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ScienceDirect
Food Chemistry 103 (2007) 766–770
Food Chemistry
www.elsevier.com/locate/foodchem

Chemical composition and antioxidant activity of the essential oil of *Clinopodium vulgare* L.

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Received 2 May 2006; received in revised form 22 June 2006; accepted 4 September 2006

Abstract

This study was designed to examine the chemical composition and *in vitro* antioxidant activity of the essential oil of *Clinopodium vulgare*. GC–MS analysis of the oil resulted in the identification of 40 compounds, representing 99.4% of the oil; thymol (38.9%), γ-terpinene (29.6%) and *p*-cymene (9.1%) were the main components. The samples were subjected to a screening for their possible antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. In the first case, IC_{50} value of the *C. vulgare* essential oil was determined as 63.0 ± 2.71 μg/ml. IC_{50} value of thymol and γ-terpinene, the major compounds of the oil, was determined as 161 ± 1.3 μg/ml and 122 ± 2.5 μg/ml, respectively, whereas *p*-cymene did not show antioxidant activity. In β-carotene-linoleic acid system, *C. vulgare* essential oil exhibited 52.3 ± 1.19% inhibition against linoleic acid oxidation. In both systems, antioxidant capacities of BHT, curcumine and ascorbic acid were also determined in parallel experiments.

Keywords: Antioxidant activity; Essential oil; *Clinopodium vulgare*

1. Introduction

Free radicals and other reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, and hydrogen peroxide are an entire class of highly reactive molecules derived from the normal metabolism of oxygen or from exogenous factors and agents. Oxidative damage to crucial cellular molecules induced by ROS has been implicated as a possible factor in the etiology of several human diseases, including cancer, cardiovascular disease, and aging (Halliwell & Gutteridge, 1990). In recent years, there is an increasing interest in finding antioxidant phytochemicals, because they can inhibit the propagation of free radical reactions, protect the human body from diseases (Kinsella, Frankel, German, & Kanner, 1993; Pryor, 1991; Terao & Piskula, 1997) and retard lipid oxidative rancidity in foods (Duthie, 1993).

The use of essential oils as functional ingredients in foods, drinks, toiletries and cosmetics is gaining momentum, both for the growing interest of consumers in ingredients from natural sources and also because of increasing concern about potentially harmful synthetic additives (Reische, Lillard, & Eitenmiller, 1998). Within the wide range of the above-mentioned products, a common need is availability of natural extracts with a pleasant taste or smell combined with a preservative action, aimed to avoid the oxidation of lipids, and spoilage by microorganisms. Those undesired phenomena are not an exclusive concern of the food industry, but a common risk wherever a lipid or perishable organic substrate is present. In fact, they induce the development of undesirable off-flavours, create toxicity and several affect the shelf-life of many goods (Farag, Ali, & Taha, 1990; Hirasa & Takemasa, 1998).

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doi:10.1016/j.foodchem.2006.09.019

Until recently, essential oils have been studied most from the viewpoint of their flavour and fragrance chemistry only for flavouring foods, drinks and other goods. Actually, however, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey, Sisalli, & Coutiere, 2001; Sawamura, 2000). Many authors, in fact, have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties (Hirasa & Takemasa, 1998) by spices and essential oils and, in some cases, a direct food-related application has been tested (Madsen & Bertelsen, 1995).

As far as our literature survey could as certain, we could reach no report on the antioxidant activity of the essential oil and/or extracts of *C. vulgare*. From this point of view, this study could be assumed as the first report on this plant.

The aims of this work were: (i) to evaluate the *in vitro* antioxidant properties of the essential oil, obtained by using a Clevenger distillation apparatus, (ii) and to determine the chemical composition of *C. vulgare* essential oil. The *in vitro* antioxidant activities were determined by using two complementary assays; namely inhibition of DPPH radical and β-carotene-linoleic acid systems. The chemical composition of the essential oil was evaluated by using Gas Chromatography – FID (GC) and Gas Chromatography–Mass Spectroscopy (GC–MS).

2. Materials and methods

2.1. Plant material

C. vulgare plants were collected from Domuzlukici, Taslidere, Sivas, Turkey, when flowering (07 July, 2004). The voucher specimen was identified by a senior plant taxonomist, Dr. H. Askin AKPULAT at the Department of Biology, Cumhuriyet University, Sivas-Turkey and has been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (CUFH-Voucher No: AA 3402).

2.2. Extraction of the essential oil

The air-dried and ground aerial parts of the plants were submitted for 3 h to water-distillation using a Clevenger-type apparatus (yield 0.75%, v/w). The obtained essential oil was dried over anhydrous sodium sulphate and after filtration, stored at +4 °C until tested and analysed.

2.3. GC analysis conditions of the essential oil

The essential oil was analyzed using Hewlett Packard 5890 II GC equipped with a FID detector and HP-5 ms capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were set at 220 °C and 290 °C, respectively. GC oven temperature was raised from 50 °C to 240 °C by a rate of 3 °C/min. Helium was the carrier gas, at a flow rate of 1 ml/min. Diluted samples (1/100 in acetone, v/v) of 1.0 μl were injected manually and in the splitless mode. Quantitative data were obtained electronically from FID area percent data without the use of correction factors.

2.4. GC–MS analysis conditions of the essential oil

The analysis of the essential oil was performed under the same conditions with GC, using a Hewlett Packard 5890 II gas chromatograph equipped with a Hewlett Packard 5972 mass selective detector in the electron impact mode (70 eV). Identification of the components was based on comparisons of their relative retention times and mass spectra with those obtained from standards and/or the NIST98 and Wiley275 library data and the literature (Adams, 2001). All standards were purchased from the Sigma–Aldrich Co. Alkanes were used as reference points in the calculation of relative retention indices (RRI).

2.5. DPPH assay

The hydrogen atoms or electrons donation ability of the essential oil and some pure compounds was measured from the bleaching of purple coloured methanol solution of DPPH. As reported elsewhere (Burits & Bucar, 2000; Cuendet, Hostettmann, & Potterat, 1997), this spectrophotometric assay uses stable radical diphenylpicrylhydrazyl (DPPH) as a reagent.

2.6. 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. This assay was carried out following a method given elsewhere (Dapkevicius, Venskutonis, Van Beek, & Linssen, 1998).

3. Results and discussion

3.1. Chemical composition of the essential oil

GC–MS analysis of the crude oil resulted in the identification of forty compounds representing 99.4% of the oil. Thymol (38.9%), γ-terpinene (29.6%), and *p*-cymene (9.1%), were the main components comprising the 77.6% of the oil (Table 1).

Based on GC and GC–MS analysis of the essential oil of *C. vulgare*, the major compounds were found as thymol, γ-terpinene and *p*-cymene. These findings are not in agreement with the study carried out by Kokdil (Kokdil, 1998). According to their report, major compounds of the essential oil of *C. vulgare* ssp. *arundanum* have been determined as germacrene-D, β-caryophyllene and β-caryophyllene oxide.

Literature is poor about the chemical composition of the essential oil from species of the genus *Clinopodium*. What is

Table 1
Chemical composition *C. vulgare* essential oil (%)

No.	K.I.[a]	Components	Composition (%)
1	890	α-Thujene	1.4
2	897	α-Pinene	3.4
3	911	Camphene	0.2
4	935	Sabinene	tr[b]
5	937	β-Pinene	3.1
6	953	β-Myrcene	2.3
7	966	α-Phelladrene	0.4
8	972	*p*-Mentha-1(7),8-diene	0.1
9	979	α-Terpinene	3.7
10	989	*p*-Cymene	9.1
11	1005	*(Z)*-β-Ocimene	0.1
12	1016	*(E)*-β-Ocimene	0.2
13	1026	γ-Terpinene	29.6
14	1040	*cis*-Sabinene hydrate	tr
15	1062	Terpinolene	0.2
16	1075	*trans*-Sabinene hydrate	tr
17	1155	Borneol	0.2
18	1168	Terpinen-4-ol	0.3
19	1187	α-Terpineol	tr
20	1242	Pulegone	tr
21	1247	Carvacrol methyl ether	0.2
22	1260	*cis*-Piperitone epoxide	0.1
23	1267	isopulegone	0.1
24	1273	*trans*-Piperitone epoxide	0.2
25	1311	Thymol	38.9
26	1330	Carvacrol	4.2
27	1369	Piperitenone	0.1
28	1377	Thymol acetate	0.2
29	1393	Piperitenone oxide	0.1
30	1448	β-Caryophyllene	0.6
31	1459	β-Copaene	tr
32	1470	Aromadendrene	tr
33	1486	α-Caryophyllene	tr
34	1511	γ-Muurolene	tr
35	1515	Germacrene D	0.1
36	1530	Bicyclogermacrene	0.1
37	1544	β-Bisabolene	0.2
38	1551	γ-Cadinene	tr
39	1560	δ-Cadinene	tr
40	1619	Spathulenol	tr
		Total	99.4

[a] K.I. = Kovats Index on HP-5 ms column in reference to *n*-alkanes.
[b] Trace(tr) $\leqslant$ 0.07%.

comes out from this study is that plants of *C. vulgare* either present a high monoterpene content and either a remarkable percentage of sesquiterpene fraction. This phenomenon is very common for species in the family Lamiaceae, for example the classical case of the *Origanum vulgare* plants is reported, where the essential oil chemotype is differentiated among the three *O. vulgare* subspecies (Kokkini, Karousou, & Vokou, 1994). On the other hand, the environmental conditions and flowering effect on the monoterpene content of plant oils have been reported elsewhere (Dudai, Putievsky, Ravid, Palevitch, & Halevy, 1992).

3.2. Antioxidant activity

The essential oil was subjected to screening for the 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 oil measured by DPPH assay are shown in Table 2. As can be seen from the table, IC_{50} value of the *C. vulgare* essential oil was determined as 63.0 ± 2.71 μg/ml. When compared to BHT, curcumine, and ascorbic acid, oil has been found less effective than these synthetic antioxidant agents. IC_{50} value of thymol and γ-terpinene, the major compounds of the oil, was determined as 161 ± 1.3 μg/ml and 122 ± 2.5 μg/ml, respectively, whereas *p*-cymene did not show any activity.

Table 2
Free radical scavenging capacities of *C. vulgare* essential oil measured in DPPH assay[a]

Extracts	IC_{50} (μg/ml)
C. vulgare essential oil	63.0 ± 2.71
Thymol	162 ± 1.3
p-cymene	N.A.[b]
γ-terpinene	122 ± 2.5
BHT	18.0 ± 0.40
Curcumine	7.80 ± 0.32
Ascorbic acid	3.80 ± 0.17

[a] Results are means of three different experiments.
[b] N.A. (Not Active).

In β-carotene/linoleic acid system, *C. vulgare* essential oil exhibited $52.3 \pm 1.19\%$ inhibition against linoleic acid oxidation (Table 3). Percent inhibition of thymol and γ-terpinene were calculated as $56.7 \pm 1.49\%$ and $79.2 \pm 1.23\%$ respectively, while the other major component, *p*-cymene, could not protect the linoleic acid against oxidation.

The literature outlines different approaches for determination of the antioxidant activities of the plant extracts. Therefore, different methodological approaches lead to scattered results, which are hardly comparable and often conflicting (Koleva, van Beek, Linssen, de Groot, & Evstatieva, 2002; Mantle et al., 1998; Ruberto & Baratta, 2000; Zygadlo, Lamarque, Maestri, & Grosso, 1995). A plethora of different antioxidant assays in available and because results rely on different mechanisms, they strictly depend on the oxidant/antioxidant models employed and on lipophilic/hydrophilic balance (Frankel, Huang, Kanner, & German, 1994). A single/substance/single-assay produces relative results and it is perceived as a reductive

Table 3
Percent inhibition of the linoleic acid oxidation by *C. vulgare* essential oil[a]

Extracts	Inhibition (%)
C. vulgare essential oil	52.3 ± 1.19
Thymol	56.7 ± 1.49
p-cymene	N.A.[b]
γ-terpinene	79.2 ± 1.23
BHT	96.6 ± 1.29
Curcumine	89.3 ± 2.14
Ascorbic acid	94.8 ± 1.86

[a] Results are means of three different experiments.
[b] N.A. (Not Active).

approach whenever a phytocomplex is involved. Therefore, antioxidant activity of the plant oil studied here was determined by two complementary test systems namely DPPH free radical scavenging and β-carotene/linoleic acid systems. As can be seen from the Tables 1 and 2, in β-carotene-linoleic acid system, antioxidant activity of the major compounds of *C. vulgare* essential oil (thymol and γ-terpinene) was determined stronger than that of the total oil, whereas the activity of these substances was found weaker in DPPH system. Thereby, by using these complementary test systems, it is possible to make a better conclusion on the antioxidative nature of the oil and its components.

As can be seen from the Tables 2 and 3, thymol and γ-terpinene, which are the major components of the essential oil, have remarkable antioxidant activity in both systems, whereas *p*-cymene has no activity. Thereby, these compounds could be assumed as major contributors of the total antioxidant activity of *C. vulgare* essential oil. Moreover, in DPPH system, antioxidant activity of the oil was determined stronger than those of the pure samples of the major compounds, thymol and γ-terpinene. This indicates also that the other compounds of the oil can act as electron donators, contributing the total activity of the oil. In the literature, there are many reports indicating the antioxidant potential of the thymol and γ-terpinene (Aeschbach et al., 1994; Ruberto & Baratta, 2000; Yanishlieva, Marinova, Gordon, & Raneva, 1999). On the other hand the key role of phenolic compounds as scavengers of free radicals is emphasised in several reports (Komali, Zheng, & Shetty, 1999; Moller, Madsen, Altonen, & Skibsted, 1999). Our results concerning the antioxidant activity of thymol and γ-terpinene are in agreement with the previous report studied by Ruberto and Baratta (2000). According to their report, monoterpenes hydrocarbons present andioxidant activity due to the presence of strongly activated methylene groups, and this is clearer in β-carotene-linoleic acid system where a competition with the activated methylene in C-11 of linoleic acid may be hypothesized. This can explain the better activity of γ-terpinene in linoleic acid oxidation.

In this study, the essential oil of *C. vulgare*, was found to possess remarkable radical-scavenging and antioxidant activities. The bioactive components of *C. vulgare* oil can act as primary and secondary antioxidants, scavenging free radicals, and can therefore inhibit the lipid peroxidation. The analysis carried out by the pure samples of the major compounds showed, the presence of thymol and γ-terpinene mainly, that could be responsible for the effects, as well as the contribution of other major compounds, but further experiments are necessary to verify the relation between chemical composition and antioxidant activity.

Selected active constituents of *C. vulgare* essential oil may be an alternative to more toxic synthetic antioxidants as additives in food, pharmaceutical and cosmetic preparations.

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