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Article

The chemical composition and antibacterial activity of a methanolic extract of *Satureja khuzistanica*

Mansooreh Davoodi[1], Samad Nejad-Ebrahimi[2,*], Abdolhossein Rustaiyan[1], Davoud Esmaeili[3]

[1]Department of Chemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran, [2]Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Evin, Tehran, Iran, [3]Department of Microbiology and Applied Microbiology Research Center, Systems biology and poisonings institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

In the present study, the metabolite profiling of methanolic extract from aerial parts of *Satureja khuzistanica* Jamzad, as an endemic medicinal plant from Iran, was evaluated using HPLC-PDA-ESI. Then, the main compound from the extract was isolated and purified by using extensive chromatographic techniques. In addition, the structure of the isolated compounds was elucidated using 1D, 2D NMR, and MS spectrometry, upon which 22 compounds were identified. The antibacterial activity of diosmetin 7-rutinoside (**6**) and linarin (**13**) in combination with carvacrol as a major compound of the essential oil was tested against *Pseudomonas aeruginosa* and *Staphylococcus aureus* through disc diffusion and minimum inhibitory concentration methods. The results indicated that the linarin, when mixed with carvacrol as the main compounds in the essential oil of the plant, has a satisfactory activity against both *Pseudomonas aeruginosa* and *Staphylococcus aureus* with MIC values of 0.16 and 0.18 µg/mL, respectively. Further, the fractional inhibitory concentration (FIC) index indicated that this compound had synergism with carvacrol.

Keywords: Lamiaceae. Caffeic acid. Flavonoid. HPLC-PDA-MS profiling. *Pseudomonas aeruginosa*. *Staphylococcus aureus*.

INTRODUCTION

The genus *Satureja* which belongs to Lamiaceae includes approximately 30 species distributed worldwide. The aerial parts of *Satureja* species have been used as spices and flavoring agents, as well as producing essential oil in the perfumery and herbal pharmaceutical industry for their various biological properties(Hadian *et al*., 2011). The plants of *Satureja* genius have been widely used as flavoring agents and spices, and they have been applied in traditional medicine to treat various illnesses including cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases (Hajhashemi *et al*., 2000). Different extracts from *Satureja* display their antispasmodic, antidiarrheal, antioxidant, sedative, and antimicrobial properties(Hajhashemi *et al*., 2000). The insecticidal and larvicidal activities of the essential oils of *Satureja hortensis*, *S. thymbra*, *S. montana*, and *S. Spigera*, which are used potentially as natural pesticides have been reported in the previous studies(Yildirim, Kordali, Yazici, 2011). The essential oil from *Satureja* had a wide range of biological properties such as antimicrobial, antiviral, and antioxidant activities(Eftekhar, Ashoori, Yousefzadi, 2017).

In Iran, the genus *Satureja* is represented by 14 species which are generally known as "Marze", with a geographical distribution in the western, northern, and southern parts of Iran. Among these species, eight are known to be endemic to Iran, one of which being *Satureja khuzistanica* Jamzad, which is mainly found in the southwestern parts of the Zagros Mountains and has

***Correspondence:** S. Nejad-Ebrahimi. Department of Phytochemistry. Medicinal Plants and Drugs Research Institute. Shahid Beheshti University, G. C. Evin, Tehran, Iran. Telefax: +98-21-22431783. E-mail address: s_ebrahimi@sbu.ac.ir. ORCID: 0000-0003-2167-8032

been used as antiseptic and an analgesic in the Iranian folk medicine (Hadian *et al*., 2011). The essential oil of *S. khuzistanica* has been reported to be a rich source of carvacrol and thymol (Hadian *et al*., 2011) with numerous biological properties such as antimicrobial (Mahboubi, Kazempour 2016), antioxidant (Ghorbanpour, Hadian 2015), antidiabetic (Mirazi, Rezaei, Mirhoseini, 2016), cytoprotective, and anti-apoptotic activities (Nasimi *et al*., 2016). Although the composition of the essential oil of *S. khuzistanica* aerial parts was considered in some studies, the phytochemical profile of the nonvolatile part of the plant extract was emphasized in a few cases. For instance, rosmarinic acid, flavonoids, and triterpenoids were reported as the main compounds of the aerial parts in *S. kuzistanica* (Malmir *et al*., 2015; Moghaddam *et al*., 2007).

In many plant-extracted materials used as natural antibiotics, synergism plays a great role in their therapeutic effect (González-Lamothe *et al*., 2009), which results in more significant biological activity compared to that of the individual compounds (Chanda, Rakholiya, 2011). Many drugs are currently used because of synergistic interactions between different antibiotics with different targets (Sibanda, Okoh, 2007). Based on the results, some flavonoids such as apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have antibacterial activity (Malmir *et al*., 2015; Moghaddam *et al*., 2007). Therefore, the antibacterial role of compounds **6** and **13** as the major compound of *S. kuzistanica* extract was evaluated, along withthe carvacrol as an active component of the essential oil. Thus, the antibacterial activity of diosmetin 7-rutinoside (**6**) and linarin (**13**), along with carvacrol, was examined against *Pseudomonas aeruginosa* and *Staphylococcus aureus* through disc diffusion and minimum inhibitory concentration techniques. The results indicated that *P. aeruginosa* and *S. aureus* were highly sensitive to both isolated flavonoids and carvacrol as major compounds found in the essential oil of the plant.

The present study aimed to evaluate the phytochemical content of the methanolic extract of *S. khuzitanica* through extensive chromatographic and spectroscopic techniques. Further, the isolation and structural elucidation of several caffeic acid derivatives and flavonoids was considered.

MATERIAL AND METHODS

Chemicals and reference compounds

Solvents for extraction and HPLC grade solvents for chromatography were prepared from Scharlau (Barcelona, Spain). HPLC grade water was obtained by an EASY-pure II water purification system (Barnstead, Dubuque IA, USA). Sephadex LH-20 was purchased from GE Healthcare (Fairfield CT, USA), and deuterated solvents were purchased from Armar Chemicals (Döttingen, Switzerland). Diaion HP-20 resin was purchased from Sigma Aldrich (Buchs, Switzerland). Finally, solvents for NMR were obtained from Armar Chemicals (Döttingen, Switzerland).

General experimental procedures

The Bruker Avance III™ spectrometer (Fällanden, Switzerland) was used to record compounds spectra. The 1-mm TXI microprobe (^{1}H and 2D NMR) operating at 500.11 MHz was utilized for measuring spectra. The analysis of spectra was conducted through Bruker TopSpin 3.0 Software. In addition, HRESI-MS spectra were recorded on a Bruker Micro TOF ESI-MS System with the scan range of *m/z* 200-1500. The isopropanol-water (1:1) containing 5 mM sodium hydroxide solution was used for MS calibration. The typical mass accuracy was ± 3 ppm. Further, HyStar 3.0 Software (Bruker Daltonics) was used for data acquisition and processing. In the next stage, ECD spectra were recorded in MeOH with a Chirascan® CD Spectrometer (Leatherhead, England). HPLC separations were performed on an 1100-series HPLC system (Agilent, Waldbronn, Germany) including a quaternary low-pressure mixing pump with degasser module, column oven, PDA detector, and autosampler. Additionally, ESIMS spectra were obtained on an Esquire 3000 plus an ion trap mass spectrometer (Bruker Daltonic, Bremen, Germany). A P 50 pump (GE Healthcare) and the fraction collector (Pharmacia Biotech) were used for column chromatography on Sephadex LH-20 (870

×70 mm). Then, all fractions monitored by using TLC (silica gel 60 F_{254}) analysis (Merck, Darmstadt, Germany). The mobile phase for monitoring the fractions was EtOAc/MeOH/HCOOH (60/40/0.05), and detection was performed at UV 254 and 366 nm.

For all of the analytical HPLC analyses, the samples were dissolved in DMSO at 5 mg/mL. In another stage, aliquots of 10 µL were injected. The mobile phase consisted of water (A) and MeCN (B) containing 0.1% formic acid. The flow rate was 0.4 mL/min. Further, 10 % B to 100 % B in 30 min, and 100 % B, 31-35 min 100% as the gradient profile was used for qualitative analysis. UV spectra were recorded at 210 to 700 nm. SunFire® C_{18} (3.5 µm, 3.0×150 mm), and SunFire Prep were used for analytical, semi-preparative, and preparative RP-HPLC separations, respectively.

Plant Material

The plant material *Satureja khuzistanica* Jamzad was collected from the cultivation site of Khorraman Pharma Co. (Khorramabad, Iran) on June 2012. Dr. Javad Hadian identified the plant material, and a voucher specimen (MPH-1582) was deposited at the Herbarium of the Department of Biology, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

Extraction and Isolation

The dried leaf material (800 g) was grounded with a ZM1 equipped with a 0.75 mm ConidUR ring sieve, and was extracted by successive percolation with CH_2Cl_2, EtOAc, and MeOH (3×5 L each) consecutively. Then, 18.2 g of MeOH extract was obtained after evaporation to dryness under reduced pressure. In addition, the extract was suspended in distilled water (1 L) and filtered to remove undissolved parts. Further, the aqueous solution was subsequently passed through a high porous styrenic adsorbent resin column (Diaion Hp-20, 9.0 × 5.0 cm). The column was washed with distilled water (3×1.5 L). Additionally, the adsorbed material was desorbed by washing with MeOH (3×1.0 L). Finally, the obtained fraction was evaporated under vacuum and produced 14.2 g gummy material. The obtained material was dissolved in 100 mL MeOH and after filtration was subjected to gel chromatography on Sephadex LH-20 column (5.5 × 88.0 cm). The column was eluted with MeOH at a flow rate of 2.5 mL/min. Every eight minutes (20 mL), one fraction was collected and finally pooled into 8 major fractions (Fr1-8) according to the TLC results (the mobile phase EtOAc/MeOH (4:6): Fr 1 (500 mL, 0.08 g), Fr 2 (320 mL, 1.1 g), Fr 3 (160 mL, 2.1 g), Fr 4 (280 mL, 4.2 g), Fr 5 (360 mL, 2.7 g), Fr 6 (540 mL, 3.29 g), Fr 7 (800 mL, 0.21 g), and Fr 8 (280 mL, 0.02 g). Fraction 7 (200 mg) was subjected to the medium pressure liquid chromatography (MPLC) separation on a glass column (26 × 460 mm) packed with LiChroprep RPC18 (25–40 µm, Merck; Darmstadt, Germany), at a flow rate of 10 mL/min. The liquid injection was carried out directly on top of the column by using a 20 mL syringe. A gradient of H_2O /MeCN (both containing 0.1% formic acid) (10:90 to 50:50 over 23 min and 50:50 to 0:100 in 7 min), the peaks 24 subfractions (7_1-7_{24}). The subfraction 7_6-7_9 were pooled (50 mg) and separated by semi-preparative RP-HPLC at a flow rate of 4.0 mL/min to afford compounds **11** (4.5 mg, t_R 11.5 min), **12** (0.3 mg, t_R 13.5 min), **17** (1.0 mg, t_R 13.9 min), **20** (0.9 mg, t_R 18.5 min), **13** (3.7 mg, t_R 17.3 min), **6** (0.8 mg, t_R 10.2 min), **18** (14.2 mg, t_R 8.5 min) and **14** (0.7 mg, t_R 11.5 min). A portion (100 mg) of fraction 5 was dissolved in MeOH and separated by preparative RP-HPLC with a gradient of H_2O/MeOH (56:44 to 0:100 over 28 min) at a flow rate of 10 mL/min afford compounds **1** (1.4mg, t_R 3.5 min), **3** (2.5 mg, t_R 3.5 min), **5** (1.3 mg, t_R 10.3 min), **19** (3.8 mg, t_R 13.1 min), **21** (3.1 mg, t_R 13.9 min) and **16** (3.2 mg, t_R 14.5 min).

Biological Activity

In this study, the microbial strains were collected in the Surgery, ICU, and Burn wards of Motahari Hospital. The susceptibility test to antibiotics including Aminoglycosides, Betalactames, and Oxacillin was performed according to Kirby Bauer's methods (CLSI2016). The resistant strains were subjected to carvacrol (prepared from Barij Essence Kashan VC), carvacrol + compound **6** and carvacrol+ compound **13**. Then, ethanol (Merck, Germany) with the concentration

1:1 was used to dissolve the compounds and diluted to the concentrations (0.25-500 µg/mL). Cultures were performed by a sterile swab, and the suspension was cultured for 24 h, which was inoculated onto Mueller Hinton agar blank discs (Merck, Germany) with a diameter of 6 mm containing 30 µL of compounds placed on Mueller Hinton agar medium (MHA). The growth inhibition zones were measured after 24 hours of incubation at 37^0C. The experiments were repeated three times. Disks containing 30 µL of ethanol were selected as the control group. The growth inhibition zones were subtracted from the control zone. MIC determination was performed with the microdilution method according to CLSI2016. Finally, the standard antibiotic discs of polymyxin E (30 µg/disc), ciprofloxacin (10 µg/disc), gentamicin (10 µg/disc), and imipenem (10 µg/disc) were prepared to evaluate the antimicrobial susceptibility from Padtanteb.

The interactions between major isolated compounds (6, 13) and carvacrol

The checkerboard method, which is typically employed to measure interactive inhibition, was used in this study to determine how the compounds **6** and **13** interact with the carvacrol (Palaniappan, Holey, 2010). Synergistic interactions between the isolated compounds (**6**, **13**) (drug a) and the carvacrol (drug b) were also examined. The agents were used with the initial concentration that was three times greater than their MIC value. The effects of the combinations were assessed by calculating the FIC (Fractional Inhibitory Concentration) index for each combination using the equations shown below:

The FIC index of the compound a (FIC_a) = MIC of the compound a in combination/MIC of the compound an alone

FIC of compound b (FIC_b) = MIC of the compound b in combination/MIC of compound b alone

The sum of FIC indices of the two compounds in the combination was calculated as follows: $FIC_a + FIC_b = FICI_s$

Synergism has been defined as an FIC index of 0.5 or less, additivity as an FIC index of more than 0.5 and less than 4, and antagonism as an FIC index of more than 4 and 1.0 indicated indifference, and between 1.0 and 0.5 was evaluated as an additive interaction.

RESULTS AND DISCUSSION

The aerial parts of *S. khuzistanica* were extracted sequentially by using dichloromethane, ethyl acetate, and methanol. In addition, the methanolic extract was analyzed by HPLC-PDA-ESIMS in the positive and negative modes. The UV trace at 330 nm and ESI base-peak chromatogram (Figure 1) revealed the phytochemical pattern of methanolic extract. The analysis of the UV spectra pattern and molecular weight indicated the presence of phenolic compounds, which is mainly related to flavonoids and caffeic acids. The methanol extract was dissolved in water and passed through a diaion HP-20 resin to obtain an enriched fraction of phenolic compounds. The enriched fraction was then passed through the Sephadex LH-20, and major peaks were purified using preparative and semi-preparative RP-HPLC. In the next stage, the structures of the compounds were elucidated by a combination of ^{1}D and ^{2}D NMR, HRMS, and UV spectroscopy, and the results were compared with the published data. In total, 22 structures were identified (Table I). The identified compounds were: erigeroside (**1**) (Tafazzoli, Ghiasi, Moridi, 2008), apigenin-7-*O*-gentiobioside (**2**) (Montoro *et al.*, 2005), zataroside A (Moses et al., 2005; Ali *et al.*, 1999), ombuside (**4**) (Deulofeu, Noire, Hug, 1952), zataroside B (**5**) (Ali *et al.*, 1999), diosmetin 7-rutinoside (**6**) (Schneider, Blaut, 2000), Rhoifolin (**7**) (Hattori, Matsuda, 1952), quercetin-7-glucuronide(**8**) (O'Leary *et al.*, 2003), Rutin (**9**) (Zhishen, Mengcheng, Jianming, 1999), Apigenin-7-*O*-*β*-D- glucuronide (**10**) (Zhishen, Mengcheng, Jianming, 1999), rosmarinic acid (**11**) (Petersen, Simmonds 2003), methyl rosmarinate (**12**) (Fecka, Turek 2008), linarin (**13**) (Ina, Iida 1981), melitric acid A (**14**) (Agata *et al.*, 1993), Poncirin (**15**) (Lee *et al.*, 2009), (7'*R*, 8'*R*, 8''*R*, 8'''*R*)-epi-salvianolic acid B(**16**), methyl melitric acid A (**17**) (Agata *et al.*, 1993), lycopic acid C (**18**) (Murata *et al.*, 2010), saturejenol (**19**) (Davoodi, Rustaiyan, Ebrahimi, 2018), diosmin (**20**) (Nieto, Gutierrez, 1986), keshonin (**21**) (Yamada *et al.*, 2010), and saturejin (**22**) (Malmir *et al.*, 2015) (See Figure 2).

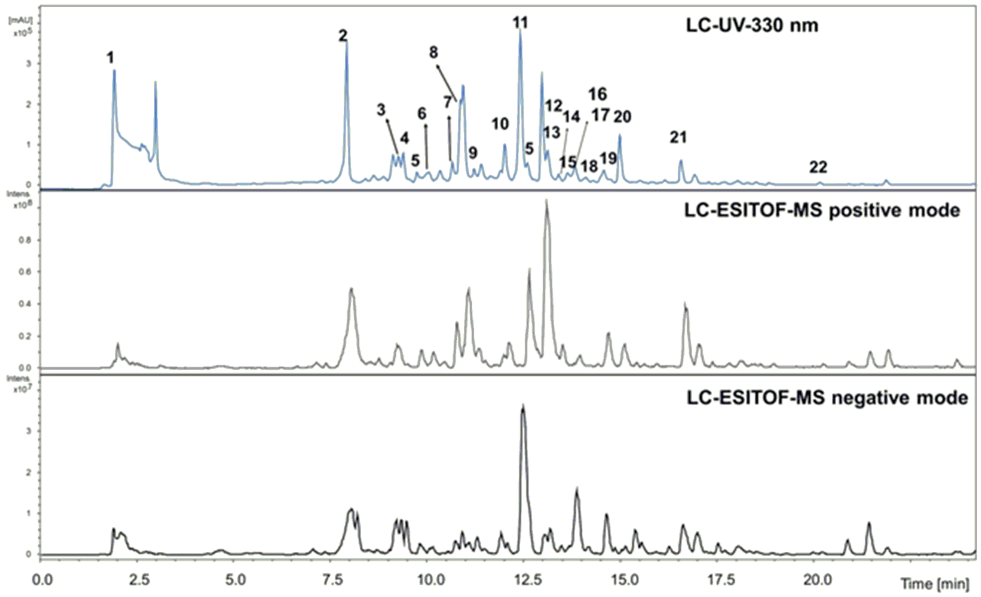

FIGURE 1 - HPLC chromatogram of the methanol extract from dried leaves of *S. khuzistanica*.

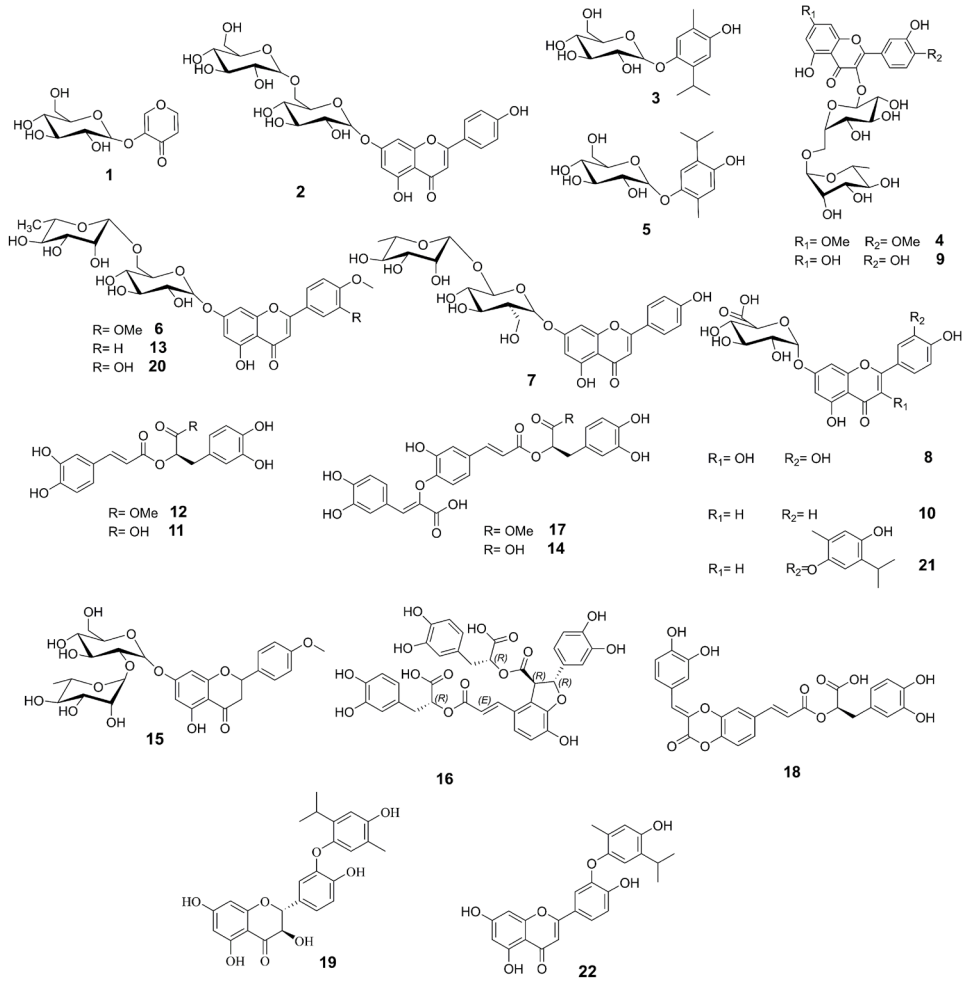

FIGURE 2 - Structures of compounds **1-22.**

TABLE I - List of identified compounds from methanolic extract of *S. khuzistanica*

No	t_R(min)	Compound	UV-vis λ_{max} (nm)	m/z positive	m/z negative	Identification Method	References
1	1.9	erigeroside	233, 261	$[M+Na]^+$ 297, $[M+K]^+$ 313 $[2M+Na]^+$ 571	-	MS-UV, NMR	(Tafazzoli, Ghiasi, Moridi, 2008)
2	7.9	apigenin-7-*O*-gentiobioside	269, 328	$[M+H]^+$ 595	$[M-H]^-$ 593	MS-UV	(Montoro, *et al.* 2005)
3	9.2	zataroside A	267	-	$[M-H]^-$ 327	MS-UV, NMR	(Ali, *et al.* 1999)
4	9.4	ombuside	261	$[M+H]^+$ 639	-	MS-UV	(Deulofeu, Noire, Hug, 1952)
5	9.7	zataroside B	265	-	$[M-H]^-$ 327	MS-UV, NMR	(Ali, *et al.* 1999)
6	10	diosmetin 7-rutinoside	262, 330	$[M+H]^+$ 623	$[M-H]^-$ 621	MS-UV, NMR	(Schneider, Blaut, 2000)
7	10.7	rhoifolin	261, 310	$[M+H]^+$579	$[M-H]^-$ 577	MS-UV	(Hattori, Matsuda, 1952)
8	10.8	quercetin-7-glucuronide	265, 351	-	$[M-H]^-$ 451	MS-UV	(O'Leary, *et al.* 2003)
9	11.2	rutin	262, 330	$[M+H]^+$ 611	$[M-H]^-$ 609	MS-UV	(Zhishen, Mengcheng, Jianming, 1999)
10	12.0	apigenin-7-*O*- β-D-glucuronide	266, 323	$[M+H]^+$ 447	$[M-H]^-$ 445, 2$[M-H]^-$ 891	MS-UV	(De Bruyne, *et al.* 1999)
11	12.4	rosmarinic acid	246, 284, 325	-	$[M-H]^-$ 359, 2$[M-H]^-$ 719	MS-UV, NMR	(Petersen, Simmonds, 2003)
12	13.1	methyl rosmarinate	269	-	$[M-H]^-$ 373	MS-UV, NMR	(Woo, Piao, 2004)
13	13.1	linarin	319	$[M+H]^+$593	$[M-H]^-$ 591	MS-UV, NMR	(Ina, Iida, 1981)
14	13.2	isomelitric acid A	253, 286, 324		$[M-H]^-$ 537	MS-UV, NMR	(Agata, *et al.* 1993)
15	13.5	poncirin	267, 321	$[M+H]^+$ 595	$[M-H]^-$ 593	MS-UV	(Lee, *et al.* 2009)
16	13.7	(7'*R*, 8' *R*, 8''*R*, 8'''*R*)-epi-salvianolic acid B	256, 285, 316	-	$[M-H]^-$ 717	MS-UV, NMR	-
17	13.7	methyl militrinate	254, 284, 313	-	$[M-H]^-$ 551	MS-UV, NMR	(Dapkevicius, *et al.* 1999)
18	13.9	lycopic acid C	255, 287, 317	$[M+H]^+$ 521	-	MS-UV, NMR	(Murata, *et al.* 2010)
19	14.4	saturejenol	275.331	-	$[M-H]^-$ 451	MS-UV, NMR	(Davoodi, Rustaiyan, Ebrahimi, 2018)
20	15.0	diosmin	279, 339	-	$[M-H]^-$ 607	MS-UV, NMR	(Nieto, Gutierrez, 1986)
21	16.6	keshonin	269, 328	$[M+H]^+$611	$[M-H]^-$ 609	MS-UV, NMR	(Yamada, *et al.* 2010)
22	21.0	saturejin	245, 266, 342	$[M+H]^+$ 435	$[M-H]^-$ 433	MS-UV	(Malmir, *et al.* 2015)

Compound **16** was isolated as gummy material with the maximum absorption at 256, 285, and 316 nm, which corresponded to caffeic acid derivatives. The negative ESI-MS showed a quasi-molecular ion peak at *m/z* 717 $[M-H]^-$, which indicated the molecular formula $C_{36}H_{30}O_{16}$. This compound gave the characteristic ^{1}H-NMR spectrum possessing the same pattern of salvianolic acid B derivatives. The ^{1}H NMR spectrum afforded signals for two –methine protons attached to ester bond at δ 5.04 (1H, dd, J =8.7, 4.2 Hz, H-8''), 4.96 (1H, dd, J =7.7, 4.9 Hz, H-8'''), 2.96, 2.99 (2H, dd, J =14.3, 7.5 Hz, H-7''a, J = 14.6, 4.7 Hz, H-7''b), and δ 2.91 (2H, dd, J =13.1, 8.7, H-7'''a, m, H-7'''b), respectively. Moreover, the two observed doublets were assigned to trans-olefinic protons at δ 7.61 (1H, d, J = 15.9 Hz, H-7) and δ 6.29 (1H, d, J = 15.9 Hz, H-8). Furthermore, the presence of a dihydrobenzofuran moiety was suggested by the signals at δ 5.72 (1H, d, J = 4.8 Hz, H-7') and δ 4.43 (1H, d, J = 4.9 Hz, H-8'). Then, 36 carbon signals were exhibited in the ^{13}C NMR spectrum, which were elucidated as four carbonyl carbons at δ 171.6 (C-9'), δ 172.9 (C-9''), δ 165.1 (C-9'''), and δ 169.5 (C-9), 26 aromatic carbons at δ 110–150, two olefinic carbons at δ 142.6(C-7), d 115.7 (C-8), two characteristic carbons for the dihydrobenzofuran ring at δ 86.4 (C-7') and d 55.7 (C-8'), two oxygenated methane carbons at δ 74.9 (C-8'') and δ 74.1 (C-8'''), and two methylene carbons at δ 36.5 (C-7'') and δ 36.5 (C-7'''). Thus, the planar structure was determined to be similar to that of salvianolic acid B compared to ^{1}H NMR data (Table II, Figures S1-S4 and S7**)**. The MSn analysis of compound **16** was performed through direct injection into Ion trap mass spectrometer. As illustrated in Figure 3, the $[M-H]^-$ at *m/z* 717 in the negative mode for compound **16** was attributed to ion-molecule. The fragmentation of *m/z* 717 in the MS^3 spectrum indicated the loss of two molecules of Danshensu (DSS) (198 u), leading to the fragment ions at 519 (-DSS) and 321 (-DSS-DSS), respectively. The ions of $[M-H-CH_2-CH_2-CO]^-$ (*m/z* 251) could be observed in the MS^4 spectrum of compound **16**. These data and the fragmentation pattern confirmed the proposed structure.

TABLE II - ^{1}H and ^{13}C NMR data (500 and 125 MHz, in CD_3OD) of compound **16** (7'R, 8' R, 8''R, 8'''R)-epi- salvianolic acid B).

position	δ_H (J in Hz)	δ_C [a]	Hey HMBC signals (H → C)
1		124.4	
3		146.4	
4		147.7	
5	6.82 (d, 8.4)	117.8	
6	7.25 (d, 8.5)	120.9	
7	7.61 (d, 15.9)	142.6	1, 9
8	6.29 (d, 15.9)	115.8	1, 9
9		169.6	
2'	6.67 (d, 1.8)	117.0	
5'	6.71 (d, 8.2)	116.0	
6'	6.51 (dd, 8.1, 1.9)	117.3	
7'	5.72 (d, 4.8)	86.4	6', 4, 9', 2'
8'	4.34 (d, 4.9)	55.7	9', 1, 3,
9'		171.6	
1''		127.6	
2''	6.59 (d, 8.0)	115.8	

(continues on the next page...)

TABLE II - ^{1}H and ^{13}C NMR data (500 and 125 MHz, in CD_3OD) of compound **16** (7'R, 8' R, 8''R, 8'''R)-epi- salvianolic acid B).

position	δ_H (J in Hz)	δ_C [a]	Hey HMBC signals (H → C)
5"	6.60 (d, 8.0)	115.8	
6"	6.42 (dd, 8, 1.8)	120.5	
$7''_a$	2.96 (dd, 14.3, 7.5)	36.5	
$7''_b$	2.99 (dd, 14.6, 4.7)	36.5	
8"	5.04 (dd, 7.7, 4.9)	74.9	9", 1",9
9"		173.9	
2"'	6.63 (d, 1.8)	116.9	
5"'	6.71 (d, 8.2)	112.9	
6"'	6.51 (dd, 8.1, 1.9)	120.3	
$7'''_a$	6.91 (dd, 13. 1,8.7)	36.5	2"'
$7'''_b$	2.88 (m)	36.5	
8"'	4.96 (dd, 8.7, 4.2)	74.1	9"'
9"'		165.1	

[a]The values obtained from HSQC and HMBC correlations

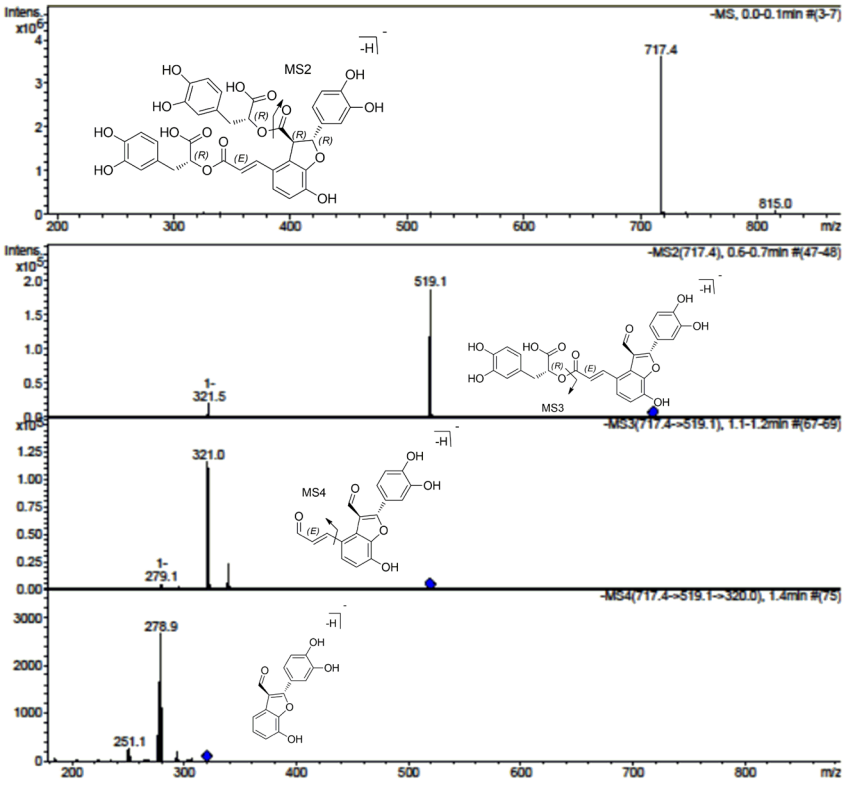

FIGURE 3 - ESI-MSn spectrum (negative ion mode) of (7'*R*, 8' *R*, 8''*R*, 8'''*R*)-epi-salvianolic acid B (**16**), and proposed fragmentation pattern.

In the experimental ECD spectrum, compound **16** revealed the presence of three sequential negative Cotton effects at 350, 265, and 240 nm, along with a positive CE at 210 nm. A comparison of these ECD data with those of salvianolic acid B which was purchased from Sigma Aldrich represented an opposite sign with three positive CEs and one negative CE, which means that compound **16** must have different stereochemistries. In order to establish the absolute configuration, compound **16** was degraded by permethylating **16** followed by methanolysis to provide a dihydro benzofuran derivative (Figure S5). The core structure was obtained and ECD spectrum was measured to compare with the results in the previously published data (Watzke *et al.*, 2006), representing that the coupling constant value of 4.8 Hz between H-7' and H-8' in the dihydro benzofuran ring is trans-oriented and two stereoisomers (7'*S*, 8'*S* or 7'*R*, 8'*R*) can be suggested (Murata *et al.*, 2013). Regarding the ECD measurement for the degraded products and comparison of the obtained values with those in the literature, the configuration of 7'*R*, 8'*R* was proposed for inner benzofuran ring (Figure S6**).** In fact, the evaluation of chemical shifts in C-8'' and C-8''' and their comparison with the published data indicated that C-8'' had a chemical shift of 74.9 in carbon NMR where C-8''' had a chemical shift of 74.1. Si *et al.* (2016) reported that the compounds with 8''R (or 8'''R) configuration displayed the chemical shifts of C-8'' (or C-8''') at about δ 74–75, and the compounds with 8''S (or 8'''S) configuration had the chemical shifts of C-8'' (or C-8''') at approximately δ 77–78. Consequently, the C-8''and C-8''' in compound **16** was observed at δ 74.9 and 74.1, respectively, indicating R as the absolute configuration of C-8'' and C-8'''. So the structure of compound **16** was finally determined to be (7'*R*, 8'*R*, 8''*R*, 8'''*R*)-epi-salvianolic acid B.

In another study, the structure of **20** was evaluated by ESI–MS_n analysis in the positive mode (Figure 4). Compound **20** represented molecular ion for $[M+H]^+$ at *m/z* 609. In order to evaluate the linkage of sugars in MS^2, the cation ion $[M+H- 146]^+$ at *m/z* 463 corresponded to the loss of a rhamnose moiety (146 amu) located in the external part of the molecule. Regarding MS^3, the fragmentation revealed that $[M+H- 162]^+$ at *m/z* 301 corresponded to neutral loss of glucose moiety as inner moiety. Finally, $[M+H-15]^+$ corresponded to the loss of methyl group in MS^4, where the molecular ion for aglycon was represented at $[M+H]^+$ at *m/z* 286. Therefore, the structure of diosmin was confirmed.

Compound **14** in the negative electrospray mass spectrum indicated a $[M-H]^-$ at *m/z* 492 (Figure 5). In the MS^2 spectrum, an ion at *m/z* 359 corresponded to the neutral loss of a 179 amu fragment of caffeic acid group, which resulted in the rosmarinic acid core structure at *m/z* 359, while the ion at *m/z* 315 was obtained by a cleavage of the second carboxyl group in MS^3.Thus, the structure of melitric acid A was confirmed.

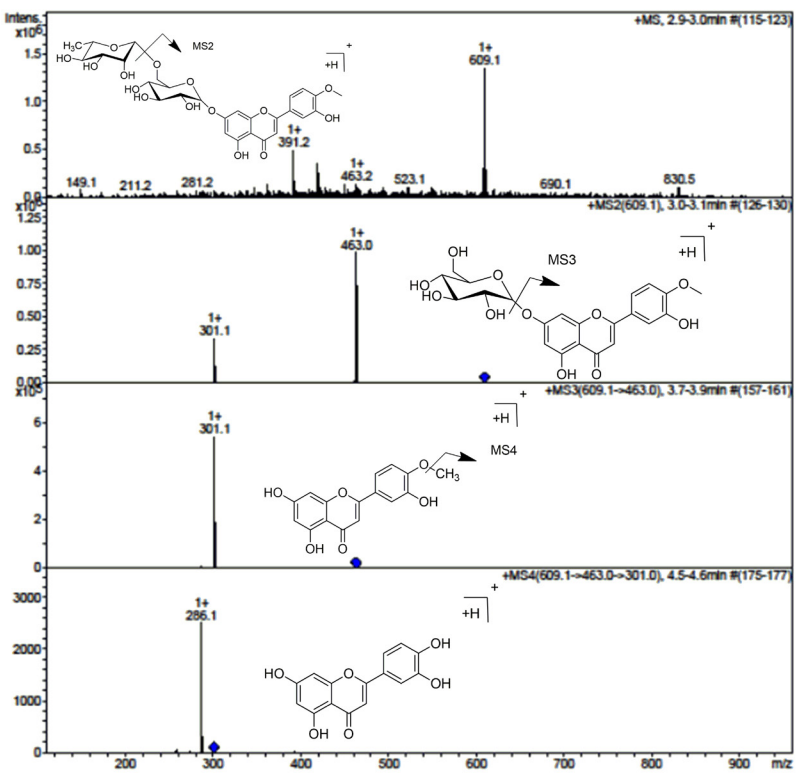

FIGURE 4 - ESI-MSn spectrum (positive ion mode) of diosmin (**20**), and proposed fragmentation pattern.

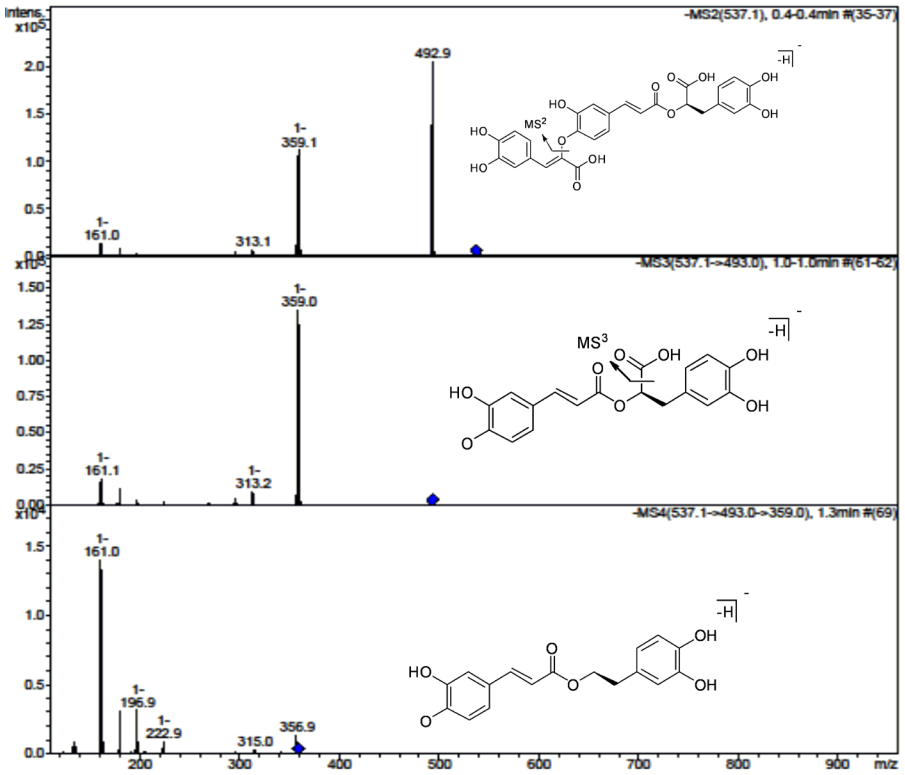

FIGURE 5 - ESI-MSn spectrum (negative ion mode) of melitric acid A (**14**), and proposed fragmentation pattern.

Biological activity

The present study sought to evaluate the combined effect of the antimicrobial potential of major isolated compounds (**6** and **13**) with carvacrol. The results of the previous studies revealed that carvacrol can affect the inhibition of the growth of *P. aeruginosa* (Tapia-Rodriguez MR *et al.*, 2017) and *S. aureus*(Knowles J *et al.*, 2005). Thus, the antibacterial activity of diosmetin 7-rutinoside (**6**) and linarin (**13**) in combination with carvacrol as a major compound of the essential oil was tested against *P. aeruginosa* and *S. aureus*. Table III indicates the results of the antibacterial activity as the mean inhibition zone for the tested compounds and a variety of antibiotics. The inhibitory effect of the major compounds from *S. kuzistanica* was investigated against *P. aeruginosa* and *S. aureus* with carvacrol by the agar disc diffusion method. In addition, the MIC values were determined by the broth dilution method according to protocol CLSI 2016. Further, the resistant strains were treated with carvacrol+compound **6** and carvacrol +compound **13**. Based on the results, the compounds **6** and **13** demonstrated a favorable activity against *P. aeruginosa* and *S. aureus*. Furthermore, MIC values for *P. aeruginosa* were equal to 0.185 µg/mL, and 0.155 µg/mL, respectively, while the MIC values for *S. aureus* were 0.205 µg/mL, and 0.17 5µg/mL, respectively. The comparison of these data with antibiotics as the positive control indicated a good activity (Table III). Additionally, the checkerboard method was employed to determine the way the carvacrol and compounds 6 and 13 may potentially interact with each other. The FICI index of carvacrol+compound **6** is 0.48, while it is 0.42 in carvacrol+compound **13**, showing the synergistic effects between the compounds with carvacrol.

TABLE III - Antimicrobial activity of carvacrol, diosmetin 7-rutinoside (**6**) and linarin (**13**

Compounds	*Pseudomonas aeruginosa*		*Staphylococcus aureus*	
	inhibition zone (mm)[a]	**MIC (µg/ mL)**	**inhibition zone (mm)[a]**	**MIC (µg/ mL)**
carvacrol [b]	20	0.34	19	0.37
carvacrol+compound **6** [c]	25	0.19	21	0.21
carvacrol+compound **13**[d]	28	0.16	23	0.18
compound **6**[e]	18	0.39	16	0.39
compound **13**[f]	19	0.38	18	0.38
polymyxin E		0.04		0.37
ciprofloxacin		0.50		No
imipeneme		4.00		1.00
gentamycin		2.00		5.00

[a] Values are the average of triplicate
[b] cultivated carvacrol (30 µg/mL)
[c] ratio of carvacrol and compound **6** (1: 1)(30 µg/ mL)
[d] ratio of carvacrol and compound **13** (1: 1)(30 µg/ mL)
[e] cultivated carvacrol (30 µg/ mL)
[f] cultivated carvacrol (30 µg/ mL)

In this study, *P. aeruginosa* was more susceptible to carvacrol and compounds **6** and **13** combined with carvacrol. *P. aeruginosa* is a gram-negative bacterium, including porins which are more permeable to these compounds. In addition, *S. aureus aureus* has thicker peptidoglycan and less permeability than these compounds. Carvacrol is a phenolic compound which is effective on membranes, nucleic acids, and proteins, and is able to control *P. aeruginosa*. Based on the MIC results, a combination of carvacrol with compounds **6** and **13** has a synergistic effect. The result of the present study is consistent with those focused on the antibacterial effect of flavonoids, especially when they are used in combination with antibacterial agents(Cushnie, Lamb 2005). Further, the antibacterial activities of flavonoids have multiple cellular targets rather than one specific site of action. As a molecular action, they can form a complex with proteins via nonspecific forces including hydrogen bonding and hydrophobic effects, as well as through covalent bond formation. Accordingly, the antimicrobial action of these flavonoids can be attributed to their ability to inactivate microbial adhesins, enzymes, and cell envelope transport proteins. Additionally, lipophilic flavonoids may lead to the disruption of microbial membranes. It was proposed that flavonoids can interfere with microbial enzymes and inhibit the growth of bacteria through their inhibition of DNA gyrase (Cushnie, Lamb 2005).

Some reported the antimicrobial activity of different *Satureja* species(Şahin *et al.*, 2003). A large number of studies focused on the antimicrobial activity of the essential oil and extraction of *S. khuzistanica,* which showed inhibitory activity against common nosocomial bacteria (Amanlou *et al.*, 2007; Zibaei *et al.*, 2012). Based on the results, the essential oil *S. khuzistanica* can play a major role in lowering the *P. aeruginosa* resistance to drugs by reducing mexA gene expression (Jalalvandi *et al.*, 2015) and the antibacterial properties of this plant can be related to the presence of this agent since carvacrol is considered as one of the major compounds in this plant (90.8%). The results indicated that a combination of major compounds from the extract with carvacrol can increase the antimicrobial activity.

CONCLUSION

The phytochemical profiling of *S. khuzistanica* was performed by comprehensive chromatography and spectroscopic methods. The analysis resulted in identifying twenty-two compounds from methanolic extract of *S. khuzistanica*. Further, this plant is considered as a rich source for caffeic acid and flavonoid derivatives. Finally, the results of the biological activity assays showed that the combination of carvacrol and polyphenol (**6** and **13**) has a high potential for eliminating pathogens, which demonstrates synergistic interactions.

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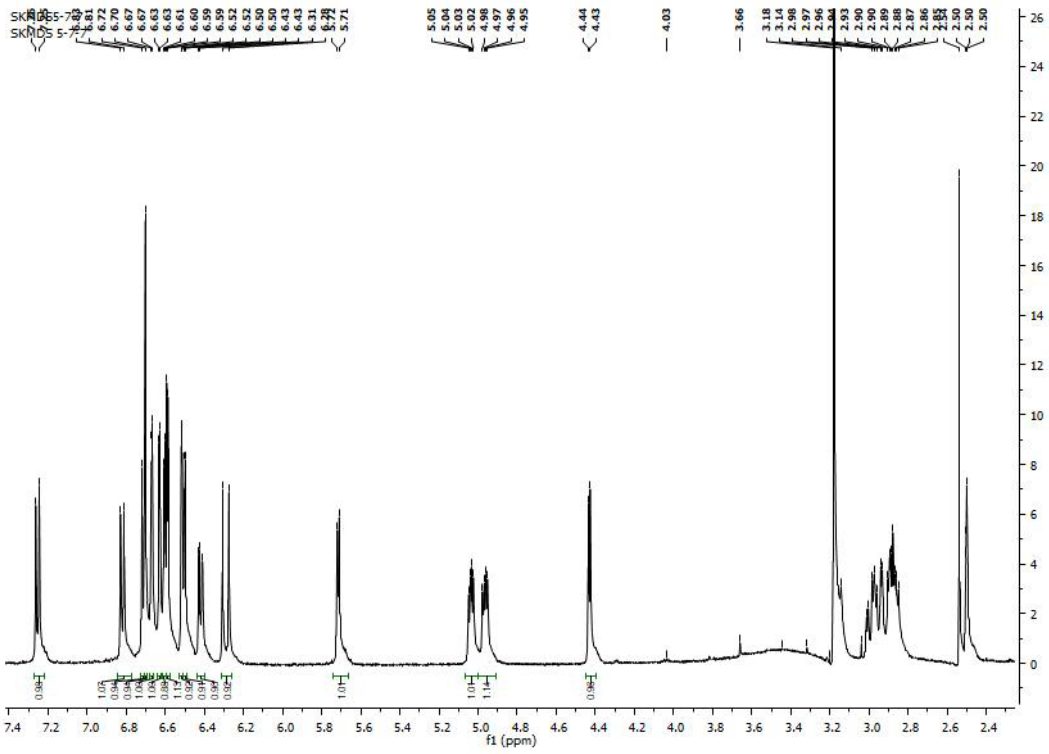
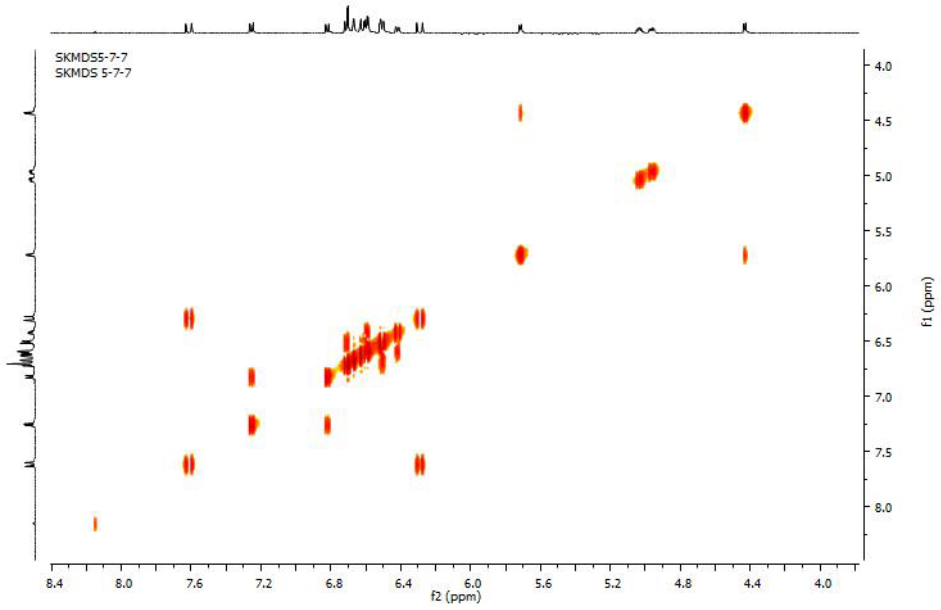
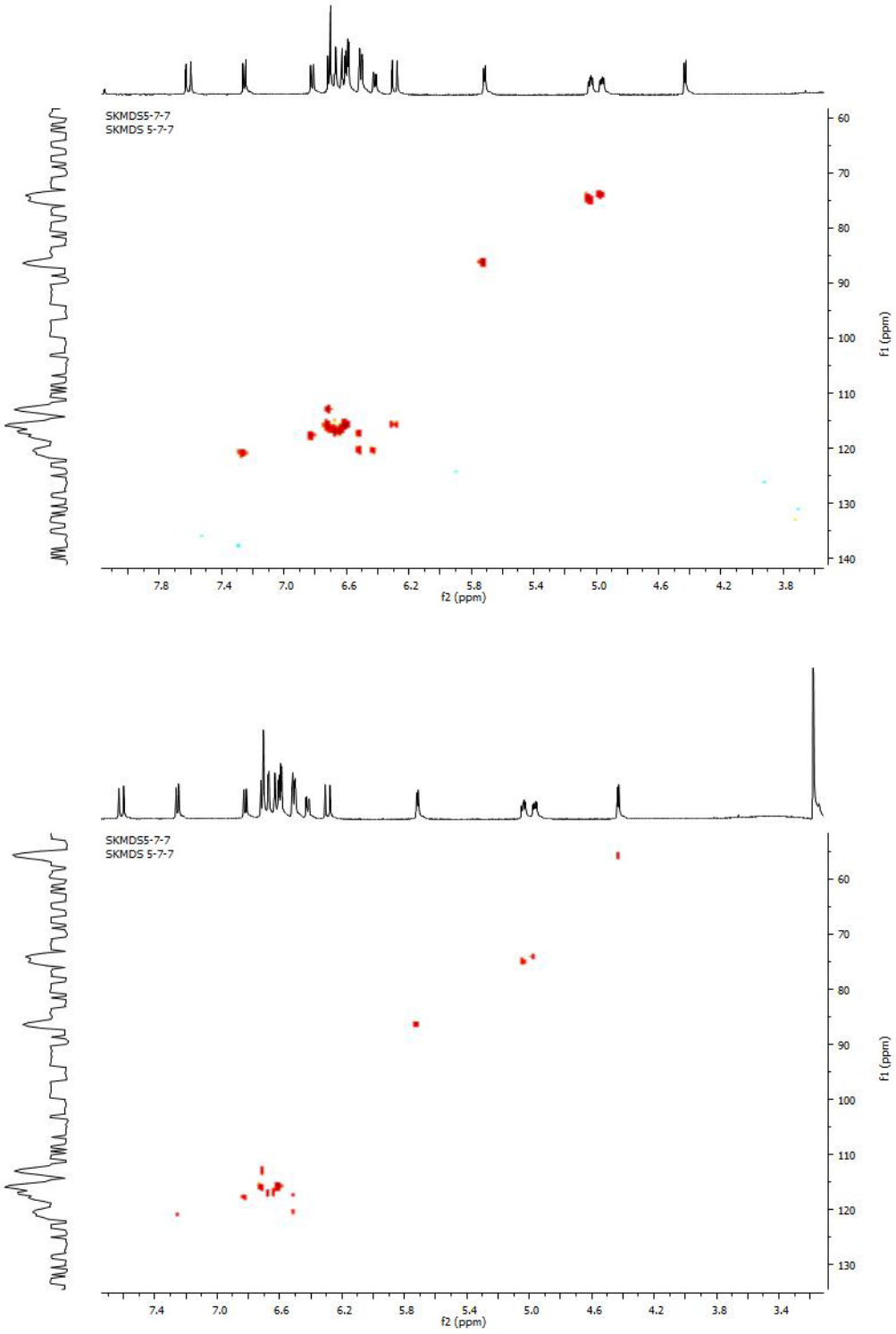
SUPPLEMENTARY MATERIAL

figuras S1 a S7



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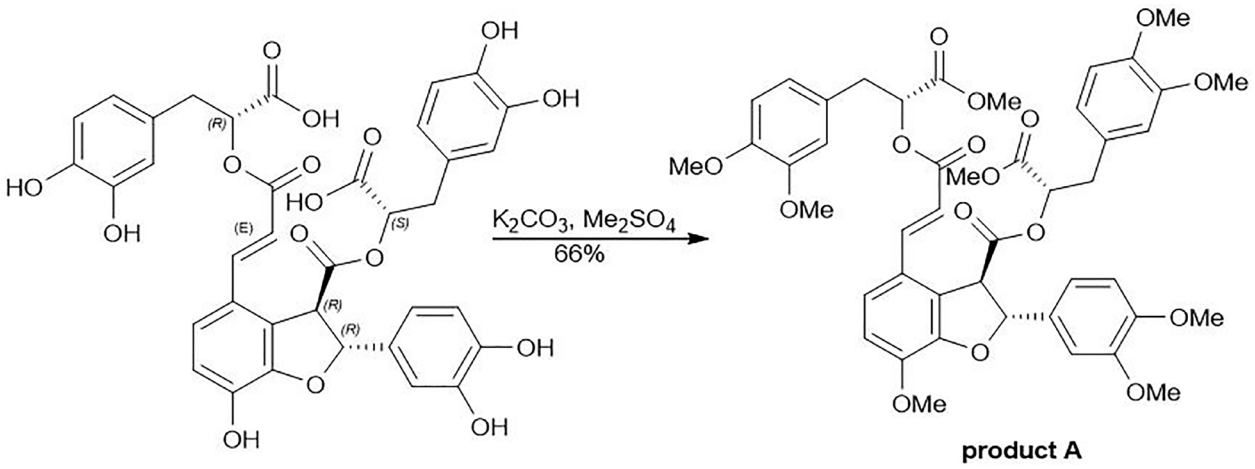
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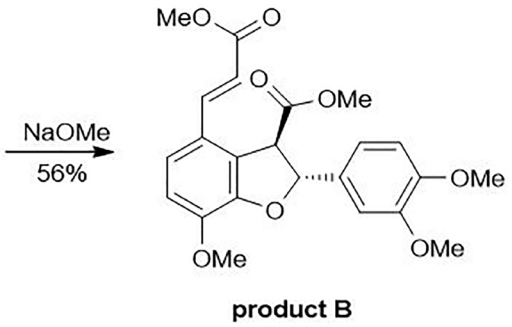
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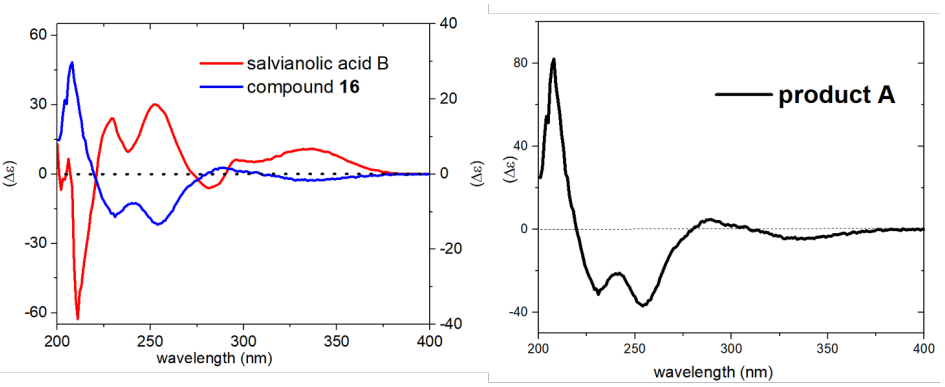
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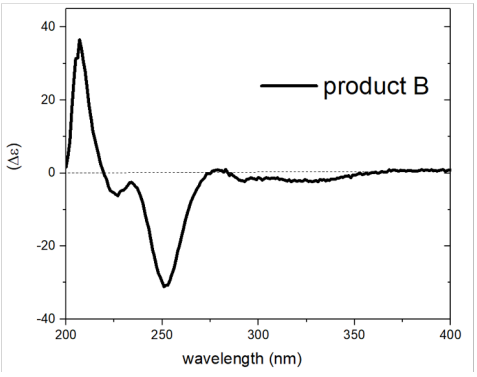
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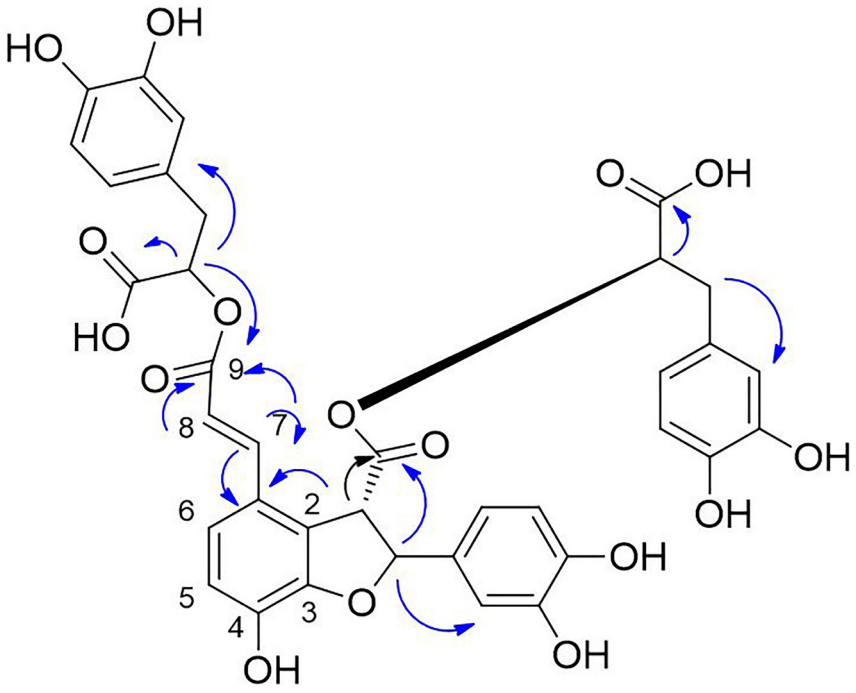

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