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CHEMICAL CONSTITUENTS ISOLATED FROM *ORİGANUM SOLYMICUM* WITH ANTIOXIDANT ACTIVITITES

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Abstract: *Origanum* genus includes 43 species and 18 hybrids. Sixteen species are endemic for Turkey. Due to the spicy fragrance, *Origanum* has been widely used in food products as flavoring agents. These species are well known for their essential oils which have been applied in food and cosmetic industries. *Origanum Solymicum* P.H. Davis was collected from Kemer, Antalya. The aerial part of plant materials were dried and powdered then boiled in water. After filtration, water phase was partitioned with ethyl acetate and n-butanol sequentially. After removal of the solvent by rotary evaporator, the chromatographic methods were applied for ethyl acetate extract to isolate the bioactive compounds. 3,4-dihdroxy benzoic acid (protocatechuic acid) (1), 3,4-dihydroxy benzaldehyde (protocatechuic aldehyde) (2), 2-*O*-*ß*-D- glycopyronozyl-4,6-dihydroxyacetophenone (myrciaphenone) (3), caffeic acid (4), rosmarinic acid (5), lithospermic acid B (6) were isolated by chromatographic techniques (column chromatography, preparative HPLC) and spectroscopic methods (1D, 2D NMR, LC-TOF/MS) were used for elucidation of isolated compounds. All isolated natural products revealed the outstanding activities on antioxidant assays including DPPH$^{\bullet}$ free radical scavenging, ABTS$^{\bullet+}$ radical cation scavenging, and reducing power (FRAP).

Keywords: Origanum Solymicum

Introduction

Natural products have been gained the great interest since ancient times due to the medicinal usage and including bioactive compounds for drug discovery and development process (Cragg, Newman, & Snader, 1997; Demirtas,

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Erenler, Elmastas, & Goktasoglu, 2013; R. Erenler, Sen, Yaglioglu, & Demirtas, 2016; R. Erenler, Sen, Yildiz, & Aydin, 2016; Topcu et al., 1999).

Origanum genus consists of forty three species and eighteen hybrids most of which distributed through Mediterranean region (Duman et al., 1995; Ietswaart & Ietswaart, 1980). *Orignaum* species have been used extensively in folk medicine to treat several diseases such as cough, gastrointestinal disease, bronchitis, flu, dizziness, diabetes, high cholesterol, stomachache, headache, toothache, and hypertension (Tepe, Cakir, & Sihoglu Tepe, 2016). The significance of *Origanum* in cosmetic and food industries results from the rich essential oil content. A plenty of research was carried out regarding the essential oils and their biological effects of *Origanum* species, the results indicated that *Origanum* essential oils revealed the significant biological activities and the main constituents were detected as carvacrol and thymol as well as the other monoterpenes (Abu-Lafi et al., 2008; Busatta et al., 2008; Orhan, Ozcelik, Kan, & Kartal, 2011; Yazdanparast & Shahriyary, 2008). *Origanum* species also comprises bioactive secondary metabolites. We isolated flavonoids and phenolic acid derivatives from *Origanum rotundifolium* and *Origanum majorana* showed the significant antioxidant and anticancer activities (Ramazan Erenler et al., 2017; R. Erenler, Sen, Aksit, et al., 2016).

Recent investigation on *Origanum* species exhibited a wide range of biological activities including antimicrobial (Walker, Santos, Schmidt, Bittencourt, & Guimarães, 2016), anticancer (Oke Altuntas & Demirtas, 2017; Soliman, Desouky, Marzouk, & Sayed, 2016), antioxidant (Yılmaz et al., 2017), antidiabetes (Bower, Hernandez, Berhow, & de Mejia, 2014), antibacterial (Karaman et al., 2017), antifungal(Waller et al., 2017), antinociceptive (Awaad, El-Meligy, Qenawy, Atta, & Soliman, 2011), and antilipase (Quiroga et al., 2013) properties. Herein, we isolated six secondary metabolites which revealed the outstanding antioxidant activities.

Methods

General Procedures

Bruker 400 MHz and 100 MHz spectrometer was used for ^{1}H and ^{13}C-NMR measurements, respectively. UV analysis was carried out with Shimadzu UV-260 spectrometer. The HRESI-MS spectra were recorded on Agilent 6210 LC-TOF/MS instrument. Sephedex LH-20 and GF254 were applied for column chromatography and thin layer chromatography (TLC), respectively. Ferrous chloride, α-tocopherol, polyoxyethylene sorbitan monolaurate (Tween20), DPPH, Ferrozine, nicotinamide adenine dinucleotide (NADH), trichloracetic acid (TCA), BHA (butylated hydroxyanisole) were bought from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium triocyanate and BHT (butylated hydroxytoluene) were supplied from E. Merck (Darmstadt, Germany).

Plant Material

O. solymicum was collected from Kemer, Kuzdere, Kesme Bogazi area of Antalya at September, altitude of 128m by Botanist Prof. Dr. Murat Aydin Sanda (MS 1004).

Extraction and Isolation

The areal parts of the plant material were dried at shade (330 g) and then boiled in water (300 mL). After cooling to room temperature, filtered and solid part was removed. 50 mL solvent was taken and lyophilized to obtain the water extract. The solvent was partitioned with ethyl acetate and n-butanol sequentially then solvents were removed by rotary evaporator to yield the ethyl acetate and n-butanol extracts. Antioxidant assays including $DPPH^{\bullet}$ scavenging, $ABTS^{\bullet+}$ scavenging, and reducing power as well as total phenolic content were executed for water, ethyl acetate and n-butanol extract. Ethyl acetate extract included the most phenolic content and revealed the most antioxidant activities among the extracts. Hence, ethyl acetate extract was subjected to chromatographic techniques to isolate the bioactive compounds. The ethyl aceate extract (2.0 g) was subjected to column chromatography (2.0 × 50 cm) using the Sephedex LH-20 (100 g) as a stationary phase and methanol as mobile phase. 60 fractions each of 10 mL were collected. After checking TLC (Thin layer chromatograph) spots, the compounds having the same R_f (Retention factor) values were combined. Further purification was carried out by semi preparative HPLC. Fractions 27-32 were combined and subjected to semi preparative HPLC, flow rate was adjusted to 5 mL/min using a gradient system of A, water with 2.5% formic acid and B, acetonitrile. The gradient program was fixed as, 0-3 min, 100% A, 4-30 min, 85% A, 31-55 min, 60% A, 56-63 min, 100%A to isolate 3,4-dihdroxy benzoic acid (**1**). The fractions 33-35 collected from column chromatography was applied to semi preparative HPLC. Flow rate was fixed as 3 mL/min. Gradient system was A, water with 2.5% formic acid and B, acetonitrile. The gradient program was adjusted as, 0-3 min, 78% A, 4-25 min, 72% A, 26-36 min, 100%

A to purify the 3,4-dihydroxy benzaldehyde (**2**). Fraction 38 was applied to HPLC, flow rate was adjusted to 3 mL/min using a gradient system of A, water with 2.5% formic acid and B, acetonitrile. The gradient program was fixed as, 0-3 min, 75% A, 4-25 min, 72% A, 26-36 min, 0%A to isolate 2-*O*-*ß*-D- glycopyronozyl-4,6-dihydroxyacetophenone (myrciaphenone) (**3**). The fractions 44-60 were combined and subjected to semi preparative HPLC, flow rate was 5 mL/min, the gradient program was 0-3 min 95% A, 4-35 min, 70% A, 36-40 min, 100% A, two compounds were detected by HPLC chromatogram. Therefore, further purification was executed to isolate caffeic acid (**4**), rosmarinic acid (**5**). Lithospermic acid (**6**) was isolated from the combined fractions of 65-70 which subjected to HPLC, with the flow rate was 3 mL/min. Same gradient system was used and gradient program was adjusted as, 0-3 min, 80% A, 4-25 min, 75% A, 26-30 min 0% A.

Antioxidant Assays

Total Phenolic Content

Total phenolic content of the water, ethyl acetate, and n-butanol extracts were determined with Folin-Ciocalteu reagent using gallic acid as standard (Singleton & Slinkard, 1977). The absorbance was measured at 760 nm in a spectrophotometer (Hitachi U-2900). The concentration of total phenolic compounds in each extract was determined as mg gallic acid equivalent.

$DPPH^{\bullet}$ Free Radical Assay

The electron or hydrogen releasing ability of antioxidant compounds was evaluated by bleaching of purple color solution of $DPPH^{\bullet}$. $DPPH^{\bullet}$ scavenging activity of natural products and standards was measured according to the literature (Blois, 1958).

$ABTS^{\bullet+}$ Scavenging Assay

2,2″-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) cation radical scavenging assay based on the decreasing the ABTS radical cation, a blue/green chromophore with absorption at 737 nm, in comparison to that of BHA, BHT and trolox. This assay was executed as in the literature (Re et al., 1999).

Ferric Ions (Fe^{3+}) Reducing Antioxidant Power Assay (FRAP)

Different concentrations of samples (2.5-10 µg/mL) in 1 mL of deionized water were mixed with sodium phosphate buffer (1.25 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1.25 mL, 1%). The reaction mixture was vortexed thoroughly and absorbance measurement at 700 nm was executed in a spectrophotometer (Oyaizu, 1986).

Results and Findings

Origanum Solymicum was boiled in water, after filtration, the solvent part was partitioned with ethyl acetate and n-butanol successively. Due to the consisting of high phenolic content and revealing the most antioxidant activity, ethyl acetate extract was subjected to chromatographic techniques to isolate the bioactive compounds (Table 1)

Table 1. Total phenolic content and antioxidant activities of *O.solymicum*

Extracts and Standards	Total phenolic contents (g GAE/Kg extract)	$DPPH^{\bullet}$ scavenging [IC_{50} (µg/mL)]	$ABTS^{\bullet+}$ Scavenging [IC_{50} (µg/mL)]	Reducing power (µmol TE/g extract)
Water ext	235.5 ± 2.3	64.3 ± 0.4	17.2 ± 0.07	170.5 ± 7.5
n-ButOH ext	307.7 ± 8.4	38.9 ± 0.6	11.5 ± 0.08	282.6 ± 4.7
EtOAc ext	494.7±15.6	22.8 ± 1.2	9.1 ± 0.04	398.4 ± 5.6
BHT	-	5.4 ± 0.4	4.3 ± 0.2	5.03 ± 0.01
BHA	-	12.8 ± 1.9	7.5 ± 0.02	7.5 ± 0.04
Trolox	-	17.3 ± 0.7	13.8 ± 0.02	-

After a series of chromatographic techniques, six compounds were isolated and elucidated from ethyl acetate extract (Figure 1). 3,4-dihdroxy benzoic acid (**1**) is the first isolated natural product (Ban, Jeon, Bae, Song, & Seong, 2006). 3,4-dihydroxy benzoic acid known as protocatechuic acid is a phenolic compound found in many food plants such as *Olea europaea*, *Hibiscus sabdafiffa*, *Eucommia ulmoides*, *Citrus microcarpa* and *Vitis vinifera.* The spectral data were in accord with the literature (Makhmoor & Choudhary, 2010). Protocatechuic acid (**1**) displayed a broad spectrum of biological activities such as antioxidant, anti-inflammatory, antihyperglycemia, antiapoptosis, and atimicroabial (Semaming, Pannengpetch, Chattipakorn, & Chattipakorn, 2015). In this work, protocatechuic acid (**1**) displayed the excellent DPPH$^{\bullet}$ scavenging (IC_{50}, 9.5), $ABTS^{\bullet+}$ scavenging (IC_{50}, 6.4) and reducing power (4.0, µmol TE/mL extract) activities.

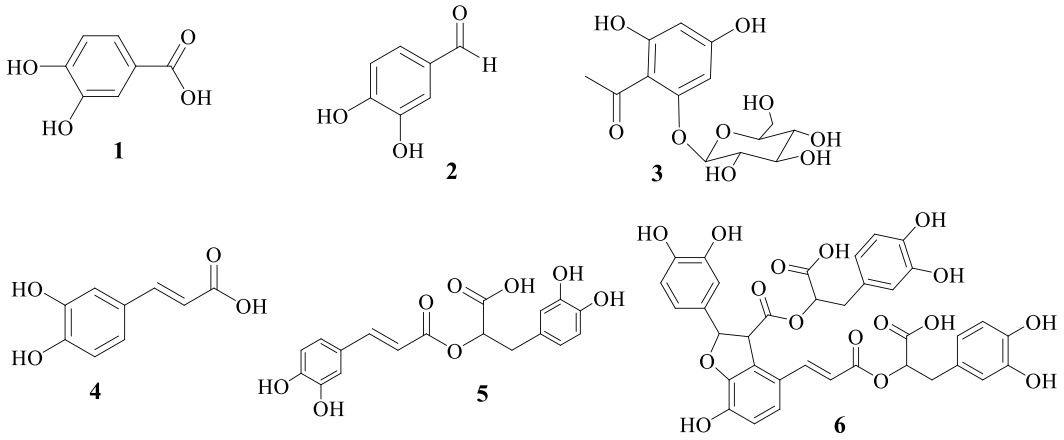

Figure 1. Isolated compounds from *Origanum Solymicum*

Since protocatechuic acid (**1**) has acidic hydrogen that can be donated easily, it displayed excellent antioxidant activity. Same trend was observed to 3,4-dihydroxy benzaldehyde (protocatechuric aldehyde) (**2**). It was displayed outstanding antioxidant effects on all assays. 3,4-dihydroxy benzaldehyde (**2**) is the common natural product found in plant kingdom abundantly (Ren et al., 2017). Protocatechuric aldehyde (**2**) was reported to exhibit a large variety of biological effects such as tumor cell inhibition on various cell lines (Zhang et al., 2017). Due to the importance of 3,4-dihydroxy benzaldehyde (**2**) in pharmaceutical and food industry, total synthesis of this compound was achieved by treatment of pyrocatechol with glyoxylic acid followed by a series of chemical reactions (Cerveny, Kovarova, & Marhoul, 1996). The structure was elucidated by the spectral data comparison with the reported literature (Ayinde, Onwukaeme, & Omogbai, 2007). 2-*O*-*ß*-D-glycopyronozyl-4,6-dihydroxyacetophenone (myrciaphenone) (**3**) was the third compound isolated by chromatographic techniques. This compound displayed the superior activities on DPPH$^{\bullet}$ (IC_{50}, 8.1), $ABTS^{\bullet+}$ (IC_{50}, 1.6) and reducing power (30.2, µmol TE/mL extract). This could be due to the bearing acidic hydrogen atoms of corresponding molecule. The comparison of spectral data with the literature confirmed the proposed structure (Ayinde et al., 2007). Caffeic acid (**4**) well known natural product revealed the wonderful $ABTS^{\bullet+}$ (IC_{50}, 0.06) and reducing power (35.3, µmol TE/mL extract) activity in this study. Caffeic acid and its derivatives have revealed a broad array of biological activities. Caffeic acid phenyl ester reveals antioxidant, anti-inflammatory, antiproliferative, antitumor, and immunomodulatory effects (Erdemli et al., 2016). Rosmarinic acid (**5**) is the main constituent of *Origanum* species (Ramazan Erenler et al., 2017). It exhibited the good antioxidant activity in all assays. Rosmarinic acid has been reported to have a number of significant biological effects such as antiviral, antibacterial, anticancer, anti-inflammatory activities (Petersen & Simmonds, 2003). The spectral data were in accordance with the literature (Aksit et al., 2014). The last compound isolated from *O. Solymicum* was lithospermic acid B (**6**). Its spectral data confirmed the proposed structure (Murata et al., 2013). Lithospermic acid B (**6**) displayed the excellent DPPH$^{\bullet}$ (IC_{50}, 15.9), $ABTS^{\bullet+}$ (IC_{50}, 0.5) and reducing power (32.0, µmol TE/mL extract) activity (Table 2).

Table 2. Antioxidant effects of isolated compounds from *O. solymicum*

Compounds	DPPH$^{\bullet}$ scavenging activity [IC_{50} (µg/mL)]	$ABTS^{\bullet+}$ scavenging activity [IC_{50} (µg/mL)]	Reducing power (µmol TE/mL extract)
3,4-dihdroxy benzoic acid (**1**)	9.5 ± 0.5	6.4 ± 0.1	4.0 ± 0.1
3,4-dihydroxy benzaldehyde (**2**)	6.4 ± 0.3	3.9 ± 0.2	4.1 ± 0.1
Myrciaphenone (**3**)	8.1 ± 3.3	1.6 ± 0.1	30.2 ± 1.2

Caffeic acid (**4**	35.4 ± 0.3	0.06 ± 0.4	35.3 ± 0.3
Rosmarinic acid (**5**)	12.2 ± 1.1	6.3 ± 0.7	47.2 ± 1.1
Lithospermic acid B (**6**)	15.9 ± 1.9	0.5 ± 0.0	32.0 ± 0.9
BHA	7.4 ± 0.3	3.4 ± 0.0	7.2 ± 0.3
BHT	20.9 ± 1.7	5.4 ± 0.2	4.3 ± 0.2
TROLOX	5.3 ± 0.3	6.1 ± 0.2	3.9 ± 0.3

Conclusion

O. Solymicum was dried and powdered then boiled in water. After filtration, water phase was partitioned with ethyl acetate and n-butanol sequentially. Ethyl acetate extract included the most phenolic content and exhibited the most antioxidant activity. Therefore, isolation of active compounds was carried out from ethyl acetate extract. Hence, the best extraction techniques were presented to yield the most antioxidant compounds. protocatechuic acid (**1**), protocatechuic aldehyde (**2**), myrciaphenone (**3**), caffeic acid (**4**), rosmarinic acid (**5**), lithospermic acid B (**6**) were isolated and identified. These compounds displayed the excellent antioxidant activity.

Recommendations

The cultivation of *Origanum* species should be extended. Phytochemical investigation of other species of *Origanum* should be executed to produce bioactive compounds. A broad spectrum of biological activity tests should be carried out for secondary metabolites isolated from *Origanum* species. *Origanum* species may be used in food as a natural antioxidant rather than synthetic antioxidant.

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