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Determination of *in vitro* antioxidant activities and phenolic compounds of different extracts of *Salvia verticillata ssp. verticillata* and *spp. amasiaca* from Turkey's flora

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ABSTRACT

This study was designed to evaluate the *in vitro* antioxidant activities of hexane, dichloromethane and methanol extracts of *Salvia verticillata* ssp. *verticillata* and spp. *amasiaca*. The samples were subjected to screening for their possible antioxidant activity by using 2,2-diphenyl-1-picriylhydrazyl (DPPH) and β-carotene/linoleic acid assays. The methanol extracts particularly found to posses strong antioxidant activities (IC_{50}= 16.0 ± 3.01 and 12.1 ± 3.82 mg ml^{-1}, respectively) whereas the dichloromethane extracts showed low activities. In the linoleic acid system, oxidation of the linoleic acid was moderately inhibited by the methanol extracts (61.3 ± 5.38 and 68.7 ± 1.82 %, respectively). Also, in methanol extracts of these plants have been determined amount of 9 phenolic compounds in different proportions and identified by the RP-HPLC method. These plants have high level of rosmarinic acid (37.1 and 24.83 mg/g, respectively) when compared with other components. Amounts of caffeic acid were determined as 9.82 and 8.69 mg/g, respectively. Our results showed that the high levels of rosmarinic and caffeic acid are more likely to be responsible for most of the observed antioxidant activities of Salvia species.

Keywords: *Salvia verticillata* ssp. *verticillata*; *Salvia verticillata* spp. *amasiaca*; Antioxidant activity; Phenolic contents, DPPH, HPLC.

INTRODUCTION

Oxidative stress may induce damage to cellular biomolecules (Halliwell and Gutteridge, 1999). Recent developments in biomedical science emphasise the involvement of free radicals in many diseases. There is increasing evidence to suggest that many degenerative diseases, such as brain dysfunction, cancer, heart disease and immune system decline could be the result of cellular damage caused by free radicals (Aruoma, 1998). Moreover antioxidants may play an important role in disease prevention. Antioxidants can quench reactive free radicals and they have health-promoting effects (Biglari et al. 2008). Phenolic compounds are known to exhibit antioxidant activities. The antioxidant activities of phenolics are mainly due to their redox properties that allow them to act as a reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans et al., 1996). Phenolic compounds from plants belong to a class of bioactive components that have received much attention during recent years, mainly owing to their positive effects on diet-health interaction in human nutrition (Rice-Evans, 2004). The Lamiaceae includes a large number of plants that are well known for their antioxidant properties. The genus Salvia consist of 900 species in the family of Lamiaceae and this genus is represented in Turkish flora by 88 species and 93 taxa of which 45 are endemic (Guner et al., 2000). In this study was to investigate the

MATERIALS AND METHODS

Collection of plant material

Salvia verticillata ssp. *verticillata* and *Salvia verticillata* spp. *amasiaca* were collected at flowering stage from Sivas-Turkey. Herbarium information of the plant species, which are individually numbered, is listed below:

1- *Salvia verticillata* ssp. *verticillata*: Ulas, meadow-steppe place, Sivas-Turkey; 15th July 2006.

2- *Salvia verticillata* spp. *amasiaca*: Cetinkaya- Divrigi, Sivas-Turkey; 15th July 2006.

The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (CUFH-Voucher NO:1 AA 4560 and 2-AA 4561, respectively).

Preparation of the extracts

The air-dried and powdered leaves of plant (50 g) were extracted successively with 500 ml of Hex, DCM and MeOH by using Soxhlet extractor for 48 h at 30 ˚C, respectively. The extracts were then concentrated in vacuo at 40 ºC using a rotary evaporator. Then the extracts were kept in the dark at +4 ºĊ until tested.

Determination of DPPH scavenging activity

The hydrogen atoms or electrons donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple coloured methanol solution of stable free radical DPPH. This spectrophotometric assay uses stable free radical 2,2-diphenylpicrylhydrazyl (DPPH) as a reagent (Cuendet et al.1997; Burits and Bucar, 2000). Fifty µl of various concentrations of the extracts in methanol was added to 5 ml of a 0.004% MeOH 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:

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

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% inhibit ion (IC_{50}) was calculated form the graph plotted inhibition percentage against extract concentration. Tests were carried out in triplicate.

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 et al, 1998). A stock solution of β–carotene-linoleic acid mixture was prepared as following: 0.5 mg β–carotene was dissolved in 1 ml of chloroform, 25 µl linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Than 100 ml distilled water saturated with oxygen (30 min 100 ml/min.) was added with a vigorous shaking. 2500 µl of this reaction mixture was dispersed to test tubes and 350 µl portions of the extracts prepared at 2g/l concentrations were added and emulsion system was incubated up to 48 hours at room temperature. Same procedure was repeated with positive control BHT and a blank. After this incubation period absorbance of the mixtures were measured at 490 nm. Antioxidant capacities of the extracts were compared with those BHT and blank.

Determination of phenolic acid contents

An HPLC system consisting of a model 600 E HPLC pump, 717 plus Autosampler, 996 photodiode array detector (PAD), and data processor of a Millenium 32 was used for the HPLC analysis (all Waters Corp. Massachusetts, USA). The liquid chromatographic apparatus (Shimadzu LC 10A*vp*) consisted of an in-line degasser, pump and controller coupled to a photodiode array detector (Shimadzu SPD 10A*vp*)equipped with an automatic injector (20 mL injection volume) interfaced to a PC running Class VP chromatography manager software (Shimadzu, Japan). Separations were performed on a reverse-phase C18 Ultra sphere analytical column operating at room temperature with a flow rate of 1 mL/min. Detection was carried out with sensitivity between the wavelengths of 200 to 550 nm. Elution was effected using a nonlinear gradient of the solvent mixture MeOH:H2O:CH3COOH (10:88:2, v/v/v) (solvent A) and MeOH:H2O:CH3COOH (90:8:2, v/v/v) (solvent B). The composition of B was increased from 0% to 15% in 15 min, increased to 50% in 5 min, increased to %70 in 9 min, then increased to %100 in 6 min, held for 10 min, and finally decreased to 0% in 1 min and held for 4 min. Components were identified by comparison of their retention times to those of authentic standards under analysis conditions and UV spectra with our in-house PDA library. A 10 min equilibrium time was allowed between injections (Öztürk et al., 2007).

RESULTS AND DISCUSSION

From aerial parts of *S. verticillata ssp. verticillata,* and *S. verticillata ssp. amasiaca* were obtained successively in values of 5.33 and 3.58 % Hex, 2.13 and 2.10 % DCM and 20.16 and 19.85 % MeOH by Soxhlet extraction, respectively.

Determination of DPPH scavenging activity

The extracts obtained by Soxhlet extraction were screened for their possible antioxidant potential by two complementary test systems, namely DPPH free radical scavenging and β-carotene/linoleic acid systems. Free radical scavenging capacities of extracts measured by DPPH assay are shown in Table 1.

According to the findings presented in the table, MeOH and DCM extracts of *S. verticillata ssp. amasiaca* (12.1 ± 3.82 mg ml^{-1}) were showed highest scavenging activity than extracts of *S. verticillata ssp. verticillata,* (16.0 ± 2.12 mg ml^{-1}). Both of plants exhibited the strongest activity when compared with synthetic antioxidant BHT. DCM extracts showed lower activity than that of the methanol extracts (Table 1) whereas Hex extracts did not show free radical scavenging activity.

Table 1. Antioxidative potentials of various extracts from *S. verticillata ssp. verticillata,* and *S. verticillata ssp. amasiaca* and positive control (BHT, Curcumin and Ascorbic acid) in DPPH.

Plants and controls	MeOH	DCM	Hex
S. verticillata ssp. verticillata,	16.0 ±3.01	21.3 ± 0.86	N.D
S. verticillata ssp. amasiaca	12.1± 3.82	20.3 ± 0.45	N.D
BHT	18.0± 0.40	18.0 ± 0.40	18.0 ± 0.40
Curcumin	7.80 ± 0.32	7.80 ± 0.32	7.80 ± 0.32
Ascorbic acid	3.30 ± 0.17	3.30 ± 0.17	3.30 ± 0.17

MeOH: Methanol; DCM: Dichloromethane; Hex: Hexane; N.D: Not Determined. Results are mean of tree different experiments.

Table 2. Antioxidative potentials of various extracts from *S. verticillata ssp. verticillata,* and *S. verticillata ssp. amasiaca* and positive control (BHT, Curcumin and Ascorbic acid) in β-carotene/linoleic acid.

Plants and controls	MeOH	DCM	Hex
S. verticillata ssp. verticillata,	61.3 ± 5.38	30.4 ± 1.21	N.D
S. verticillata ssp. amasiaca	68.7 ± 1.82	33.5 ± 0.50	N.D
BHT	96.6 ± 1.29	96.6 ± 1.29	96.6 ± 1.29
Curcumin	89.3 ± 2.14	89.3 ± 2.14	89.3 ± 2.14
Ascorbic acid	94.8 ± 1.86	94.8 ± 1.86	94.8 ± 1.86

MeOH: Methanol; DCM: Dichloromethane; Hex: Hexane; N.D: Not Determined. Results are mean of tree different experiments.

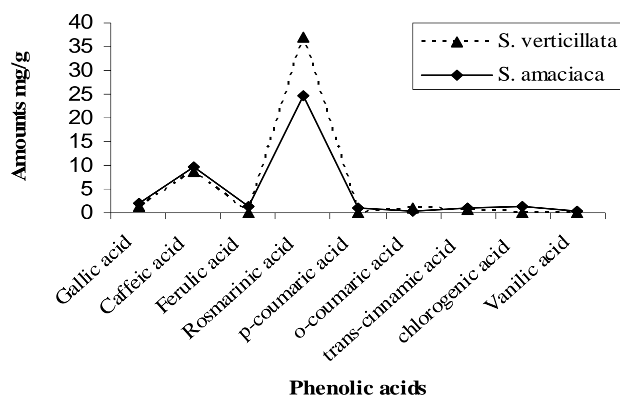

Fig 1. The phenolic acid amounts (mg/g) of *S. verticilllata* ssp. *verticillata* and *S. verticillata ssp. amasiaca.*

β –Carotene/linoleic acid assay

In β-carotene/linoleic acid system are shown in Table 2. As can be seen from the table, both MeOH and DCM of *S. verticillata ssp. amasiaca* were more active than extracts of *S. verticillata ssp. verticillata,* extracts (inhibition value is 68.7 ± 5.38 and 33.5 ± 0.50, respectively) . Additionally, antioxidant activities of BHT, ascorbic acid and curcumin were determined in parallel experiments. Hex extracts did not show any antioxidant activity in both test systems.

Determination of phenolic acids

In the MeOH extracts prepared from *S. verticilllata* ssp. *verticillata* and *S. verticillata ssp. amasiaca* have determined amount of 9 phenolic compounds in different proportions, separated and identified by the RP-HPLC method, are shown in Fig 1. Considerable variation was found in phenolic compounds of both subspecies belonging to *S. verticillata.* According to the results presented in the Fig. 1, these plants have high level of rosmarinic acid (37.1 and 24.83 mg/g, respectively) when compared with the other components. Rosmarinic acid is a natural phenolic compound. The presence of this phytochemical in the members of the genus Salvia is well known. It contains two phenolic rings of which both have the ortho-position hydroxyl groups. There is a carbonyl, 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 the HIV-1, antitumor, antihepatitis and protecting the liver, inhibiting the blood clots and anti-inflammation. Some experiments have reported the strong capacity of rosmarinic acid scavenging the free radicals, which showed that the antioxidant activity is over three times than trolox that rosmarinic acid can inhibit the activity of Xanhine Oxidase, and it is used to scavenge the surplus free radicals in the body. In addition, rosmarinic acid can reduce Mo(VI) to Mo(V), preventing the product of free radicals caused by the metal (Peterson and Simmonds, 2003). Also, it was determined in values of 9.82 and 8.69 mg/g amount of caffeic acid which it is a hydroxycinnamide, respectively. Caffeic acid (3,4-dihydroxycinnamic acid, CA) is a polyphenol catechol with antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic activities (Challis and Bartlett, 1975; Koshihara et al, 1984; Tanaka et al, 1993; Chen et al., 2004). It is found naturally in a wide variety of plant-derived materials such as fruits, vegetables, tea, wine, olive oil, and coffee bean (Shahidi and Naczk, 1995). Cytotoxicity and oxidative damage are prevented by catechols, such as CA, presumably as a result of their free radical-scavenging activities (Shahidi and Naczk, 1995). However, there are also reports of CA-induced cytotoxicity at high concentrations of CA (Nardini et al., 1998). The quality of antioxidant activity is highly correlated with phenolic compounds (e.g., carnosic acid, rosmarinic acid and caffeic acid) (Thorsen and Hildebrandt, 2003). It was determined that amount of gallic acid was low in these plants (1.22 and 1.87, respectively). Vanilic acid, ferulic acid, *p*-coumaric acid and chlorogenic acid (0.46, 3.41, 0.86 and 1.30 µg/mg, respectively) were determined in *S. verticillata ssp. amasiaca* whereas these phenolic acids were not determined in *S. verticillata ssp. verticillata,* (Fig. 1). Though the methanol extract of *S. verticillata ssp. verticillata,* has higher amount of rosmarinic and caffeic acid than *S. verticillata ssp. amasiaca,* in the antioxidant assays *S. verticillata ssp. amasiaca* was exhibited higher activity than *S. verticillata ssp. verticillata,* . This might be due to present of other phenolic acids such as vanilic acid, ferulic acid, *p*- coumaric acid and chlorogenic acid in *S. verticillata ssp. amasiaca*. Standards of caffeic acid and rosmarinic acid exhibited good antioxidant activity using the DPPH assay (Kamatou, 2006).

CONCLUSION

In conclusion, antioxidant activities of the selected plants and their phenolic components have been investigated. These plants exhibited strong antioxidant activity and have some various of phenolic acids, especially rosmarinic acid and caffeic acid.

These plants might be used in many diseases due to pharmaceutical functions. According to our screening, there is not any information about phenolic acids of the selected plants in literature; hence, it might be used as a new source for literature.

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