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Comparison of Chemical Compositions and Antioxidant Activities of the Essential Oils of Two *Ziziphora* Taxa from Anatolia

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Abstract

The aim of the current study was to investigate the protective effects of essential oils, used in traditional Turkish food and medicine, prepared from two *Ziziphora* subspecies against H_2O_2-induced oxidative damage in human erythrocytes. Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities, and effects of lipid peroxidation (LPO) and reduced glutathione (GSH) levels of the essential oils on erythrocyte were assessed. The results indicated that both essential oils of *Ziziphora* species were effective on antioxidant enzyme systems of erythrocytes when compared with H_2O_2 group. *Ziziphora taurica* Bieb. subsp. *taurica* was more effective than endemic *Ziziphora taurica* Bieb. subsp. *cleonioides* (Boiss) P. H. Davis on CAT, GPx, and SOD enzyme systems of erythrocytes. In this study, we established that the essential oils of *Z. taurica subsp. taurica* contained caryophyllene oxide (26.16%), β-caryophyllene (24.80%), and germacrene-D (7.92%), and *Z. taurica subsp. cleonioides* contained (+)-pulegone (69.24%), piperitenone (6.47%), and limonene (3.59%). Results presented here may suggest that the essential oils possess antioxidant properties, and therefore they can be used in the nutraceutical or pharmaceutical industry.

Keywords: Antioxidant enzymes, β-caryophyllene, erythrocytes, essential oil, pulegone, *Ziziphora*.

Introduction

In the past two decades, much emphasis has been given to natural antioxidants. This is due to a general trend toward natural ingredients in food and the relation of antioxidants to free-radical mechanisms in the human body and to protection from chronic disease. The protection that antioxidants provide against diseases, including cancer and cardio- and cerebrovascular diseases, has been attributed to the various antioxidants, especially antioxidant vitamins, and flavonoids. Essential oils are important natural antioxidants. Their antioxidant activity has been attributed to the presence of polar phenol compounds. As a result, naturally occurring nutritive and non-nutritive antioxidants have become a major area of scientific research (Cao et al., 1996; Demo et al., 1998; Mantle et al., 1998).

The genus *Ziziphora* L. (Lamiaceae) is represented in the Turkish flora with six different taxa of five species, and one of them is endemic. *Z. taurica* Bieb., an annual; strongly aromatic, 5–35 cm in height, with reddish-violet, lilac, or white flowers. There are two subspecies, *Z. taurica subsp. taurica* and endemic *Z. taurica subsp. cleonioides* (Boiss) P. H. Davis, both distributed throughout the western Anatolian region.

Z. taurica, *Z. capitata* L., and *Z. tenuior* L. species are used for the treatment of various diseases in Anatolia. These are used for their aperitive, carminative, and antiseptic effects. Especially, infusions of *Z. taurica* subsp. *cleonioides*, known as "filiskin otu," and *Z. taurica subsp. taurica*, known as "nane ruhu" (mint spirit), have been used internally to treat gastrointestinal symptoms such as stomach pains, and also *Z. taurica subsp. taurica* has been used externally for its wound-healing effect (Baser et al., 1992; Baytop, 1999).

Chemical compositions of the essential oils of *Z. taurica subsp. taurica* (Sezik & Tumen, 1986) and *Z. taurica subsp. cleonioides* (Sezik & Tumen, 1988, 1990) have been

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studied with GLC (Gas liquid chromatography). The exact compositions of the essential oils of all Turkish *Ziziphora* taxa have been identified and compared with GC and GC-MS (Gas Chromatography-Mass Spectroscopy) (Baser et al., 1992). The composition of the essential oil of *Z. taurica subsp. cleonioides* that had been collected from a different locality was reported to have pulegone as its major component (81.86%) (Meral et al., 2002). Differences in the essential oil composition, between and within the species of *Ziziphora*, have been reported (Baser et al., 1992).

The aim of the current study was to compare the chemical compositions and the antioxidant activities of the essential oils of two *Ziziphora* subspecies that are traditionally used as folk medicine and food in Anatolia.

Materials and Methods

Plant material

Aerial parts of fresh plant material from wild collections were gathered at the start of the flowering season (June 2003), from the Babadag, Altindere village at an altitude of 750 m, Denizli, Turkey (*Z. taurica* Bieb. subsp. *taurica*), and from the Gume Mountain at an altitude of 800 m, Tire, Izmir, Turkey [*Z. taurica* Bieb. subsp. *cleonioides* (Boiss) P. H. Davis)]. The plants were identified at IZEF Herbarium (http://www.izef.ege.edu.tr), Ege University, Faculty of Pharmacy, Department of Pharmaceutical Botany (Izmir, Turkey), and voucher specimens were deposited at IZEF Herbarium: *Ziziphora taurica* Bieb. subsp. *taurica* (IZEF no. 5751), *Ziziphora taurica* Bieb. subsp. *cleonioides* (Boiss) P. H. Davis (IZEF no. 5752).

Preparation of essential oils of *Ziziphora* species

The dried-in-shade at ambient temperature aerial parts of two *Ziziphora* taxa were hydrodistilled for 3 h using a modified Clevenger-type apparatus according to the European Pharmacopoeia (1996). The essential oils were dried over anhydrous sodium sulfate and stored at 4°C before analysis.

GC-MS analysis conditions

The analysis of the essential oils was performed using a Hewlett-Packard (HP) (Kyoto, Japan) 6890 gas chromatograph, coupled with an HP 5973 mass selective detector and HP-5 capillary column (60 m × 0.25 mm i.d., film thickness 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed from 50°C to 300°C at 10°C/min. Injector temperature was 150°C and detector temperature was 250°C. Diluted samples (1/100 in ethylacetate, v/v) of 1.0 μl were injected by autosampler in the non-split mode (Baser et al., 1992; Adams, 2001).

Identification of essential oil compounds was based on comparison of their relative retention time and mass spectra with those of commercial standards (for the main components) and retention indices (RI) relative to C_8–C_{32} *n*-alkanes mixture. The results were also confirmed by computer matching of mass spectra with the Wiley 275 L mass spectra data library (Baser et al., 1992; Adams, 2001). The relative percentage of the essential oil constituents was calculated from the GC peak areas.

Chemicals

RANDOX-Ransel and RANDOX-Ransod enzyme kits were used for glotathione peroxidase (GPx) and superoxide dismutas (SOD) assays, respectively. Hydrogen peroxide (3% solution), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals used were of analytical grade. Essential oil components were determined using commercially available standards from Sigma Chemical Co.

Erythrocyte isolation

Fresh blood samples from normal healthy donors were centrifuged at 3000 rpm for 15 min, and plasma and buffy coats were removed. Red cells were washed with phosphate buffered saline (PBS; pH 7.00, containing 140 mM NaCl) three-times.

Preparation incubation with essential oils of *Ziziphora* species

Erythrocytes were divided control group, H_2O_2 group (oxidative stress group), and essential oils of *Ziziphora* species group.

Incubation mixtures of erythrocytes were prepared as follows:

Control group: Erythrocyte 750 μl, PBS 1000 μl, distilled water 250 μl.
H_2O_2 group: Erythrocyte 750 μl, H_2O_2 (10 mM) 50 μl, PBS 950 μl, distilled water 250 μl.
Ziziphora species group: Erythrocyte 750 μl, H_2O_2 (10 mM) 50 μl, essential oils of *Ziziphora* species 250 μl, PBS 950 μl.

These experimental groups were incubated in a shaking water-bath (60 revolutions per minute) for 1 h at 37°C. After the incubation, PBS (including tested essential oils) was removed by centrifugation (2500 rpm for 5 min), and red cells were washed twice with PBS. They were hemolyzed by adding 2 volumes of cold distilled and deionized water and vortexed for 15 s. Antioxidant enzymes activities, (LPO, GSH, and Hb levels) were

determined. Three measurements were performed on each group (control, H_2O_2, and essential oil groups).

Antioxidant enzyme activity

CAT activity

Catalase (CAT) activity was determined using the method of Aebi (Aebi, 1984). The reaction mixture consisted of 1 ml phosphate-buffered saline (50 mM, pH 7.00), and 2 ml diluted erythrocytes. The mixture was incubated at 25°C for 3 min, and the reaction was started by the addition of 1 ml of 30 mM H_2O_2. The decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm at 25°C in a Shimadzu UV-1601 (Kyoto, Japan) spectrophotometer with temperature-control. The results are expressed for erythroctyes as U/g Hb.

SOD activity

SOD activity was determined using a commercially available enzyme kit (Ransod, RANDOX/SD-125 supplied by Randox Laboratories, Crumlin, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium-chloride to form the red formazan dye. The superoxide dismutase activity was measured by the degree of inhibition of this reaction. The results are expressed for erythroctes as U/g Hb.

GPx activity

GPx activity was determined using a commercially available enzyme kit (Ransel, RANDOX/RS-504 supplied by Randox Laboratories). This method is based on Paglia and Valentine's method. In this method, GPx catalyses the oxidation of glutathione by hydrogen peroxide. In the presence of glutathione reductase (GR) and reduced nicotinamide adenin diphosphate (NADPH), the oxidized glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$ (oxidized form). The decrease in absorbance at 340 nm was measured. The results are expressed for erythroctes as U/g Hb.

GSH determination

Reduced glutathione (GSH) levels were determined in leukocyte and erythrocyte homogenate by using Ellman's reagent (DTNB). The assay is based on the reduction of DTNB to NMBA (2-nitro-5-mercaptobenzoic acid) by GSH. NMBA is deep yellow and this color, correlated with –SH groups was measured spectrophotometrically at 412 nm (Sedlak, 1968).

TCA (4 ml, 5%) was added to erythrocyte hemogenate samples (1 ml) in centrifuge tubes. These mixtures were centrifuged at 1000 rpm for 15 min. Phosphate-buffered saline (50 mM, pH 8.00, 2 ml) and DTNB (5 μM, 250 μl) were mixed with 200-μl aliqouts of erythrocyte supernatant. Absorbances of these mixtures were measured against a blank tube [distilled water (200 μl) was added instead of supernatant] at 412 nm. The results are expressed for erythrocytes as μg/g Hb.

LPO determination

Lipid peroxidation (LPO) was measured by the thiobarbituric acid method (Satoh, 1978; Yagi, 1984). This method evaluates oxidative stress by measuring malondialdehyde (MDA), the last product of lipid breakdown caused by oxidative stress. In all experimental groups, erythrocytes hemogenate samples were used. Test solutions (0.5 ml aliqouts, samples and standards) were added to 4 ml of N/12 H_2SO_4, and 0.5 ml of 10% phosphotungstic acid was added and allowed to stay at room temperature for 5 min. The mixture was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. N/12 H_2SO_4 (2.5 ml) was added to each tube. Tubes were centrifuged at 3000 rpm for 10 min and again the supernatant was discarded. N/12 H_2SO_4 (2.5 ml) and 0.2% thiobarbituric acid were added to each tube, and the tubes were allowed to stand in a boiling water-bath for 60 min. After cooling in a cold water-bath, each tube was treated with 3 ml of the mixture of *n*-butanol and HCl (15:1, v/v) and the mixture was shaken vigorously. After centrifugation at 3000 rpm for 5 min, absorbance of the organic layer (upper layer) was measured at 532 nm.

Hemoglobin determinations

Hemoglobin concentrations, in erythrocyte hemolysates, were determined by the cyanmethemoglobin method (Bauer et al., 1974).

Statistical analysis

Antioxidant enzymes activities, LPO and GSH levels of H_2O_2 and of both *Ziziphora* species groups were compared with the control group. The results were analyzed by Student's *t*-test (SPSS for Windows, release 11.0). Differences were considered significant at $p < 0.05$, $p < 0.01$, and all results were expressed as mean $\pm$SE.

Results

Chemical composition of the essential oils

The yields of the isolated pale yellow essential oils amounted to 0.8% and 1.4% (v/w), on a dried weight basis, for *Z. taurica subsp. taurica* and *Z. taurica subsp. cleonioides*. The components of essential oils of both *Ziziphora* taxa are listed in Table 1. Fifteen components were identified representing 88.49% of essential oil of *Z. taurica subsp. cleonioides*. The main components of the

Table 1. Quantitive compositions of the essential oils of two *Ziziphora* subspecies.

Compounds	RI	*Ziziphora taurica subsp. cleonioides* %	*Ziziphora taurica subsp. taurica* %
α-Pinene[a]	940	0.54	—
β-Pinene[a]	982	0.71	—
Yomogi alcohol[b]	1000	3.27	—
p-Cymene[a]	1025	0.10	0.54
Limonene[a]	1032	**3.59**	0.54
Linalool[a]	1100	—	**7.65**
Isomenton[a]	1167	0.21	—
Isopulegon	1181	1.70	—
α-Terpineol[a]	1189	—	1.31
(+)-Pulegone[a]	1223	**69.24**	0.36
Geranial	1273	—	0.54
Thymol[a]	1294	—	2.54
Carvacrol[a]	1301	0.49	—
Piperitenone	1318	**6.47**	—
α-Copaene	1376	0.09	1.97
β-Bourbonene	1388	—	1.14
β-Caryophyllene[a]	1421	0.18	**24.80**
Aromadendrene	1439	0.37	—
β-Farnesene	1446	—	0.76
α-Humulene[a]	1457	—	1.55
Germacrene-D[a]	1482	—	**7.92**
cis-Calamenene	1523	0.15	—
Delta-Cadinene	1528	—	1.31
Germacrene-B[b]	1559	—	0.47
Nerolidol	1569	—	1.04
Spathulenol	1581	—	1.45
Caryophyllene oxide[a]	1587	1.32	**26.16**
Cedrol	1603	—	0.64
% Total		**88.49**	**82.69**

Major compounds listed in bold.
[a]Comparison with commercial standards.
[b]Based on only MS data.

essential oil were (+)-pulegone (69.24%), piperitenone (6.47%), and limonene (3.59%). Nineteen components were identified representing 82.69% of essential oil of *Z. taurica subsp. taurica*. The main components of the essential oil were caryophyllene oxide (26.16%), β-caryophyllene (24.80%), and germacrene-D (7.92%).

Erythrocytes antioxidant enzymes, LPO, and GSH levels

In this study, we investigated the *in vitro* effects of two *Ziziphora* subspecies on the levels of human erythrocytes antioxidant enzymes, LPO and GSH. The results of our study are shown in Table 2. Antioxidant enzyme activities, LPO and GSH levels for the H_2O_2 and both *Ziziphora* species treated groups are compared with levels from the control group. It has been found that the antioxidant effects of *Z. taurica subsp. taurica* oils were higher than those of *Z. taurica subsp. cleonioides* oils.

Discussion

Reactive oxygen species (ROS), which include nonradical species such as hydrogen peroxide, are highly reactive transient chemical species formed in all tissues during normal aerobic cellular metabolism. ROS have the potential to initiate damage to various intracellular components (nucleic acids, lipids, proteins) on which normal cell functioning depends (Mantle et al., 1998).

Cells have developed a comprehensive set of antioxidant defense mechanisms to prevent ROS formation and to limit their damaging effects. These mechanisms include enzymes to inactivate peroxides, proteins to sequester transition metals, and a range of compounds to scavenge free radicals. These include a number of specific enzymes, viz. catalase (CAT; EC 1.11.1.6), which reduces the H_2O_2 via $2H_2O_2 = 2H_2O + O_2$; superoxide dismutase (SOD; EC1.15.1.1), which reduces O_2^- via $2O_2^- + 2H^+ = H_2O_2 + O_2$; and glutathione peroxidase (GPx, EC 1.11.1.9), which reduces peroxides (H_2O_2 or organic peroxides) via 2 GSH (reduced glutathione) + ROOH leading to GSSG (oxidized glutathione) + ROH + 2 H_2O, where ROOH is peroxide and ROH is the corresponding alcohol (or water for 2 H_2O_2 as substrate) (Guemouri et al., 1991; Amstad et al., 1993; Isamah et al., 2004). Antioxidant enzyme capacity of erythrocytes can be a suitable model because erythrocytes can be easily obtained, homogenized, and stored. Due to these properties, antioxidant enzyme capacity determination was chosen (Nagababu et al., 2003).

This paper is focused on the investigation of the effects of two *Ziziphora* species on antioxidant enzyme activities, LPO and GSH levels in human erythrocytes. According to this study, high levels of all antioxidant enzyme activities and LPO levels were found in the H_2O_2 group in erythrocytes. Both *Ziziphora* species treated groups have decreased LPO levels and antioxidant enzyme activities. However, LPO levels and antioxidant enzyme activities of *Ziziphora taurica subsp. taurica* group were found lower than *Ziziphora taurica subsp. cleonioides* group. Lipid peroxidation and PUFA (polyunsaturated fatty acids) oxidation, is highly deleterious, resulting in damage to cellular biomembranes as a consequence of oxidative deteriorated membrane lipids. LPO mediates membrane degradation in a variety of oxidative stress conditions. Among biomarkers of stress, the alteration of membrane phospholipids through lipid peroxidation is considered to be one of the primary key events in oxidative damage (Townsend, 2003; Elhassaneen et al., 2004; Ozturk et al., 2004).

GSH is a tripeptide thiol found in millimolar concentrations in virtually all cells. It is a cofactor in many enzymatic reactions and important in intracellular cysteine storage. GSH is the reduced form, which can be oxidized to a disulfide form (GSSG) or to form mixed disulfides with other thiol-containing reactants (GSSR). GSH is released by cells in response to oxidative stress,

Table 2. The results of LPO, GSH levels and antioxidant enzyme activities.

Groups (n = 20)	LPO (nmol/g Hb)	GSH (μg/g Hb)	CAT (AU/g Hb)	SOD (U/g Hb)	GPx (U/g Hb)
Control group	9.260 ± 0.874	76.100 ± 3.990	24.620 ± 1.774	1364.139 ± 87.824	110.539 ± 11.285
H_2O_2 group	20,968* ± 2.310	12.900** ± 0.820	42.392* ± 1.956	3826.294** ± 131.217	257.084* ± 14.833
Ziziphora taurica subsp. *cleonioides* group	12.968 ± 1.995 (ns)	69.900 ± 1.171 (ns)	28.544 ± 1.947 (ns)	1396.770 ± 154.865 (ns)	120.540 ± 6.480 (ns)
Ziziphora taurica subsp. *taurica* group	10.619 ± 1.848 (ns)	70.000 ± 2.80 (ns)	27.157 ± 1.578 (ns)	1260.850 ± 189.265 (ns)	119.156 ± 12.172(ns)

LPD, lipid peroxidation; GSH, reduced glotathione; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase.
*$p < 0.05$; **$p < 0.01$; (ns); nonsignificant.

presumably to protect the essential thiol groups on the membrane surface. The increased use of GSH and export of GSH catabolic products indicates an increased need for GSH in cells (Dumaswala et al., 1999; Ault et al., 2003; Pastore, 2003; Townsend, 2003). The highest GSH levels were found in *Ziziphora taurica subsp. taurica* group.

The results indicated that essential oils of both the *Ziziphora* species were effective as antioxidants on the enzyme systems of erythrocytes when compared with the H_2O_2 group. *Ziziphora taurica subsp. taurica* was more effective than the endemic *Ziziphora taurica subsp. cleonioides* on CAT, GPx, and SOD enzyme systems of erythrocytes. This antioxidant activity may be correlated with the composition of the essential oils of *Ziziphora taurica subsp. taurica*. Main components of the essential oil of this species were caryophyllene oxide, β-caryophyllene, and germacrene-D. Results presented here may suggest that the essential oils possess antioxidant properties and can be used in the nutraceutical or pharmaceutical industry.

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