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RESEARCH ARTICLE

Comparative Study of The Anti-Inflammatory Pathway Enzyme Activities of Selected Plant Extracts from Lamiaceae Family

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Comparative Study of The Anti-Inflammatory Pathway Enzyme Activities of Selected Plant Extracts from Lamiaceae Family

SUMMARY

In this study, the effects of selected plants from the Lamiaceae family (Mentha piperita L., Salvia officinalis L., Lavandula officinalis Chaix., Scutellaria orientalis L. and Melissa officinalis L.) on the activities of lipoxygenase- 12/15 (LOX-12/15), cyclooxygenase-2 (COX-2) and acetylcholinesterase (AChE) enzymes, which have an essential place in the inflammation pathway, were determined. They have been studied as an alternative to inhibitor drugs that have many side effects. The metabolomic profiles of the extracts were defined by GC-MS and LC-qTOF-MS methods. The antioxidant parameters of the extracts were investigated by DPPH+ radical scavenging activity and TAC methods. The time-dependent scavenging capacity of extracts for DPPH+ radicals varies depending on the extracts, time, and concentration. The potential inhibitory effects of the extracts on 12-15-LOX, COX-2, and AChE enzymes were compared with metabolomics analysis. Our combined results suggest that the extracts have potential use as anti-inflammatory agents.

Key Words: *Anti-inflammatory, Lamiaceae, lipoxygenase, cyclooxygenase, acetylcholinesterase, metabolomic.*

Lamiaceae Familyasından Seçilen Bitki Ekstrelerinin Anti-İnflamatuar Yolak Enzim Aktivitelerinin Karşılaştırmalı Çalışması

ÖZ

Bu çalışmada Lamiaceae familyasından seçilmiş bitkilerin (Mentha piperita L., Salvia officinalis L., Lavandula officinalis Chaix., Scutellaria orientalis L. ve Melissa officinalis L.) inflamasyon yolağında önemli bir yeri olduğu bilinen lipoksijenaz-12/15 (LOX-12/15), siklooksijenaz-2 (COX-2) ve asetilkolinesteraz (AChE) aktiviteleri üzerine etkileri araştırılmıştır. İnflamasyon sürecinde yan etkileri fazla bulunan inhibitör ilaçlara alternatif olmaları açısından bu bitki ektstelerinin etkinlikleri incelenmiştir. Ekstrelerin metabolomik profilleri GC-MS ve LC-qTOF-MS yöntemleriyle tanımlanmıştır. Ekstrelerin antioksidan potansiyelleri, DPPH+ radikal süpürücü etki ve TAC yöntemleriyle araştırılmıştır. DPPH+ radikalinin zamana bağlı süpürücü etkileri incelendiğinde, ekstrelerin zamana ve konsantrasyona bağlı olarak etkilerinin değiştiği bulunmuştur. Ekstrelerin 12-15-LOX, COX-2 ve AChE enzimleri üzerindeki potansiyel inhibitör etkileri incelenmiş ve elde edilen sonuçların metabolomik profilleri korelasyon analizi ile karşılaştırılmıştır. Elde edilen sonuçlar, ekstrelerin potansiyel anti-inflamatuvar ajanlar olarak kullanıma sahip olduğunu düşündürmektedir.

Anahtar Kelimeler: *Anti-inflamatuvar, Lamiaceae, lipoksijenaz, siklooksijenaz, asetilkolinesteraz, metabolomik.*

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INTRODUCTION

Tissue damage, destruction, and prolonged persistent infection are associated with chronic inflammation. In this process, tissue damage occurs through imbalances in blood flow, increased permeability in vascular tissues, synthesis of reactive oxygen derivatives (ROS), and activation and migration of leukocytes (Shah, 2008).

Inflammation is a very complex process, and various mediators such as prostaglandins (PGs), leukotrienes (LTs), phospholipase A_2 (PLA_2), cyclooxygenases (COXs), and lipoxygenases (LOXs) are involved (Busse, 1998). COX and LOX enzymes are convert arachidonic acid (AA) to biologically active leukotrienes and prostaglandins (Gilroy, Tomlinson, & Willoughby, 1998). The formation of the inflammatory response also has various effects on the nervous system, where acetylcholine (ACh) is the neurotransmitter that plays a critical role (Massoulié, Pezzementi, Bon, Krejci, & Vallette, 1993). Borovikova et al. (2000) reported that ACh effectively inhibited peripheral macrophages and mediated the inflammatory response by inhibiting the release of proinflammatory mediators, including tumor necrosis factor (TNF-α) (Borovikova et al., 2000). These results show that acetylcholinesterase (AChE) may induce ACh-dependent macrophage deactivation, hence forming an important of the cholinergic anti-inflammatory (CAI) pathway (Figure 1).

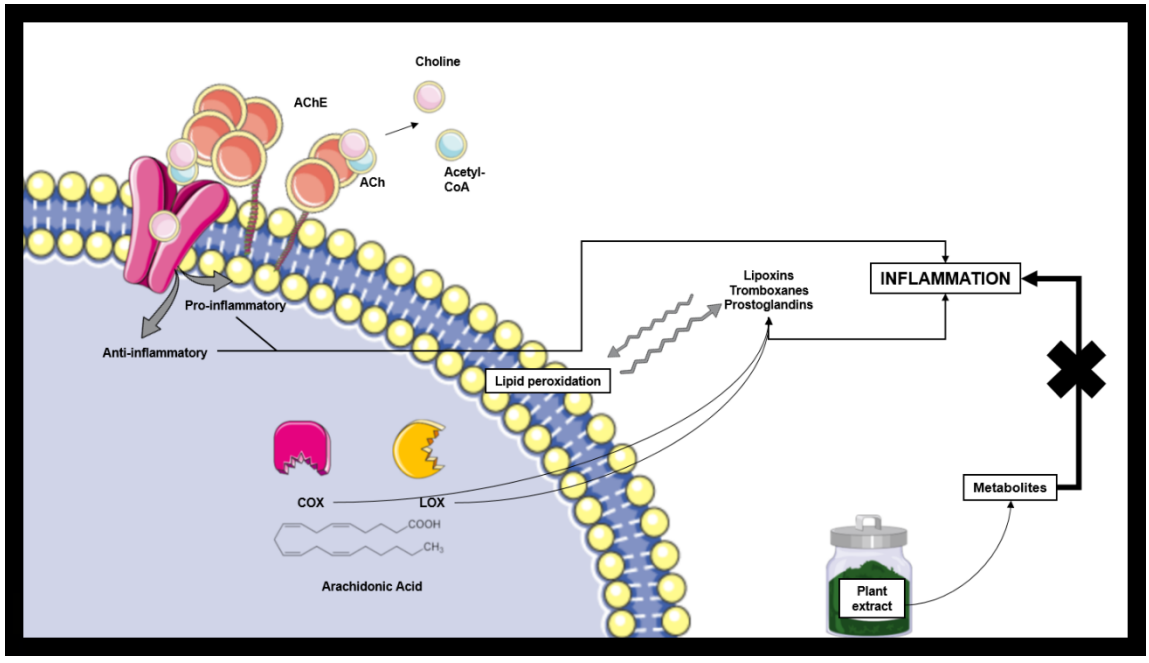

Figure 1. Potential inflammation pathways associated with AChE, LOX, and COX

Steroidal glucocorticoids can reduce cytokine-induced gene expression, and non-steroidal anti-inflammatory (NSAID) drugs, which target cyclooxygenase (COX) enzyme isoforms, can regulate the uncontrolled inflammatory response (Barnes, 1998; Vane, 1971). However, long-term use of these drugs causes undesirable side effects, including ulcers and cardiovascular disorders (Baron et al., 2008; Rostom et al., 2002). The discovery of less toxic dual LOX/COX and AChE inhibitors is essential to overcome these disadvantages.

Since the beginning of human history, medicinal and aromatic plants have been used for many purposes, such as food, medicine, cosmetics, and spices. The first records of the use of plants for medicinal purposes date back to B.C. (Demirezer, 2010). Turkey has a very diverse ecosystem waiting to be discovered. Advances in the biological sciences have enabled

them to evaluate the therapeutic potential of various plant species (Mesquita et al., 2019).

The Lamiaceae (Labiatae) family is a cosmopolitan family that includes plants in herbaceous, shrub, or tree forms and is of great economic importance in many parts of the world. It is mainly distributed in the Mediterranean and Central Asia (Suddee, Paton, & Parnell, 2004). The Lamiaceae family also has a vital role in the flora of Turkey and is the third most affluent family, with a 44.2% endemism rate (Baser & Kırımer, 2014). Plants of the Lamiaceae family have a rich content of secondary metabolites and are used in phytotherapy (Fecka & Turek, 2008). These secondary metabolites display many biological activities such as antioxidant, antimicrobial, antiviral (Sökmen et al., 2004), anti-inflammatory effects –especially as inhibitors of LOX-COX enzymes (Juergens, Stöber, & Vetter, 1998) and AChE (Orhan, Senol, Ozturk, Akaydin, & Sener, 2012).

The development of technology leads to the increased use of omics technologies. Metabolomics, an omics technology that investigates all metabolic components of the organism rather than scanning a single metabolite, is beginning to be used in the content analysis of plant extracts, especially in pharmacology (Ulrich-Merzenich et al., 2007). Metabolomics research leads to pharmacologically active substances obtained by scanning the content analysis of materials such as plant extracts. These have formed new ways to treat various diseases (Kamatou, Makunga, Ramogola, & Viljoen, 2008). Investigating the correlation of biologically active components determined by metabolomics studies with bio-activity studies is a new approach and necessary for further studies.

In this study, plant species of *Mentha piperita* L., *Salvia officinalis* L., *Lavandula officinalis* Chaix., *Scutellaria orientalis* L., and *Melissa officinalis* L. from the Lamiaceae family, known to have biologically active secondary metabolites, were selected. Given these selected plant extracts' antioxidant and anti-inflammatory potential, we aimed to determine their inhibitory effects on COX-2, 12/15-LOX, and AChE enzymes, if any. Determination of the possible double/triple enzyme inhibitor potential of these plant extracts would enable a new approach to develop ligands in the inflammation pathway.

Our combined results display that *S. officinalis* L. and *M. officinalis* methanol extracts have LOX/COX dual inhibitor potentials. *M. officinalis L.* extract may be a potential natural triple LOX/COX/AChE enzyme inhibitor.

MATERIALS AND METHOD

Chemicals

Trizma hydrochloride, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipoxidase (*Glycine max* Type I), dimethyl sulfoxide (DMSO), Quercetin and Tween-20 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Linoleic acid was purchased from Calbiochem, Germany. Total Antioxidant Capacity (TAC) Assay Kit and COX-2 Inhibitor Screening Assay Kit (Fluorometric) were obtained from Biovision (USA). All other chemicals used were of the best analytical grade.

Plant Materials and Extraction Procedures

Plants (*Lavandula officinalis* Chaix., *Melissa officinalis* L., *Mentha piperita* L., and *Salvia officinalis* L.) were collected from the culture area of Selçuk University Faculty of Agriculture, Department of Field Crops. *Scutellaria orientalis* L. was collected from the Ankara-Beytepe region during flowering periods. The herbarium specimen numbers of the plants are as follows, respectively (TBÇ-L-001, TBÇ-M-001, TBÇ-M-002, TBÇ-S-001 and HUEF 20016).

The parts to be used were separated after the plant samples were dried at room temperature, free from moisture, in a sun-free environment. The aerial parts of the plants were used in this study. These parts were powdered with the help of a grinder. 5 g of dried and powdered plant samples were weighed, extracted at 40°C under reflux for 30 minutes with

50 ml of methanol, and then filtered. After the filtrate was separated, the remaining solid phase was extracted a second time at the same temperature, with 30 ml of methanol under reflux for 15 minutes. The filtrates were combined, a rotary evaporator removed the solvent, and a dry extract was obtained by lyophilization. For DPPH radical scavenging assays, powdered plant extracts were weighed and dissolved in methanol. For Trolox equivalent determination, the solvent used was absolute ethanol. The extracts were dissolved in 1% dimethylsulfoxide (DMSO) for enzymatic assays.

DPPH$^+$ radical scavenging activity

The radical scavenging capacity of extracts was determined by DPPH$^+$ (1,1-diphenyl-2-picrylhydrazyl) assay according to Brand-Williams Cuvelier and Berset method (Brand-Williams, Cuvelier, & Berset, 1995). Briefly, 50 µl of the extracts dissolved in MeOH (200-1.56 µg/ml, in the final) was transferred to 96-well plates. 1 mM 150 µl DPPH$^+$ radical was added to samples and standard. After 30 min of incubation, the absorbance was read at 517 nm with the microplate reader SpectraMax i3. In addition, the radical scavenging effects of the samples were investigated over time (0-90 minutes). Quercetin was used as the standard for all radical scavenging activity assays. All assays were carried out in triplicate. The antioxidant activity values of all materials were calculated according to the following formula:

$$\%Inhibition: \frac{A0 - Asample}{A0} \times 100 \qquad (1)$$

where A_0 is the absorbance value consisting of MeOH and DPPH$^+$ for extracts and Quercetin, A_{sample} is the absorbance with extracts or standard. The EC_{50} value was determined by a linear regression curve.

Total antioxidant capacity: Trolox equivalent

The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•$^+$) radical is used to measure the total radical scavenging capacity. This method is based on the discoloration of ABTS•$^+$ by antioxidant compounds. The stable free radical scavenging ability of the molecules is compared with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). Determination of the total antioxidant capacity of the extracts was performed using the TAC Assay Kit (Biovision, Catalog No: #K274-100, USA) according to the manufacturer's instructions. Trolox was used as a standard in the concentration range of 0-20 nmol/µl. The total antioxidant capacities of the extracts are given as nmol/µl Trolox equivalent value. All assays were carried out in triplicate. All samples (Trolox and extracts) were prepared in absolute methanol.

COX-2 activity assay

A commercial COX-2 Inhibitor Screening Kit (Biovision, Catalog #K547-100, U.S.A) was used to determine the effects of extracts on COX-2 (EC: 1.14.99.1) enzyme activity. Generally, this assay is based on the fluorometric detection of prostaglandin G_2 produced by the COX enzyme in AA metabolism. The extracts were dissolved in 1% DMSO and were transferred to the microplate with 80 µl of substrate-free activity medium. The reaction was initiated by adding 10 µl of AA as the substrate. The change in fluorescence intensity was measured at Ex/Em = 535/587 nm wavelengths, kinetically at 25°C for 10 minutes in a microplate reader (SpectraMax i3). All assays were carried out in triplicate. % Inhibition values were given according to Formula (1): where A_0 is the initial, -uninhibited- enzyme activity consisting of enzyme, substrate and solvent; A_{sample} is an enzyme activity with extracts. A linear regression curve determined the IC_{50} value.

12-15/LOX activity assay

The protocol used to determine the effects of extracts on 12/15-LOX enzyme activity was carried out with some modifications (Tappel, 1962; Ulusu, Ercil, Sakar, & Tezcan, 2002). The activity mixture was prepared as 1 ml. It contained 1.5 µg/ml of the enzyme (Lipoxidase from Glycine max, Type-1, EC: 1.13.11.12) and various concentrations of extracts in

50 mM sodium borate buffer (pH 9.0) in the final. The reaction was started by adding substrate (Linoleic acid, LA) after extracts were incubated with enzyme for 5 minutes at 25°C. The change in absorbance was monitored with quartz cuvettes in Schimadzu UV1700 UV-Vis spectrophotometer at 234 nm, kinetically at 25°C for 1 minute. While all extracts were dissolved in 5% DMSO, LA was suspended in 50 mM sodium borate buffer pH 9.0 containing % 0.2 Tween-20 in the final. % Inhibition values were given according to Formula (1): where A_0 is the initial, -uninhibited-enzyme activity consisting of enzyme, substrate, and solvent; A_{sample} is an enzyme activity with extracts. A linear regression curve determined the IC_{50} value.

AChE activity assay

The Ellman assay in 96-well plates determined Acetylcholinesterase (AChE, EC 3.1.1.7) activity with a final volume of 200 µl (Ellman, Courtney, Andres Jr, & Featherstone, 1961; Gok, Zeybek, & Bodur, 2016). AChE activity was measured using acetylthiocholine (ATCh) as substrate at a final concentration of 1 mM in 50 mM 3-(N-morpholino) propanesulfonic acid buffer (MOPS) pH 7.4, 0.25 mM 5,5'-Dithiobis (2-Nitro Benzoic Acid (Ellman's Reagent, DTNB) and varying concentration of extracts (containing %0.05 DMSO in the final) at 412 nm for 1 minute, kinetically. % Inhibition values were given according to Formula (1): where A_0 is the initial, -uninhibited-enzyme activity consisting of enzyme, substrate and, solvent; A_{sample} is an enzyme activity with extracts. A linear regression curve determined the IC_{50} value.

Metabolomics analysis

GC-MS

Gas chromatography-mass spectrometry (GC–MS) analysis was carried out as described previously (Nemutlu et al., 2015). 100 µg/ml plant sample was prepared with methanol. GC–MS (Shimadzu GCMS-QP2010 Ultra) was used for metabolomic profiles, and DB-5MS was chosen as the stationary phase column. After the procedure, the data were analyzed with AMDIS and SpectConnect software. Myristic acid was used as an internal standard in this metabolomics analysis.

LC-qTOF-MS

LC-qTOF-MS metabolomic analysis was performed as described previously with minor modifications (Gonulalan et al., 2020). Metabolites were separated by C18 column in LC-qTOF-MS system (Agilent 6530). Natural product databases [Universal Natural Products Database (UNPD), KNAPpSAcK, and PlantCyc] were scanned for metabolite identification. The mass tolerance is fitted at ten ppm. Only structures with a score greater than six were correctly identified. Phenylalanine was used as an internal standard in this metabolomics analysis.

Statistical analysis

Statistical analyses of enzymatic and scavenging activity assays were performed with GraphPad Prism 8.4.2 software. Values are given as means ± SEM (Standard error mean) of at least triplicate experiments. Statistical analyses for all experiments were calculated using one-way ANOVA analysis, followed by Post-Hoc tests. Values of $p \leq 0.05$ were considered statistically significant. Microsoft Excel was used for the correlation analysis between metabolomic and activity data.

RESULTS and DISCUSSION

$DPPH^+$ radical scavenging activity

In comparing the results of the $DPPH^+$ radical scavenging activity of the plant extracts, Quercetin was used as a control. The EC_{50} of quercetin was found as 2.92±8.54 µg/ml. *M. officinalis* L. displayed the highest $DPPH^+$ radical scavenging activity; EC_{50}: 25.75±13.41 µg/ml (Figure 2). EC_{50} values of all extracts were statistically significant as compared to Quercetin ($P \leq 0.05$, Figure 2).

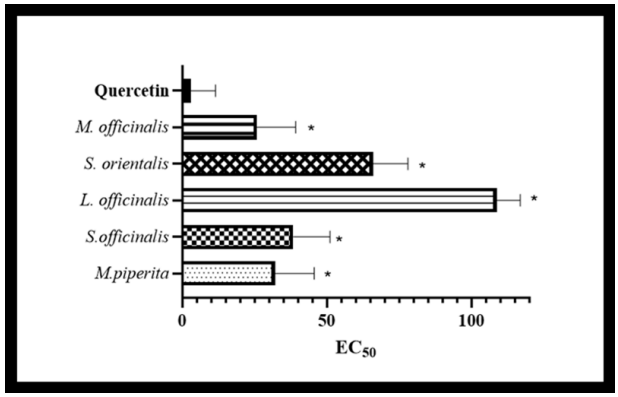

Figure 2. DPPH⁺ radical scavenging effect of the Lamiaceae extracts
n=3, EC_{50} values are given ±SEM * Compared to quercetin $p<0.05$.

The results confirm that all extracts have antioxidant properties comparable to Quercetin. Next, we evaluated the time-dependent radical scavenging effects of extracts. When the time-dependent scavenging capacities of DPPH⁺ radicals were examined, reliant on the extracts, time, and concentration, the effects were varied (Figure 3). Generally, all extracts displayed a steady inhibition over the observed period. *M.piperita* L., *S. officinalis.* L and *S. orientalis* L. extracts displayed a concentration-dependent increase in DPPH⁺ scavenging activity. *M. officinalis* L. and *S. officinalis* L. displayed the highest DPPH⁺ scavenging activity (Figure 3).

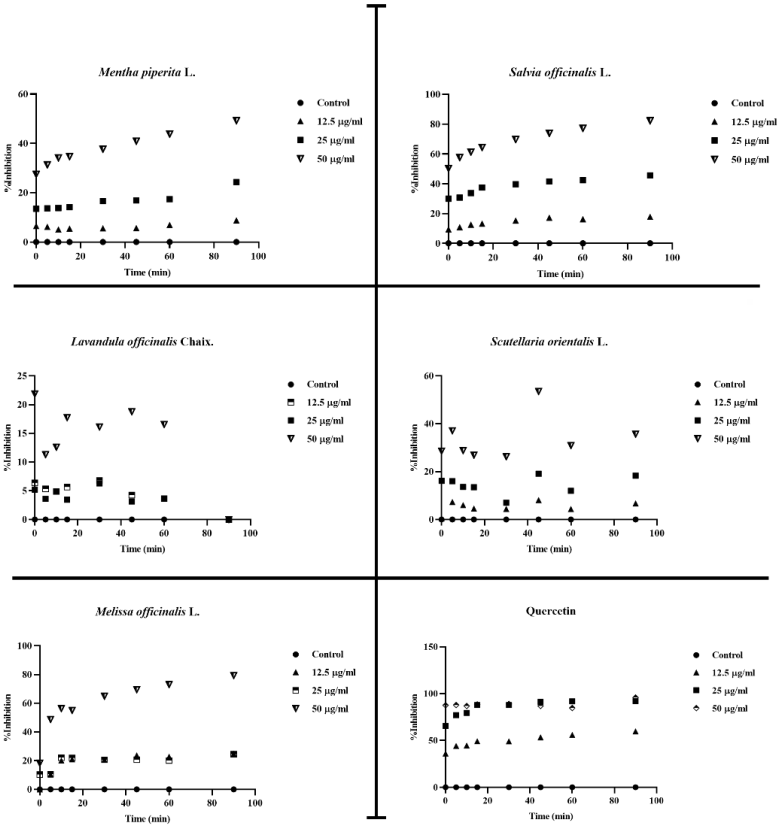

Figure 3. Time-dependent DPPH⁺ radical scavenging effect of the Lamiaceae extracts

Total antioxidant capacity: Trolox equivalent

The total antioxidant capacities of the extracts are given as Trolox Equivalent values (nmol/µL). The highest antioxidant activity belonged to *M. piperita* L. (Table 1; 50 µg/ml: 726.62±0.02 nmol/ µl Trolox Equivalent).

Table 1. Total antioxidant capacity of Lamiaceae extracts as Trolox equivalent

Concentration (µg/ml)	*M.piperita* L.	*S.officinalis* L.	*L.officinalis* Chaix.	*S. orientalis* L.	*M. officinalis* L.
	nmol/ µl Trolox Equivalent				
6.25	128.15±0.01	103.87±0.01	53.06±0.02	132.64±0.01	222.57±0.02
12.5	298.08±0.01	171.31±0.01	59.80±0.02	137.14±0.01	312.50±0.01
25	578.24±0.02	336.33±0.01	60.25±0.01	192.00±0.01	553.51±0.01
50	726.62±0.02	400.63±0.02	73.29±0.02	260.34±0.01	676.26±0.01

n=3, Trolox equivalent values are given ±SEM

COX-2 enzyme activity assay

Inhibitory potentials of the extracts were screened against COX-2 enzyme activity at 0-400 µg/ml. The results are given in Table 2 as IC_{50} values. According to our results, all of the extracts displayed COX-2 enzyme inhibition in varying degrees. The highest inhibitory activity belonged to *L. officinalis* Chaix. (IC_{50}: 6.24±13.70 µg/ml), which was followed by *M. officinalis* L., with the IC_{50} value of 16.65±9.09 µg/ml. The plant extract with the highest IC_{50} value and the lowest inhibitor potential was determined as *M. piperita* L. for the COX-2 enzyme (Table 2).

12-15/LOX enzyme activity assay

The IC_{50} values indicating the effects of Lamiaceae extracts at different concentrations on 12/15 Lipoxygenase enzyme activity are in Table 2. The highest inhibitory activity belonged to *M. piperita* L. which was 14.98±9.23 µg/ml (Table 2). *M. officinalis* L. and *S. officinalis L.* extracts displaying similar IC_{50} values also have high 12/15 LOX inhibitory potential (Table 2).

AChE enzyme activity assay

In addition to COX-2 and 12-15/LOX enzymes, the effects of Lamiaceae extracts on AChE enzyme were investigated. According to our results, all extracts except S. orientalis L. displayed AChE inhibition. The highest AChE inhibitory potential was displayed by *M. officinalis* L., which had an IC_{50} value of 157.97±1.62 µg/ml. The other three plant extracts displayed IC_{50} values in 345-455 µg/ml (Table 2).

Table 2. Inhibitory effects (IC_{50} values) of Lamiaceae extracts on COX-2, 12-15/LOX, and AChE enzyme activities

Extracts	Enzymes, IC_{50} (µg/ml)		
	COX-2	12-15/LOX	AChE
*M. piperit*a L.	69.98±13.70	14.98±9.23	455.55±6.77
S.officinalis L.	26.44±17.68	42.54±37.70	432.31±5.22
L. officinalis Chaix.	6.23±13.25	329.48±44.77	345.52±1.86
S. orientalis L.	30.43±12.32	96.63±55.70	ND*
M. officinalis L.	16.65±9.09	35.73±16.75	157.97±1.62

*Not detected.

GC-MS

After analysis of GC-MS chromatograms, 1745 mass spectral properties were found, of which 295 were analyzed using retention index libraries. We determined 81 organic acids and derivates, 69 organic compounds, 48 lipids and lipid-like molecules, 37 benzonoids, and 28 organoheterocylic-type compounds (Supplementary data 1a).

LC-qTOF-MS

Secondary metabolites were determined with LC-qTOF-MS analysis, and 362 metabolites were identified using MS/MS databases. Among the

metabolites identified, 60 flavonoid glycosides and aglycones with flavone, isoflavone, flavonol, methylated flavonoids, and flavonol skeletons were the most common group. Fourteen anthocyanidin glycosides were determined as the other major secondary metabolite group. In addition, coumarin, catechin, terpenoids, and hydroxycinnamic acid-type compounds were detected (Supplementary data 2a).

Also, we have found that metabolites belong to fatty acyls (fatty acid esters, fatty acids and conjugates, fatty acyl glycosides, fatty amides, fatty alcohols, and linoleic acids and derivatives) as a result of GC-MS and LC-QTOF-MS analyses (Table 3).

Table 3. Fatty acyls determined in extracts

Class	Sub Class	Compound Name	Presence in membrane
Fatty Acyls	Fatty acid esters	Methyl Stearate	+
	Fatty acyl glycosides	Lactobionic acid	-
		Lactitol	-
		Maltitol	-
		Turanose	-
		Palatinitol	+
	Fatty amides	Adipamide	-
	Fatty alcohols	1-hexadecanol	-
	Lineolic acids and derivatives	Linoleic acid	+
		Methyl linolenate	-
	Fatty acids and conjugates	Oleic acid	+
		Capric acid	+
		Pimelic acid	+
		Maleamic acid	-
		Methyl oleate	-
		2-isopropylmalic acid	-
		3-hydroxy-3-methylglutaric acid (dicrotalic acid)	-
		Citramalic acid	+
		Methyl palmitate	+
		Methyl palmitoleate	-
		Palmitic acid	+
		Stearic acid	+
		Arachidic acid	+
		Heptadecanoic acid	+
		Myristic acid	+
		Palmitoleic acid	+
		4-acetylbutyric acid	-
		Lauric acid	-
		Trans,trans-muconic acid	+
		2,3-dimethylsuccinic acid	-
		Behenic acid	+
		Methyl hexacosanoate	-
		Itaconic acid	+
		Tetracosanoic acid	+
		16-Hydroxyhexadecanoic acid	+
		cis,cis-Muconic acid	+
		Sebacate	+
		Suberic acid	+
		Methyl octadecanoate	-
		3-Hydroxyisovaleric acid	+

Correlation analysis

The findings obtained by correlation analyses of LC-QTOF-MS and GC-MS analysis are shown in Table 4. LC-qTOF-MS correlation analyses show that 13, 24, and 77 positively correlated metabolites with 12-15/LOX, COX-2, and AChE enzymes, respectively. On the other hand, GC-MS correlation analyses show that 22, 13, and 66 positively correlated metabolites with 12-15/LOX, COX-2, and AChE enzymes, respectively ($r \geq 0.70$; Table 4).

Table 4. Correlation analyses between metabolites and enzyme activities

	Number of positively correlated metabolites ($r \geq 0.70$)			Number of negatively correlated metabolites ($r \leq - 0.70$)		
	12-15/LOX	COX-2	AChE	12-15/LOX	COX-2	AChE
LC-QTOF-MS	13	24	77	63	47	33
GC-MS	22	13	66	44	24	29

*r: Correlation coefficient

Antioxidant activity is widely used to characterize biological materials (López-Alarcón & Denicola, 2013). Plants contain various phytochemicals or bioactive molecules that can neutralize free radicals and delay the progression of many chronic diseases associated with oxidative stress and ROS (Ani, Varadaraj, & Naidu, 2006). Lamiaceae family plants have rich secondary metabolite content (Fecka & Turek, 2008). It is known that these secondary metabolites have many antioxidant properties and are effective in many anti-inflammatory pathways (Paun et al., 2018).

This study determined the radical scavenging effects and the total antioxidant capacity of the selected Lamiaceae extracts. When the radical scavenging effects of *M. piperita* L., *S. officinalis* L., and *M. officinalis* L. extracts were compared with the literature our extracts were found to have higher antioxidant activity than previous studies (Albano et al., 2012; Derwich, Chabir, Taouil, & Senhaji, 2011; Koksal, Bursal, Dikici, Tozoglu, & Gulcin, 2011). Moreover, *L. officinalis* Chaix. results are very close to those reported in literature (Spiridon et al., 2011).

In addition, time-dependent (0-90 minutes) radical scavenging capacities of the extracts' at determined concentrations (12.5, 25, and 50 µg/ml) were investigated. Generally, all extracts displayed a steady scavenging effect over the observed period. While the radical scavenging capacities of the extracts did not change over time, they were nevertheless concentration-dependent. All plant extracts displayed a higher scavenging effect with higher (50 µg/ml) extract concentrations. *M. officinalis* L. and *Salvia officinalis* L. plant extracts displayed the highest $DPPH^+$ scavenging activity. These two plants displayed an 80 % inhibition on the $DPPH^+$ radical formation over a period of 90 minutes (Figure 3). It is thought that the high efficiency of our extracts in antioxidant and radical scavenging activities may be related to the secondary metabolite components in their content. A comparison of the radical scavenging effects with Quercetin and the total antioxidant capacity with Trolox suggests that our extracts have strong antioxidant properties (Table 1).

Given the extracts' potential to inhibit cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, the results of *S. officinalis* L. and *M. officinalis* L. extracts are promising. The IC_{50} values of *S. officinalis* L. and *M. officinalis* L. for COX-2 enzyme were 26.44±17.68 and 16.65±9.09 µg/ml, respectively. Moreover,12/15-LOX enzyme IC_{50} values of the *S. officinalis* L. and *M. officinalis* L. were 42.54±37.70 and 35.73±16.75 µg/ml, respectively (Table 2). It is seen that the inhibition values of both extracts for both enzymes are very close. These findings may be related to the similarity of secondary compounds

and, or the synergistic and antagonistic effects of the secondary metabolites in the extract. Metabolomics analyses show that our extracts are rich in flavonoids and anthocyanidins. Coumarin, catechin, terpenoids, and hydroxycinnamic acid-type compounds are commonly found in these extracts.

Plants from the Lamiaceae family display inhibitory effects on the inflammation pathway enzymes. Alonazi et al. (2021) showed that *Salvia lanigera* ethanol and water extracts inhibited COX-2 and 5-LOX enzymes in human colorectal cancer cell lines (HCT-116 and Lovo) without causing any cytotoxicity (Alonazi et al., 2021). Another study on COX enzyme showed that hydroalcoholic extracts of *L. officinalis* inhibited inflammation through and COX-1 COX-2 activity (Husseini et al., 2016). Also, the anti-inflammatory activity of menthol and peppermint oil was investigated on human monocytes *in vitro*. Juergens et al. found that menthol and peppermint oil are potential inhibitors for LOX and COX pathways (Juergens et al., 1998). In another study with rats, 800 mg/kg of *M. officinalis* L. was found to cause 50% inhibition of COX-2 levels in tissues (Hamza, Ahmed, Elwey, & Amin, 2016). *In vitro* study with *S. baicalensis* L., the IC_{50} value for the COX-2 and 5-LOX enzyme of the extract was found to be 15 μg/ml and 25 μg/ml, respectively (Burnett, Jia, Zhao, & Levy, 2007).

Acetylcholinesterase (AChE) enzyme is the neurotransmitter in cholinergic synapses (Massoulié et al., 1993). Several plants and their metabolites are used in folk medicine to alter cognitive function, alleviate other neurodegenerative disorder symptoms, and prevent memory loss (Howes, Perry, & Houghton, 2003). Literature findings show that *Salvia* and *Melissa* genus have promising key metabolites that inhibit AChE and butyrylcholinesterase enzymes (Castro et al., 2021; Gülçin et al., 2016). In addition, the large-scale study by Miyazawa et al. showed that *Mentha* essential oils and their metabolites also have a strong AChE inhibitory potential (Miyazawa, Watanabe, Umemoto, & Kameoka, 1998).

This is the first study that incorporates both the metabolomic profiles and correlation of the inhibition potentials of these metabolites on LOX COX and AChE enzymes, especially fatty acyl metabolites. Through correlation analyses of LC-qTOF-MS and GC-MS, it was seen that a group of metabolites were positively correlated with the inhibitions of AChE>12-15/LOX>COX-2 enzymes, respectively (Table 4). We found 77 and 66 metabolites positively correlated with the AChE enzyme with LC-qTOF-MS and GC-MS analyses, respectively. Surprisingly, correlation analyses revealed that through LC-qTOF-MS and GC-MS analyses, 63 and 44 negatively correlated metabolites were found for 12-15/LOX enzyme. The number of metabolites correlated with COX-2 enzyme by LC-qTOF-MS and GC-MS analyses were 47 and 24, respectively. The correlation type was negative, as it was with the 12/15/ LOX enzyme (Table 4).

The inhibition studies of our extracts show promising results. The extracts contain many primary and secondary metabolites that display antioxidant and anti-inflammatory activity. We believe that these activities of plant extracts may be due to the synergistic/antagonistic effects of different metabolites instead of single active metabolites.

It is known that the fatty acyls linoleic and arachidonic acid are involved in anti-inflammatory effects. Considering AChE is a member of the cholinergic anti-inflammatory pathway, we have previously investigated their potential as acetylcholinesterase inhibitors. We found that linoleic acid, linolenic acid, and arachidonic acid have inhibitory effects on human erythrocyte AChE and characterized the interaction of these fatty acyls with the AChE enzyme through docking studies (Akay, Şener, Sari, & Bodur, 2023). In this study, we determined that the Lamiaceae feature fatty acyl metabolites (fatty acid esters, fatty acids, and conjugates, fatty acyl glycosides, fatty amides, fatty alcohols, and linoleic acids and derivatives) as a result of GC-MS and LC-QTOF-MS analyses. Through

these analyses, we find that the dual LOX/COX inhibitory potential of *S. officinalis* L. and the triple LOX/COX/AChE inhibitory potential of *M. officinalis* positively correlate with fatty acyl type compounds. *M. officinalis* contains relatively more of these compounds than others, which may be the source of the triple effect. Therefore, further studies are needed to clarify the metabolite or metabolites responsible for the activity.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

EB and ME conceived the study; KS performed the experiments, collected and analyzed the data, EMG provided the plant extracts and performed the metabolic analysis, EB and KS wrote the manuscript; EMG and ME made manuscript revisions.

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