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Food Chemistry 100 (2007) 985–989
Food Chemistry
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Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of *Salvia verticillata* (L.) subsp. *verticillata* and *S. verticillata* (L.) subsp. *amasiaca* (Freyn & Bornm.) Bornm

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Received 4 July 2005; accepted 28 October 2005

Abstract

This study was designed to examine the in vitro antioxidant activities and rosmarinic acid levels of the methanol extracts of *Salvia verticillata* subsp. *verticillata* and *S. verticillata* subsp. *amasiaca*. The extracts were screened for their possible antioxidant activity by two complementary test systems, namely DPPH free radical-scavenging and β-carotene/linoleic acid systems. In the first case, *S. verticillata* subsp. *verticillata* was superior to the subsp. *amasiaca* with an IC_{50} value of 14.5 ± 1.21 μg mg^{-1}. In the β-carotene/linoleic acid test system, inhibition capacity of *S. verticillata* subsp. *verticillata* was $74.4 \pm 1.29\%$. Antioxidant activities of BHT, ascorbic acid, curcumin and α-tocopherol were determined in parallel experiments. Activity of rosmarinic acid was also screened for better establishing the relationship between rosmarinic acid level and antioxidant activity for the plant extracts. *S. verticillata* subsp. *verticillata* had the highest rosmarinic acid level with a value of 28.7 ± 0.89 μg mg^{-1}. There is a strong correlation between the rosmarinic acid level and antioxidant activity potential. Our results showed that rosmarinic acid and its derivatives are more likely to be responsible for most of the observed antioxidant activities of *Salvia* species.

Keywords: *Salvia verticillata* subsp. *verticillata*; *Salvia verticillata* subsp. *amasiaca*; Antioxidant activity; DPPH; β-Carotene/linoleic acid test; Rosmarinic acid

1. Introduction

Free radicals can result in food sourness, oil rottenness, and most industrial product aging. BHT, BHA and TBH are extensively used as antioxidants, at present, in order to reduce the harm caused by free radicals. However, these antioxidants are unfavourable for use in the fields of foods and health because they may cause some tumors and other toxicity in the animal body (Scott, 1988). Many experiments have indicate that free radicals are necessary to support life, though they are also dangerous in biological tissues. Under normal physiological conditions, free radicals in the body will undergo a process of production and continuous scavenging so as to sustain physiological equilibrium. Even when the free radicals generated in the body are in low concentrations, the body metabolism may be disordered and some diseases can be caused (Pietta, 2000).

Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyllactic acid) is mainly found in species of Boraginaceae and the subfamily Nepetoidae of the Lamiaceae (Petersen & Simmonds, 2003). It contains two phenolic rings, both of which have two *ortho*-position hydroxyl groups. There are a carbonyl group, an unsaturated double bond and a carboxylic acid between the two phenolic rings. Its structure is different from the flavonoids, which have been studied extensively. It has many biological activities, such as inhibiting of HIV-1, antitumor, antihepatitis, and protecting the liver, inhibiting blood clots and anti-inflammation.

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0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.10.062

Some experiments have reported the strong capacity of RA for scavenging free radicals, and showed that the antioxidant activity was over three times that of trolox, that RA can inhibit the activity of xanthine oxidase, and it can be used to scavenge the surplus free radicals in the body. In addition, RA can reduce Mo (VI) to Mo (V), preventing the production of free radicals caused by the metal (Petersen & Simmonds, 2003).

The genus *Salvia*, with about 700 species, is one of the most widespread members of the Lamiaceae family. An unusually large number of useful secondary metabolites, belonging to various chemical groups, such as essential oils, terpenoid compounds and phenolic derivatives, have been isolated from the genus, which features prominently in the pharmacopoeias of many countries throughout the world (Banthorpe, Bilyard, & Brown, 1989; Gibbs, 1974; Luis, Gonzalez, Andrews, & Mederos, 1992; Ulubelen & Topcu, 1992).

Many *Salvia* species and their isolated constituents possess significant antioxidant activity in enzyme-dependent and enzyme-independent systems (Dorman, Deans, & Noble, 1995; Hohmann et al., 1999; Lu & Foo, 2001; Malencic, Gasic, Popovic, & Boza, 2000; Zupko et al., 2001). *S. lavandulaefolia* ethanolic extracts (both the water-soluble and chloroform-soluble fractions), individual constituents of the essential oil (the monoterpenoids, 1,8-cineole, linalool, α- and β-pinene) and herb (the phenolic monoterpenoid carvacrol, the flavone luteolin, and the phenolic rosmarinic acid) have been reported to be antioxidants (Adam, Sivropoulou, Kokkini, Lanaras, & Arsenakis, 1998; Dorman et al., 1995; Lu & Foo, 2001; Malencic et al., 2000; Perry et al., 2001; Zupko et al., 2001), while camphor (20–30% of essential oil) has demonstrated pro-oxidant effects in a liposome peroxidation preparation (Perry et al., 2001).

Antioxidant activities of the many members of the genus *Salvia* were reported elsewhere. Additionally, our previous papers concerning the biological activities of *Salvia* species (Tepe et al., 2004; Tepe, Daferera, Sokmen, Sokmen, & Polissiou, 2005; Tepe, Sokmen, Akpulat, & Sokmen, 2006) confirm that this genus has great potential, especially in antioxidant systems, for the food and cosmetic industries.

In this paper, antioxidant potentials of the methanolic extracts of *Salvia verticillata* subsp. *verticillata* and *S. verticillata* subsp. *amasiaca* were determined in relation to their rosmarinic acid levels.

2. Materials and methods

2.1. Plant material

Herbarium information of the plant species, which are individually numbered, is listed below:

1. *S. verticillata* subsp. *verticillata*: Seyitler Village, Artvin-Turkey; 13th August 2004.
2. *S. verticillata* subsp. *amasiaca*: Seyitler Village, Artvin-Turkey; 13th August 2004.

The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (CUFH-Voucher No: 1-AA 3491 and 2-AA 3494, respectively).

2.2. Preparation of the methanolic extracts

The air-dried and finely ground samples were extracted by using the method described elsewhere (Sokmen et al., 1999). Briefly, the sample, weighing about 100 g, was extracted in a Soxhlet apparatus with methanol (MeOH) at 60 °C for 6 h (10.42, and 9.54%, w/w, respectively), extract was then lyophilised and kept in the dark at +4 °C until tested.

2.3. Antioxidant activity

2.3.1. DPPH assay

The hydrogen atom- or electron-donation abilities of the corresponding extracts and some pure compounds was measured by the bleaching of a purple-coloured methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 2,2′-diphenylpicrylhydrazyl (DPPH), as a reagent (Burits & Bucar, 2000; Cuendet, Hostettmann, & Potterat, 1997). 50 μl of various concentrations of the extracts in methanol were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I%) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated form the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.3.2. β-Carotene/linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius, Venskutonis, Van Beek, & Linssen, 1998). A stock solution of β-carotene/linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade), 25 μl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml of distilled water saturated with oxygen (30 min 100 ml/min) were added with vigorous shaking. 2.5 ml of this reaction mixture were dispensed into test tubes and 350 μl portions of the extracts, prepared at 2 g l^{-1} concentrations, were added and emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with the synthetic antioxi-

dant, butylated hydroxytoluene (BHT), as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. Antioxidative capacities of the extracts were compared with those BHT and blank.

2.4. Determination of the rosmarinic acid levels

The rosmarinic acid was isolated from the dried methanolic extracts of the plants studied. Isolation was done with 50% (v/v) ethanol (for 1 h) at 70 °C. The extract was evaporated to dryness, the dry residue was dissolved in 70% (v/v) ethanol and then it was stored for 24 h at −10 °C. The precipitate was filtered off and the filtrate was used to determine rosmarinic acid from its absorbance at 327 nm (Lopez-Arnaldos, Lopez-Serrano, Ros Barcelo, Calderon, & Zapata, 1995). The data presented are averages of two independent experiments.

3. Results and discussion

The extracts obtained by Soxhlet extraction were screened for their possible antioxidant activity by two complementary test systems, namely DPPH free radical-scavenging and β-carotene/linoleic acid systems. Free radical-scavenging capacities of the corresponding extracts, measured by DPPH assay, are shown in Table 1. According to the findings presented in the Table, *S. verticillata* subsp. *verticillata* was superior to the subsp. *amasiaca* with an IC_{50} value of 14.5 ± 1.21 μg mg^{-1}.

Similar results were obtained from the β-carotene/linoleic acid system for both plants. Inhibition capacity of the linoleic acid of *S. verticillata* subsp. *verticillata* was $74.4 \pm 1.29\%$. Additionally, antioxidant activities of BHT, ascorbic acid, curcumin and α-tocopherol were determined in parallel experiments. Antioxidant activity of rosmarinic acid was also screened for better establishing the relationship between the rosmarinic acid contents and antioxidant activities of the plant extracts.

As can be seen from Table 2, there is a strong correlation between the rosmarinic acid level and antioxidant activity potential. According to the Table, *S. verticillata* subsp. *verticillata* has the highest rosmarinic acid level with a value of 28.7 ± 0.89 μg mg^{-1}.

In light of the differences among the wide number of test systems available, the results of a single-assay can give only a reductive suggestion of the antioxidant properties of extracts toward food matrices and must be interpreted with some caution. Moreover, the chemical complexity of extracts, often a mixture of dozens of compounds with differences in functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays in screening work is highly advisable. Among the plethora of methods that can be used for the evaluation of the antioxidant activity (TEAC, TRAP, LDL, DMPD, FRAP, ORAC, DPPH, PCL and β-carotene bleaching), very few of them (TEAC, DPPH, PCL) are useful for determining the activity of both hydrophilic and lipophilic species, thus ensuring a better comparison of the results and covering a wider range of possible applications (Sacchetti et al., 2005). Taking this into account, the in vitro antioxidant activities of the extracts tested, compared to those of BHT, ascorbic acid, curcumin and α-tocopherol, were assessed by two different tests, DPPH free radical-scavenging and β-carotene/linoleic acid systems.

Rosmarinic acid is a natural phenolic compound extracted from *Rosemarinus officinalis* L. It contains two phenolic rings, both of which have the two *ortho*-position hydroxyl groups. There are a carbonyl group, an unsaturated double bond and a carboxylic acid between the two phenolic rings. Its structure is different from the flavonoids, which have been studied extensively. It has many biological activities, such as inhibiting HIV-I, antitumor, antihepatitis, and protection of the liver, inhibiting blood clots and antiinflammation. Some experiments have reported the strong capacity of rosmarinic acid for scavenging free radicals, which showed that the antioxidant activity was over three times that of trolox, that rosmarinic acid can inhibit xanthine oxidase, and it can be used to scavenge the surplus free radicals in the body. In addition, rosmarinic acid can reduce Mo (VI) to Mo (V), preventing the production of free radicals caused by the metal (Petersen & Simmonds, 2003).

As far as our literature survey could ascertain, there are no reports concerning the in vitro antioxidant activities of these sub-species. Chemical compositions and/or phytochemical compositions of *S. verticillata* were reported elsewhere (Chalchat, Gorunovic, & Petrovic, 2001; Nagy et al.,

Table 1
Free radical-scavenging capacities and the inhibition ratio of linoleic acid oxidation by extracts measured in DPPH and β-carotene/linoleic acid assays[a]

Plants	Results in DPPH system (μg mg^{-1})	Results in β-carotene/linoleic acid system (inhibition %)
S. verticillata subsp. *verticillata*	14.5 ± 1.21	74.4 ± 1.29
S. verticillata subsp. *amasiaca*	15.0 ± 0.24	62.1 ± 1.14
BHT	18.8 ± 1.21	96.0 ± 0.23
Ascorbic acid	3.80 ± 0.10	94.5 ± 2.14
Curcumin	7.80 ± 0.30	89.3 ± 1.86
α-Tocopherol	6.50 ± 0.70	96.7 ± 1.72
Rosmarinic acid	2.90 ± 0.30	100 ± 0.27

[a] Results are means of three different experiments.

Table 2
Amounts of rosmarinic acid in the methanol extracts of *Salvia* species[a]

Plants	Amount of rosmarinic acid (μg mg^{-1})
S. verticillata subsp. *verticillata*	28.7 ± 0.89
S. verticillata subsp. *amasiaca*	24.1 ± 1.67

[a] Results are means of three different experiments.

1999; Sefidkon & Khajavi, 1999; Sonmez, Topcu, & Ulubelen, 1997; Ulubelen & Topcu, 1984).

Relationships between antioxidant activities of plant extracts and phenolic contents have been previously reported (Maillard & Berset, 1995). In earlier studies, sage and rosemary were shown to have similar patterns of phenolic compounds and the antioxidative activity was attributed mainly to carnosic acid and rosmarinic acid (Brieskorn & Domling, 1969; Cuvelier, Richard, & Berset, 1996). Studies on the antioxidant activity of sage had also been limited to the diterpenoid compounds (Cuvelier, Berset, & Richard, 1994; Zhang, Bao, Wu, Rosen, & Ho, 1990). Based on some studies (Wang et al., 1998, 1999), two sage phenolic glycosides showed moderate antioxidant activities. Lu and Foo (1999, 2000) and Lu et al. (1999) previously characterized a number of flavonoids and phenolic acids, including the novel rosmarinic acid derivatives, sagecoumarin and sagerinic acid, as new potential antioxidant substances.

As a result, the potency of these compounds could provide a chemical basis for some of the health benefits claimed for sage in folk medicine and warrant further studies to assess their potential as effective natural remedies.

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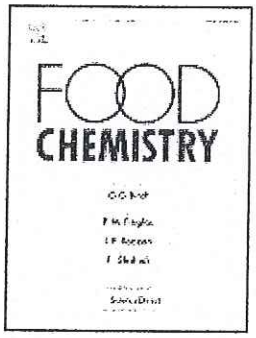

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