South African Journal of Botany 141 (2021) 177–182

Contents lists available at ScienceDirect

South African Journal of Botany

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

Isolation of rosmarinic acid and methyl rosmarinate as lipoxygenase inhibitors from *Salvia palaestina* Benth. by activity-guided fractionation

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ARTICLE INFO

Article History:
Received 15 October 2020
Revised 5 April 2021
Accepted 27 April 2021
Available online 30 May 2021

Edited by Dr L. Rarova

Keywords:
Salvia palaestina
Lipoxygenase
Activity-guided fractionation
Rosmarinate
Anti-inflammatory activity

ABSTRACT

Salvia palaestina aqueous and methanol extracts were prepared from the aerial parts, which were evaluated for the *in vitro* anti-inflammatory properties using the lipoxygenase (LO) enzyme inhibition assay. While the aqueous extract showed no activity at test concentrations, a significant ($p < 0.001$) lipoxygenase inhibition was detected for the methanol extract with 29% inhibition. Activity guided fractionation was carried out on the methanol extract via liquid-liquid partitioning using *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol. The ethyl acetate fraction showed statistically the best inhibition among the sub-fractions with 70% inhibition ($p < 0.0001$). The compounds responsible for the activity were purified, and their structures were established as rosmarinic acid, and methyl rosmarinate by spectroscopic methods. IC_{50} values of rosmarinic acid, and methyl rosmarinate were calculated as 0.21 and 0.02 μM, respectively. In conclusion, the *in vitro* anti-inflammatory potential of *S. palaestina* was associated to rosmarinic acid, and methyl rosmarinate, for the first time to the best of our knowledge.

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1. Introduction

Cyclooxygenase (COX), which plays a key role in the biosynthesis of prostaglandins, controls one of the main pathways of arachidonic acid metabolism and it is the target of non-steroidal anti-inflammatory (NSAI) drugs together with the lipoxygenase (LO) enzymes, which are known as the other main pathways of arachidonic acid metabolism (Araico et al., 2007). LO's are responsible for the biosynthesis of various biological regulatory compounds such as leukotriene, hydroxyl eicosatetraenoic acid, lipoxin etc. and commonly found in animals and plants (Rehman et al., 2005). By inducing inflammation, LO mediators were reported to be related with many diseases (especially asthma) and they also had an impact on the development of certain types of cancer (Steinhilber 1999; Ding et al., 2001). Although there are many drugs for treating inflammatory diseases, they are inconvenient for long-term use due to side effects and high costs. Thus, there is an immense need for the development of novel anti-inflammatory compounds with low side effects and costs (Yeşilada et al., 1997).

Salvia L. is the largest genus of the Lamiaceae family and is represented by approximately 1000 species in the world. The *Salvia* genus was represented by 107 taxa, and 100 species, where its endemism rate was reported 54% in Turkey (Celep and Dirmenci 2017). *Salvia* species are used traditionally against inflammatory diseases (Yeşilada et al., 1993; Fujita et al., 1995; Gürbüz et al., 2019; Karcı et al., 2017). However, detailed studies evaluating the anti-inflammatory effect of *Salvia* species are rather limited.

Salvia palaestina Benth. is a perennial herbaceous plant, which is localized in south-eastern region of Turkey (Hedge 1982). It was reported that the leaves were traditionally used in wound healing remedies (Miski et al., 1983). According to a recent study, the *in vivo* topical anti-inflammatory effect of *S. palaestina n*-hexane, and ethyl acetate extracts were remarkable (Shehadeh et al., 2014).

Therefore, the aim of this present study was to investigate the effect of *S. palaestina* on 15-LO inhibition, one of the major inflammation pathways, and to determine the active compounds by activity-guided fractionation.

2. Materials and methods

2.1. General experimental procedures

The isolated pure substances were analyzed by LC-TOF-MS for determination of molecular masses. Spectra were obtained using a Waters LCT Premier XE model ultra performance liquid

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https://doi.org/10.1016/j.sajb.2021.04.030
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chromatography-time of flight-mass spectrometer (UPLC-TOF-MS) system, and MassLynx 4.1 software. Acquity BEH C18 column (2.1 × 100 mm 1.7 Mm, flow rate: 0.25 mL/min) was used as column and acetonitrile:water linear gradient (1:99 – 90:10) system containing formic acid (0.1%) was used as the mobile phase. Agilent G6550A model LC-QTOF-MS device was used to determine the molecular masses of the purified substances and their fragments. Chromatographic separation was performed on Poroshell 120 EC C18 column (3 × 50 mm, flow rate: 0.4 mL/min). The injection volume was 1 mL, the analysis time was 15 min, and the column temperature was 35°C. ^{1}H NMR spectra of the isolated compounds were taken with 600 MHz Bruker Avance III HD spectrometer using appropriate deuteron solvents. TMS was used as internal standard. Column chromatography was performed on Sephadex LH-20 (Sigma, Germany), LiChroprep (C18) RP-18 (Fluka, Germany; Merck, Germany), and silica gel (0.063-0.200 mm J.T. Baker, Deventer, Netherlands). Lipoxygenase enzyme (from *Glycine max*) [EC 1.13.11.12] and linoleic acid were purchased from Sigma (Sigma Chemical Co. St. Louis, MO, USA). Methanol (99.8% J.T. Baker, Deventer, Holland; EMSURE® ACS, ISO, Reag. Ph Eur Sigma, Germany), *n*-hexane (≥95% Sigma, Germany; ACS Reagent, Reag. Ph. Eur., ≥ 99% Riedel-de Haën, Germany), dichloromethane (≥ 99.8%, Sigma, Germany; EMSURE® ACS, ISO, Reag. Ph Eur Merck, Darmstadt, Germany), ethyl acetate (ACS Reagent, ≥ 99.5% Riedel-de Haën, Germany; ACS reagent, ≥99.5% Sigma, France), *n*-butanol (EMSURE® ACS, ISO, Reag. Ph Eur Merck, Darmstadt, Germany), acetonitrile (%99.9 Dop, Ankara, Turkey) were purchased for the extraction and isolation procedures.

2.2. Plant material

The flowering aerial parts of *S. palaestina* were collected in May 2014 on the southern slopes of Inönü University campus. The collection coordinates were N: 38°19′29″, E: 38°25′34″. The plant was identified by Prof. Dr. Turan ARABACI at Inonu University Faculty of Pharmacy, Department of Pharmaceutical Botany. A specimen was deposited (no:3483) in the Gazi University, Faculty of Pharmacy Herbarium (GUEF).

2.3. Preparation of extracts and sub-extracts

Aerial parts of the plant were dried under shade at room temperature until further use. The coarsely ground 10 g powdered material was separately macerated using 300 mL of water, or methanol for 24 hours, and then filtered. This process was repeated three times, and resulting filtrates were combined. The methanol extract was evaporated to dryness under reduced pressure to yield 1.7 g, also the aqueous extract was partly evaporated under reduced pressure at 45°C, and then further dried using a freeze dryer to yield 2.9 g extract, respectively. Considering the result of the activity studies, it was decided to proceed fractionation using the methanol extract. For this purpose, dried and coarsely ground 500 g plant material was macerated using 5 L methanol for 24 hours and filtered subsequently. This process was repeated eight times, and the filtrates were combined which was evaporated to dryness under reduced pressure at 45°C to yield a final 97.5 g methanol extract. The methanolic residue (95.5 g) was dissolved in 500 mL 90% methanol (MeOH/H_2O, 9:1), and extracted with *n*-hexane (19 × 300 mL), dichloromethane (CH_2Cl_2) (11 × 300 mL), ethyl acetate (EtOAc) (13 × 300 mL), *n*-butanol (*n*-BuOH) (16 × 300 mL), respectively using a separatory funnel. After extracting with *n*-hexane, the methanol extract was evaporated at 45°C to dryness, and suspended in distilled water (500 mL) to continue the process. Each partition solvent was evaporated to dryness under reduced pressure at 45°C to yield first the *n*-hexane (18.2 g), CH_2Cl_2 (10.2 g), EtOAc (5.4g), *n*-BuOH (12.8 g), and final remaining H_2O (35.2 g) fractions, respectively.

2.4. Activity-guided fractionation and isolation of active compounds

The CH_2Cl_2 (9.0 g) and EtOAc (4.9 g) fractions, which showed significant activity, were eluted separately using the Sephadex LH-20 column (85 g, Sigma, 4 × 40 cm, flow rate 7.5 mL/min) with methanol. Fractions were collected in 150 mL volumes each. Fifty fractions from CH_2Cl_2, and 30 fractions from EtOAc were collected. According to the thin layer chromatograms (TLC, Silica Gel, $60F_{254}$), the fractions with similar composition were combined. SP-CH_2Cl_2/LH1/Fr.A (870 mg), SP-CH_2Cl_2/LH1/Fr.B (6660 mg), and SP-CH_2Cl_2/LH1/Fr.C (1392 mg) were obtained from the elution of the CH_2Cl_2 fraction. From the EtOAc fraction, SP-EtOAc/LH2/Fr.A (26 mg), SP-EtOAc/LH2/Fr.B (1505 mg), SP-EtOAc/LH2/Fr.C (3012 mg), SP-EtOAc/LH2/Fr.D (166 mg), and SP-EtOAc/LH2/Fr.E (74 mg) were obtained. Activity of each fraction obtained from Sephadex LH-20 column chromatography was evaluated *in vitro* on the LO enzyme. The fraction with the highest activity (SP-EtOAc/LH2/Fr.C), was applied onto the C18 column (150 g, Fluka, 4 × 50 cm, flow rate 10 mL/min), and eluted with H_2O:MeOH (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35: 65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95, 0:100) gradient. Fractions were collected in 100 mL volumes. The fractions were combined into seven groups, and their LO enzyme inhibition activity was evaluated. One of the fractions, which showed the highest activity, (SP/RP1/Fr.65-192 - 100 mg), was subjected to a preparative HPLC (Combi flash EZ prep) using C18 column (Redisep Prep C18, 100 A, 5 μm, 250 × 20 mm), and eluted with linear H_2O:MeOH gradient (flow rate 5 mL/min), respectively. Fractions were collected in 5-15 mL volumes, resulting in the isolation of compound **1** (35.6 mg). The other highly active fraction (SP/RP1/Fr.222-228) was further chromatographed on a silica gel column (J.T.Baker, 45 g, 2.5 × 35 cm, flow rate 5 mL/min) with chloroform:methanol [9:1 (600 mL), 8:2 (400 mL)] elution to afford compound **2** (14.6 mg). Fractions were collected in volumes of 10 mL. To identify other potentially active compounds or chemotaxonomical constituents, the isolation process was continued. SP/RP1/Fr.229-235 was chromatographed over a C18 MPLC system (Spectra/Chrom LC column, Thermo scientific FH 100 peristaltic pump, Fluka, 60 g C18, 2.8 × 30 cm, flow rate 2 mL/min, 10 mL fraction volume) using a H_2O:acetonitrile gradient (80:20, 700 mL; 75:25 800 mL) to afford **3** (4.0 mg). The remaining portion (SP/RP1/Fr.65-192) was fractionated over a Sephadex LH-20 column (Sigma, 85 g, 4 × 40 cm, flow rate 5 mL/min, 100 mL fraction volume) with methanol. Thus, overall, a total of 3 fractions were obtained. SP/LH3/Fr.C was chromatographed over C18 column (Fluka, 75 g, 4 × 25 cm, flow rate 10 mL/min, 50-100 mL fraction volume) using a H_2O:MeOH gradient (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35: 65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95, 0:100). The resulting eluents were combined into 11 sub-fractions, where the SP/RP2/Fr.36-44 fraction was applied for further preparative HPLC (Combi flash EZ prep) purification using a C18 column (Redisep Prep C18, 100 A, 5 μm, 250 × 20 mm, 5 mL fraction volume), which was eluted using linear H_2O:MeOH gradient with 5 mL/min flow rate to yield the compound **4** (4.0 mg).

2.5. Lipoxygenase inhibition assay

The activity of the extracts, fractions and pure compounds (**1-4**) from *S. palaestina* were evaluated by the Soya bean [*Glycine max* (L.) Merr.] Lipoxygenase enzyme. The spectrophotometric method with some minor modifications (Baylac and Racine, 2003) was applied, where linoleic acid was used as the substrate. Enzyme inhibition (%) was assessed by spectrometric kinetic absorbance at 234 nm/min for 10 min, calculated according to the following formula:

Inhibition% = [(A-B) / A] x 100

A: Control (3. min absorbance - 1. min absorbance)

B: Sample (3. min absorbance - 1. min absorbance)

2.6. Statistical analysis of data

GraphPad Prism 6.0. software (one way ANOVA) was used for statistical analysis of enzyme inhibition values. IC_{50} values of the isolated compounds were calculated at Microsoft Excel by the dose-response curves. P values less than 0.05 were considered as statistically significant [$^{*}(p < 0.05)$, $^{**}(p < 0.01)$, $^{***}(p < 0.001)$, $^{****}(p < 0.0001)$]. In the enzyme inhibition studies, dose-response graphs mean and standard deviations (n=3) were calculated using Microsoft Excel.

3. Results and discussion

In the present work, *in vitro* LO inhibitory activity of *S. palaestina* aqueous, and methanol extracts were evaluated at a concentration of 100 μg/mL. It was observed that the aqueous extract was ineffective on LO enzyme; however, the methanol extract showed reasonable inhibitory activity (29%, $p < 0.001$). Thus, the methanol extract was fractioned by liquid-liquid extraction method to obtain *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH and H_2O fractions, respectively, and their inhibitory activities were evaluated further. The highest inhibition rates were observed with fraction SP-EtOAc (almost 70%, $p < 0.0001$), and SP-CH_2Cl_2 (approx. 58%, $p < 0.0001$), respectively. IC_{50} values of SP-EtOAc and SP-CH_2Cl_2 were determined as 93.37 and 83.25 μg/mL, respectively. According to a previous study (Shehadeh et al., 2014), the *in vivo* topical anti-inflammatory effect of the *n*-hexane, ethyl acetate, and methanol extracts prepared from some *Salvia* species, including *S. palaestina*, were evaluated by croton oil-induced mouse ear oedema, where the *n*-hexane and ethyl acetate extracts showed remarkable activity (160 and 47 μg/cm^2 ID_{50} values, respectively); however, compounds responsible for the activity could not identified. Therefore, one of the aims of this present study was to show the LO inhibitory properties of *S. palaestina*, which one of the major inflammation mechanisms, and to characterize the compound(s) responsible for bioactivity *via* activity-guided fractionation.

Due to comparable enzyme inhibitory results with the literature, it was decided to perform activity-guided fractionation on both active fractions mainly focusing on the ethyl acetate fraction. The activity-guided fractionation scheme is given below (Fig. 1). Initially, the abovementioned two fractions were subjected to Sephadex LH-20 column, followed by LO inhibitory activities (as listed in Table 1). The SP-EtOAc/LH2/Fr.C fraction showed the highest activity with almost 72% LO enzyme inhibition ($p < 0.0001$). As a result, it was decided to continue with SP-EtOAc/LH2/Fr.C, which was re-chromatographed over the C18 column to yield 7 subfractions. The inhibitory effects of these fractions were also determined, where the highest activity was observed in SP/RP1/Fr.65-192, and SP/RP1/Fr.222-228 fractions (86% and 89% inhibitions, respectively). Thereafter, SP/RP1/Fr.65-192 (100 mg) was subjected to preparative HPLC affording SP/p.HPLC1/Fr.61-73 (**1**). In fact, in SP/p.HPLC1/Fr.61-106, the major constituent was **1**. SP/p.HPLC1/Fr.61-73 and SP/p.HPLC1/Fr.93-106 had compound **1** in relatively high purity; however, a minor impurity in SP/p.HPLC1/Fr.74-92 was present. Therefore, the fraction was kept separate. The activity experiments, and data also supported this observation. When compound **1** was tested, approx. 78% LO inhibitory activity was observed at a concentration of 100 μg/mL. The IC_{50} values of **1,** and

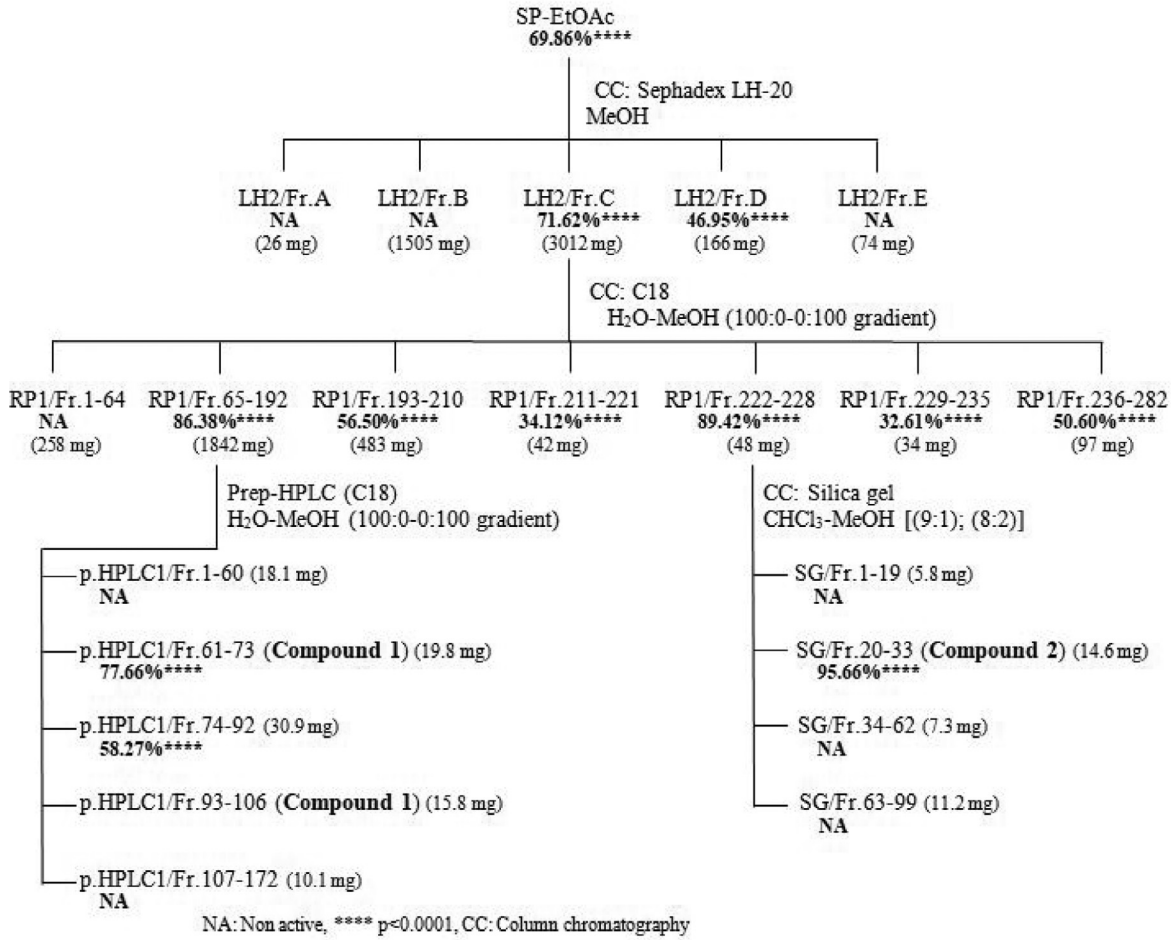


Fig. 1. Isolation of the active compounds by activity-guided fractionation.

Table 1
LO Inhibitory effects of the extracts, fractions and pure compounds.

Extract/fraction/compound	Concentration (μg/mL)	% Inhibition ± S.E.M	IC_{50} (μg/mL) ± S.E.M
SP- H_2O	100	NA	-
SP-MeOH	100	**28.63 ±1.4*** **	-
NDGA	20	85.26 ±4.9****	8.70 ±0.6
SP- Hexane	100	33.73 ±5.6****	-
SP-CH_2Cl_2	100	**57.81 ±4.6**** **	83.25 ±3.4
SP-EtOAc	100	**69.86 ±0.6**** **	93.37 ±0.8
SP-BuOH	100	NA	-
SP-R H_2O	100	NA	-
NDGA	20	82.22 ±3.2****	10.80 ±0.5
CH_2Cl_2 /LH1/Fr.A	100	NA	-
CH_2Cl_2 /LH1/Fr.B	100	NA	-
CH_2Cl_2 /LH1/Fr.C	100	46.21 ±2.2****	-
NDGA	20	83.02 ±1.8****	9.98 ±0.7
EtOAc /LH2/Fr.A	100	NA	-
EtOAc /LH2/Fr.B	100	NA	-
EtOAc /LH2/Fr.C	100	**71.62 ±3.2**** **	-
EtOAc /LH2/Fr.D	100	46.95 ±3.2****	-
EtOAc /LH2/Fr.E	100	NA	-
NDGA	20	83.02 ±1.8****	9.98 ±0.7
RP1/Fr.1-64	100	NA	-
RP1/Fr.65-192	100	**86.38 ±2.6**** **	-
RP1/Fr.193-210	100	56.50 ±7.6****	-
RP1/Fr.211-221	100	34.12 ±0.8****	-
RP1/Fr.222-228	100	**89.42 ±3.9**** **	-
RP1/Fr.229-235	100	32.61 ±2.0****	-
RP1/Fr.236-282	100	50.60 ±10.1****	-
NDGA	20	87.72 ±1.5****	8.43 ±0.5
p.HPLC1/Fr.1-60	100	NA	-
p.HPLC1/Fr.61-73 (1)	100	**77.66 ±2.1**** **	**76.00 ±0.9 (0.21 μM)**
p.HPLC1/Fr.74-92	100	58.27 ±3.7****	-
p.HPLC1/Fr.107-172	100	NA	-
NDGA	20	86.89 ±1.4****	11.62 ±1.1 (0.03 μM)
SG/Fr.1-19	100	NA	-
SG/Fr.20-33 (2)	100	**95.66 ±2.4**** **	**8.23 ±0.4 (0.02 μM)**
SG/Fr.34-62	100	NA	-
SG/Fr.63-99	100	NA	-
NDGA	20	99.49 ±1.7****	4.03 ±0.2 (0.01 μM)
MPLC/Fr.72-78 (**3**)	100	NA	-
NDGA	20	86.00 ±3.5***	10.53 ±0.8
p.HPLC2/Fr.60-72 (**4**)	100	NA	-
NDGA	20	89.26 ±4.9***	9.42 ±0.7

S.E.M: Standard error of the mean; NA: Not active; *** $p < 0.001$, **** $p < 0.0001$

the reference nordihydroguaiaretic acid (NDGA) comparatively were determined as 0.21 μM and 0.03 μM, respectively. Thereafter, SP/RP1/Fr.222-228 was applied to a silica gel column, and fraction SP/SG/Fr.20-33 (**2**) was further purified. Compound **2** showed approx. 96% LO inhibitory activity at a concentration of 100 μg/mL. The IC_{50} values of compound **2,** and NDGA were found as 0.02 and 0.01 μM, respectively. The IC_{50} values of the compounds were converted to μM after purity check.

After obtaining the major bioactive compounds, to contribute to the phytochemistry of *S. palaestina,* and pursue the search for other compounds with bioactivity, fractionation process was continued with other active subfractions. For this purpose, SP/RP1/Fr.229-235 was subjected to a MPLC system using C18 column, which resulted in the isolation of SP/MPLC/Fr.72-81 (**3**). Nevertheless, **3** did not show LO inhibitory activity, when compared to the standard, and other compounds. To isolate and identify the other impurities from SP/p.HPLC1/Fr.74-92, and evaluate their LO inhibitory activities, the remaining portion of SP/RP1/Fr.65-192 was chromatographed over Sephadex LH-20, which yielded 3 subfractions, and in turn, one of them (SP/LH3/Fr.C) was applied to the C18 column chromatography. Among the obtained 11 fractions, SP/RP2/Fr.36-44 was applied to further separation by preparative HPLC, to afford SP/p.HPLC2/Fr.60-72 (**4**). The LO inhibitory activity of compound **4** was also evaluated, which showed no inhibition (Table 1).

The structures of the isolated compounds (**1-4**) were elucidated using various spectrophotometric and chromatographic methods (^{1}H NMR, LC-TOF-MS and LC-QTOF-MS). On the basis of the spectral and chromatographic data, SP/p.HPLC1/Fr.61-73 (**1**), SP/SG/Fr.20-33 (**2**), SP/MPLC/Fr.72-81 (**3**) and SP/p.HPLC2/Fr.60-72 (**4**) were identified as rosmarinic acid, methyl rosmarinate, apigenin and apigenin 7-*O*-glucuronide, respectively. Structures of the isolated and purified compounds are shown in Fig. 2.

Rosmarinic acid (1); Amorphous solid, QTOF-MS/MS $[M-H]^-$ *m/z*= 359.0775 ($C_{18}H_{16}O_8$, 360.08), (fragments): 197.0466, 179.0360, 161.0252, 135.0459. ^{1}H NMR (DMSO-d_6, 600 MHz/ δ ppm, *J* Hz): 7.02 (*d*, 2.0 Hz, H-2), 6.73 (*d*, 8.1 Hz, H-5), 6.92 (*dd*, 8.2 Hz, 2.1 Hz, H-6), 7.35 (*d*, 15.9 Hz, H-7), 6.16 (*d*, 15.9 Hz, H-8), 6.65 (*d*, 2.1 Hz, H-2′), 6.58 (*d*, 8.0 Hz, H-5′), 6.47 (*dd*, 8.0 Hz, 2.0 Hz, H-6′), 2.73 (*dd*, 14.4 Hz, 10.1 Hz, H-7′), 2.99 (*dd*, 14.4 Hz, 3.1 Hz, H-7′), 4.81 (*dd*, 10.0 Hz, 3.1 Hz, H-8′) (Ticli et al., 2005).

Methyl rosmarinate (2); Amorphous solid, TOF-MS $[M-H]^+$ *m/z*= 375.1074 ($C_{19}H_{18}O_8$, 374.10). ^{1}H NMR (CD_3OD, 600 MHz/ δ ppm, *J* Hz): 7.04 (*d*, 1.7 Hz, H-2), 6.77 (*d*, 8.2 Hz, H-5), 6.95 (*dd*, 8.2 Hz, 1.7 Hz, H-6), 7.55 (*d*, 15.9 Hz, H-7), 6.26 (*d*, 15.9 Hz, H-8), 6.70 (*d*,

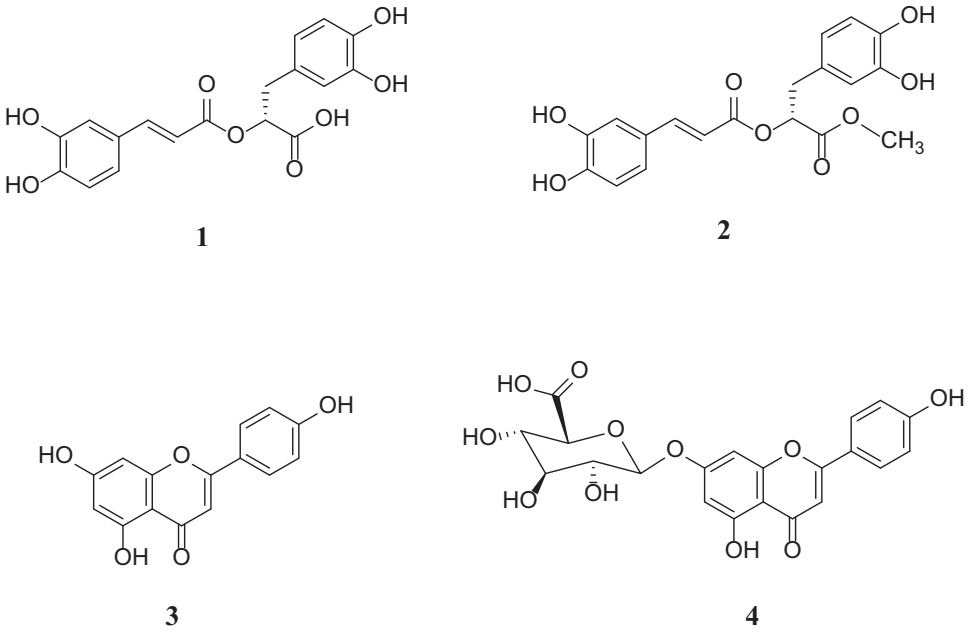


Fig. 2. Structures of purified compounds 1-4.

2.0 Hz, H-2′), 6.69 (*d*, 8.1 Hz, H-5′), 6.57 (*dd*, 8.1 Hz, 2.0 Hz, H-6′), 3.01 (*dd*, 14.1 Hz, 7.8 Hz, H-7′), 5.18 (*dd*, 7.6 Hz, 5.1 Hz, H-8′), 3.70 (3H, *s*, OCH_3) (Woo and Piao, 2004).

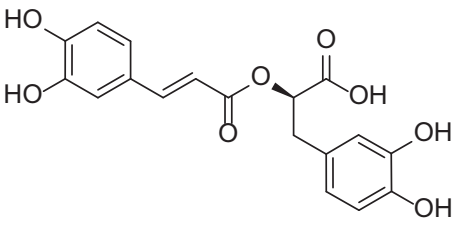
Apigenin (3); Amorphous yellow solid, QTOF-MS/MS $[M-H]^-$ *m/z*= 269.0456 ($C_{15}H_{10}O_5$, 270.05), (fragments): 225.0559, 151.0040, 117.0349. ^{1}H NMR (CD_3OD, 600 MHz/ δ ppm, *J* Hz): 6.39 (1H, *s*, H-3), 6.00 (*d*, 2.1 Hz, H-6), 6.19 (*d*, 2.0 Hz, H-8), 7.76 (2H, *d*, 8.8 Hz, H-2′, 6′), 6.84 (2H, *d*, 8.8 Hz, H-3′, 5′) (De la Torre-Carbot et al., 2005; Ha et al., 2012).

Apigenin 7-*O*-glucuronide (4); Amorphous yellow solid, QTOF-MS/MS $[M-H]^-$ *m/z*= 445.0788 ($C_{21}H_{18}O_{11}$, 446.08), (fragments): 269.0474, 225.0561, 175.0251, 113.0243. ^{1}H NMR (DMSO-d_6, 600 MHz/ δ ppm, *J* Hz): 6.85 (1H, *s*, H-3), 6.42 (*d*, 2.0 Hz, H-6), 6.82 (*d*, 2.0 Hz, H-8), 7.94 (2H, *d*, 8.4 Hz, H-2′, 6′), 6.93 (2H, *d*, 8.5 Hz, H-3′, 5′), 5.05 (*d*, 7.7 Hz, H-1″), 12.96 (1H, *s*, 5-OH), 8.43 (1H, *s*, 4′-OH) (Qu et al., 2001; Hase et al., 1995).

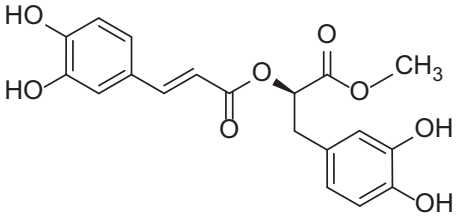
Rosmarinic acid, apigenin and apigenin 7-*O*-glucuronide were previously reported from *S. palaestina* (Miski et al., 1983; Cioffi et al., 2008; Al-Jaber et al., 2012). To the best of our knowledge, methyl rosmarinate was reported from *S. palaestina* herein for the first time.

In a previous study evaluating the effects of various phytochemicals on 5-LO enzyme, it was reported that rosmarinic acid showed strong inhibition, similar to our work, and the IC_{50} was below 10 μM (Chen 2011). In another study, the anti-inflammatory effect of *Perilla* L. seeds was studied, where was reported that rosmarinic acid, and methyl rosmarinate significantly inhibited the 5-LO enzyme with IC_{50} values 6.2 and 0.6 μM, respectively (Yamamoto et al., 1998).

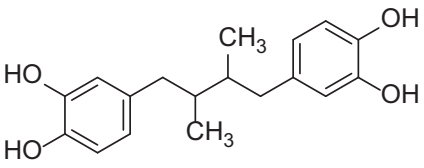
It is known that methyl rosmarinate, the methyl ester of rosmarinic acid, is generally present in plants together with rosmarinic acid. The anti-inflammatory effect of rosmarinic acid and methyl rosmarinate isolated from *Ehretia obtusifolia* Hochst. Ex A.DC. was evaluated *via* inhibition of the same type of LO enzyme used in this study. The most active compounds were determined as methyl rosmarinate (IC_{50}= 0.3 μM), and rosmarinic acid (IC_{50} = 0.6 μM), respectively (Iqbal et al., 2005). Consistent with previous experimental findings, methyl rosmarinate was found as relatively more active than rosmarinic acid, in the present study, too. Since the present work is an *in vitro* study, the LO inhibition potential emphasized herein is known; compounds affecting 15-LO may also have the potential in other varieties such as 5-LO and 12-LO, respectively (Chen 2011; Yamamoto et al., 1998). In a previous study (Thammason et al., 2018), this issue was evaluated through structure-activity relationship studies, where the ester analogues of rosmarinic acid were synthesized, and their anti-inflammatory effects were tested on lipopolysaccharide induced alveolar macrophages. Among the synthesized analogues, the highest activity was observed by ethyl rosmarinate. This activity was



Compound 1: Rosmarinic acid (SP/p.HPLC1/Fr.61-73)



Compound 2: Methyl rosmarinate (SP/SG/Fr.20-33)



Nordihydroguaiaretic acid (NDGA)

Fig. 3. Structural similarities of the active compounds with NDGA

followed by methyl rosmarinate, and hydrophobic ester groups were reported to be important for increasing the overall anti-inflammatory effect.

In the present LO enzyme inhibition experiments, NDGA used as a reference was found to be seven-, and two-fold more active than rosmarinic acid, and methyl rosmarinate, respectively. When chemical structures of NDGA and rosmarinic acid are considered, it can be proposed that the compounds are similar in certain frameworks. In particular, the presence of 3,4-dihydroxy phenyl groups such as shown in Fig. 3 was noteworthy for rosmarinic acid, methyl rosmarinate, and NDGA. The LO enzyme inhibitory activities of methyl rosmarinate and rosmarinic acid is probably different, due to the methyl ester functionality in methyl rosmarinate, which increases the hydrophobicity and may prevent ionization of carboxylic acid group. The absence of the ester linkage in the side chain, and the more hydrophobic character are possibly responsible for higher activity of the standard NDGA, which is more stable compared to the active compounds **1** and **2**.

Several studies (Raso et al., 2001; Hu et al., 2016) reported that apigenin, and apigenin 7-*O*-glucuronide showed anti-inflammatory activity; however, no *in vitro* LO inhibitory activity was observed for both compounds in the present work. This suggest that apigenin and apigenin 7-*O*-glucuronide may demonstrate their anti-inflammatory effects through other anti-inflammatory pathways.

4. Conclusions

In this present study, rosmarinic acid (**1**) and methyl rosmarinate (**2**) were purified from *S. palaestina* as the active compounds showing inhibitory properties on 15-LO enzyme. In addition, the observed inhibition on the inflammatory enzyme 15-LO, partially supports the folkloric use of *S. palaestina* as a wound healing remedy in Turkey (Miski et al., 1983; Guimaraes et al., 2018). Finally, further detailed studies such as phytochemical / *in vitro* / *in vivo* / molecular studies are required to explain the complex phytochemistry of the species and its relationship with bioactivity.

Declaration of Competing Interest

There is no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2021.04.030.

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