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Antioxidant activity of extracts from *in vitro* cultures of *Salvia officinalis* L.

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Abstract

Methanolic and acetone extracts from organ (shoots and hairy roots) and undifferentiated (cell and callus) cultures of *Salvia officinalis*, as well as from shoots and roots of *in vitro* regenerated plants were evaluated for their antioxidant properties using three various *in vitro* models: scavenging of the free radicals using DPPH·, transition metal reduction in phosphomolybdenum assay and inhibition of lipid oxidation. The concentrations of rosmarinic acid, diterpenoids (carnosic acid and carnosol) and total phenolic compounds in each extract were determined. The methanolic hairy root and root regenerated plant extracts possessed the strongest effects on reducing Mo and DPPH· radical scavenging. On the other hand the best protective effect against linoleic acid oxidation was observed for acetone extracts of shoots obtained from *in vitro* culture followed by the extracts of shoots of intact plants grown in the field, without statistically significant differences between them.

Keywords: Antioxidant activity; *Salvia officinalis*; *In vitro* cultures

1. Introduction

In recent years considerable attention has been devoted to medicinal plants with antioxidant properties. The properties are commonly postulated to play an important role in preventing diseases caused by oxidative stress, such as cancer, coronary arteriosclerosis, and the ageing processes (Haraguchi, Saito, Okamura, & Yagi, 1995). There is much literature concerning the antioxidant properties of many species of genus *Salvia*. For example, Lamaison, Petitjean-Freytet, and Carnat (1990) investigated hydroalkoholic extracts of several *Salvia* species for their 1,1-diphenyl-2-picrylhydrazyl (DPPH·) free radical scavenging activities. Among them, *Salvia officinalis* leaf extracts have been shown to be the most active with effective dose (EC_{50}) of 41 μg/ml, followed by *Salvia sclarea* ($EC_{50} = 45$ μg/ml), *Salvia triloba* ($EC_{50} = 50$ μg/ml) and *Salvia lavandulifolia* ($EC_{50} = 80$ μg/ml). For its high antioxidant activity, *S. officinalis* besides rosemary is widely used commercially in foodstuffs (Ternes & Schwarz, 1995). There is evidence that antioxidant properties of sage extracts are mainly attributed to the abietane-type diterpenoids (carnosic acid and carnosol) and caffeic acid derivatives (e.g., rosmarinic acid) (Cuvelier, Berset, & Richard, 1994). Flavonoids and certain components of essential oil are of less significance (Lu & Foo, 2001).

Biotechnological methods based on *in vitro* culture of tissues and plants are considered to give the possibility of producing standardized material, independent from environmental factors. The production of secondary metabolites in cultured *in vitro* plants can be increased by genetic transformation with the soil-borne pathogen *Agrobacterium rhizogenes*. In earlier studies we have established protocols for the following systems for sage *in vitro* cultures: cell and callus cultures, shoot and hairy root cultures and *in vitro* propagated plants (Grzegorczyk, Bilichowski, Mikiciuk-Olasik, & Wysokińska, 2005, 2006). We have

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also shown that the tissues and plants produce compounds with antioxidant activity (rosmarinic acid, carnosic acid and carnosol) (Grzegorczyk et al., 2005).

In the present paper we evaluated the antioxidant capacities of acetone and methanolic extracts obtained from different *in vitro* cultures and *in vitro* propagated plants of sage. Three different assays (DPPH· free radical scavenging activity, reduction of transition metal ions by of phosphomolybdenum complex and inhibition of lipid peroxidation) were used. Antioxidant activities of the extracts from *in vitro* cultures were compared with those of extracts of *S. officinalis* shoots and roots from plants obtained from the seeds and grown in the field. All extracts were chemically characterized by HPLC method in order to find the connection between their activity and chemical composition of extracts.

2. Material and methods

2.1. Plant material

For antioxidant assay the following plant materials of *S. officinalis* were used:

- callus and cell cultures (passage 17). Both cultures were maintained on MS (Murashige & Skoog, 1962) medium containing 0.1 mg/l NAA (α-naphthaleneacetic acid), 0.5 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.2 mg/l BAP (6-benzylaminopurine). They were subcultured every 21 days (callus) or 14 days (suspension culture);
- shoot culture (passage 18); it was cultured on MS agar (0.7%) medium supplemented with 0.1 mg/l IAA (indole-3-acetic acid), 0.45 mg/l BAP and subcultured every 5 weeks;
- hairy root culture (passage 20) obtained after infection of shoots with *A. rhizogenes* strain ATCC 15834. The cultures were maintained in WP (Lloyd & McCown, 1980) liquid medium without growth regulators and transferred routinely to fresh medium every 4 weeks. The root culture was confirmed to be transformed by PCR method (Grzegorczyk, Królicka, & Wysokińska, 2006);
- shoots and roots of micropropagated plants after 10 weeks of growth in greenhouse.

The procedures for establishment of callus, cell suspension, shoot culture and hairy root culture as well as regeneration of *S. officinalis* plantlets were described in our previous works (Grzegorczyk et al., 2005, 2006). All sage cultures tested were kept at 26 ± 2 °C under light conditions (40 μM m^{-2} s^{-1}) with a photoperiod of 16 h light/8 h dark.

For the comparison, shoots and roots of plants derived from the same seeds as *in vitro* cultures and cultivated for 10 weeks in the experimental field of the Department of Pharmacognosy at the Medical University of Łódź were used. Identification of the plant was established by Prof. J. Siciński (Department of Plant Ecology and Phytosociology, University of Łódź).

2.2. Preparation of extracts

The dried and powdered materials tested (2 g, each) were separately extracted with methanol (2 × 50 ml of boiling solvent for 2 h) or acetone (3 × 50 ml at room temperature for 15 min). Both methanolic and acetone extracts were treated in the same way. The extracts were filtered and the solvent was removed on a rotary evaporator at 40 °C. The residues were dissolved in an appropriate volume of methanol to obtain 10 mg/ml concentration and stored closed at −20 °C until studied.

2.3. HPLC analysis

Dried and powdered plant materials (250 mg) were extracted with methanol or acetone as described above. Acetone and methanolic extracts were analyzed by HPLC on a Waters (Milford, USA) Symetry® C18 column. The details of the extraction procedure and HPLC analysis were described in our previous work (Grzegorczyk et al., 2005). CA, Car and RA were identified by comparing their retention times and UV spectra with authentic samples under the identical conditions. The amounts of the compounds were expressed in mg g^{-1} of dry weight.

2.4. Antioxidant assays

2.4.1. DPPH· radical scavenging

This experimental procedure was adopted from Brand-Williams, Cuvelier, and Berset (1995). The sage extracts at different concentrations (2.5, 5.0, 12.5, 25.0, 50.0 and 250 μg/ml) were mixed with the same volume of 0.2 mM methanolic solution of DPPH· from Sigma. The disappearance of DPPH· was read spectrophotometrically (the Shimadzu 1601 UV/VIS spectrophotometer, Shimadzu Corp., Japan) at 517 nm immediately after mixing and after 1, 5 and 30 min of incubation at room temperature. Free radical scavenging capacity was calculated by the following equation:

$$\%\ \text{scavenging} = \left(100 - \frac{\text{Abs sample}}{\text{Abs DPPH}}\right) \times 100\%$$

Abs sample = Abs measured − Abs control (i.e., absorbance of the sample tested without DPPH·).

From the obtained values, the EC_{50} (defined as the concentration of sample at which 50% of maximum scavenging activity was recorded) was calculated for each sample.

2.4.2. Phosphomolybdenum reduction

The method of Prieto, Pineda, and Aguilar (1999) was used. Four hundred microlitres of the extract tested (at the concentration of 50 μg/ml) was mixed with the reagent solution (3.6 ml) containing ammonium molybdate

(4 mM), sodium phosphate (28 mM) and sulfuric acid (600 mM). The reaction mixture was incubated in a water bath shaker at 95 °C for 90 min. After cooling at room temperature the absorbance of the green phosphomolybdenum complex was measured at 695 nm against a blank. The reducing capacity of extract was calculated using the following equation:

$$\text{Abs final} = \text{Abs sample} - \text{Abs blank} - \text{Abs extract}$$

Abs extract – absorbance of sample where molybdate solution was replaced by water;
Abs blank – absorbance of blank containing methanol (400 μl) instead of sage sample.

2.4.3. Linoleic acid peroxidation inhibition

The procedure was performed according to modified method of Choi et al. (2002). The sage extract at the concentration of 100 μg/ml was mixed with 550 μl of linoleic acid (40 μM), 500 μl of phosphate buffer (100 μM, pH 7.4) and 150 μl of ascorbic acid (10 μM). Linoleic acid peroxidation was initiated by the addition of $FeSO_4$. The reaction mixture was incubated for 60 min at 37 °C. The reaction was terminated by the addition of 1.5 ml of ice cold 10% trichloroacetic acid (TCA) (Ubichem) in 0.5% HCl. Then, 3 ml of TBA (Aldrich) in 50 mM NaOH was added. The TBA/sample mixture was heated in the water bath at 95 °C for 60 min. After cooling down 3 ml aliquots were taken from each sample and vortexed with equal volume of butanol, centrifuged at 450*g* for 30 min and the upper colored layer was decanted to the spectrophotometric cuvettes. The absorbance was read at 532 nm and percentage of linoleic acid peroxidation inhibition was calculated using the following equation:

$$\%\ \text{inhibition} = (\text{Abs control} - \text{Abs sample} - \text{Abs extract}) \times 100/\text{Abs control}$$

Abs control includes ethanol instead of sage sample; Abs extract – absorbance of sample where linoleic acid solution was replaced by ethanol.

2.5. Total phenolic determination

Total phenolic compound amount in extracts was determined by Folin–Ciocalteu method. The procedure of Singleton and Rossi (1965) has been used. The extracts at the concentration of 100 μg/ml were added to the reaction mixture (POCh, Gliwice, Poland) and the absorbance read at 765 nm. The results are means of three repetitions expressed in the form of gallic acid (GA) equivalents per g of extract.

2.6. Statistical analysis

The estimated values are the means of three samples for each plant material. EC_{50} was calculated using nonlinear regression module of STATISTICA 5.0. Significance of treatment effects was determined using Duncan's multiple range at a 5% probability level (Statistica 5.0, Statsoft, Poland).

3. Results and discussion

3.1. General

The antioxidant effects of methanolic and acetone extracts of various *in vitro* cultures of sage (callus, cell suspension, shoots and hairy roots) as well as shoots and roots of *in vitro* propagated plants were measured. The activity of the extracts was compared with that of methanolic and acetone extracts of sage plants obtained from seeds and grown in the field. For the chemical characterization, amounts of the total phenolic compounds and the contents of carnosic acid, carnosol and rosmarinic acid in all extracts were determined (Table 1) (Fig. 1). These compounds are generally known to protect tissues and cells against various oxidative stresses (Haraguchi et al., 1995). Since the active substances of plant extracts tested are different, the antioxidant activities of these extracts cannot be evaluated by only a single method. Therefore, three different models were used in this study.

3.2. DPPH· radical scavenging

The radical scavenging activity was expressed as the percentage of reduction of the initial DPPH· absorbance by the extracts studied. EC_{50} values for these extracts were also calculated (Table 2). The highest radical scavenging activity was detected for RR methanolic extract (from roots of *in vitro* regenerated sage plants) and HR methanolic extract (from transformed roots grown in *in vitro* culture). The free radicals amount in the test sample with these extracts at the concentration of 50 μg/ml after 30 min incubation decreased, respectively, by 94% and 88%. Their EC_{50} values were 18.4 ± 5.5 μg/ml and 20.4 ± 4.2 μg/ml, respectively. The values are comparable to EC_{50} for the synthetic antioxidant agent BHT (19.8 ± 0.5 μg/ml) in the same test (Güllüce et al., 2003). The high antiradical activity of sage untransformed (RR) and transformed (HR) root methanolic extracts might be explained by the presence of RA (derivative of caffeic acid with an orthohydroxybenzene structure). It had been earlier reported that radical scavenging activity of plant extracts may be attributed to the compounds with catechol moieties (Lu & Foo, 2001). The presence of RA is probably responsible for the inhibition of DPPH· by methanolic extract of undifferentiated cell culture (LC) of *S. officinalis*. From the estimated EC_{50} value it could be seen that the extract exhibited almost half the activity of the strongest RR methanolic extract. The antiradical activity of *in vitro* culture callus was not practically observed (Table 2). The SF and SR shoot methanolic extracts (from shoots of *in vivo* and *in vitro* regenerated plants) were also good radical scavengers with DPPH· inhi-

Table 1
Rosmarinic acid, carnosic acid and carnosol contents in various extracts of *S. officinalis*

Plant material	Name	Extract	Compound contents (mg/g dry wt.)		
			RA	CA	Car
Shoot culture	SC	MeOH	12.3 ± 0.1	2.75 ± 0.02	0.50 ± 0.07
		AcO	Trace	4.37 ± 0.22	0.88 ± 0.02
Hairy root culture	HR	MeOH	30.9 ± 1.0	n.d.	n.d.
		AcO	Trace	n.d.	n.d.
Callus culture	CC	MeOH	15.8 ± 0.2	n.d.	n.d.
		AcO	Trace	n.d.	0.04 ± 0.0004
Suspension culture	LC	MeOH	18.6 ± 0.3	n.d.	n.d.
		AcO	Trace	n.d.	0.05 ± 0.0003
In vitro regenerated plants					
Shoots	SR	MeOH	12.2 ± 0.2	10.1 ± 0.1	0.91 ± 0.01
		AcO	Trace	11.4 ± 0.2	1.09 ± 0.01
Roots	RR	MeOH	7.23 ± 0.20	n.d.	n.d.
		AcO	Trace	0.07 ± 0.01	0.09 ± 0.004
In vivo plants					
Shoots	SF	MeOH	18.5 ± 0.2	8.68 ± 0.02	1.78 ± 0.01
		AcO	Trace	10.1 ± 0.2	1.14 ± 0.04
Roots	RF	MeOH	8.18 ± 0.36	n.d.	n.d.
		AcO	Trace	n.d.	0.07 ± 0.002

MeOH – methanolic extracts, AcO – acetone extracts.
n.d. – not detected; Values are the means of three experiments ± standard error (SE).

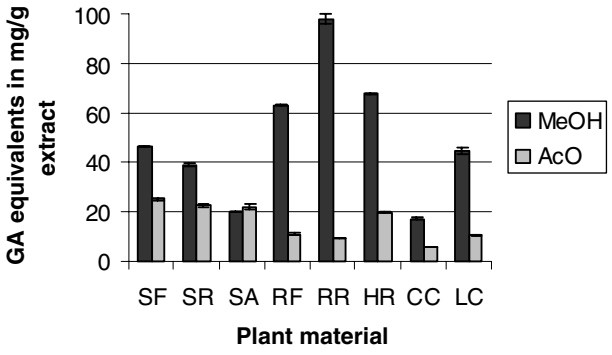

Fig. 1. The results of total phenols determination by the Folin-Ciocalteu method expressed as gallic acid equivalents in mg per gram of extract. MeOH – methanolic extracts, AcO – acetone extracts. The values are the means ± SD. SF – shoots *in vivo* grown plants, SR – shoots *in vitro* regenerated plants, SC – shoot culture, RF – roots *in vivo* grown plants, SR – roots *in vitro* regenerated plants, HR – hairy root culture, CC – callus culture, LC – suspension culture.

bition of 81% and 72%, respectively, at 50 μg/ml. The lowest scavenging activity (with EC_{50} values of 70.1 ± 4.0) exhibited SC (from shoot culture) methanolic extract. As shown by HPLC all three analyzed compounds, i.e., RA, CA and Car were identified in the methanolic shoot extracts and their scavenging effects were related to their total content (Table 1).

The acetone extracts of sage were found to be less active in DPPH· system compared to the parallel methanolic extracts. Among them, the strongest effect was observed for shoot extracts in the following order: SF > SR > SC. When the extracts were tested at the concentration of 50 μg/ml after 30 min of incubation, their capacity to reduce DPPH· radicals ranged between 25% and 40% (Table 2). It should be also noticed that acetone extracts of roots (RF, RR and HR) and *in vitro* cultures of undifferentiated tissues (CC and LC), in which no peak of carnosic acid or carnosol could be detected (or only trace of Car were found) exhibited an evident scavenging effect only at a very high concentration (250 μg/ml). These results as well as the overall weaker action of shoot extracts rich in CA and Car, in comparison to the root methanolic extracts (HR, RR, RF) not containing these diterpenoids, suggest the limited contribution of the abietanes to the DPPH· scavenging activity. Also RA is likely not the sole regulator such activity. The better correlation with antiradical activity of sage extracts seems to have the total phenolic content (Fig. 1). Therefore, the synergistic effect of RA and other phenolics, such as salvianolic acid or/and flavonoids might be taken into account.

3.3. Phosphomolybdenum assay

The different sage extracts were also used to determine their antioxidant capacities by the formation of green phosphomolybdenum complex. The formation of the complex was measured by the intensity of absorbance in extracts at the concentration of 50 μg/ml at 90 °C. The results indicate that under these conditions both methanolic and acetone extracts of sage are powerful antioxidants (Fig. 2). However, the differences in the degree of Mo

Table 2
DPPH· radical scavenging activity of methanolic and acetone extracts of *S. officinalis*

Plant material	MeOH extracts		AcO extracts	
	I (%)[A]	EC_{50}[B]	I (%)[A]	EC_{50}[B]
SF	81.4 ± 0.8	23.2 ± 3.3[a]	39.6 ± 2.8	62.5 ± 8.5[ce]
SR	72.3 ± 2.2	39.6 ± 6.6[b]	29.5 ± 0.2	61.8 ± 9.1[ce]
SC	39.2 ± 0.04	70.1 ± 4.0[c]	24.3 ± 1.9	142 ± 8
RF	69.8 ± 0.6	32.5 ± 4.1[b]	3.16 ± 0.87	>5000
RR	94.3 ± 4.0	18.4 ± 5.5[a]	5.38 ± 0.36	>1000
HR	88.1 ± 3.0	20.4 ± 4.2[a]	14.3 ± 0.1	169 ± 1
CC	23.8 ± 1.0	81.7 ± 2.8[d]	5.70 ± 1.04	>5000
LC	57.6 ± 0.4	34.1 ± 2.7[b]	14.3 ± 0.2	178 ± 1

MeOH – methanolic extracts, AcO – acetone extracts.
The means with the same letter do not differ statistically according to Duncan's multiple range test ($p \leqslant 0.05$).
SF – shoots *in vivo* grown plants, SR – shoots *in vitro* regenerated plants, SC – shoot culture, RF – roots *in vivo* grown plants, SR – roots *in vitro* regenerated plants, HR – hairy root culture, CC – callus culture, LC – suspension culture.
[A] Percentage of inhibition at 50 μg/ml; 30 min of incubation. The values are the means ± standard deviation (SD).
[B] EC_{50} – the concentration of sample (μg/ml) showing 50% of maximal DPPH· radical scavenging activity. The values are the means ± SE.

reduction between two types of extracts (methanolic and acetone) were observed. Similarly as in DPPH· assay, methanol was a more efficient solvent to extract antioxidants from sage roots (RF, RR and HR extracts) compared to acetone. Mo reduction capacity of root methanolic extracts seems to depend mainly on their RA content. Thus, methanolic extract of hairy roots with highest amount of RA was also characterized by the greatest reducing potential (Fig. 2). However, the acetone extracts of shoots (SF, SC and SR), in which carnosic acid and carnosol were found, showed quite similar reducing capacities as the shoot methanolic extracts containing RA in addition to diterpenoids. It is likely that carnosic acid and carnosol are more important factors at work in this assay than they were in the DPPH· scavenging assay.

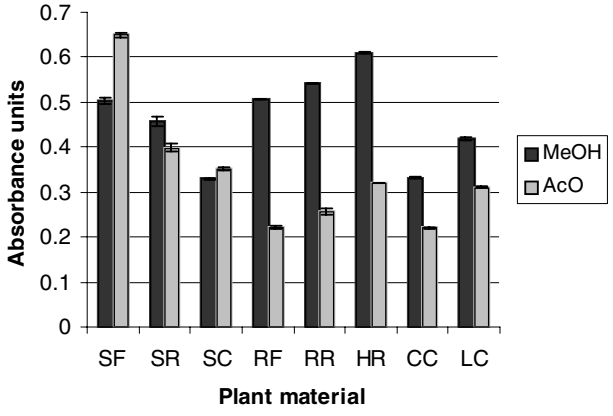

Fig. 2. The reducing power (expressed as absorbance units) of different *S. officinalis* methanolic and acetone extracts in phosphomolybdenum assay at the extract concentration of 50 μg/ml in incubation temperature: 90 °C. MeOH – methanolic extracts, AcO – acetone extracts. The values are the means ± SD. SF – shoots *in vivo* grown plants, SR – shoots *in vitro* regenerated plants, SC – shoot culture, RF – roots *in vivo* grown plants, SR – roots *in vitro* regenerated plants, HR – hairy root culture, CC – callus culture, LC – suspension culture.

3.4. Linoleic acid peroxidation

The inhibition of hydroxyl radical-induced linoleic acid peroxidation (LPO) by various sage extracts was assayed by the TBARS test. In this system, the oxidation activity was different when compared to the results of DPPH· scavenging model. In LPO method, shoot acetone extracts (SF, SR and SC) showed the highest activity with inhibitory effect of 70–78% at a concentration of 100 μg/ml (Fig. 3). The acetone extracts contained higher amount of carnosic acid compared to the other extracts tested. It suggests that the diterpenoid has the greatest participation in lipid protection. Methanolic extracts of the shoots resulted in 53–56% linoleic acid peroxidation inhibition. Methanolic extracts of sage roots (RR, HR and RF) with rosmarinic acid as the main compound showed a similar inhibitory effect (48–56%) (Table 1). The acetone extracts of sage roots and undifferentiated callus and cell cultures of *S. officinalis* indicated weak activity. These extracts contained only a trace of abietane diterpenoids (Table 1). The higher ability of the less polar compounds extracted by acetone to

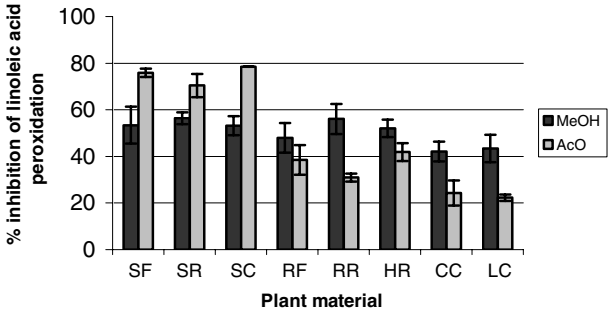

Fig. 3. Inhibition of linoleic acid peroxidation by *S. officinalis* methanolic and acetone extracts at the concentration of 100 μg/ml in the Fe/AA system using TBARS detection. MeOH – methanolic extracts, AcO – acetone extracts; The values are the means ± SD. SF – shoots *in vivo* grown plants, SR – shoots *in vitro* regenerated plants, SC – shoot culture, RF – roots *in vivo* grown plants, SR – roots *in vitro* regenerated plants, HR – hairy root culture, CC – callus culture, LC – suspension culture.

interact directly with the fatty acid molecules may account for the better efficiency of these extracts in breaking the free radical propagation chain reaction (Halliwell & Gutteridge, 1999). The lower activity of methanolic extracts despite the higher content of RA, in addition to the diterpenoids in case of shoots, is hard to interpret with the present data.

4. Conclusions

The results of our work showed antioxidant effects of *S. officinalis* extracts. Such properties have been previously reported for leaf extracts of the plant (Lamaison et al., 1990). We found that also extracts of sage roots exhibited strong antioxidant activity. It was observed that due to variety of antioxidant compounds presented in sage, the activity of the extracts was attributed to the assay method and the method of sample extraction (e.g., the polarity of solvent used for sample extraction). For example, less polar acetone extracts characterized mainly by the presence of carnosic acid and carnosol showed strong activity in LPO method, whereas they exhibited less DPPH$^{\cdot}$ radical scavenging properties. On the other hand, the antioxidant properties measured by DPPH$^{\cdot}$ method were greater in polar fractions, i.e., methanolic extracts, containing both diterpenoids and rosmarinic acid. Attempts to correlate the observed antioxidant activity with chemical nature or content of compounds in the extracts did not reveal direct association between the two parameters. Matsingou, Petrakis, Kapsokefalou, and Salifoglou (2003) have reported that differences in antioxidant properties of plant in different biological system may be attributed to the presence of different substrates as well as to the variable nature of product generated by the reaction system.

The main finding of this work is the fact that extracts of sage organ cultures, especially hairy roots, obtained as a result of transformation through *A. rhizogenes* possessed a high antioxidant activity comparable to or even higher than that of extracts of shoots and roots of field-collected *S. officinalis*. With respect to this, it is possible to consider the culture as a potential source of natural antioxidants. An attraction of transformed roots is their biochemical and genetic stability and relatively rapid growth in the absence of exogenous growth regulators. They are also suitable for bioreactor systems. As expected, the extracts of undifferentiated callus and cell cultures of sage proved to be less active in all methods tested. It is accepted that the dedifferentiation of plant tissues during establishment of callus and cell cultures is often connected with reduction of content of secondary metabolites.

Acknowledgements

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