

Inhibitory effect of *Calamintha nepeta* subsp. *glandulosa* essential oil on lipoxygenase*

[*Calamintha nepeta* subsp. *glandulosa* uçucu yağının lipoksijenaz üzerindeki inhibisyon etkisi]

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* This work was presented at the 9th International Symposium on Pharmaceutical Sciences (ISOPS-9) on June 23-26, 2009 in Ankara, Turkey [Bu çalışma 9. Uluslararası Farmasötik Bilimler Kongresi, Ankara'da sunulmuştur.]

Registered: 4 July 2011; Accepted: 19 September 2011

[Kayıt Tarihi : 4 Temmuz 2011; Kabul Tarihi : 19 Eylül 2011]

ABSTRACT

Objective: Scavenging of free radicals and inhibition of lipoxygenase are important target in the treatment of a variety of inflammatory diseases. *Calamintha nepeta* (L.) Savi subsp. *glandulosa* (Req.) P.W. Ball of Lamiaceae was previously reported in the treatment of several diseases in folk medicine. In this study, the essential oil of *C. nepeta* subsp. *glandulosa* was investigated for its radical scavenging activity and inhibitory effect on lipoxygenase.

Material and Method: The essential oil of *Calamintha nepeta* (L.) Savi subsp. *glandulosa* (Req.) P.W. Ball was obtained by hydrodistillation and analyzed by gas chromatography – mass spectrometry. Essential oil and its major component of biological activity was determined using an UV-spectrophotometric assay.

Results: Oxygenated monoterpenes pulegone (54%) and menthone (16%) were found to be the major constituents of the oil. *C. nepeta* essential oil demonstrated inhibition on lipoxygenase at $IC_{50} = 69.6 \pm 9.1 \mu\text{g/mL}$, whereas its main component pulegone showed no effect at the same tested concentration. Neither the essential oil nor pulegone displayed radical scavenging activity ($>0.5 \text{ mg/mL}$). Major component pulegone of *C. nepeta* essential oil showed no inhibitory effect on lipoxygenase activity, but other components besides of their low concentration demonstrated an inhibitory effect on lipoxygenase activity in 1/29 ratio when compared to the standard substance.

Conclusion: Data of our study indicated that essential oil of *C. nepeta* may contain potent lipoxygenase inhibitor substances. Further, investigation of other components except the major constituent pulegone on lipoxygenase activity may result in the identification of effective lipoxygenase inhibitors.

Key words: Lipoxygenase (LOX), antioxidant activity, essential oil, GC-MS, *Calamintha nepeta*

ÖZET

Amaç: Çeşitli inflamatuvar hastalıkların tedavisinde lipoksijenaz inhibisyonu ve serbest radikallerin temizlenmesi önemli hedeflerdir. Lamiaceae familyasından *Calamintha nepeta* (L.) Savi subsp. *glandulosa* (Req.) P.W. Ball halk arasında çeşitli hastalıkların tedavisinde kullanılmaktadır. Bu çalışmada *Calamintha nepeta* (L.) Savi subsp. *glandulosa* (Req.) P.W. Ball uçucu yağının radikal temizleyici etkinliği ve lipoksijenaz enzimi üzerindeki etkileri araştırıldı.

Materyal ve Metod: *Calamintha nepeta* (L.) Savi subsp. *glandulosa* (Req.) P.W. Ball uçucu yağı hidrodistilasyon ile elde edildi ve uçucu yağın içeriği gaz kromatografisi- kütle spektroskopisi ile belirlendi. Uçucu yağ ve temel bileşenin biyolojik aktivitesi UV-spektrofotometrik yöntemler kullanılarak belirlendi.

Bulgular: Uçucu yağın temel bileşenlerinin, oksijenlenmiş monoterpen olan pulegon (%54) ve menton (%16) olduğu saptandı. *C. Nepeta* uçucu yağının test edilen konsantrasyonda lipoksijenaz aktivitesini inhibe ettiği ($IC_{50} = 69.6 \pm 9.1 \mu\text{g/mL}$) belirlendi. Fakat ana bileşik olan pulegon test edilen konsantrasyonda enzim aktivitesini inhibe edici etki göstermedi. Uçucu yağ ve pulegon radikal temizleyici etkinlik göstermedi ($>0.5 \text{ mg/mL}$). *C. nepeta* uçucu yağının major bileşeni pulegonun lipoksijenaz aktivitesi üzerinde inhibisyon etki göstermedi ancak diğer bileşenler düşük konsantrasyonda bulunmalarına rağmen standart maddenin 1/29'u oranında lipoksijenaz inhibisyonu göstermiştir.

Sonuç: Sonuçlarımız *Calamintha nepeta* uçucu yağının güçlü lipoksijenaz inhibitörü maddeler içerebileceğini göstermektedir. Pulegon haricindeki bileşenlerin lipoksijenaz aktivitesi üzerindeki etkilerinin araştırılması farmakolojik olarak anlamlı lipoksijenaz inhibitörlerinin bulunması ile sonuçlanabilir.

Anahtar kelimeler: Lipoksijenaz (LOX), antioksidan aktivite, uçucu yağ, GC-MS, *Calamintha nepeta*

Introduction

Oxidative stress and inflammation play an important role in different pathophysiological conditions [1]. If there is an insufficiency in enzymatic antioxidant defenses, and excess productions of free radicals or a drop in the level of the antioxidants; molecules will lead to an imbalance and may cause deleterious effects, situations such as oxidative stress [2].

Nitric oxide, superoxide radicals as well as the product of lipid peroxidation, which occurs as a result of increasing oxidative stress are associated with inflammation [3]. Inflammation is a complex set of interactions among soluble factors and cells that can arise in a tissue in response to traumatic, infectious, post ischemic, toxic or autoimmune injury [4]. Lipoxygenases (LOX) are an important group of enzymes associated with various inflammatory processes and for the production of free radicals. Free radicals prime the immune response, recruit inflammatory cells, and are also innately bactericidal in nature.

There are several important oxidative enzymes such as xanthine oxidase (XOD), lipoxygenases and cyclooxygenases (COX-1 and COX-2), which are involved in normal biological processes and also in pathological conditions. LOX involved in the biosynthetic pathways leading to formation of leukotrienes, hydroxyeicosatetraenoic acids, prostaglandins and induction of oxidation of LDL, which has also been implicated in the progression of both atherosclerosis and cancer [5]. Most of the LOX inhibitors are antioxidants of free radical scavengers, since lipoxygenation occurs *via* a carbon centered radical [6].

Lipoxygenases can be found in a wide variety of microorganism, plant and animal tissues. Long carbon chain fatty acids such as linoleic acid are the primary substrates of the plant lipoxygenases. Soybean lipoxygenase isozymes have significantly different properties [7]. Newly developed anti-inflammatory drugs inhibit both COX and the 5-LOX metabolic pathways, during the formation of prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs). The inhibition of the LT synthesis increases the anti-inflammatory efficacy, especially in pneumological and rheumatological diseases [8]. Lipoxygenases are therefore potential targets for the rational drug design and discovery of mechanism based inhibitors for the treatment of a variety of disorders including bronchial asthma, inflammation, cancer and autoimmune disease. Thus, search for new lipoxygenase inhibitors appears to be a promising approach for the development of new drugs [9].

The genus *Calamintha* (Lamiaceae) is represented by eight species, of which four are endemic in the Flora of Turkey [10]. *Calamintha* species are used in folk medicine like mints, mainly as stimulant, digestive, tonic, anti-septic *etc* [11]. Investigations showed that leaves and flowers of *Calamintha* species are effective as an antiseptic, antispasmodic and tonic [12], the essential oils of plant have antimicrobial and antispasmodic activity [13, 14].

Essential oils and extracts of several medicinal plant aeriels have been tested for their antioxidant activity and anti-inflammatory activity [15, 16]. Also, *C. nepeta* (L.) Savi is well known as a spice, which is used in food flavouring, and it is employed in folk medicine, for the treatment of respiratory and gastroenteric diseases. In several parts of Sicily it is used for the disinfection and cicatrization of wounds [17]. Boiled *C. nepeta* broth against gout, cough up slime and an external application of the leaves against hip pains was reported [15].

In this present study, the essential oil of *C. nepeta* was obtained by hydrodistillation. The chemical composition of the essential oil was determined both by gas chromatography (GC) and gas chromatography – mass spectrometry (GC-MS) methods, simultaneously. Furthermore, the essential oil and its major compound pulegone were subjected spectrophotometrically both to LOX enzyme inhibition and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to evaluate anti-inflammatory activity as well as their anti-oxidant activity, respectively.

Material and Methods

Plant material

C. nepeta was collected from Kayışdağı, Istanbul, Northwest Anatolia, Turkey on 20.09.07. A voucher specimen (Akaydın 11631) has been deposited at the Herbarium of the Faculty of Education, Hacettepe University, Ankara, Turkey.

Isolation of essential oil

The essential oil from 100 g air-dried plant material was isolated by hydrodistillation using 600 mL water for 2 h, using a Clevenger-type apparatus according to the method recommended in the European Pharmacopoeia [18]. The obtained oil (0.9 mL) was dried over anhydrous sodium sulphate and stored at +4°C in the dark until analyzed.

Gas chromatography – mass spectrometry (GC–MS analysis)

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450.

GC analysis

The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same co-

lumn applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The analysis results are expressed as mean percentage \pm standard deviation (SD) ($n=3$) as listed in Table 1.

Identification of components

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC-MS Library, Adams Library, MassFinder 3 Library) [19-20], and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data [21-23], was used for the identification.

Free radical scavenging activity

Stock solutions of each test sample were prepared to obtain a final concentration of 0.5 mg/mL in methanol which were transferred in aliquots of 100 μ l of each test solution to the first row of the 96 well plate. 10 fold serial dilutions in an equal amount of methanol (MeOH) were prepared using a multichannel pipette and vortexed for 5 min. 2 mg of DPPH was dissolved in 25 mL of methanol to obtain a stock reagent solution with final concentration of 80 μ g/mL. To initiate the reaction 100 μ l of DPPH solution was added into each well and left for 30 minutes in dark. Vitamin C was used as positive control at the same concentration. DPPH+MeOH served as negative control and only MeOH as blank on the same test plate. The UV absorbance was read at 517 nm using a microplate reader (Bio-Tek, Powerwave) at room temperature. Half maximal of the inhibitory concentration (IC_{50}) values were calculated using the formula inhibition percent I (%) below where A_0 is the absorbance of the control and A_1 the absorbance of the test sample [24].

$$I(\%) = 100 (A_0 - A_1) / A_0$$

Lipoxygenase inhibition assay

LOX inhibiting activity was measured by modifying the spectrophotometric method developed by Baylac and Racine [25]. LOX (1.13.11.12, type I-B, Soybean), linoleic acid and test compounds were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. Potassium phosphate buffer (1,94 mL; 100mM; pH 9.0), 40 μ L of test compound solution and 20 μ L of lipoxygenase solution were mixed and incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 μ L linoleic acid solution, the change of absorbance at 234 nm was followed for 10 min. Test compounds and the positive control Nordihydroguaiaretic acid (NDGA) were dissolved in MeOH. All the kinetic experiments were performed in quartz cuvette. The concentration that gave 50% inhibition (IC_{50}) for test compounds was calculated. Each tested compound and

control were run in triplicate at each concentration and the results. All test and control (without test compound) assays were corrected by blanks for non-enzymatic hydrolysis. The absorbance change per minute was determined. Percentage of inhibition was calculated as the absorbance change per minute of enzyme activity (without any inhibitor) compared to absorbance change per minute of test compound. The experiments were conducted in triplicate.

Results

In the present study, the main constituents identified by GC and GC-MS were the monoterpenes pulegone (54 \pm 2.6%) and menthone (16 \pm 0.8%) (Table 1), respectively. Overall, 35 constituents of the oil were identified representing 99.9% of the total. The analysis of the essential oil of our study shows that it had a chemical composition close to that reported by other studies. In previous studies, the essential oil of *C. nepeta* subsp. *glandulosa* showed that the major constituents were piperitone oxide, *trans*-piperitone oxide and pulegone in different percentages [26, 27]. Data reported earlier demonstrated that the main constituent of oil obtained from *C. nepeta* by supercritical fluid extraction (SFE) and hydrodistillation (HD) was pulegone (25.2% and 21.4%, respectively) [28].

Furthermore, the essential oil and its major constituent were subjected to biological evaluation by LOX inhibition and DPPH radical scavenging assays. The tested oil showed relatively weak LOX inhibitory activity (IC_{50} 69.6 \pm 9.1 μ g/mL) when compared to standard NDGA (IC_{50} 2.4 \pm 0.5 μ g/mL) (Table 2). On the other hand, pulegone the major constituent of essential oil did not show inhibition on LOX (Table 2). Additionally, antioxidant efficacy of the essential oil and pulegone was tested. Our data obtained from DPPH assay demonstrated that free radical scavenging activity of *C. nepeta* subsp. *glandulosa* essential oil and the major constituent pulegone was higher than 0.5 mg/mL suggesting that they are not active at the tested concentrations compared to the standard test substances (see Table 2). To the best of our knowledge the biological evaluation of *C. nepeta* essential oil by LOX inhibitory and antioxidant activity is reported here for the first time.

Discussion

The biological activity studies on *C. nepeta* are limited as our research and one of study [29] shows that the essential oil and its major constituent pulegone has antimicrobial and antifungal activity. Recently, antifungal activity of *C. nepeta* oil against dermatophytes was reported [30]

The traditional uses of the essential oil suggested that it may poses anti-inflammatory activities through antioxidant mechanism. Hence, we tested both the essential oil and its major component, pulegone for antioxidant and anti-inflammatory activity against standard active substances.

Table 1. The Composition of the Essential Oil of *Calamintha nepeta* subsp. *glandulosa*

RRI ^a	Compound	%	identification method
1032	α -Pinene	0.30±0 ^b	c,d
1118	β -Pinene	0.33±0.06	c,d
1132	Sabinene	0.20±0	c,d
1174	Myrcene	0.333±0.06	c,d
1203	Limonene	5.77±0.29	c,d
1213	1,8-Cineole	0.20±0	c,d
1246	(Z)- β -Ocimene	0.10±0	c
1255	γ -Terpinene	0.10±0	c,d
1280	<i>p</i> -Cymene	0.13±0.06	c,d
1290	Terpinolene	0.10±0	c,d
1393	3-Octanol	1.23±0.06	c
1474	<i>trans</i> -Sabinene hydrate	0.23±0.06	c
1475	Menthone	16.23±0.78	c,d
1503	Isomenthone	1.77±0.12	c,d
1553	Linalool	0.20±0	c,d
1582	<i>cis</i> -Isopulegone	1.03±0.06	c
1598	<i>trans</i> -Isopulegone	0.53±0.06	c
1606	<i>iso</i> -Isopulegol	0.10±0	c
1611	Terpinen-4-ol	0.70±0	c,d
1612	β -Caryophyllene	0.37±0.12	c,d
1638	Menthol	1.70±0.17	c,d
1639	<i>trans-p</i> -Mentha-2,8-dien-1-ol	0.10±0	c
1662	Pulegone	53.93±2.64	c,d
1706	α -Terpineol	0.23±0.06	c,d
1719	Borneol	0.1±0	c,d
1726	Germacrene D	0.57±0.12	c,d
1733	<i>cis</i> -Piperitone oxide	1.07±0.06	c
1754	<i>trans</i> -Piperitone oxide	4.83±0.32	c
1865	Isopiperitenone	0.13±0.06	c
1916	<i>trans-p</i> -Mentha-8-methylthio-3-one	0.10±0	c
1949	Piperitenone	1.50±0.27	c,d
1983	Piperitenone oxide	5.43±0.36	c,d
2006	8,9-Dehydrothymol	0.10±0	c
2045	Carotol	0.13±0.06	c
2239	Carvacrol	0.13±0.06	c,d
	Total	99.87±0.06	

^a RRI Relative retention indices calculated against n-alkanes on the HP Innowax column; ^b mean % calculated from Flame Ionization Detector (FID) data ± SD (n= 3); c, comparison of mass spectra with the Wiley and Mass Finder libraries and retention times; MI: Method of Identification; d, comparison with genuine compounds on the HP Innowax column.

Table 2. Lipoxygenase inhibitory and antioxidant activities of essential oil of *Calamintha nepeta* subsp. *glandulosa* and its major constituent pulegone

	LOX IC ₅₀ (μg/mL)	Antioxidant activity IC ₅₀ (mg/mL)
Essential oil	69.6±9.1	>0,5
Pulegone	na	>0,5
Vitamin C ^a	nt	0,02±0,002
NDGA ^b	2.4±0.5	nt

^apositive control used in antioxidant assays; ^bpositive control used in LOX inhibiting assay, na:Not active, nt, not tested

Inhibition rate of 5-lipoxygenase is used as an indicator of anti-inflammatory activity, resulting in the inhibition of prostaglandin and leukotriene synthesis. This present study, clearly demonstrated that the essential oil of *C. nepeta* subsp. *glandulosa* showed an *in vitro* inhibitory effect on the soybean LOX activity. But it should be noted that there are different pathways involved in inflammation process and the essential oils may exhibit variable activity on the other inflammatory related assays (e.g. COX-1 and COX-2 inhibition) [31].

It has been previously reported that the petrol ether fraction of *C. grandiflora* and *C. nepeta* subsp. *glandulosa* showed significant inhibitory effect on the production of NO, which is an inflammatory mediator in macrophages as compared to indomethacin. Furthermore, the extracts of species showed antioxidant efficacy in the same study. Additionally, *C. nepeta* subsp. *glandulosa* and *C. grandiflora* displayed no cytotoxicity up to 500 µg/mL concentration [32]. This recent findings may support the anti-inflammatory effect and mode of action of *Calamintha* species.

As known, free radicals are involved in several disorders. The harmful action of the free radicals, however, can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism [33]. Also it is well known that medicinal plants, foods and compounds have antioxidant activity play an essential role prevention of disease and anti-aging process. Therefore, antioxidant effect of essential oils, natural compounds and extracts obtained from medicinal plants is now receiving a special attention.

It is well documented that (+)-pulegone is a potent inhibitor of acetylcholinesterase activity among ketones [34]. Studies have shown that metabolites of pulegone were responsible for the toxicity. Liver is the target organ most closely associated with pulegone toxicity. The direct conjugation of pulegone with glutathione might partially explain the glutathione-depleting effect of pulegone. A single i.p. dose of 150 mg/kg pulegone has been shown to deplete glutathione in plasma as well as liver of rats and plays an important role in the toxicity [35, 36]. Glutathione is an important antioxidant molecule. The effect of pulegone on glutathione is to affect antioxidant balance negatively.

As a conclusion, no significant radical scavenging activity was observed at tested concentration of the oil and its major component, respectively. However, when compared to the standard substance, the essential oil of *C. nepeta* showed LOX inhibitory effect. Major component of essential oil pulegone, showed no inhibitory effect on LOX. As the major component of essential oil did not inhibit the LOX enzyme activity, it's presumed that other components of essential oil rather may responsible for LOX inhibitory activity. Data obtained from this study might support the possible effects of *C. nepeta* species in certain inflammatory processes.

Further research on *C. nepeta* essential oil components and other fractions may result in LOX inhibition with anti-inflammatory effect. Also other inflammatory and related assays of the plant material may be worthwhile to investigate.

Acknowledgement

The authors would like to thank Tubitak (project no 106T117), for partial support. The authors also wish to thank Dr. Galip Akaydin (Hacettepe University, Department of Biology Education, Ankara, Turkey) for authentication of the plant material.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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