Food Chemistry 134 (2012) 48–54

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Anticholinesterase and antioxidant activities of Savoury (*Satureja thymbra* L.) with identified major terpenes of the essential oil

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ARTICLE INFO

Article history:
Received 27 January 2011
Received in revised form 5 January 2012
Accepted 9 February 2012
Available online 18 February 2012

Keywords:
Satureja thymbra
Essential oil
GC-MS
γ-Terpinene
Antioxidant activity
Anticholinesterase activity

ABSTRACT

Satureja thymbra was used as a spice in Anatolia and Greece. The essential oil and the methanol extract of *Satureja thymbra* were evaluated for cholinesterase inhibitory effects against acetylcholinesterase and butyrylcholinesterase which are the chief enzymes of Alzheimer's disease. The antioxidative effects of the essential oil and the extract were also determined by using β-carotene-linoleic acid, DPPH•-scavenging, $ABTS^{\bullet+}$-scavenging, and CUPRAC assays. The GC and GC–MS analyses of the essential oil afforded twenty-five compounds. The identified main compounds of the essential oil, carvacrol (34.6%), γ-terpinene (22.9%), *p*-cymene (13.0%) and thymol (12.8%) were also tested in the same manner. The experimental findings indicated that the compounds, except *p*-cymene, were active in both activity tests. Moreover, the extract (IC_{50}:13.1 ± 0.23 μg/ml), the oil (IC_{50}:26.7 ± 0.56 μg/ml) and γ-terpinene (IC_{50}:11.9 ± 0.21 μg/ml) exhibited a good lipid peroxidation inhibitory activity. In addition, the possible mechanism of lipid peroxidation inhibitory activity of γ-terpinene was also discussed.

1. Introduction

The leaves of some plants, such as oregano, thyme and savoury, belonging to the Lamiaceae family, have been added to meat, fish and food products for many years. *Satureja* species have been widely used as folk medicines and locally known as sivri kekik (thyme or savoury) in Turkey. In general, *Satureja* species are widely grown in Mediterranean areas of the world. *Satureja thymbra*, *Satureja cuneifolia*, *Satureja wiedemanniana*, *Satureja spicigera*, *Satureja hortensis* and *Satureja cilicica* have commercial importance due to their usage as spices. The essential oils of these *Satureja* species are also used in pharmaceutical and cosmetic industries. Therefore, *Satureja*, *Thymus*, *Origanum* and *Coridothymus* species are exported (8000 tons per year). The exportation quantity of *Satureja thymbra* is 100 tons per a year (Satıl, Dirmenci, Tümen, & Turan, 2008).

In Turkey, there are 15 *Satureja* species and five of them are endemic (Tümen, Satil, Duman, & Başer, 2000). Among them, the *S. thymbra, S. spicigera, S.cuneifolia, S. boissieri*, *S. coerulea*, *S. pilosa*, *S. icarica*, *S. wiedemanniana*, *S. hortensis* and *S. cilicica* are consumed as spices or herbal teas by the local people (Satıl et al., 2008; Baytop, 1999). Literature survey shows that *Satureja* species have antibacterial (Gören et al., 2004; Azaz, Kürkçüoğlu, Satıl, Başer, & Tümen, 2005; Vagionas, Graikou, Ngassapa, Runyoro, & Chinou, 2007), antifungal (Müller-Riebau, Berger, & Yegen, 1995), antiviral (Loizzo et al., 2008), antinociceptive and analgesic (Karabay-Yavaşoğlu, Baykan, Öztürk, Apaydın, & Tuglular, 2006) properties.

Several studies on the essential oil of *S. thymbra* have been carried out. These studies indicated that the major compounds of the essential oil are carvacrol, thymol, *p*-cymene and γ-terpinene (Gören et al., 2004; Chorianopoulos et al., 2004,2006; and Loizzo et al., 2008; Azaz et al., 2005). The fatty acid composition and the surface flavonoids of *S. thymbra* were also investigated (Gören, Bilsel, Altun, Satil, & Dirmenci, 2003; Skoula, Grayer, & Kite, 2005). Besides, the essential oil of *S. thymbra* indicates valuable larvicidal activity against *Culex pipiens* biotype *molestus* (Michaelakis, Theotokatos, Koliopoulos, & Chorianopoulos, 2007). It has significant antifungal and antibacterial activities (Gören et al., 2004; Chorianopoulos et al., 2006), and it also shows activity against food borne pathogens (Chorianopoulos et al., 2004). The antiviral activity of the oil was also tested and meaningful results have been found against HSV-1 (Loizzo et al., 2008). The insecticidal and genotoxic activities of the essential oil of Greek and Turkish *S. thymbra* were reported (Michaelakis et al., 2007; Ayvaz, Sagdic, Karaborklu, & Öztürk, 2010).

The phenolic constituents of *S. thymbra*, particularly their flavonoids and flavonoid glycosides, may provide a potential source of antioxidants. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) have been widely used in the food industry to prevent oxidative deterioration. However, BHA and BHT are suspected of being responsible for liver damage and carcinogenesis (Grice, 1988).

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0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2012.02.054

Some chemicals that occur naturally in plants have begun to receive much attention as safe antioxidants, since they may be used to protect humans from oxidative stress damage (Scalbert, Manach, Morand, & Remesy, 2005). On the other hand, the use of antioxidants may slow the progression of Alzheimer's disease and minimise neuronal degeneration (Atta-ur-Rahman & Choudhary, 2001). To date, the pathogenesis of Alzheimer's disease has not been fully clarified. However, the valid hypothesis being accepted is lack of acetylcholine which is a neuromediator (Grossberg, 2003). Thus, acetylcholinesterase inhibitor drugs have been used for the treatment of Alzheimer's disease. However, most of these drugs have side effects. Therefore, the development and utilisation of more effective antioxidants and anticholinesterase compounds of natural origin are desired. It is very advantageous for any food or any compound to have both cholinesterase inhibitory effects and antioxidant activity.

Though the essential oil of *S. thymbra* has been studied chemically, the anticholinesterase, and the antioxidant activities of the essential oil and methanol extract of *S. thymbra* have not yet been published. Regarding the consumption of *S. thymbra* in rural areas of Turkey and Greece, it was aimed to investigate the antioxidant and anticholinesterase activities of the essential oil and the methanol extract of *S. thymbra,* which was collected from the south west of Anatolia. The identified main compounds of the essential oil; namely, carvacrol, thymol, *p*-cymene and γ-terpinene, were also evaluated for their antioxidant and anticholinesterase activities. Identification of the constituents of the essential oil was achieved by using GC and GC–MS analytical methods. The objective of this study was to compare antioxidant and anticholinesterase activities of *S. thymbra* with those of commercial antioxidants and galantamine. In addition, the possible oxygen-scavenging mechanism of γ-terpinene was also discussed.

2. Materials and methods

2.1. Plant materials

Whole plant material of *Satureja thymbra* L. (Lamiaceae), collected from the Marmaris region (Muğla), Turkey, in May 2009, was purchased from a local market. The plant sample was identified by Dr. Tuncay Dirmenci. A voucher specimen has been deposited in the Herbarium of Chemistry, Faculty of Arts and Science, Mugla University, Turkey.

2.2. Spectral measurements and chemicals

GC analyses were performed on a Shimadzu GC-17 AAF, V3, 230 V series gas chromatograph (Japan); GC–MS analyses were on a Varian Saturn 2100T (USA) system at the Department of Chemistry, Muğla University. Both antioxidant and anticholinesterase activity measurements were carried out on a 96-well microplate reader, SpectraMax 340PC^{384}, Molecular Devices (USA). The measurements and calculations were evaluated by Softmax PRO v5.2 software.

Ammonium acetate, copper (II) chloride and potassium persulfate were obtained from E. Merck (Darmstadt, Germany). Terpenes (authentic compounds used for co-injection) were purchased from Sigma–Aldrich and Fluka. As the main compounds of the essential oil, carvacrol and thymol were purchased from Sigma–Aldrich and *p*-cymene and γ-terpinene from Fluka. β-Carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), neocuproine, α-tocopherol, butylatedhydroxyl anisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\cdot}$), 2,2′-Azinobis (3-ethylbenzothiazoline-6-suphonic acid) diammonium salt (ABTS$^{\cdot+}$), electric eel acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 426 U/mg, Sigma), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U/mg, Sigma), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide, butyrylthiocholine chloride and galantamine were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents used were of analytical grade.

2.3. Isolation of the essential oil and preparation of the methanol extract

The essential oil of dried aerial parts of *S. thymbra* (500 g) was obtained via hydrodistillation by using a Clevenger-type apparatus for 4 h. The oil was dried with anhydrous sodium sulphate and stored under nitrogen at −20 °C until required.

The dried and powdered aerial parts of *S. thymbra* (50 g) were extracted with 200 ml of methanol (4 × 24 h) at room temperature. After filtration, the solvent was evaporated to dryness *in vacuum* to give 2.80 g yield of extract. The extract was used for antioxidant and anticholinesterase activities.

2.4. Analysis of the essential oil

2.4.1. Gas chromatography (GC)

A flame ionisation detector (FID) and a DB-1 fused silica capillary non-polar column (30 m × 0.25 id., film thickness 0.25 μm) were used for GC analyses. The injector temperature and detector temperature were adjusted to 250 and 270 °C, respectively. Carrier gas was He at a flow rate of 1.4 ml/min. Sample size was 1.0 μl with a split ratio of 50:1. The initial oven temperature was held at 60 °C for 5 min, then increased up to 240 °C with 4 °C/min increments and held at this temperature for 10 min. The percentage compositions of the essential oil were determined with the Class-GC10 GC computer programme.

2.4.2. Gas chromatography–mass spectrometry (GC–MS)

An ion trap MS spectrometer and a DB-1 MS fused silica non-polar capillary column (30 m × 0.25 mm ID, film thickness 0.25 μm) were used for the GC–MS analyses. Carrier gas was helium at a flow rate of 1.4 ml/min. The oven temperature was held at 60 °C for 5 min, then increased up to 240 °C with 4 °C/min increments and held at this temperature for 10 min.

Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. Ion source temperature was 200 °C. The injection volume was 0.2 μl with a split ratio of 1:30. EI-MS measurements were taken at 70 eV ionisation energy. Mass range was from *m/z* 28 to 650 amu. Scan time was 0.5 s with 0.1 s inter scan delays.

Identification of components of the essential oils was based on GC retention indices and computer matching with the Wiley, NIST-2005 and TRLIB Library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature (Adams, 1989) and, whenever possible, by co-injection with authentic compounds.

2.5. Determination of anticholinesterase activity

Acetylcholinesterase and butyrylcholinesterase inhibitory activities were measured, by slightly modifying the spectrophotometric method developed by Ellman, Courtney, Andres, and Featherston (1961). AChE from electric eel (5.32×10^{-3} U in 20 μl of phosphate buffer for each well) and BChE from horse serum (6.85×10^{-3} U in 20 μl of phosphate buffer for each well) were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. DTNB (5,5′-dithio-bis(2-nitrobenzoic)acid was used for the measurement of the cholinesterase activity. All conditions were the same as those described in our