

Radical Quenching by Rosmarinic Acid from *Lavandula vera* MM Cell Culture

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This study was conducted to evaluate the radical scavenging capacities of extracts and preparations from a *Lavandula vera* MM plant cell culture with different rosmarinic acid content and to compare them with pure rosmarinic and caffeic acids as well. The methods, which were used are superoxide anion and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radicals scavenging assays. Results showed that extracts and preparations from *Lavandula vera* MM possess strong radical scavengers, as the best both radical scavengers appeared to be the fractions with enriched rosmarinic acid content, obtained after ethylacetate fractioning (47.7% inhibition of superoxide radicals and 14.2 μ M 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid equivalents, respectively). These data reveal the possibilities for application of these preparations as antioxidants.

Key words: Rosmarinic Acid, *Lavandula vera* MM Cell Culture, Superoxide Anion

Introduction

Recently there has been considerable interest in finding naturally occurring antioxidants with the aim to replace synthetic ones (such as butylated hydroxyanisole and butylated hydroxytoluene), since it was found that the latter possess toxic and mutagenic effects (Akowuah *et al.*, 2004). Sources of natural antioxidants are different plant species. However, utilization of the latter is connected with some difficulties – treatment of huge plant masses, their storage, strong purification systems, influence of seasonal, geographical and agrotechnical factors on the secondary metabolites content etc. An alternative of classical technologies as well as a possible solution of the listed problems are offered plant biotechnologies. In our previous reports, it was found that *Lavandula vera* MM cell suspension culture is a good producer of rosmarinic acid (RA) and the achieved yields of it are higher in comparison with plant species grown in nature (such as *Melissa officinalis*, *Salvia officinalis*, *Rosmarinus officinalis*) (Georgiev *et al.*, 2004; Pavlov *et al.*, 2005). This led to the possibility for obtain-

ing the crude extracts and preparations with enhanced RA content from *Lavandula vera* MM cell biomass (Georgiev *et al.*, 2006). Further these extracts and preparations were successfully applied for inhibition of oxidation of plant oils and food products (Kovacheva and Georgiev, 2005). The final aim is to use these products as additives in food and cosmetic industries, and with respect to this the extracts and preparations should be characterized through different methods and from different points of view relating to their chemical nature and activity (Frankel and Meyer, 2000).

The purpose of the present report is to determine the total antioxidant activity of extracts and enriched RA preparations from a *Lavandula vera* MM cell culture using ABTS^{•+} [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] (whose radical gives information about Trolox equivalent antioxidant capacity) and superoxide radical, which is important for processes in food systems and in living cells as well.

Materials and Methods

Chemicals

Rosmarinic acid (RA), caffeic acid (CA), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and potassium peroxodisulfate were purchased from Aldrich Chemicals (Milwaukee, USA). Superoxide dismutase (SOD) from bovine erythrocytes and nitro blue tetrazolium (NBT) were supplied by Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). ABTS^{•+} was purchased from Sigma (St. Louis, USA). All other chemicals used in this work were of analytical grade.

Preparation of starting material

A *Lavandula vera* MM plant cell suspension was cultivated in a 3-l bioreactor (BioFlo 110, New Brunswick, Edison, USA) for 13 d under conditions previously described (Pavlov *et al.*, 2005). The *L. vera* MM cell biomass was filtered and frozen, then it was extracted with 50% ethanol according to Georgiev *et al.* (2004) and the obtained extract was lyophilized (lyophilizer Alpha 1–2, Martin Christ, Germany). For experiments on radical scavenging activities the lyophilized crude ethanolic extract (E1) and preparations of RA purified by further adsorption on Amberlite XAD-7 (E2) and ethylacetate (E3) were used according to the purification procedure described by Georgiev *et al.* (2006).

Determination of total phenolic and rosmarinic acid content

The samples were dissolved in 70% ethanol and the phenolic content was determined by Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965). The total phenolic content was present as gallic acid equivalents.

Rosmarinic acid content in the extract and preparations was determined spectrophotometrically at 327 nm (spectrophotometer Shimadzu UV/vis 1240), as it has been previously described (Georgiev *et al.*, 2004).

ABTS^{•+} radical scavenging activity

Generation of ABTS^{•+} was realized through mixing of 7 mM ABTS^{•+} (dissolved in PBS with pH 7.4) with 2.45 mM potassium peroxodisulfate (1:0.5 v/v) at room temperature for 16–17 h. A 1.0 ml solution of ABTS^{•+} (dissolved in ethanol to absorption 0.7 ± 0.02 at 734 nm) was mixed with

10 μ l of samples to be investigated (dissolved in ethanol at the concentration 0.05 mg/ml) or with a solution of Trolox also dissolved in ethanol (final concentration 0–20 μ M was used for standard curve) at 30 °C after 2 min. The effect of each sample on scavenging ABTS^{•+} was expressed as Trolox equivalent antioxidant capacity (TEAC) and defined as the concentration of Trolox (μ M) with equivalent antioxidant activity to a 0.05 mg/ml sample solution ($R^2 = 0.99$).

Superoxide anion radical scavenging activity

The superoxide anion ($O_2^{\bullet-}$) scavenging activity was determined using the method based on the inhibition of NBT-reduction by photochemically generated $O_2^{\bullet-}$ (Beauchamp and Fridovich, 1971). The sample solution, containing 4.9 μ g/ml, was added to 56 μ M NBT, 0.01 M methionine, 1.17 μ M riboflavin, 20 μ M NaCN and 0.05 M phosphate buffer, pH 7.8. The resulting mixture was incubated at 30 °C and after 6 min absorbance at 560 nm was measured. Inhibition of superoxide anion radicals was calculated according to the following equation:

$$\text{Inhibition (\%)} = [1 - A_{560 \text{ nm}}(\text{sample}) / A_{560 \text{ nm}}(\text{control})] \times 100.$$

For detection of mutually influence of both superoxide dismutase and the investigated samples, commercial SOD from bovine erythrocytes (4780 U/mg protein) was added to the above-mentioned.

Statistical analysis

All experiments were carried out fourfold; the reported results are the averages of at least three measurements, and the coefficients of variations (CV), expressed as the percentage ratio between standard deviations (SD) and the mean values, were found to be <15% in all cases.

Results and Discussion

Total phenolics and rosmarinic acid content in extracts and preparations of RA from *L. vera* MM

The obtaining of enriched rosmarinic acid preparations from crude extract (E1) was performed following two variants of purification: adsorption on Amberlite XAD-7 and further elution with 80% ethanolic solution (E2) and through ethylacetate fractioning (E3). Obtained extracts and

	Total phenolics [mg gallic acid/g dry weight]	Correlation	Rosmarinic acid [mg/g dry weight]	Correlation
E1	42.3 ± 4.2	1.0	106.3 ± 5.7	1.0
E2	140.2 ± 15.6	3.3	321.4 ± 11.9	3.0
E3	310.1 ± 21.2	7.3	748.6 ± 9.3	7.0

Table I. Content of total phenolics and rosmarinic acid in crude extract (E1), Amberlite XAD-7 eluate (E2) and ethylacetate fraction (E3) ($\bar{X} \pm \text{SD}$).

RA preparations were checked for their phenolics content and rosmarinic acid content as well (Table I). The correlation between E1:E2:E3 expressed as total phenolics and as rosmarinic acid was established and it was found that in purified E2 and E3 preparations the amounts of RA are 3 and 7 times higher compared to the crude extract, and the content of RA in purified preparations reached 32.1% (in E2 preparations) and 74.9% (in E3 preparations) (Table I).

ABTS^{•+} radical scavenging activity

The ABTS^{•+} radical scavenging is based on decolorization of the blue-green radical, when it reacts with radical scavengers (Re *et al.*, 1999; Hu and Skibsted, 2002). The ABTS^{•+} radical decolorization assay is applicable for the study of both water-soluble and lipid-soluble antioxidants (Re *et al.*, 1999). This method is known for its high sensitivity (Koleva *et al.*, 2001) and is used for determination of total antioxidant activities. Before addition of antioxidants potassium peroxodisulfate was used for forming of ABTS^{•+} free radicals.

The results (Fig. 1) demonstrated that the pure RA standard and E3 showed almost the same ABTS^{•+} radical scavenging activities, according to the TEAC value (14.53 and 14.19 μM Trolox, respectively) versus 19.02 μM Trolox of CA. These

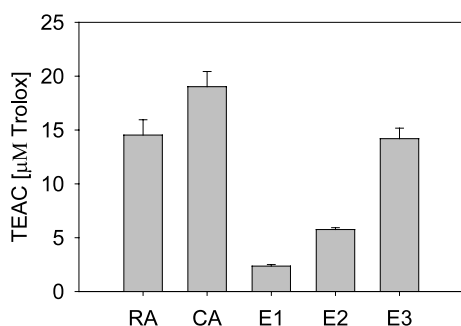


Fig. 1. Total antioxidant activity of rosmarinic acid (RA), caffeic acid (CA) and extracts (E1–E3) expressed as Trolox equivalent (TEAC) in μM . Error bars represent standard deviations.

results support the statement of Nenadis *et al.* (2004) that an increased number of hydroxy groups do not necessarily lead to an increase of the TEAC values. Probably the ABTS^{•+} radical scavenging potential is due to the caffeoyl moiety of the RA molecule.

The antioxidant capacities of E1 and E2 extracts were lower (2.36 and 5.76 μM Trolox, respectively), which is due to their lower RA content in comparison with ethyl acetate preparations.

Superoxide anion radical scavenging activity

Superoxide anion radicals ($\text{O}_2^{\cdot-}$) are generated *in vivo*, which is dismutated very effectively by different superoxide dismutase forms (SOD, EC 1.15.1.1).

The $\text{O}_2^{\cdot-}$ scavenging capacity of extracts from *L. vera* MM and pure compounds (as standards) was investigated and the obtained results are presented in Fig. 2 (black bars). The order of scavenging effectiveness was: E3 (47.7% inhibition) \geq RA (about 46%) $>$ E1 (42.5%) = CA (42.3%) $>$ E2 (33.2%). Crude extract from *L. vera* MM (E1) showed better $\text{O}_2^{\cdot-}$ scavenging activity in comparison with the preparation purified on Amberlite XAD-7 resin (E2), which is higher in RA content.

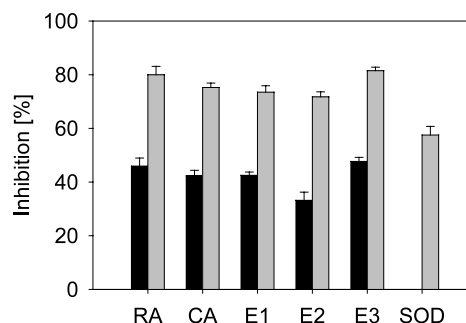


Fig. 2. Superoxide anion radical scavenging activity of rosmarinic acid (RA), caffeic acid (CA) and extracts (E1–E3) from *Lavandula vera* MM (black bars) and mutual scavenging activity with added superoxide dismutase (SOD) from bovine erythrocytes (grey bars). Error bars represent standard deviations.

This means that the $O_2^{\bullet-}$ scavenging activities are correlated notably with the RA content of samples, but also with other compounds present in the crude extracts (and not in the column eluate). Perhaps these compounds are sugars, which are known to serve as donors of hydrogen and by this reason they possess antioxidant activities (Ako-uwah *et al.*, 2004).

For a detailed study of radical scavenging capacity, we investigated the $O_2^{\bullet-}$ scavenging activities of samples at different concentrations, and dose-response curves were formed (Fig. 3). It was established that at higher concentrations (13.3–66.6 $\mu\text{g}/\text{ml}$) the best results were obtained for CA, while RA and ethylacetate fraction E3 showed the same superoxide anion disproportionation activities as SOD. These results are in agreement with previous reported results (Lu and Foo, 2001).

Further, we investigated the $O_2^{\bullet-}$ scavenging activities of samples (4.9 $\mu\text{g}/\text{ml}$) in which commercial SOD was added (5.5 U/ml) (Fig. 2 grey bars). Using this combination the relation between the investigated samples remains the same as without enzyme addition, but in all investigated variants significant inhibition of superoxide radicals was observed. The strongest inhibition of superoxide radicals was observed, when E3 and pure RA were used (81.5% and 80%, respectively), which is about 23% higher compared to the variant when

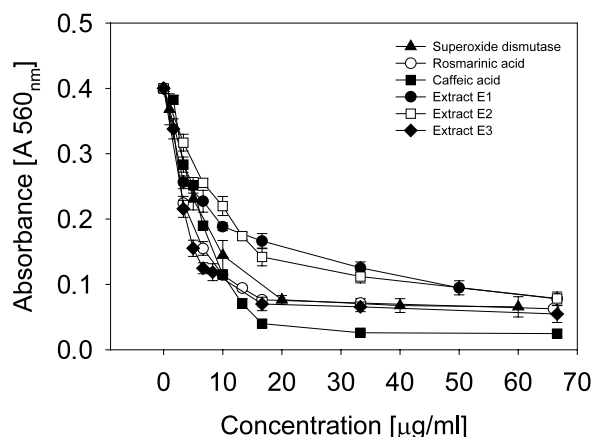


Fig. 3. Dose-response curves of rosmarinic acid, caffeic acid, superoxide dismutase and extracts from *Lavandula vera* MM against superoxide anion radicals. Error bars represent standard deviations.

only SOD was used. The results outlined the possibilities to use these antioxidants (rosmarinic acid and RA-containing preparations) alone or in combination with SOD effectively in cases, when the cells are exposed to oxidative stress.

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