Arch Pharm Res Vol 29, No 2, 159-164, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Antigenotoxic Effects of *Satureja hortensis* L. on Rat Lymphocytes Exposed to Oxidative Stress

Fatemeh Mosaffa[1,2], Javad Behravan[1,2], Gholamreza Karimi[3], and Mehrdad Iranshahi[2]

[1]Biotechnology Laboratory, Biotechnology and Pharmaceutical Sciences Research Centers, [2]Department of Pharmacognosy and Biotechnology, School of Pharmacy, and [3]Department of Pharmacodinamy and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

(Received October 27, 2005)

The protective properties of *Satureja hortensis* L. on the rat lymphocytes DNA lesions were tested. Lymphocytes were isolated from blood samples taken from healthy rats. DNA breaks and resistance to H_2O_2-induced damage were measured with the comet assay. Rat lymphocytes were incubated in *S. hortensis* ethanolic extract (SHE) (0.05, 0.1, 0.5, 1.0, and 2.5 mg/mL), essential oil (SHEO)(0.05, 0.1, 0.5, 1.0, and 2.5 μL/mL), H_2O_2 (50, 100, and 200 μM), a combination of H_2O_2 (200 mM) with either SHE (1.0, 2.5 mg/mL) or SHEO (1.0, 2.5 μL/mL) at 4°C for 30 min, and the extent of DNA migration was measured using a single-cell microgel electrophoresis technique under alkaline conditions. Treatment of rat lymphocytes with SHE or SHEO resulted in significant reduction of H_2O_2-induced DNA damage compared to controls. SHE exhibited a significant (P<0.01) inhibitory effect on oxidative DNA damage at 2.5 mg/mL. SHEO (1.0 and 2.5 μL/mL) also showed significant inhibitory effects (P <0.01) on H_2O_2 induced chromosomal damage. In conclusion both the ethanolic extract and the essential oil of the plant reversed the oxidative damage to rat lymphocytes induced by hydrogen peroxide.

Key words: Antigenotoxicity, Antioxidant, DNA damage, *Satureja hortensis*

INTRODUCTION

Satureja hortensis L. (summer savory) is a well known aromatic and medicinal plant widely cultivated throughout the Middle East., The aerial stems and leaves of *S. hortensis* have frequently been used in traditional Iranian medicines to treat muscle pains, cramps, nausea, infectious diseases and diarrhea (Zargari, 1990). Extracts of *S. hortensis* have been shown to have antimicrobial, antioxidant, antispasmodic and sedative properties (Hajhashemi *et al.*, 2000; Gulluce *et al.*, 2003; Dorman *et al.*, 2004; Souri *et al.*, 2004). Also, because of its aroma, *S. hortensis* is used as a flavoring additive in cookery (Amin, 1991).

DNA damage and oxidative stresses are accepted to be major factors in many degenerative diseases and in the aging process (Ames and Gold, 1990; Ceruti, 1985; Wiseman *et al.*, 1995). Numerous reports have demonstrated that certain plant products, when studied *in vitro*, have a variety of antioxidant properties and further, that the consumption of foods or beverages rich in antioxidant phytochemicals have positive effects on human health and aging (Hertog *et al.*, 1993; Joseph *et al.*, 1999; Willcox *et al.*, 2004).

In this study, we report on our evaluation the inhibitory effects of an ethanol extract and the essential oils of *S. hortensis* L on the oxidative damage that is caused by H_2O_2 on isolated rat lymphocytes.

Correspondence to: J. Behravan, Dept. Pharmacognosy and Biotechnology, School of Pharmacy, P. O. Box 91775-1365, Mashhad, Iran
Tel: 98-511-8823255, Fax: 98-511-8823251
E-mail: behravanj@yahoo.com

MATERIALS AND METHODS

Plant material

Aerial parts (stems and leaves) of *S. hortensis* were collected from the suburbs of Mashhad (northeastern Iran) in July, 2004. A voucher specimen was deposited in the herbarium of the School of Pharmacy, Mashhad University of Medical Sciences (MUMS), Iran (153-1908-1).

Preparation of ethanol extracts and essential oils

Dried aerial parts of *S. hortensis* (115.28 g) were homogenized and extracted with ethanol using a standard

Soxhlet apparatus. The ethanol was removed under reduced pressure yielding 16.71 g of a viscous extract.

The plant essential oils (SHEO) were obtained from aerial parts of *S. hortensis* (100 g) by a hydrodistillation method using a Clevenger type of apparatus (Clevenger, 1928). SHEO was separated from the aqueous layer, dried over anhydrous sodium sulfate and stored at 4°C until further use. The SHEO yield was 0.6 % (v/w).

Collection of blood samples and cell isolation

Male Sprague-Dawley rats (180-250 g) were obtained from the Razi Institute (Mashhad, Iran) and housed in groups of five under standard laboratory conditions of constant temperature (21±2°C) and a 12/12 h light/dark cycle for at least 10 days prior to testing. Commercial food pellets and tap water was freely available. Animals were transferred to the testing laboratory at least 1 h before the start of the experiments. The use of the animals was carried out in accordance with the regulations of the MUMS Ethics Committee.

Blood samples were obtained by venipuncture from tail veins. Five mL of the whole blood was diluted 1:1 with phosphate-buffered saline (PBS) and carefully layered on the top of a lymphocyte separation medium having a density of 1.077 g/mL (aqueous solution of Ficoll, 57 g/L) in a centrifugation tube and in a ratio of 1:1. After centrifugation for 20 min at 1,000 × *g*, gradient-separated lymphocytes were recovered, diluted 1:1 with PBS and centrifuged a second time at 1,000 × *g* for 5 min. The cell pellets were resuspended in 0.5 mL of PBS and the cells counted in a Neobauer chamber. The cell concentration was adjusted to 5,000 cells/μL in preparation for the comet assay. Cell viability was determined by using the trypan blue dye exclusion technique of (Philips (1973) and was seen to be more than 98%.

Comet assay

The comet assay was performed under alkaline conditions according to the method described by Singh *et al.* (1988) with slight modifications. 10 μL aliquots of a cell suspension was mixed with 90 μL of preheated 0.5% (w/v) low melting point agarose (LMP) in PBS at 37°C and the mixtures then added to frosted glass microscopic slides pre-coated with a 1.5% normal melting point agarose. After solidification of the second layer, a third layer of 100 μL LMP agarose (0.5% w/v) was applied. Immediately after the addition of the cells and their inclusion into the agarose and the solidification of the gel, the slides were separately immersed in a large volume (50 mL per slide) of different concentrations of SHE (0.05, 0.1, 0.5, 1.0, and 2.5 mg/mL), SHEO (0.05, 0.1, 0.5, 1.0, and 2.5 μL/mL), H_2O_2 (50, 100, and 200 μM) and a combination of H_2O_2 (200 μM) with either SHE (1.0 and 2.5 mg/mL) or SHEO (1.0 and 2.5 μL/mL) and incubated at 4°C for 30 min.

The microscopic slides were then washed with PBS, immersed in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% triton X-100, 10% dimethyl sulfoxide, pH 10.0) and incubated overnight at 4°C. At the end of the incubation period, the slides were washed with PBS and placed in an electrophoresis tank. DNA was allowed to unwind for 30 min in freshly prepared alkaline electrophoresis buffer (1 mM Na_2EDTA, 0.3 N NaOH, pH 13.0). Electrophoresis was conducted at 300 mA at 4°C for 30 min; all procedural steps were performed under yellow light conditions to minimize additional DNA damage. The slides were then neutralized with by three times washing Tris-HCl buffer (0.4 M, pH 7.5) and stained with ethidium bromide (20 μg/mL). The slides were studied using a fluorescent microscope (Nikon100) attached to a CCD camera connected to a personal computer. Fifty individual cells were selected for calculations for each analysis, and four separate experiments (four slides for each experimental point) were conducted for each series. Single cells were analyzed with "Casp 1.2.2." software. The DNA damage was expressed as % Tail DNA, where % Tail DNA= [Tail DNA/ (Head DNA + Tail DNA)] × 100. A higher % Tail DNA indicated a higher level of DNA damage.

Statistical analysis

Differences between groups were evaluated by means of one-way analysis of variance (ANOVA) followed by the Dunnett test .Values were expressed as mean ± standard error (SE). Statistical significance was accepted at the $p< 0.05$ level.

Chromatographic fingerprint analyses of the SHE

The liquid chromatographic system used (Knauer) consisted of an in-line degasser and a controller coupled to a UV detector (Knauer) interfaced with a PC running Eurochrom (Knauer). Sample separations were performed on a reverse-phase nucleosil -100 C18 analytical column (125 × 4.0 mm) operating at room temperature and a flow rate of 1 mL/min. Detection was carried out at 450 nm. Elution was by a ternary nonlinear gradient of the solvent mixture $MeOH/H_2O/CH_3COOH$ (10:88:2, v/v/v) (solvent A), $MeOH/H_2O/CH_3COOH$ (90:8:2, v/v/v) (solvent B) and MeOH (solvent C). The composition of the mobile phase was changed from 85:15:0 (A/B/C) to 70:30:0 (A/B/C) in 15 min, changed to 60:40:0 (A/B/C) in 3 min, held for 12 min, changed to 0:100:0 (A/B/C) in 5 min, 0:85:15 (A/B/C) in a further 2 min, and to 0:70:30 (A/B/C) in 11 min, and returned to the initial conditions in 2 min.

RESULTS

Isolated rat lymphocytes were treated with various con-

centrations (50, 100, and 200 μM) of hydrogen peroxide (Figs. 1 and 2), SHE (0.05, 0.1, 0.5, 1.0, and 2.5 mg/mL) or SHEO (0.05, 0.1, 0.5, 1.0, and 2.5 mL/mL) at 4°C for 30 min to evaluate chromosomal; damage. The induced effects of oxidative stress were measured by means of a single-cell gel electrophoresis (SCGE) assay. A significant increase in % tail DNA was observed with increasing concentrations of H_2O_2 (50 to 200 μM; $P<0.001$; Fig. 1). The representative photographic images of the rat lymphocytes exposed to PBS and H_2O_2 are shown in Fig. 2. The experiments using the control cell groups (SHE and SHEO with no H_2O_2 included) resulted in very slight chromosomal damage with the tail DNA distributed

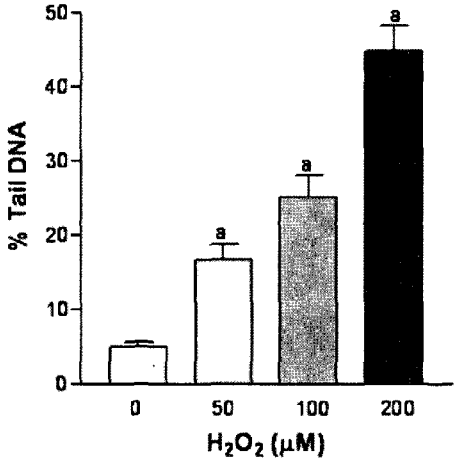

Fig. 1. Rat peripheral blood lymphocyte DNA damage (breaks) after exposure to different concentrations of H_2O_2 (30 min at 4°C and processed by SCGE). The level of the DNA breaks are expressed as the mean ± SE (n=4 slides × 50 lymphocytes) of the percentage of DNA migrated in the tail of the comet (percent DNA in Tail). $^aP < 0.001$ is statistically different compared to the negative control sample (0 μM H_2O_2) (ANOVA test).

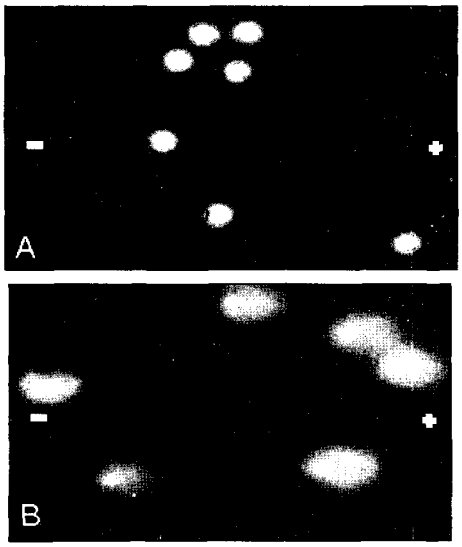

Fig. 2. DNA comet images of lymphocytes: (A) control cells with no induced damage; (B) test cells with H_2O_2 (200 mM) induced DNA damage.

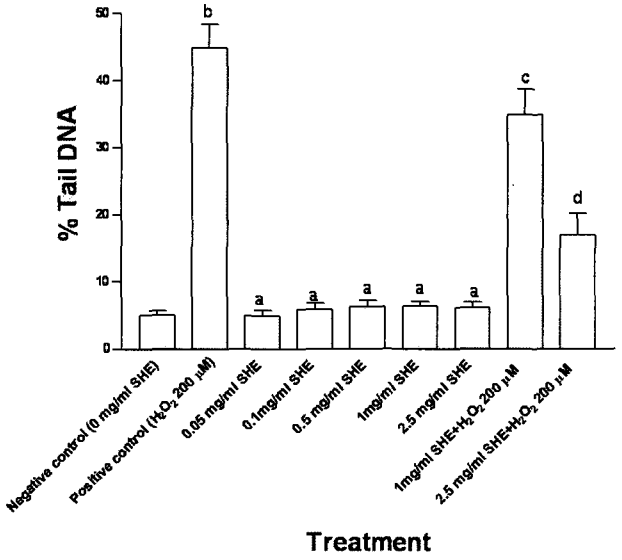

Fig. 3. DNA damage of rat peripheral blood lymphocytes exposed to different concentrations of *Satureja hortensis* ethanol extract (SHE), H_2O_2 (200 mM) or a combination of H_2O_2 (200 μM) with SHE (1.0 and 2.5 mg/mL) at 4°C for 30 min in the SCGE assay. DNA damage is expressed as the mean ± SE (n=4 slides × 50 lymphocytes) of % DNA in tail. $^aP > 0.05$, $^bP < 0.001$, compared with the negative control (0 mg/mL SHE); $^cP > 0.05$, $^dP < 0.01$ compared with the positive control (200 μM H_2O_2) (ANOVA test).

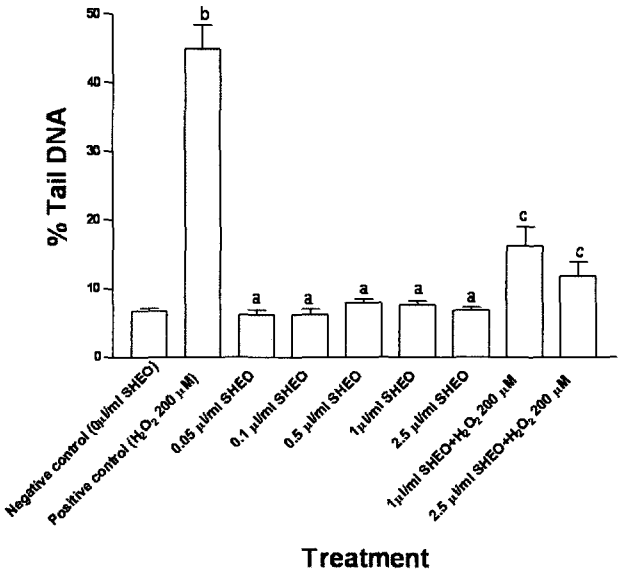

Fig. 4. DNA damage of rat peripheral blood lymphocytes exposed to different concentrations of *Satureja hortensis* essential oil (SHEO), H_2O_2 (200 mM) or a combination of H_2O_2 (200 μM) with SHEO (1.0 and 2.5 μL/mL) at 4°C for 30 min in the SCGE assay. DNA damage is expressed as the mean ± SE (n=4 slides × 50 lymphocytes) of % DNA in the tail. $^aP > 0.05$, $^bP < 0.001$, compared with the negative control (0 μL/mL SHEO); $^cP < 0.01$ compared with the positive control (200 μM H_2O_2) (ANOVA test).

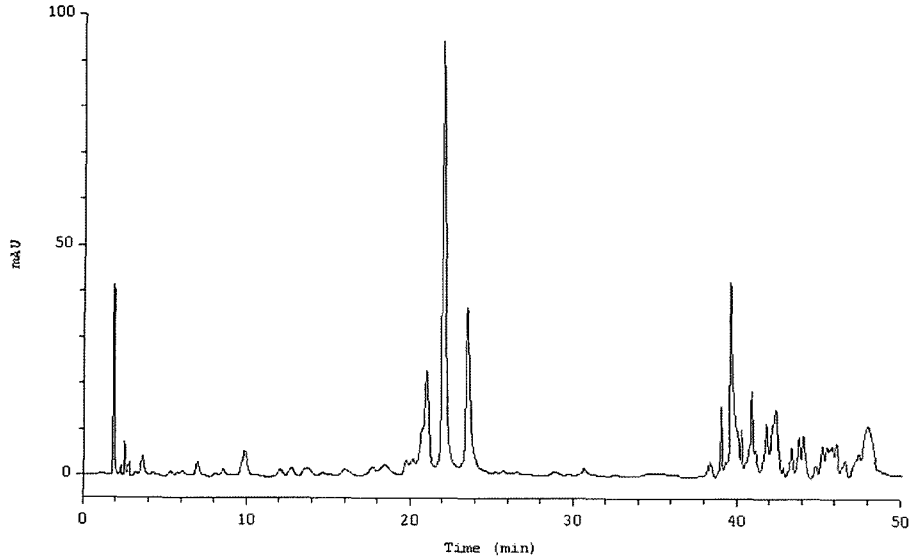

Fig. 5. HPLC fingerprint analyses of a Satureja *hortensis* ethanol extract

between 0 and 10% (P>0.05; Fig. 3 and 4). The protective effects of SHE and SHEO against H_2O_2 induced chromosomal damage were measured and as shown in Fig. 3, SHE had a significant (P<0.01) inhibitory effect on H_2O_2 induced DNA damage at the relatively low concentration of 2.5 mg/mL. The SHEO at concentrations of 1.0 and 2.5 µL/mL exhibited significant inhibitory effects (P <0.01) on H_2O_2 induced chromosomal damage (Fig. 4). The SHE was also analyzed by HPLC and yielded 13 major. A representative SHE chromatogram is shown in Fig. 5.

DISCUSSION

Many plants growing in the wild or cultivated have been used as sources of different classes of useful chemicals, including natural antioxidants (Nakatani, 2002, 2003; Chipault *et al.*, 1956; Bracco *et al.*, 1981; Madsen *et al.*, 1996). Among the various medicinal herbs and plants, some species are endemic and important to that region since they may be used to produce raw materials as well as finished pharmaceutical products containing antioxidant phytochemicals that provide significant health benefits.

The observed biological activities of the essential oils or extracts of a particular plant are attributed to their major components or to the interactions of multiple phytochemicals. The synergistic or antagonistic effects of other compounds present as a minor percentage of the mixture, however, must be considered. Nonetheless, it is well known that the contents of essential oil in particular, and extracts of medicinal herbs in general contain antioxidants and other useful biological factors that may change due to different conditions e.g. the origins and the stage of development of the collected plant material and their immediate environment (Uslu *et al.*, 2003).

The introduction of synthetic antioxidants, e.g., butylated hydroxyanisole and butylated hydroxytoluene (BHT), as potential inhibitors of lipid peroxidation in the food industry has caused serious problems because of their extreme volatility, an instability at elevated temperatures, and strict legal and regulatory limitations, resulting in increasingly high demand for naturally occurring antioxidant sources (Dapkevicius *et al.*, 1998).

Antioxidants act by different mechanisms, one being the prevention of reactive oxygen species (ROS) formation. The ROS are formed during normal cell aerobic respiration and they are the main causes of cellular damage that has been related to cancer (ref?) and cardiovascular diseases (ref?). Antioxidants are well known to play an important role in the protection of cells against oxidative damage that is caused by ROS (Koleva *et al.*, 2002; Ou *et al.*, 2002). For example, it has been speculated that enzymatic oxidation of linoleic acid produces a range of products that may cause cancer (Bull *et al.*, 2002).

In this study, we provide an initial report on the antigenotoxic properties of ethanolic extracts and the essential oils of *S. hortensis,* isolated from the intact plant,. The essential oils from different *Satureja* species have been found to differ qualitatively and quantitatively (Milos *et al.*, 2001; Tumen *et al.*, 1998; Gulluce *et al.*, 2003). The essential oil of the cultivated *S. hortensis* plant has been found to be rich in ç-terpinene and carvacrol. It has also been reported as a source of gamma terpinen, thymol, carvacrol and other phenols (Go'ra *et al.*, 1996; Milos *et al.*, 2001; Muller-Riebau *et al.*, 1997; Madsen *et al.*, 1996; Zargari, 1990; Gulluce *et al.*, 2003). The antioxidant properties of thymol, carvacrol, and γ-terpinene have been reported by other investigators (Daferera *et al.*,

2000; Gulluce *et al.*, 2003; Didry *et al.*, 1993). Therefore, we suggest that the observed antigenotoxic activites of the essential oil is thought to be related to the high content of these components in the oil. The presence of phenolic acids such as rosmarinic acid derivatives in *S. hortensis* have also been reported (Bertelsen *et al.*, 1995), and these polyphenols ultimately were shown to possess antioxidant activities in different test systems (Lu and Foo, 2001). The activity observed in the antigenotoxicity assay, therefore, may be related to the presence of rosmarinic acid derivatives in the extract. Further analytical studies on the composition and on the antioxidative activities of SHE and SHEO should help to better understand the mechanisms responsible for the observed antigenotoxic effects.

ACKNOWLEDGEMENT

The authors are indebted to the research committee of the School of Pharmacy and Research Council of Mashhad University of Medical Sciences, (city,) Iran for approval and financial support of this project.

REFERENCES

Ames, B. N. and Gold, L. S., Dietary Carcinogens, Environmental-Pollution, and Cancer - Some Misconceptions. *Med. Oncol. Tumor Phar.,* 7, 69-85 (1990).

Amin, G., Popular Medicinal Plants of Iran. Iranian Ministry of Health Press, Tehran, pp. 103-104 (1991).

Bertelsen, G., Christophersen, C., Nielsen, P. H., Madsen, H. L., and Stadel, P., Chromatographic Isolation of Antioxidants Guided by a Methyl Linoleate Assay. *J. Agr. Food Chem.,* 43, 1272-1275 (1995).

Bracco, U., J, L., and J, V., Production and use of natural antioxidants. *J. Am. Oil Chem.,* 58, 686-690 (1981).

Bull, A. W., Seeley, S. K., Geno, J., and Mannervik, B., Conjugation of the linoleic acid oxidation product, 13-oxooctadeca-9,11-dienoic acid, a bioactive endogenous substrate for mammalian glutathione transferase. *Biochim. Biophys. Acta,* 1571, 77-82 (2002).

Ceruti, P., Prooxidant states and tumorpromotion. *Science,* 227, 375-381 (1985).

Chipault, J., GR, M., and WO, L., The antioxidant properties of spices in foods. *Food Technol.,* 10, 209-211 (1956).

Clevenger, J., Aparatus for determination of volatile oil. *J. Am. Pharm. Assoc.,* 17, 346 (1928).

Daferera, D. J., Ziogas, B. N., and Polissiou, M. G., GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on Penicillium digitatum. *J. Agr. Food Chem.,* 48, 2576-2581 (2000).

Dapkevicius, A., Venskutonis, R., VanBeek, T. A., and Linssen, P. H., Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *Food Agric.,* 77, 140-146 (1998).

Didry, N., Dubreuil, L., and Pinkas, M., Antimicrobial Activity of Thymol, Carvacrol and cinnamaldehyde alone or in combination. *Pharmazie,* 48, 301-304 (1993).

Dorman, H. J., Bachmayer, O., Kosar, M., and Hiltunen, R., Antioxidant properties of aqueous extracts from selected lamiaceae species grown in Turkey. *J. Agric. Food Chem.,* 52, 762-770 (2004).

Go'ra, J., Liz, A., and Lewandowski, A., Chemical composition of the essential oil of cultivated summer savory (*Satureja hortensis* L. cv. Saturn). *J. Essent. Oil Res.,* 8, 427-428 (1996).

Gulluce, M., Sokmen, M., Daferera, D., Agar, G., Ozkan, H., Kartal, N., Polissiou, M., Sokmen, A., and Sahin, F., *In vitro* antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of Satureja hortensis L. *J. Agric. Food Chem.,* 51, 3958-3965 (2003).

Hajhashemi, V., Sadraei, H., Ghannadi, A. R., and Mohseni, M., Antispasmodic and anti-diarrhoeal effect of Satureja hortensis L. essential oil. *J. Ethnopharmacol.,* 71, 187-192 (2000).

Hertog, M. G., Feskens, E. J., Hollman, P. C., Katan, M. B., and Kromhout, D., Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet,* 342, 1007-1011 (1993).

Joseph, J. A., Shukitt-Hale, B., Denisova, N. A., Bielinski, D., Martin, A., McEwen, J. J., and Bickford, P. C., Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *J. Neurosci.,* 19, 8114-8121 (1999).

Koleva, I. I., van Beek, T. A., Linssen, J. P. H., de Groot, A., and Evstatieva, L. N., Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochem. Anal.,* 13, 8-17 (2002).

Lu, Y. R. and Foo, L. Y., Antioxidant activities of polyphenols from sage (Salvia officinalis). *Food Chem.,* 75, 197-202 (2001).

Madsen, H. L., Andersen, L., Christiansen, L., Brockhoff, P., and Bertelsen, G., Antioxidative activity of summer savory (*Satureja hortensis* L) and rosemary (*Rosmarinus officinalis* L) in minced, cooked pork meat. *Z Lebensm Unters For,* 203, 333-338 (1996).

Milos, M., Radonic, A., Bezic, N., and Dunkic, V., Localities and seasonal variations in the chemical composition of essential oils of Satureja montana L. and S. cuneifolia Ten. *Flavour Frag. J.,* 16, 157-160 (2001).

Muller-Riebau, F. J., Berger, B. M., Yegen, O., and Cakir, C., Seasonal variations in the chemical compositions of essential oils of selected aromatic plants growing wild in Turkey. *J. Agr. Food Chem.,* 45, 4821-4825 (1997).

Nakatani, N., Antioxidants in herbs of Okinawa islands. *Abstr.*

Pap. Am. Chem. S, 223, U45-U45 (2002).

Nakatani, N., Antioxidants in herbs of Okinawa islands. *Acs. Sym. Ser.,* 859, 166-175 (2003).

Ou, B. X., Huang, D. J., Hampsch-Woodill, M., Flanagan, J. A., and Deemer, E. K., Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *J. Agr. Food Chem.,* 50, 3122-3128 (2002).

Philips, H., In Tissue Culture. Methods and Applications(Eds, Kruse, P. and M. Patterson) Academic press, New York, pp. 406-408 (1973).

Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L., A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.,* 175, 184-191 (1988).

Souri, E., Amin, G., Farsam, H., and Andaji, S., The antioxidant activity of some commonly used vegetables in Iranian diet. *Fitoter.,* 75, 585-588 (2004).

Tumen, G., Kirimer, N., Ermin, N., and Baser, K. H. C., The essential oil of Satureja cuneifolia. *Planta Medica,* 64, 81-83 (1998).

Uslu, C., Murat Karasen, R., Sahin, F., Taysi, S., and Akcay, F., Effects of aqueous extracts of *Satureja hortensis* L. on rhinosinusitis treatment in rabbit. *J. Ethnopharmacol.,* 88, 225-228 (2003).

Willcox, J. K., Ash, S. L., and Catignani, G. L., Antioxidants and prevention of chronic disease. *Crit. Rev. Food Sci. Nutr.,* 44, 275-295 (2004).

Wiseman, H., Kaur, H., and Halliwell, B., DNA damage and cancer: measurement and mechanism. *Cancer Lett.,* 93, 113-120 (1995).

Zargari, A., Medicinal Plants. Tehran University Press, Tehran, pp. 42-45 (1990).