation of medium pH for morphogenesis (Owen *et al.,* 1991) and the creation of an environment that simulates soil dark conditions for the plant (Dumas *et al.,* 1995). Also 5-hydroxymethyl-furfural (a growth inhibitory chemical) produced during autoclaving from dehydration of sucrose will be removed by AC (Pan *et al.,* 1998).

Table 1. Total phenol, total flavonoid and antioxidant activity in MeOH extracts of callus exposed to AC and MWCNT-COOH.

Treatment	Total phenol ± STD μ(g galic acid in ml)	Total Flavonoid± STD (μg rutin in ml)	IC50± STD
AC=25	35.21± 0.04	233.33 ±0.05	21.97 ± 1.2
AC=50	37.63 ± 0.03	235.41 ±0.7	14.8 ± 0.81
AC=100	45.38 ± 0.1	289.58 ±0.8	10.9 ± 0.7
AC=250	37.3 ± 0.06	437.5 ±1.11	9.1 ±0.3
AC=500	32.96 ± 0.08	314.58 ±0.45	18.99 ±0.68
Control	36.63 ± 0.05	366.66 ±0.56	18.95 ±0.56
CNT=25	32.55 ± 0.11	147.91±0.35	20.97± 0.9
CNT=50	41.85 ± 0.2	364.58 ±0.95	9 ±0.45
CNT=100	42.63 ± 0.03	345.83 ±0.46	8.04 ±0.78
CNT=250	35.8 ± 0.05	254.16 ±0.75	13.08 ±1.3
CNT=500	48.38 ± 0.06	308.33 ±0.39	10.24±0.91

These data are consistent with the data obtained by Khodakovskaya *et al.* (2012) and Villagarcia *et al.* (2012), and disagree with some previous results (Lin *et al.,* 2009; Tan *et al.,* 2009). That different results have been obtained about the impact of carbon nanotubes on plant growth is attributable to several factors such as type of plant and medium, chemical and physical properties of nanotubes, the dose used, culture conditions and the duration that the plant was exposed to nanotubes.

AC and MWCNT-COOH both enhanced rosmarinic acid compared to control. Also carbon nanotubes indicated a further increase in the amount of rosmarinic acid in comparison with activated charcoal. AC concentrations up to 250 μg/ml and MWCNT-COOH concentrations up to 100 μg/ml, increased the amount of rosmarinic acid (Fig. 2).

The involvement of Phenylalanine ammonia-lyase (PAL) in RA biosynthesis has been supported in several previous studies by an increase in the PAL activity preceding the RA accumulation induced by fungal (or yeast) elicitors and methyl jasmonate (MJ) in cell cultures of the Boraginaceae and Lamiaceae species (Mizukami *et al.,* 1992; Sumaryono *et al.,* 1991; Mizukami *et al.,* 1993; Szabo *et al.,* 1999). Yan *et al.* (2006) indicated that yeast extract and Ag^{+} induced biosynthesis of RA and phenolic compounds in the *Salvia miltiorrhiza* hairy roots and this

increase correlated with the tyrosine aminotransferase (TAT) activity.

It appears that AC and MWCNT-COOH as elicitors cause rosmarinic acid and caffeic acid to increase. Since these carbon materials express a number of genes, they may increase the expression of genes involved in the biosynthesis of rosmarinic acid and caffeic acid. The mechanism of elicitation by AC and MWCNT-COOH should be further investigated.

Table 2. Correlations among traits.

IC 50	Total flavonoid	Total phenol	RA content	CA content	
CA content	1				
RA content	0.363	1			
Total phenol	0.291	0.693*	1		
Total flavonoid	-0.67	0.701*	0.33	1	
IC 50	-0.328	-0.94**	-0.74**	-0.59	1

*.Correlation Correlation is significant at the 0.05 level.

**.Correlation is significant at the 0.01 level.

Total phenol, total flavonoid and antioxidant activity of MeOH extracts of callus after one month of treatment with AC and MWCNT-COOH (Table 1) were measured. Then, the correlation between these traits with the amount of rosmarinic acid and caffeic acid were studied (Table 2).

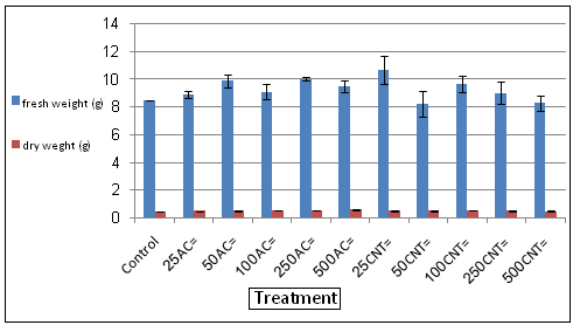

Fig. 1. Fresh and dry weight of calluses exposed to activated charcoal (AC) and multi-wall carbon nanotubes with carboxyl functional group (MWCNT-COOH).

Sloley *et al.* (2000) showed that the free-radical scavenging capacity of extracts of leaves and flowers of *Hypericum perforatum* correlated with the content of several flavonoids including quercetin and hyperoside. It has also been shown that there is a linear correlation between total phenolic content and ORAC (oxygen radical absorbance capacity) capacity of blackberry fruits and leaves (Wang and Lin, 2000). In this study, a positive correlation between antioxidant activity and total phenol content was observed. In addition, a stronger positive correlation between the antioxidant activity and rosmarinic acid content was found. These data underline the role of rosmarinic acid as a potent antioxidant in plants.

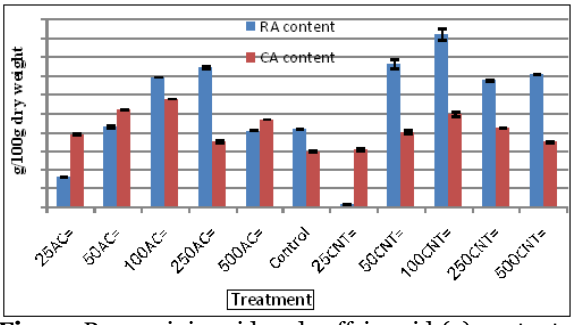

Fig. 2. Rosmarinic acid and caffeic acid (g) contents in 100 g dry weight of callus after one month of treatment with activated charcoal (AC) and multi-wall carbon nanotubes with carboxyl functional group (MWCNT-COOH).

Since the first characteristic of the influence of stress on plants is growth reduction, the nanotubes and activated carbon at these concentrations cannot act as tension materials because they have a positive effect on callus growth. It showed that the AC and MWCNT-COOH can act as elicitors enhancing rosmarinic acid. These materials may increase primary metabolites such as amino acids tyrosine and phenylalanine which in turn increase the synthesis of rosmarinic

acid. To further understand the process of increasing secondary metabolites such as rosmarinic acid and caffeic acid treated with AC and MWCNT-COOH it is required to have further studies at molecular level. On the other hand, the safety of nanotubes is still debated. However, since secondary metabolites are used, they can be used to increase the production of secondary metabolites in tissue culture conditions even if the nanotubes are not safe. However, their use in soil must be safe for the ecosystem and the environment.

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