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Origanum minutiflorum O. Schwarz et P. H. Davis essential oil: enzyme inhibitory activities and chemical composition

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ABSTRACT: The essential oil (EO) of *Origanum minutiflorum* O. Schwarz et P. H. Davis was obtained by the hydrodistillation method. The analysis of the EO was conducted using Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC/MS). The yield of the EO was determined to be 3.9%. Fifty-six compounds were identified, constituting 97.8% of the EO. The EO was rich in carvacrol at a rate of 83.3%, and the other major compounds were *p*-cymene (3.0%), *β*-caryophyllene (1.3%), *trans*-sabinene hydrate (1.1%), *γ*-terpinene (1.1%), borneol (1.1%), and terpinene-4-ol (1.0%). While the EO (100 µg/mL) demonstrated the highest inhibition on cyclooxygenase-1 (COX-1) with 55.26%, the inhibitory activities on the other enzymes were as follows: cyclooxygenase-2 (COX-2); 33.10%, and matrix metalloproteinase-9 (MMP-9); 12.87%. The EO had no inhibition on lipoxygenase (LOX). In this research, inhibitory activity of the EO of *O. minutiflorum* on COXs and MMP-9 enzymes was reported for the first time.

KEYWORDS: *Origanum minutiflorum*; Lamiaceae; essential oil; enzyme inhibitory activity

1. INTRODUCTION

Origanum (Lamiaceae) species are among the important medicinal and aromatic plants. They are mostly consumed in daily life as a condiment in meals [1]. Besides, they are used as traditional medicine in Anatolia against gastrointestinal disorders, asthma, headache, toothache, kidney disorders, and rheumatism [2-4]. The chemical content of *Origanum* species includes terpens (carvacrol, thymol, *p*-cymen etc.), phenols (arbutin, hydroquinone etc.), phenolic acids (caffeic acid, rosmarinic acid, chlorogenic acid etc.), and flavonoids (apigenin, luteolin, naringenin etc.) [4]. Carvacrol, the main component of the essential oil (EO) of *Origanum* species, has been described as antimicrobial [5], antioxidant [6], cyctoxic [7], and anti-inflammatory [8] properties. Other major compounds of the EO of *Origanum* species are thymol, *p*-cymene, *γ*-terpinene, and linalool [9-12]. In Türkiye, the species that have commercial significance and are used for EO production are as follows; *Origanum onites* L., *Origanum vulgare* L. subsp. *hirtum* (Link) Ietswaart, *Origanum minutiflorum* O. Schwarz et P.H. Davis (Endemic), *Origanum dubium* Boiss., *Origanum syriacum* L. var. *bevanii* (Holmes) Ietswaart [13-15]. They are commonly provided from wild growing areas as well as cultivated fields [1,13]. *O. minutiflorum* is an endemic species and is named as 'Sütçüler kekiği', 'Yayla kekiği', 'Toka kekiği'. *O. minutiflorum* is distributed in the Western Taurus Mountains, which lies in a narrow band between the provinces of Isparta and Antalya. The plant has been harvested in a sustainable and conscious way at the end of August and the beginning of September every year in Isparta, mostly in Sütçüler and Çandır districts. This time interval coincides with the plant mature period [13]. *O. minutiflorum* is used as a herbal tea and flavoring spice in Türkiye. Some secondary metabolites obtained from various extracts of *O. minutiflorum* were found to be responsible for antioxidant, cytotoxic, and antimicrobial activities [16-18]. It has been reported that EO of *O. minutiflorum*'s various biological activities such as wound-healing, bactericidal, anticandidal [19], immunostimulant, antioxidant [20], and cytotoxic effects [21-24].

Inflammation is the response to infection, injury, and metabolic stress. The regulation of the inflammatory response is controlled by pro-inflammatory mediators such as prostaglandins and leukotrienes. However, excessive inflammatory response creates undesirable situations [25-28]. Arachidonic acid is the most important polyunsaturated fatty acids, which are the precursors of many mediators such as thromboxane A2, prostaglandins, leukotrienes, as well as protecting membrane integrity. Arachidonic acid is generally oxidized

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by three types of enzymes; cyclooxygenase, cytochrome P45 and lipoxygenase [29]. Among them, LOXs are enzymes that are responsible for the formation of various physiologically active compounds, which have key roles that regulate cell metabolism and apoptosis [30]. Another enzyme, COX, catalyzes the biosynthesis of prostanoids from arachidonic acid. There are two isoforms of COX enzymes [31]. While COX-1 provides prostaglandins necessary for hemostasis, gastric protective and homeostatic functions, COX-2 plays a dominant role in prostaglandin formation in pathophysiological conditions such as inflammation and tumor formation [32]. COX-1 selective inhibition is undesirable as it can cause serious side effects such as ulcers, especially in the gastrointestinal tract. Because of these side effects, selective COX-2 inhibitors are sought after. Therefore, scientific studies have focused on selective COX-1/COX-2 inhibitors in recent years [33]. Overproduction of LOXs and COXs enzyme metabolites has been associated with cancer, stroke, myocardial infarction, ischemia and/or post-ischemia inflammation, arthritis, asthma, and many other diseases and pathological conditions [30, 34]. MMP-9, another inflammatory enzyme, is produced in macrophage, neutrophil and eosinophil inflammatory cells [35]. It is well known that increased MMP-9 expression is associated with many inflammatory pathologies including cardiovascular diseases, rheumatoid arthritis, liver fibrosis and periodontal diseases. It has been determined that MMP-9 has a role in tissue destruction and remodeling during tumor growth and angiogenesis [36]. It has been reported that the MMP-9 inhibitor doxycycline which was approved by the FDA attenuates the cardiac-damaging infection of *Trypanosoma cruzi* [37-39]. MMP inhibitors have been found to be effective in animal experiments, whereas broad-spectrum MMP inhibitors have not been found to be successful in humans owing to their undesirable effects on the musculoskeletal system. The reason for the failures with MMPs to date is the lack of selective inhibitors for MMP enzymes [36]. To summarize, a new bioactive source with selective, effective and low side effects appears to be a goal in drug discovery as an inhibitor of LOX, COXs and MMP enzymes.

The structure-activity relationship of the anti-inflammatory effect of plant-derived compounds is quite complex. Because natural constituents with anti-inflammatory effects can act through many different mechanisms of action. Anti-inflammatory compound research has focused more on flavonoids. Flavonoids generally inhibit COX-1 rather than COX-2. Only very few of the flavonoids selectively inhibit COX-2. Moreover, there are not enough studies on LOX and MMP enzymes of bioactive compounds obtained from a natural origin [40, 41]. On the other hand, another natural source, EOs, are known as a mixture of the bioactive compounds responsible for many biological activities. Although EOs are used in various fields, their potential pharmacological mechanisms of action are still not fully known.

In this study, we aimed to investigate the chemical composition of the EO of *O. minutiflorum* and the screening for its inhibitory activity on inflammation-associated LOX, COX, and MMP-9 enzymes.

2. RESULTS AND DISCUSSION

2. 1. Chemical profile of the EO of *O. minutiflorum* by GC and GC-MS

The yield of the EO was found to be 3.9%. Fifty-six components were identified, which make up 97.8% of the EO. Carvacrol, a phenolic monoterpene, was found to be the prominent component, constituting 83.3% of the EO. The other major components of the EO were *p*-cymene (3.0%), *β*-caryophyllene (1.3%), *trans*-sabinene hydrate (1.1%), *γ*-terpinene (1.1%), borneol (1.1%), and terpinene-4-ol (1.0%) [Table 1].

In previous studies, the yields of the EOs of *O. minutiflorum* were between 1.7-4.9%, and the rate of carvacrol was between 44-92% [42-45]. In another study, six different samples of *O. minutiflorum* were examined and the yields of the EOs ranged from 1.1-3.8%; the most abundant components were carvacrol (42-84%), thymol (0.02-3%), *γ*-terpinene (3-11%), *p*-cymene (4-17%), and linalool (0.006-0.2%) [13]. Özkum et al. [46] compared the EO components of two samples of *O. minutiflorum* obtained by micropropagation and from nature. The rate of the EO main compounds obtained by micropropagation were carvacrol 85.7%, *p*-cymene 4.1%, and *γ*-terpinene 3.9%, while the rate of the main compounds of the EO collected from nature were carvacrol 78.6%, *p*-cymene 7.7%, and *γ*-terpinene 2.2%. In both samples, the thymol content was 0.4%. It has been reported that the content of carvacrol increased in the EO obtained by the *in vitro* propagation method, but other compounds were the same as EO collected from nature. In another research, the effect of distillation time on the composition and the yield of the EO of *O. minutiflorum* was investigated. It was determined that 60-minute distillation had the highest EO yield and carvacrol content, and the optimum time for distillation was determined as 60 min [47]. Previous studies displayed that many factors affect the EO composition and yield. When our analysis results are compared with the literature, we can conclude that there are certain differences in terms of minor and/or major compound ratios in the EO of *O. minutiflorum*.

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Table 1. Chemical components of the aerial parts of the EO of *O. minutiflorum*

[b]RRI	[a]RRI	Compound	%	[b]RRI	[a]RRI	Compound	%
(998-1029)[c]	1014	Tricyclene	tr	(1555-1645)[c]	1638	*cis*-*p*-Menth-2-en-1-ol	tr
	1024	Methyl isovalerate	tr	(1637-1689)[c]	1687	*a*-Humulene	0.1
(1008-1039)[c]	1032	*a*-Pinene	0.3	(1665-1691)[c]	1683	*trans*-Verbenol	tr
(1012-1039)[c]	1035	*a*-Thujene	0.4	(1659–1724)[c]	1706	*a*-Terpineol	0.5
	1051	2,5-Diethyl tetrahydrofuran	tr	(1653-1758)[c]	1719	Borneol	**1.1**
(1043–1086)[c]	1076	Camphene	0.2	(1698-1748)[c]	1737	*β*-Bisabolene	0.2
(1085–1130)[c]	1118	*β*-Pinene	0.1	(161699-1751)[c]	1751	Carvone	0.1
(1140-1175)[c]	1174	Myrcene	0.5	(1655-1714)[c]	1704	*γ*-Muurolene	tr
(1148–1186)[c]	1176	*a*-Phellandrene	0.1	(1763-1786)[c]	1784	(*E*)-*a*-Bisabolene	tr
(1122–1169)[c]	1151	*δ*-Carene	0.3	(1727-1809)[c]	1798	Methyl salicylate	tr
(1178-1219)[c]	1203	Limonene	0.1	(1747-1805)[c]	1802	Cumin aldehyde	tr
(1186-1231)[c]	1213	1,8-Cineole	0.7	(1805-1850)[c]	1845	*trans*-Carveol	tr
(1222-1266)[c]	1255	*γ*-Terpinene	**1.1**	(1813-1865)[c]	1864	*p*-Cymene-8-ol	0.1
(1246-1291)[c]	1280	*p*-Cymene	**3.0**		1940	4-Isopropyl salicyl aldehyde	tr
(1261-1300)[c]	1290	Terpinolene	0.1	(1936–2023)[c]	2008	Caryophyllene oxide	0.6
(11372-1408)[c]	1393	3-Octanol	tr	(1961-2033)[c]	2030	Methyl eugenol	0.1

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(1411-1465)[c]	1459	1-Octen-3-ol	0.2	(2003-2071)[c]	2050	Humulene epoxide II	tr
(1526-1565)[c]	1474	*trans*-Sabinene hydrate	**1.1**	(2049-2104)[c]	2098	Globulol	tr
	1505	Dihydroedulane II	tr	(2070-2114)c	2113	Cumin alcohol	tr
(1507-1564)[c]	1553	Linalool	0.1	(2096-2131)[c]	2144	Spathulenol	0.3
(1425-1478)[c]	1556	*cis*-Sabinene hidrate	0.3	(2100-2198)[c]	2186	Eugenol	tr
(1557-1625)[c]	1571	*trans-p*-Menth-2-en-1-ol	0.1	(2100-2205)[c]	2198	Thymol	0.2
(1564-1630)[c]	1611	Terpinen-4-ol	**1.0**	(2153-2209)[c]	2209	T-Muurolol	tr
(1569-1632)[c]	1612	*β*-Caryophyllene	**1.3**		2221	Isocarvacrol	tr
(1576-1614)[c]	1614	Carvacrol methyl ether	0.1	(2140-2246)[c]	2239	Carvacrol	**83.3**
(1583-1668)[c]	1628	Aromadendrene	0.1		2289	*cis*-Isoelemicin	0.1
	1634	*cis*-Isodihydrocarvone	tr	(2392-2396)[c]	2392	Caryophyllenol II	0.1
(1600-650)[c]	1614	*trans*-Dihydrocarvone	tr			**Total**	**97.9**

[a]RRI Relative retention indices calculated against *n*-alkanes; [b]RRI from the literature on the polar column [c][48]; % calculated based on FID data; tr: Trace amount (< 0.1%)

2. 2. Enzyme inhibitory activity

The inhibition effect of the EO on all enzymes was screened at a concentration of 100 µg/mL as preliminary work. EO exhibited the highest inhibition on COX-1 with 55.26%, while inhibition values on COX-2 and MMP-9 enzymes were 33.10% and 95.33%, respectively. EO demonstrated no inhibition on LOX. The positive control agents and their inhibition values on COX-1, COX-2, MMP-9, and LOX are as follows: SC-560; 99.00% at 0.1 µg/mL, rofecoxib; 95.33% at 10 µg/mL, NNGH; 90.06% at 1.3 µM, NDGA; 99.00% at 20 µg/mL. In view of these results, it can be said that EO of *O. minutiflorum* shows its anti-inflammatory effect through these enzyme pathways. The results are in line with the literature and support the anti-inflammatory effect of the EO of *Origanum* species [49-51]. The EO of *O. compactum* Benth. was investigated *in vitro* 5-LOX inhibition experiment, and *in vivo* carrageenan-induced paw edema test. The LOX-5 inhibitory activity of the EO was calculated within the range IC_{50} = 0.68 ± 0.02 -1.33 ± 0.01 µg/mL. Besides, EO diminished the inflammation in the mice [52]. In a previous study, Demirci et al. [53] observed the potential inhibitory effect of the commercial EOs belonging to *Origanum* ssp. (*O. majorana, O. minutiflorum*, *O. vulgare*, *O. onites*) against LOX and

angiotensin-converting enzyme-2 at a 20 µg/mL concentration. *O. majorana* exhibited the highest inhibition on LOX with 89.4%, while *O. minutiflorum* showed inhibition with 78.9%. It was determined that carvacrol had an inhibitory effect on LOX with 74.8% at a 5 µg/mL concentration. In the same study, the ratio of the carvacrol in EOs of *Origanum* subspecies was calculated in the range of 62-81%. In the literature, carvacrol has been found to have an anti-inflammatory effect by inhibiting COX-1 and COX-2 enzymes [54, 55]. Also, it has been reported that carvacrol in combination with ibuprofen creates a synergistic effect and reduces inflammatory parameters [56]. Furthermore, it has been determined that carvacrol inhibits MMP-2 and MMP-9 enzymes and shows anticancer activity in hepatocarcinoma studied in rats [57]. It is known that there is a difference in the expression of LOX and COXs enzymes in the formation of Alzheimer's disease (AD). However, the role of LOX and COX enzymes in the development of neuroinflammation in AD is unclear. Nevertheless, these enzymes are considered to be a potential new target for AD treatment [58]. Orhan et al. [59] evaluated the inhibitory activities of the EO of *O. minutiflorum* on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes associated with AD. EO (1 mg/mL) displayed extremely significant inhibition on AChE and BChE as 100% and 95%, respectively.

In a review, it was compiled that bioactive monoterpenes show their anti-inflammatory effect through mediators such as prostaglandin, leukotriene, cytokines etc. While EOs rich in terpinene-4-ol prevent prostaglandin release by inhibiting COXs. γ-Terpinene was determined as an inhibitor of leukotriene production [60]. *p*-Cymene and β-caryophyllene reduced the inflammatory mediators such as TNFα, prostaglandins [61-63]. EO has a complex mixture including various volatile components. Therefore, it can be thought that the difference in the ratios of the minor and major compounds in the EO may have affected each other synergistically or suppressively, thereby altering the results.

Recently, there have been micro- and nano-encapsulation formulations for the EO of *Origanum* species, especially for topical applications, due to its antioxidant, antimicrobial and anti-inflammatory properties. The EO of *Origanum* has been tested *in vivo* wound healing effect and positive results have been obtained. Although its role in wound healing is not fully known, the possibility of its mechanism of action has been attributed to the formation of collagenase and angiogenesis [64, 65]. Nano-emulsion of the EO of *Origanum vulgare* was found to reduce apoptosis of prostate cancer cells *in vitro* [66]. These studies, both *in vitro* and *in vivo*, are promising in terms of the effective applicability of lipophilic bioactive compounds such as EO of *Origanum*'s on human health.

3. CONCLUSION

Synthetic anti-inflammatory chemical drugs are known to have side effects. In this respect, it is of great importance to investigate the herbal products consumed by the public in order to detect new bioactive compounds with fewer side effects. We conclude that this research provides new insights into the mechanism of anti-inflammatory activity of the EO of *O. minutiflorum* by inhibiting LOX, COXs and MMP-9. The anti-inflammatory effect of the EO may be related to the fact that it contains main components such as carvacrol, *p*-cymene, β-caryophyllene, *trans*-sabinene hydrate, γ-terpinene, borneol, and terpinene-4-ol. This is the first study to evaluate COX-1, COX-2, and MMP-9 inhibitory properties of the EO of *O. minutiflorum*. These findings are promising and suggest that further studies are required for other biological activities of the EO of *O. minutiflorum*.

4. MATERIALS AND METHODS

4. 1. Materials

Origanum minutiflorum was supplied from Sütçüler, Isparta, Türkiye, in September 2017. A voucher material was stored in the Herbarium of the Faculty of Pharmacy at Anadolu University (ESSE no: 15441). The enzymes used in experiments; LOX (type I-B; *Glycine max*, Sigma-Aldrich), COXs (item No. 701050, Cayman chemical), and, MMP-9 (BML-AK410, Enzo) colorimetric inhibitor screening assay kit. The chemicals and solvents were purchased from Merck (Germany), Sigma (Germany), and Fluka (Germany). The enzyme inhibitory experiments were analyzed with ultra-pure water.

4. 2. Extraction of essential oil

20 g of aerial parts of the plant were subjected to hydrodistillation for 3 h using the Clevenger apparatus. This procedure was performed in triplicate. The yield of the EO was calculated and stored in an amber vial in a refrigerator at +4°C for analysis procedures and activity analysis.

4.3. Chemical analysis of essential oil

The analysis of the EO was carried out simultaneously with the GC and GC/MS systems. The relative percentages of the compounds detected by the FID detector in the GC system were calculated from the FID chromatograms. Mass spectra of the components were taken with the GC/MS system. Evaluation processes were executed using the "Baser Essential Oil Components Library" as well as Wiley, Adams, and MassFinder 3 Library, and also by comparing the retention indexes [67, 68].

GC analysis is performed using an Agilent 6890N GC system. The FID detector temperature was 300°C. To achieve the same elution order as for GC/MS, simultaneous automatic injection was performed on a duplicate of the same column, applying the same operating conditions. The relative percentage amounts of the separated compounds were calculated from the FID chromatograms.

GC/MS analysis was performed with an Agilent 5975 GC/MSD system. An Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with Helium (0.8 mL/min) as carrier gas. The GC oven temperature was programmed at 60°C for 10 min, to 220°C at a rate of 4°C/min, and to 240°C at a rate of 1°C/min after being kept constant at 220°C for 10 min. The injector temperature was set to 250°C. Mass spectra were recorded at 70 eV. The mass range was from m/z 35 to 450.

4. 4. Evaluation of enzyme inhibitory activity

LOX enzyme activity was performed spectrophotometrically by modifying the Baylac and Racine method [69]. Potassium phosphate buffer (1.94 mL; 100 mM; pH 8.8) was mixed with 40 µL of sample solution and 20 µL of LOX solution and incubated for 10 min at 25°C, and the reaction was initiated by adding 50 µL of linoleic acid. The change in absorbance was recorded for 10 min at 234 nm. Samples and nordihydroguaretic acid (NDGA, positive control) were prepared by dissolving appropriate concentrations in the appropriate solvent. Quartz microplate was used for the kinetic measurements in the experiment.

COX enzyme inhibition of the samples was made by adapting the "Cayman COX Colorimetric Inhibitor Screening Assay" measurement method. The buffer solution was prepared as a mixture of 3 mL of 0.1 M Tris-HCl (pH 8, 37 ^{o}C), 27 mL of dH2O. COX enzyme solution and Hem solution were diluted using this buffer. 100 µL of arachidonic acid was mixed with 100 µl of 0.1 M KOH and diluted with dH2O. Enzyme was added to all wells of the microplate except the blank. 10 µL of COX enzyme solution was added, 10 µL of sample was added for 5 min. It was incubated at 25°C. After incubation, 20 µL of colorimetric substrate (TMPD) and arachidonic acid (substrate) was added to all wells. After the final volume of each well was made up to 220 µL, the microplate was incubated for 2 min. It was incubated at 25°C. The incubation process was carried out in the incubator. After incubation, absorbances were measured at 590 nm. 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560) was used as a standard for COX-1; rofecoxib was used for the COX-2.

The MMP-9 inhibition study was performed according to the "MMP-9 Colorimetric Screening Kit" procedure. According to the kit procedure, the standard inhibitor NNGH (N-isobutyl-N-[4-methoxyphenylsulfonyl]glycyl hydroxyamic acid) was diluted with the chromogenic thiopeptide used as the substrate and the enzyme buffer. 20 µL of diluted enzyme was added to all the wells of the microplate except for the blind wells. The inhibitor and samples were applied to the corresponding wells of the microplate as 20 µL. Then incubation at 37°C for 30 min continued. After incubation, 10 µL of substrate was added to all wells and absorbance values were determined at 412 nm for 10 min and the absorbance change per minute was determined.

96-well microplates of the kit were used for COXs and MMP-9 analysis. All enzyme experiments were run in triplicate and results were given as % mean ± standard error. % Inhibition results of LOX, COX, and MMP-9 assays were calculated using the following equation:

% Inhibition = [(A control – A sample)/A control] x 100

A control: Absorbance of control, A sample: Absorbance of sample

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