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Antioxidant activity of extracts from *Lavandula vera* MM cell cultures

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Abstract

The antioxidant activity of methanolic and ethyl acetate extracts from *Lavandula vera* MM cell culture were evaluated by the Schaal oven test in bulk sunflower oil and by the DPPH radical method. The oil oxidation was followed by measuring the quantity of primary oxidation products (peroxide value). Authentic rosmarinic acid, caffeic acid and BHT were tested in parallel for comparison. Ethyl acetate extract much better protected the oil from oxidation than methanolic extract and its antioxidant efficiency was comparable to that of pure rosmarinic and caffeic acids and much stronger than that of BHT. Both cell culture extracts and the authentic phenolic acids were much stronger scavengers of DPPH free radical than BHT on an equimolar basis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Lavandula vera*; Cell culture; Rosmarinic acid; Caffeic acid; Schaal oven test; Peroxide value; DPPH

1. Introduction

Lipid oxidation causes a decrease in nutritional value of lipids, in their safety and appearance. Nowadays, various synthetic and natural antioxidants are used in prevention or retardation of lipid oxidation (Branen, Davidson & Salminen, 1990; Hudson, 1990). Recently, some negative side effects of the commonly used synthetic antioxidants have been established that has shifted the consumer interest to the natural products as a less harmful alternative to the synthetic ones (Khal, 1991; Ramarathnam, Osawa, Ochi & Kawakishi, 1995; Shahidi & Wanasundara, 1992; Branen, 1975; Hudson, 1990). Various medicinal plants and herbs (Abdalla and Roozen, 1999; Economou, Oreopoulou & Thomopoulos, 1991; Miyke & Shibamoto, 1997), spices (Madsen & Bertelsen, 1995; Pokorny, Nguyen & Korczak, 1997), tea (Von Gadow, Joubert & Hansmann, 1997); grains, fruits and vegetables (Crozier, Lean, McDonald & Black, 1997; Velioglu, Mazza, Gao & Oomach, 1998) are the main sources of natural antioxidants. Cell cultures purposefully producing bioactive substances represent an alternative source for producing natural antioxidants as well.

In a previous study, we have shown that *L. vera* MM cell culture contains mainly rosmarinic and caffeic acids (Kovatcheva, Pavlov, Koleva, Ilieva & Mihneva, 1996). Rosmarinic and caffeic acids as well as rosemary plant extracts have been found to be powerful antioxidants in different substrates, established by various methods (Banias, Oreopoulou & Thomopoulos, 1992; Brand-Williams, Cuvelier & Berset, 1995; Chen & Ho, 1997; Chen, Shi & Ho, 1992; Cuvelier, Berset & Richard, 1992; Frankel, Huang, Aeschbach & Prior, 1996; Marinova & Yanishlieva, 1992, & 1996; Shahidi & Wanasundara, 1992;).

The aim of this study was to evaluate the antioxidant and radical scavenging properties of extracts from *L. vera* MM cell culture and to compare them to that of some well-known synthetic (BHT) and natural (authentic rosmarinic and caffeic acids) antioxidants.

2. Materials and methods

2.1. Materials

Traditional sunflower oil, free of added synthetic antioxidants, was purchased from "Biser Oliva" Co., Stara Zagora, Bulgaria. Its initial peroxide value was below 2 meq O_2 kg^{-1} oil. Fatty acid composition (in%)

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determined by GC of their methyl esters was as follows: C16:0 5.90; C18:0 4.50; C18:1 27.26; C18:2 63.64.

The reagents used were purchased from: Fluka, Buchs, Switzerland [Butylated hydroxytoluene (BHT), p.a.]; Sigma-Aldrich Chemie GmbH, Steinheim, Germany [2,2′-diphenyl-1-picrylhydrazyl (DPPH), 95% and caffeic acid (CA), 97%]; Extrasynthèse, Genay, France [rosmarinic acid (RA)].

All other reagents and solvents used were of analytical grade.

2.1.1. Preparation of extracts from L. vera MM cell culture

The cell culture *L. vera* MM was cultivated as described by Ilieva and Pavlov (1997). Methanolic and ethyl acetate extracts from the cell culture with reproducible content of rosmarinic acid (Pavlov, Ilieva & Panchev, in press) were prepared as follows: *L. vera* MM fresh biomass (20.0 g) was refluxed with MeOH (3×100 cm^3). For the antioxidant activity assays the combined methanol extracts were concentrated in vacuo to 50 cm^3. Ethyl acetate extract was prepared from concentrated to dryness methanol extract (in vacuo) and subsequent partitioning between ethyl acetate (3×100 cm^3) and 10% NaCl. The combined ethyl acetate extracts were evaporated to dryness and reconstituted with MeOH for further use. Phenolic acids concentrations in the extracts, determined by RP-HPLC under the chromatographic conditions described by Kovatcheva et al. (1996) were as follows: Methanol extract (ME)–RA: 9.30 mg cm^{-3}, CA: 0.74 mg cm^{-3}, unidentified phenolic components: *ca.* 20%; Ethyl acetate extract (EAcE)–RA: 24.06 mg cm^{-3}, CA: 1.52 mg cm^{-3}, *p*-coumaric and ferulic acids: in traces.

2.2. Methods

2.2.1. Determination of antioxidant activity according to the Schaal oven test

The Schaal oven test was applied to follow the oxidation of commercial bulk sunflower oil with and without added antioxidants. Oil samples (80 g), placed into Petri dishes (15 cm diameter), were incubated at 60 ± 1°C in the dark, at free access of air for two weeks. Samples (1.0 g) were withdrawn at suitable time intervals and their peroxide values (POV) were iodometrically determined according to IUPAC Standard Methods (Paquot & Hautfenne, 1987). The studied pure antioxidants were added to the oil in concentrations 0.01, 0.02 and 0.04% (w/w). The extracts were added in amounts equivalent to 0.01, 0.02 and 0.04% (w/w) rosmarinic acid, namely: 0.45, 0.90 and 1.80 cm^3 ethyl acetate extract (26.0 mg cm^{-3}) and 0.85, 1.70 and 3.40 cm^3 methanol extract (12.0 mg cm^{-3}), respectively. A control sample (without added antioxidant) was also run.

The antioxidant efficiency (*AOE*) was calculated according to the formula:

$$AOE = IP_A/IP_0$$

where IP_0, IP_A are the induction periods (the time needed to reach a POV of 20 meq O_2 kg^{-1} oil) for the control sample and the sample with added antioxidants, respectively (Ivanov, Tanchev & Obretenov, 1984). All determinations were carried out in triplicate.

2.2.2. Determination of radical scavenging activity according to the DPPH method

Radical scavenging activity of extracts from *L. vera* MM cell culture, authentic rosmarinic (RA) and caffeic (CA) acids and BHT was determined using the method described by Von Gadow et al. (1997) slightly modified as follows. Ethanolic solutions of DPPH• (10^{-4} M), cell culture extracts, BHT, RA and CA were prepared. DPPH and sample solutions were mixed in a disposable plastic half-microcuvette (EMERGO, Landsmeer, The Netherlands, 1 cm path length). Pure compounds were studied at two molar ratios toward DPPH: 0.5 and 1.0 mol antioxidant/mol DPPH•. The extracts were also studied at molar ratios 0.5 and 1.0 mol antioxidant/mol DPPH•, calculated on the basis of their RA content. The samples were incubated for 15 min in the dark at 30°C and the decrease in absorbance at 515 nm was measured against ethanol using a Specol 11 (Carl Zeiss Jena) spectrophotometer. A blank sample (without antioxidant) containing the same amount of ethanol and DPPH• was prepared and measured daily. DPPH• standard solution was freshly prepared daily and kept in a flask protected from the light with aluminum foil and stored at 4°C between the measurements. All determinations were performed in four replicates. The radical scavenging activity of the tested samples, expressed as% Inhibition against DPPH•, was calculated according to the formula (Yen & Duh, 1994):

$$\%\text{Inhibition} = [(A_B - A_A)/A_B] * 100$$

where A_B is the absorbance of the blank sample and A_A is the absorbance of the tested sample after 15 min.

3. Results and discussion

3.1. Antioxidant activity of L. vera MM cell culture extracts according to the Schaal oven test

In assessment of antioxidant activity various methods of lipid oxidation as well as oxidizable substrates have been used (Brand-Williams et al., 1995; Cuvelier, Berset & Richard, 1990; Meyer, 1994; Simic & Karel, 1980). To accelerate and simplify the study elevated temperatures and/or model systems have been applied. One of the methods often used is the Schaal oven test. The oxidation of lipid substrates (bulk oils and fats) is car-

ried out at ambient or not very high temperatures (up to 60°C). At these temperatures, undesirable alterations of the studied antioxidants such as decomposition, evaporation, etc. are less probable.

Since the antioxidant activity of rosmarinic and caffeic acids has been long recognized we aimed at establishment of the activity of the total cell culture extracts. For this reason, the studied extracts were "equalized" on the basis of their RA content. Thus, not only the contribution of RA and CA to the exhibited activity will be considered but also the effect of the other extract components. Such information would be more beneficial with respect to the further implementation of the extracts in real lipid-containing systems.

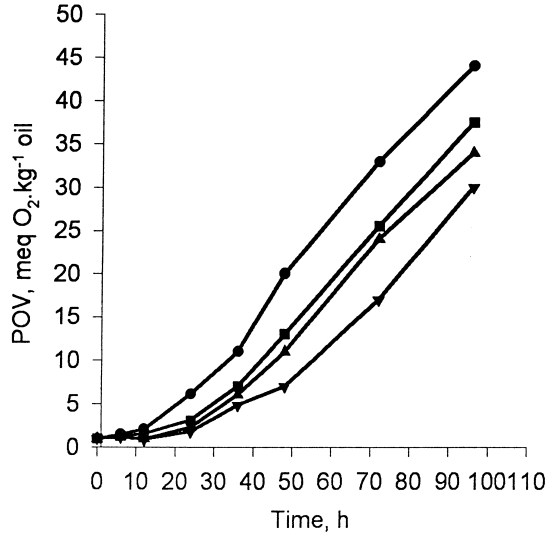

Fig. 1. Effect of BHT on sunflower oil oxidation expressed as peroxide formation (meq O_2 kg^{-1} oil) at three different concentrations. —●—, control; —■—, 0.01%; —▲—, 0.02%; —▼—0.04%.

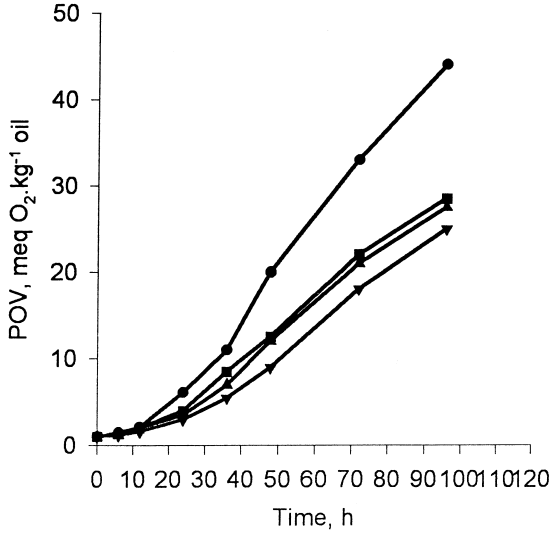

Fig. 2. Effect of caffeic acid on sunflower oil oxidation expressed as peroxide formation (meq O_2 kg^{-1} oil) at three different concentrations. —●—, control; —■—, 0.01%; —▲—, 0.02%; —▼—0.04%.

The oxidation of commercial sunflower oil under the conditions of Schaal oven test, expressed as POV at different concentrations of the added antioxidants, is presented in Figs. 1–5. On the basis of the obtained curves POV–time, induction periods (IP) were determined and the AOE of the studied antioxidants were calculated (Table 1). The longest IP and the highest AOE were observed for the samples with added ethyl acetate extract (EAcE). The obtained values were close to those for the samples with added rosmarinic acid (RA) that can be explained by the composition of the extract. Its main component was RA while the other detected constituents — caffeic (CA), ferulic and *p*-comaric acid,

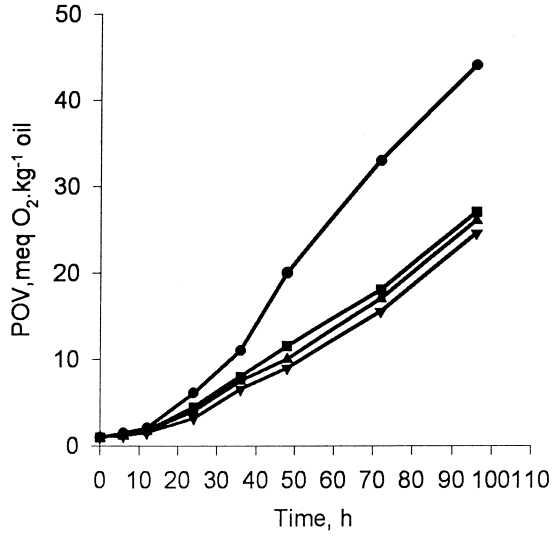

Fig. 3. Effect of rosmarinic acid on sunflower oil oxidation expressed as peroxide formation (meq O_2 kg^{-1} oil) at three different concentrations. —●—, control; —■—, 0.01%; —▲—, 0.02%; —▼—0.04%.

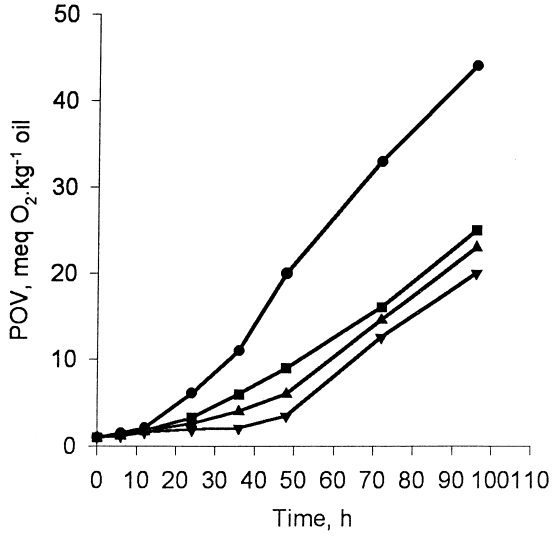

Fig. 4. Effect of ethyl acetate extract from *L. vera* MM cell culture on sunflower oil oxidation expressed as peroxide formation (meq O_2kg^{-1} oil) at three different concentrations. —●—, control; —■—, 0.01%; —▲—, 0.02%; —▼—0.04%.

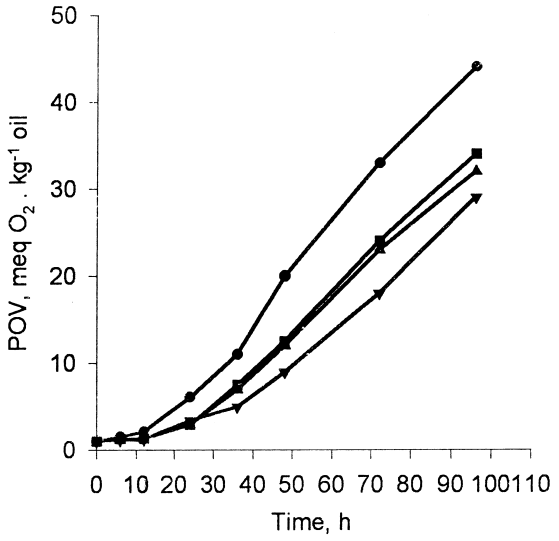

Fig. 5. Effect of methanolic extract from *L. vera* MM cell culture on sunflower oil oxidation expressed as peroxide formation (meq O_2 kg^{-1} oil) at three different concentrations. —●—, control; —■—, 0.01%; —▲—, 0.02%; —▼—0.04%.

Table 1
Induction periods (IP) of sunflower oil and antioxidant efficiency (AOE) of the studied antioxidants[a]

Sample	0.01%		0.02%		0.04%	
	IP (h)	AOE	IP (h)	AOE	IP (h)	AOE
BHT	61.5	1.28	65.0	1.35	77.0	1.58
ME	63.5	1.32	66.0	1.38	79.0	1.60
CA	66.0	1.38	69.0	1.44	79.0	1.65
RA	77.0	1.60	79.5	1.66	85.0	1.77
EAcE	83.0	1.73	88.0	1.83	96.0	2.00

[a] $IP_{control} = 48.0$ h; $AOE_{control} = 1.00$.

were in minor to trace quantities. Caffeic acid and methanolic extract of *L. vera* cell culture (ME) were weaker inhibitors of oil oxidation in comparison to EAcE and pure RA. Their AOE was comparable to that of BHT. In other studies RA has been found to be slightly more active than CA and both more active than BHT (Chen & Ho, 1997; Cuvelier et al., 1992). However, a direct comparison of these results with those obtained in our experiment is difficult because of the different substrate type, experimental conditions, the measured parameter and the AOE is highly dependent on such variables. For example, Chen and Ho (1997) have reported that CA better stabilizes lard than RA but the opposite was observed in corn oil. Cuvelier et al. (1992) have established slightly higher AOE of RA than of CA assessed by accelerated autoxidation of methyl linoleate in a lipophilic medium (dodecane). Though, in studies performed in apolar, lipophilic medium BHT is much less potent antioxidant than RA and CA. Such behavior can be explained by the "polar paradox" phenomenon: polar, hydrophilic antioxidants are more active in apolar substrates (oils, fats) while apolar, lipophilic antioxidants are more potent in polar (oil-in-water emulsions) media (Frankel, Huang, Kanner & German, 1994; Porter, 1980).

The exhibited lower antioxidant activity of ME compared to that of EAcE might be due to the presence of the other constituents (unidentified) that may have inhibiting effect on the activity of its antioxidative components or to change in the interfacial phenomena in the medium. Only after elucidation of their molecular structures and properties, a hypothesis about their mode of action can be suggested. Pokorny et al. (1997) have studied the AOE of different in polarity rosemary plant extracts (including ethyl acetate and ethanolic) in sunflower oil by the Schaal oven test. The AOE of ethyl acetate extract was about 1.3 times higher than that of the ethanolic extract. Similar ratio of the activity was observed for the studied EAcE and ME from *L. vera* MM cell culture. It should be noted that the exact composition of the plant extracts is possibly different from those of cell culture extracts and direct comparisons between both sets of data should be made with caution. However, some confirmation for the relative protective properties of alcoholic and ethyl acetate rosemary extracts on sunflower oil in this test can be found.

It is known that antioxidant concentration can greatly affect the exhibited activity. The data presented in Table 1 also show the effect of concentration on the AOE value of the studied antioxidants. The relationship AOE–antioxidant concentration was linear for RA and EAcE while for BHT, CA and ME a double increase in the antioxidant concentration from 0.02 to 0.04% affected stronger the AOE value than the same increase from 0.01 to 0.02%.

Because the maximum allowed level for the synthetic antioxidants applied in practice is 0.02% (Shahidi & Wanasundara, 1992), we further carried out our study at this concentration. The kinetics of sunflower oil oxidation at 60°C was followed for 15 days in order to establish the long-term effect of the studied antioxidants on sunflower oil oxidation. (Fig. 6). Rosmarinic acid, caffeic acid and EAcE decreased their antioxidant activity much slower than BHT and ME i.e. their capacity to retard lipid oxidation was higher than that of BHT according to this method and for this substrate. Since no negative health effects of RA and CA have been established so far, ethyl acetate extract studied here could be also applied at concentrations higher than 0.02% if stronger protection is necessary. Moreover, inhibitory properties of rosemary extracts on carcinogenesis have been reported (Chen, Shi & Ho, 1992). The AOE determined for EAcE from *L. vera* MM cell culture was higher than that of the pure phenolic acids probably due to some synergetic or additive effects between its components. Furthermore, the easier preparation of extracts with reproducible content of the active components compared to the pure-substances preparation is a premise for

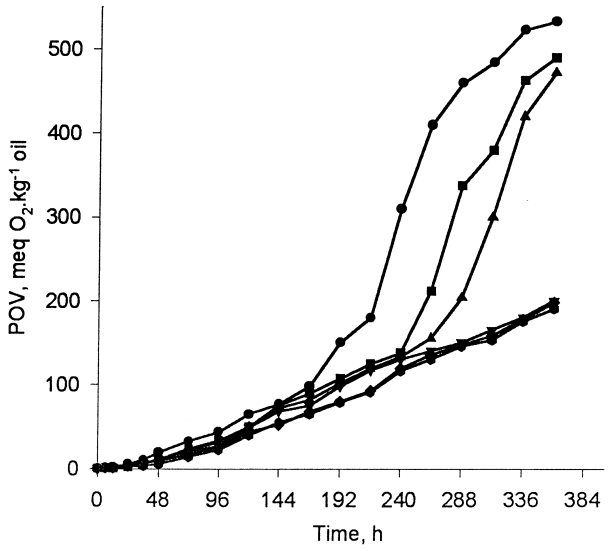

Fig. 6. Long-term effect of BHT, caffeic acid (CA), rosmarinic acid (RA), methanolic (ME) and ethyl acetate (EacE) *L. vera* MM cell culture extracts on the lipid oxidation of sunflower oil. —●—, control; —■—, BHT; —▲—, ME; —▼—, CA; —◆—, RA; —⬢—, EAcE.

a lower cost. Thus, the cell culture extract may serve as an alternative antioxidative additive instead of pure CA and RA.

3.2. *Radical scavenging activity of* L. vera *cell culture extracts determined by the DPPH method*

During lipid oxidation various reactive species (radicals) are formed. They have been proved to be the main cause for the oxidative health damage and aging (Finley & Otterburn, 1993). Hence, it is important to evaluate antioxidants on the basis of their ability to scavenge free radicals. A stable free radical 2,2′-diphenyl-1-pycrylhydrazyl (DPPH•) has widely been used in assessment of radical scavenging activity of different samples: pure synthetic and natural compounds (Abdalla, Tirzite, Tirzitis & Roozen, 1999; Brand-Williams et al., 1995), plant extracts (Yen & Chen, 1995; Yen & Duh, 1994), foods (Yamaguchi, Takamura, Matoba & Terao, 1998).

The radical scavenging activity of the studied antioxidants against the stable DPPH• free radical in terms of hydrogen donating ability is presented in Fig. 7. On an equimolar basis and under the applied experimental conditions, RA and CA showed high and similar radical scavenging activities, while BHT was much less effective inhibitor of DPPH• than them. These results are in agreement with the results reported by other authors (Brand-Williams et al., 1995; Chen & Ho, 1997; Von Gadow et al., 1997). The weaker radical quenching abilities observed for BHT in this test are due to its molecular structure (it contains only one phenolic hydroxyl group able to donate hydrogen) and to its slower kinetic behavior compared to that of RA and CA. The acids possess four and two hydroxyl groups,

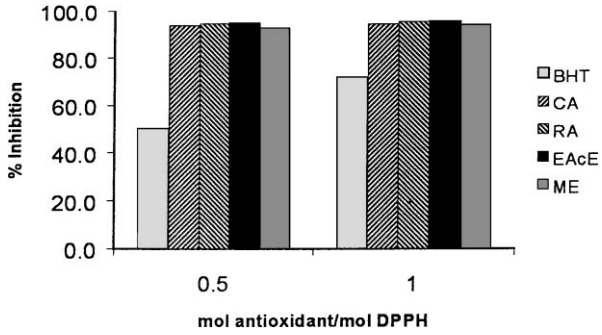

Fig. 7. Radical scavenging activity of the studied antioxidants against DPPH•, expressed as% inhibition (means of four replicates; relative S.D. is less than 1%).

respectively able to donate hydrogen and their *ortho* positions contribute to a better electron delocalization and stabilization of the formed phenoxyl radicals. The radical scavenging activity of EAcE was, as expected, close to that of the pure RA. Methanolic extract also exhibited a strong inhibition of DPPH•.

4. Conclusion

Ethyl acetate- and methanolic extracts from *L. vera* MM cell cultures were found to possess antioxidant, including radical scavenging activity determined by the Schaal oven test and the DPPH• radical method, EtOAc extract being more active. Its antioxidative potency was comparable to that of pure rosmarinic and caffeic acids and higher than that of the widely used synthetic antioxidant BHT, thus presenting an alternative source for natural antioxidative additives.

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