ORIGINAL ARTICLE

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Rec. Nat. Prod. 5:1 (2011)12-21

records of natural products

Relationship Between Chemical Structure and Antioxidant Activity of Luteolin and Its Glycosides Isolated from *Thymus sipyleus* subsp. *sipyleus* var. *sipyleus*

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(Received March 18, 2010; Revised September 28, 2010; Accepted September 30, 2010)

Abstract: One triterpenic acid (ursolic acid), one phenolic acid (rosmarinic acid), and four flavonoids (luteolin, luteolin 7-*O*-(6”-feruloyl)-*β*-glucopyranoside, luteolin 5-*O*-*β*-glucopyranoside, and luteolin 7-*O*-*β*-glucuronide) were isolated from the aerial parts of *Thymus sipyleus* subsp. *sipyleus* var. *sipyleus* and identified by spectroscopic methods. In vitro lipid peroxidation inhibition effects of the compounds were determined using TBA test method in a bovine brain liposome system. All compounds inhibited lipid peroxidation in various degrees except for ursolic acid. The order of the lipid peroxidation activities of luteolin, its glycosides and rosmarinic acid were: Luteolin 7-*O*-*β*-glucuronide> luteolin 5-*O*-*β*-glucopyranoside> luteolin 7-*O*-(6”-feruloyl)-*β*-glucopyranoside > rosmarinic acid >luteolin. However, the activity order of the compounds was completely different in DPPH radical-scavenging activity. None of the compounds shows Fe^{2+} chelating activity. The results were discussed based on their chemical structures and polarities.

Keywords: *Thymus sipyleus* subsp. *sipyleus* var. *sipyleus*; Lamiaceae; luteolin; luteolin glycosides, antioxidant activity.

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published15/12/2010 EISSN:1307-6167

1. Introduction

The family Lamiaceae is represented by about 200 genera containing 3300 species. Most of the species are aromatic, annual or perennial, herbaceous plants or shrublets. Species belonging to Lamiaceae contain flavonoids, phenolic acids, terpenes, saponins, polyphenols, tannins, iridoids, and quinones [1].

The genus *Thymus* (Lamiaceae) is represented by about 200 species worldwide [1]. There are 39 (64 taxa) *Thymus* species in Turkey, 27 taxa of which are endemic [2-4]. *Thymus* species known as "kekik", "nemamulotu", and "sater" are popularly used in Turkey for their antibacterial, secretolytic, and bronchospasmolytic effects [5]. In traditional medicine all over the world, *Thymus* extracts are used orally to treat dyspepsia and other gastrointestinal disturbances, coughs due to colds, bronchitis, and pertussis. In addition, *Thymus* extracts are also gargled to treat laryngitis and tonsillitis. Topical applications of thyme extracts have been used in the treatment of minor wounds, the common cold, and disorders of the oral cavity, and as an antibacterial agent in oral hygiene. Both the essential oil and thymol are ingredients of a number of proprietary drugs including antiseptic and healing ointments, syrups for the treatment of respiratory disorders, and preparations for inhalation [6]. *Thymus sipyleus* Boiss. subsp. *sipyleus* var. *sipyleus* is an endemic species which grows widely in Turkey [2] and is used as a spice in Turkey [7, 8]. It is known as "kekik otu" or "keklik otu" and the decoction of its aerial parts is used to treat stomachache, gastric ulcer, tonsillitis, urinary system diseases, internal diseases, dyspnea, eczema, and hemorrhoids by villagers of Ilıca District (Erzurum Province, Turkey). In a recently published study, the essential oil of *T. sipyleus* subsp. *sipyleus* var. *sipyleus* was reported to have no remarkable antioxidant activity [9].

In living organisms, various reactive oxygen and nitrogen species can be formed by different mechanisms. Endogenous antioxidants can protect biomolecules against the harmful actions of the reactive species. However, endogenous antioxidants cannot be efficient in some cases; thus exogenous antioxidant consumption may be important. Therefore, it is important to determine the antioxidant activities of the especially plants used by public traditionally. For this purpose, we studied the antioxidant activities of the compounds isolated from *T. sipyleus* subsp. *sipyleus* var. *sipyleus*.

2. Materials and Methods

2.1. Instruments and materials

^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Varian Mercury plus spectrometer at 400 and 100 MHz. Mass spectra were recorded with Thermofinnigan Trace GC/Trace DSG/A1300. Sephadex LH-20 (Sigma-Aldrich), silica gel (Kieselgel 60, 0.063-0.2 mm Merck 7734 and 0.040-0.063 mm Merck 9385 and LiChroprep RP-18, 25-40 μm, Merck 9303) for column chromatography, and silica gel 60 F_{254} (Merck, 5554) for TLC (Thin Layer Chromatography) were used. TLC spots were detected with a UV lamp and 1% vanillin/H_2SO_4 following heating at 120 °C for 1-2 min. The solvents used in the isolation and solvent systems were butanone, n-butanol, ethyl acetate, formic acid, methanol, H_2SO_4, toluene (Riedel-de Haen), n-hexane, chloroform (Merck), and vanillin (Fluka).

2.2.Plant material

Aerial parts of *T. sipyleus* subsp. *sipyleus* var. *sipyleus* were collected from Kop Mountain, Bozburun Village, at 2000 m altitude (Aşkale District, Erzurum, Turkey) in 2004. They were identified by Dr. Yusuf Kaya. A voucher specimen of *T. sipyleus* subsp. *sipyleus* var. *sipyleus* is deposited at Atatürk University Faculty of Science Herbarium (ATA 9718).

2.3. Extraction and isolation

Dried and powdered aerial parts of *T. sipyleus* (400 g) were extracted with methanol (2 liters x 3) under reflux for 3 hours, and the resulting solution was concentrated under reduced pressure to yield a viscous extract (67.7 g). The methanol extract was suspended using 300 mL of water:methanol (9:1). This mixture was partitioned against *n*-hexane (300 mL x 3), chloroform (300 mL x 3), ethyl acetate (300 mL x 3), and *n*-butanol (300 mL x 3), consecutively. *n*-Hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous phases were evaporated at reduced pressure at 40 °C. The *n*-hexane phase was 8.9 g, chloroform phase 6 g, ethyl acetate phase 4.4 g, *n*-butanol phase 11 g, and aqueous phase 36 g.

The chloroform extract (6 g) was subjected into Sephadex LH-20 eluting with methanol:water (10:0, 8:2, 6:4, 4:6 and 0:10). Fractions 15-18 (1.6 g) [water:methanol (80:20)] were subjected to a Sephadex LH-20 column, eluting with methanol. Fractions 4-9 (18 mg) gave ursolic acid (**1**).

The EtOAc extract (4.2 g) was subjected to a reversed phase silica gel column and eluted with increasing amounts of MeOH. Three fractions were obtained, Fr. A (Fr. 21-25), H_2O:MeOH (70:30); Fr. B (74-86), H_2O:MeOH (50:50); and Fr. C (87-102), H_2O:MeOH (20:80). Fr. A (250 mg) was subjected to a silica gel column, eluting with $CHCl_3$:MeOH:H_2O (70:30:3). Fr. 14-47 (Fr. A1) gave rosmarinic acid (**2**) (50 mg). Fr. B (40 mg) was subjected to a Sephadex LH-20 column, eluting with MeOH. Fr. 8 (Fr. B1) gave luteolin 7-*O*-(6"-feruloyl)-*β*-glucopyranoside (**3**) (10 mg). Fr. C (50 mg) was subjected to a Sephadex LH-20 column, eluting with MeOH. Fr. 15-31 gave luteolin (**4**) (17 mg).

The *n*-butanol extract (10 g) was subjected to a reversed phase silica gel column and eluted with increasing amounts of MeOH. Fr. 9-10 (Fr. B1) (30 mg) were subjected to a Sephadex LH-20 column, eluting with MeOH. Fr. 12-13 (Fr. B1a) gave luteolin 5-*O*-*β*-glucopyranoside (**5**) (10 mg).

Aqueous extract (30 g) was subjected to a Sephadex LH-20 column, eluting with water:methanol (80:20). Fr. 5-8 (11 g) were subjected to a Sephadex LH-20 column, eluting with water:methanol (90:10). Fr. 9-11 gave luteolin 7-*O*-*β*-glucuronide (**6**) (14 mg).

2.4. Structure analysis

Ursolic acid **(1):** White amorphous powder; EI-MS (*m/z*, %) 456.2 [M^+] (2), 249.1 (32), 248 (100), 219.0 (17), 207.1 (40), 203.1 (47); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 5.11 (*t*, 1H, H-12, *J*= 3.3 Hz), 3.00 (*dd*, 1H, H-3, *J*= 11.0 Hz, *J*= 5.0 Hz), 2.08 (*d*, 1H, H-18, *J*= 11.4 Hz), 1.02 (*s*, 3H, CH_3), 0.88 (*d*, 3H, *J*= 8.4 Hz, CH_3), 0.87 (*s*, 3H, CH_3), 0.84 (*s*, 3H, CH_3), 0.78 (*d*, 3H, *J*= 6.2 Hz, CH_3), 0.73 (*s*, 3H, CH_3), 0.65 (*s*, 3H, CH_3), 1.97-1.21 (*m*, 24H); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 179.0 (s), 138.9 (s), 125.3 (d), 77.5 (d), 55.5 (d), 53.1 (d), 47.7 (d), 47.5 (s), 42.3 (s), 39.8, (s), 39.2 (s), 39.1 (d), 39.1 (d), 38.9 (t), 37.2 (s), 37.0 (t), 33.4 (t), 30.9 (t), 28.9 (t), 28.2 (q), 27.7 (t), 24.5 (t), 24.0 (q), 23.5 (t), 21.8 (q), 18.7 (t), 17.7 (q), 17.6 (q), 16.8 (q), 15.9 (q). ^{1}H- and ^{3}C-NMR data were agreement with data given in the literature [10,11].

Rosmarinic acid **(2):** Colorless amourphous solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 7.50 (*d*, 1H, H-7, *J*= 16.0 Hz), 7.02 (*d*, 1H, H-2, *J*= 2.2 Hz), 6.92 (*dd*, 1H, H-6, *J*= 8.1 Hz, *J*= 2.2 Hz), 6.76 (*d*, 1H, H-5, *J*= 8.1 Hz), 6.75 (*d*, 1H, H-2', *J*= 1.8 Hz), 6.67 (*d*, 1H, H-5', *J*= 8.1 Hz), 6.62 (*dd*, 1H, H-6', *J*= 8.1 Hz, *J*= 1.8 Hz), 6.26 (*d*, 1H, H-8, *J*= 16.0 Hz), 5.10 (*dd*, 1H, H-8', *J*= 9.4 Hz, *J*= 3.6 Hz), 3.09 (*dd*, 1H, H-7a', *J*= 14.3 Hz, *J*= 3.6 Hz), 2.94 (*dd*, 1H, H-7b', *J*= 14.3 Hz, *J*= 9.4 Hz); ^{13}C-NMR (100 MHz, CD_3OD): δ 175.9 (C-9'), 167.8 (C-9), 148.2 (C-4), 145.6 (C-3), 145.5 (C-7), 144.8 (C-3'), 143.7 (C-4'), 129.7 (C-1'), 126.8 (C-1), 121.8 (C-6), 120.6 (C-6'), 116.3 (C-2'), 115.3 (C-5), 115.0 (C-5'), 114.3 (C-2), 113.9 (C-8), 76.0 (C-8'), 37.5 (C-7'). ^{1}H- and ^{3}C-NMR data were agreement with data given in the literature [12, 13].

Luteolin 7-O-(6''-feruloyl)-β-glucopyranoside **(3):** Yellow amorphous powder, EI-MS (*m/z*, %) 624.7 [M^+] (6), 435.9 (100). ^{1}H-NMR (400 MHz, Acetone-d_6): δ 7.56 (*d*, 1H, H-7''', *J*= 15.9 Hz), 7.48 (*d*, 1H, H-2', *J*= 2.2 Hz), 7.45 (*dd*, 1H, H-6', *J*= 8.4 Hz, *J*= 2.2 Hz), 7.21 (*d*, 1H, H-2''', *J*= 2.2 Hz), 6.99 (*dd*, 1H, H-6''', *J*= 8.4 Hz, *J*= 2.2 Hz), 6.97 (*d*, 1H, H-5', *J*= 8.4 Hz), 6.77 (*d*, 1H, H-8, *J*= 2.2 Hz), 6.75 (*d*, 1H, H-5''', *J*= 8.4 Hz), 6.58 (*s*, 1H, H-3), 6.51 (*d*, 1H, H-6, *J*= 2.2 Hz), 6.40 (*d*, 1H, H-8''', *J*= 15.9 Hz), 5.23 (*d*, 1H, H-1'', *J*= 7.3 Hz), 4.65 (*dd*, 1H, H-6a'', *J*= 11.7 Hz, *J*= 2.4 Hz), 4.30 (*dd*, 1H, H-6b'', *J*= 11.7 Hz, *J*= 7.1 Hz), 3.95 (*ddd*, 1H, H-5'', *J*= 9.2 Hz, *J*= 7.1 Hz, *J*= 2.4 Hz), 3.85 (*s*, 3H, OCH_3), 3.63 (*dd*, 1H, H-3'', *J*= 8.8 Hz, *J*= 8.5 Hz), 3.58 (*dd*, 1H, H-2'', *J*= 8.5 Hz, *J*= 7.3 Hz), 3.51 (*dd*, 1H, H-4'', *J*= 9.2 Hz, *J*= 8.8 Hz); ^{13}C-NMR (100 MHz, Acetone-d_6): δ 182.5 (C-4), 166.7 (C-9'''), 164.8 (C-2), 163.5 (C-7), 162.3 (C-5), 157.6 (C-9), 149.6 (C-4'), 149.4 (C-3'''), 147.9 (C-4'''), 145.8 (C-3'), 145.4 (C-7'''), 126.6 (C-1'''), 123.2 (C-6'''), 122.9 (C-1'), 119.6 (C-6'), 115.9 (C-5'), 115.3 (C-5'''), 114.7 (C-8'''), 113.5 (C-2'), 110.5 (C-2'''), 106.2 (C-10), 103.6 (C-3), 100.5 (C-1''), 99.9 (C-6), 95.0 (C-8), 77.1 (C-3''), 74.6 (C-5''), 73.8 (C-2''), 70.7 (C-4''), 63.5 (6''), 55.6 (OCH_3). EI-MS data, ^{1}H- and ^{13}C-NMR data were agreement with data given in the literature [14].

Luteolin **(4):** Yellow powder, EI-MS (*m/z*, %) 286.0 [M^+], (75), 174.0 (100), 148 (70), 145.0 (45). ^{1}H-NMR (400 MHz, Acetone-d_6): δ 7.51 (*d*, 1H, H-2', *J*= 2.2 Hz), 7.48 (*dd*, 1H, H-6', *J*= 8.2 Hz, *J*= 2.2 Hz), 7.00 (*d*, 1H, H-5', *J*= 8.2 Hz), 6.59 (*s*, 1H, H-3), 6.52 (*d*, 1H, H-8, *J*= 2.0 Hz), 6.25 (*d*, 1H, H-6, *J*= 2.0 Hz), ^{13}C-NMR (100 MHz, Acetone-d_6): δ 182.4 (C-4), 164.5 (C-7), 164.2 (C-2), 162.7 (C-5), 158.1 (C-9), 149.4 (C-4'), 145.8 (C-3'), 123.1 (C-1'), 119.5 (C-6'), 116.0 (C-5'), 113.5 (C-2'), 104.7 (C-10), 103.6 (C-3), 99.0 (C-6), 94.0 (C-8). ^{1}H- and ^{13}C-NMR data were agreement with data given in the literature [12, 15].

Luteolin 5-O-β-glucopyranoside **(5):** Yellow amourphous powder, EI-MS (*m/z*, %) 447.2 [M-H] (100). ^{1}H-NMR (400 MHz, CD_3OD): δ 7.37 (*dd*, 1H, H-6', *J*= 8.8 Hz, *J*= 2.2 Hz), 7.36 (*bs*, 1H, H-2'), 6.90 (*d*, 1H, H-5', *J*= 8.8 Hz), 6.83 (*d*, 1H, H-8, *J*= 2.2 Hz), 6.70 (*d*, 1H, H-6, *J*= 2.2 Hz), 6.54 (*s*, 1H, H-3), 4.84 (*d*, 1H, H-1'', *J*= 7.7 Hz), 3.94 (*dd*, 1H, H-6a'', *J*= 12.1 Hz, *J*= 1.8 Hz), 3.75 (*dd*, 1H, H-6b'', *J*= 12.1 Hz, *J*= 4.9 Hz), 3.60 (*t*, 1H, one H of sugar, *J*= 8.2 Hz), 3.52-3.42 (*m*, 3H of sugar); ^{13}C-NMR (100 MHz, CD_3OD): δ 179.2 (C-4), 163.6 (C-2), 163.2 (C-7), 159.5 (C-5), 158.9 (C-9), 149.6 (C-4'), 145.8 (C-3'), 122.3 (C-1'), 118.9 (C-6'), 115.6 (C-5'), 112.8 (C-2'), 108.2 (C-10), 105.3 (C-3), 103.8 (C-6), 103.8 (C-1''), 98.0 (C-8), 77.4 (C-5''), 76.1 (C-3''), 73.5 (C-2''), 70.0 (C-4''), 61.3 (C-6''). EI-MS data, ^{1}H- and ^{13}C-NMR data were agreement with data given in the literature [16,17].

Luteolin 7-O-β-glucuronide **(6):** EI-MS (m/z %) 461.1 [M-H] (88). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 12.96 (*bs*, COOH), 7.40 (*d*, 1H, H-2', *J*= 2.0 Hz), 7.36 (*dd*, 1H, H-6', *J*= 8.4 Hz, *J*= 2.0 Hz), 6.84 (*d*, 1H, H-5', *J*= 8.4 Hz), 6.74 (*d*, 1H, H-8, *J*= 1.9 Hz), 6.69 (*s*, 1H, H-3), 6.39 (*d*, 1H, H-6, *J*=1.9 Hz), 5.06 (*d*, 1H, H-1'', *J*= 7.3 Hz), 3.60 (*d*, 1H, H-5'', *J*= 9.9 Hz), 3.39-3.14 (*m*, 3H, H-2'', H-3'', H-4''); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 182.5 (C-4), 172.5 (C-6''), 165.1 (C-2), 163.6 (C-7), 161.7 (C-5),157.6 (C-9), 150.8 (C-4'), 146.6 (C-3'), 121.7 (C-1'), 119.7 (C-6'), 116.7 (C-5'), 114.1 (C-2'), 105.9 (C-10), 103.6 (C-3), 100.2 (2C; C-6 and C-1''), 95.2 (C-8), 77.1 (C-3''), 74.5 (C-5''), 73.6 (C-2''), 72.6 (C-4''). EI-MS data, ^{1}H- and ^{13}C-NMR data were agreement with data given in the literature [18, 19].

2.5. Determination of Lipid Peroxidation Inhibition and DPPH Radical Scavenging Activity

Lipid peroxidation inhibition (Thiobarbituric acid test -TBA test-) and DPPH radical scavenging activity were measured by the known methods described in our previous study [20].

Thiobarbituric acid test -TBA test- TBA test, an in vitro antioxidant activity assay, was carried out using the lipid peroxidation of liposomes. The compounds have been assessed to protect liposomes from lipid peroxidation. In the TBA reaction, the peroxidation of most membrane systems

leads to formation of small amounts of free malonaldehyde (MDA). One molecule of MDA reacts with two molecules of TBA to yield a colored product. This colored product, absorbing light at 532 nm in the acidic conditions, is easily extractable into organic solvents. Thus, it can be measured and quantified spectrophotometrically. The intensity of color is a measure of MDA concentration. If any antioxidant compound incorporates in the lipid peroxidation assay, the peroxidation decreases.

Absorbance at 532 nm was determined on a Helios β UV/VIS spectrophotometer. Liposomes were prepared from bovine brain extract in phosphate buffered saline (5 mg/mL). The isolated compounds from the plant were tested for their antioxidant activity against the lipid peroxidation of liposomes. After the peroxidation was started by adding $FeCl_3$ and ascorbic acid, the incubation was maintained at 37 °C for 20 min (contrary to the well known anti-oxidant property of ascorbic acid, in the presence of certain transition metal ions, such as Fe or Cu, it has also pro-oxidant property). After the incubation was finished, a solution of BHT in EtOH was added to prevent further lipid peroxidation. Propyl gallate was used as a positive control. Data were given as IC_{50} (µg/mL) extract concentration required for 50% peroxidation inhibition.

The in vitro DPPH radical-scavenging activity test was carried out according to slightly modified Blois method. In this assay, 1 mM solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical solution in methanol was prepared and then, 1 mL of this solution was mixed with 3 mL of the solution of the tested compounds in methanol at the concentration of 0.1 mM.
After incubation in dark during 30 minutes, the absorbance was measured at 517 nm. This activity is given as % DPPH radical scavenging activity.

% DPPH radical scavenging is calculated in the following equation;

% DPPH Radical Scavenging = ((Control Absorbance - Extract Absorbance)/(Control Absorbance)) x100

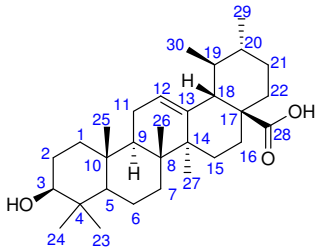
Control DPPH solution was prepared by adding of 3 mL of MeOH into the 1 mL of 1 mM DPPH solution in MeOH.

2.6. Ferrous ion (Fe^{2+}) chelating activity

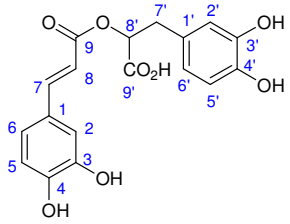
Ferrous ion (Fe^{2+}) chelating activity was determined as described in our previous study [21].

3. Results and Discussion

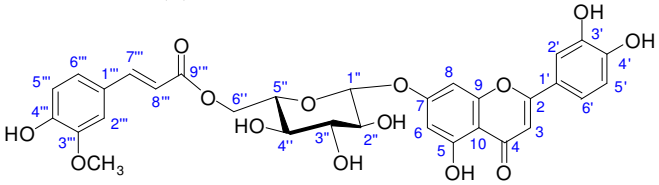
One triterpenic acid [ursolic acid (**1**)], one phenolic acid [rosmarinic acid (**2**)], and four flavonoids [luteolin (**4**), luteolin 7-*O*-(6”-feruloyl)-*β*-glucopyranoside (**3**), luteolin 5-*O*-*β*-glucoside (**5**), and luteolin 7-*O*-*β*-glucuronide (**6**)] were isolated using several chromatographic methods (Figure 1). Ursolic acid (**1**), rosmarinic acid (**2**), luteolin (**4**), luteolin 5-*O*-*β*-glucopyranoside (**5**), and luteolin 7-*O*-*β*-glucuronide (**6**) are characteristic compounds for *Thymus* species. Interestingly, luteolin 7-*O*-(6”-feruloyl)-*β*-glucopyranoside (**3**) was isolated for the first time from a *Thymus* species (Figure 1).



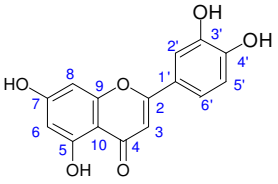
Ursolic acid (**1**)



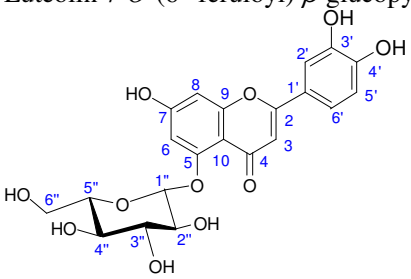
Rosmarinic acid (**2**)



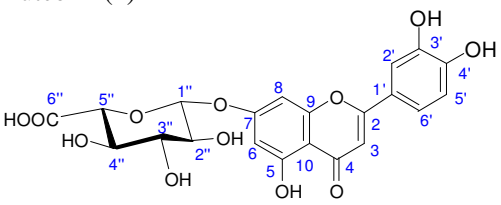
Luteolin 7-*O*-(6"-feruloyl) *β*-glucopyranoside (**3**)



Luteolin (**4**)



Luteolin 5-*O*-*β*-glucopyranoside (**5**)



Luteolin 7-*O*-*β*-glucuronide (**6**)

Figure 1. Str17uctures of isolated compounds

3.1. Relationship between antioxidant activity and chemical structure

Antioxidant activity of compounds **1-6** were tested by measuring MDA (Malondialdehyde) levels in bovine brain liposome system.

The phenolic compounds **2-6** mentioned above showed lipid peroxidation inhibition effects in various degrees (Figure 2). The antioxidant activities of the compounds **2-6** may be attributed to their phenolic structures.

As known, plant antioxidants have generally phenolic moiety. Phenolic compounds can easily donate electrons to reactive radicals because of the resonance stability of phenoxy radical and thus retard radical chain reactions. A newly formed phenoxy radical is not more reactive than a former radical. By a similar approach, unreactivity of ursolic acid (**1**) in lipid peroxidation may be explained by lacking a phenolic moiety.

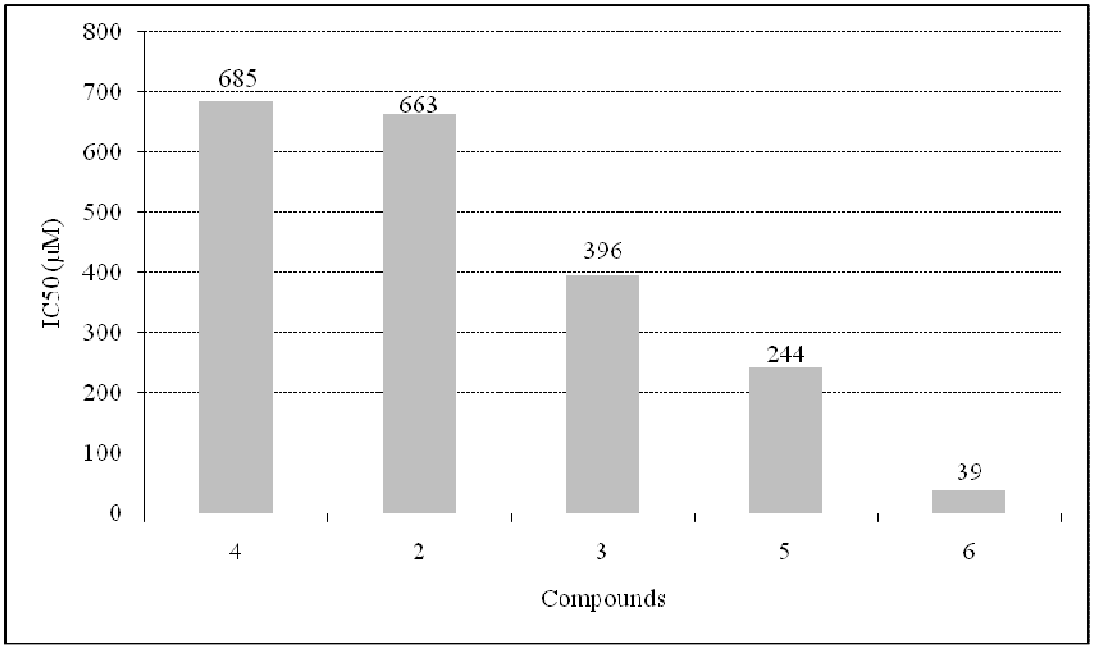


Figure 2. IC_{50} (μM) Values of isolated compounds for lipid peroxidation inhibition.
Rosmarinic acid (**2**); Luteolin 7-*O*-(6''-feruloyl)-*β*-glucopyranoside (**3**); Luteolin (**4**); Luteolin 5-*O*-*β*-glucopyranoside (**5**); Luteolin 7-*O*-*β*-glucuronide (**6**)

As can be seen in Figure 2, rosmarinic acid (**2**) and luteolin (**4**) having four phenolic hydroxyl groups showed similar lipid peroxidation inhibition. Interestingly, luteolin derivatives containing sugar group showed more lipid peroxidation inhibition. At first glance, it was very surprising for us because of the opposite findings reported in the literature [22-24]. For example, Rice-Evans et al. [24] reported that glycosylation of flavonoids reduces their antioxidant activities when it is compared to the corresponding aglycones. However, this difference may be caused from the antioxidant test methods used in the experiments. While Rice-Evans et al. [24] measured ABTS radical scavenging activity, we measured lipid peroxidation inhibition in bovine brain liposome. Therefore, we also decided to measure radical scavenging activities of the compounds.

As can be seen from Figure 3, the isolated compounds have DPPH radical scavenging activities at various degrees. However, radical scavenging activities were not similar to lipid peroxidation inhibition activities. For example, luteolin 7-*O*-*β*-glucuronide (**6**) is not relatively effective radical scavenger although it suppresses lipid peroxidation well. These findings show that radical scavenging activity is not unique factor to suppress lipid peroxidation.

In the liposome system used, the peroxidation is expedited by adding Fe^{3+} and ascorbic acid as shown in Scheme 1.

$$Fe^{3+} + \text{Ascorbic acid} \rightarrow Fe^{2+} + \text{Ascorbic acid}^{\bullet}$$
$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$$
$$2O_2^{\bullet-} + 2H^{+} \rightarrow H_2O_2 + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet}$$

Scheme 1. Proposed mechanism for production of $OH^{\bullet}$ radical

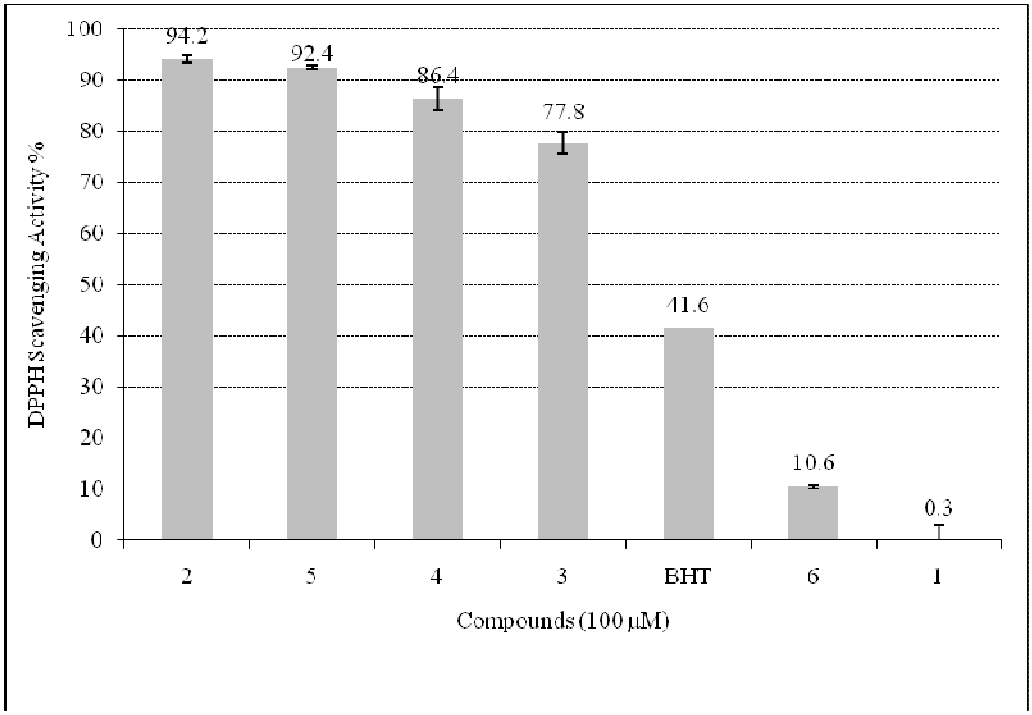


Figure 3. DPPH Scavenging activities of isolated compounds and BHT (100 µM final concentrations) Ursolic acid (**1**), Rosmarinic acid (**2**), Luteolin 7-*O*-(6"-feruloyl)-*β*-glucopyranoside (**3**), Luteolin (**4**) Luteolin 5-*O*-*β*-glucopyranoside (**5**), Luteolin 7-*O*-*β*-glucuronide (**6**) BHT : Butylated hydroxytoluene

As can be seen in the Scheme 1, ascorbic acid reduces Fe^{3+} to Fe^{2+} which promotes the Fenton reaction to produce $OH^{\bullet}$ radicals, which are one of the most reactive radicals [25-26]. These formed $OH^{\bullet}$ radicals increase the lipid oxidation rate. In the light of this proposed mechanism, masking of Fe^{3+}/Fe^{2+} by chelating with phenolic compounds may reduce peroxidation rate in the liposome test system. Therefore, we decided to detect separately ferrous chelating activities of the compounds **2-6**. However, the compounds **2-6** did not show a notable chelating activity when compared with EDTA. Van Acker et al. also reported that iron chelation does not play a role in the antioxidant activity in microsomal lipid peroxidation [27].

As reported in the literature recently, sugars themselves efficiently remove reactive oxygen species such as peroxy or hydroxyl radicals [28]. Therefore at this stage the observed results may be explained by solubility-activity relation of the compounds in the used test system. Liposomes have two components: Lipophilic part (inside) and hydrophilic part (outside) (Figure 4).

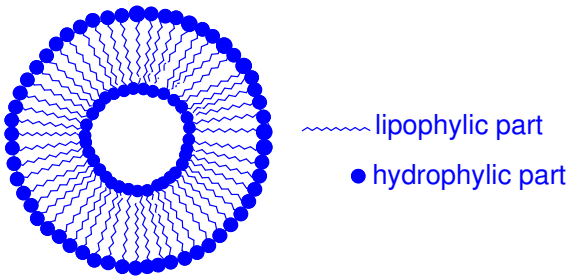


Figure 4. A representative picture of liposome.

By this approach, it is expected that the more polar compounds (sugar derivatives in these experiments) would prefer aqueous phase. Because reactive oxygen species are also produced in the aqueous phase, these radicals can be readily scavenged by the polar antioxidant molecules in the aqueous phase. Therefore, oxidation of lipid phase will relatively decrease.

In conclusion, sugar group addition to luteolin increases its antioxidant ability in the liposome system. A reasonable mechanism for this fact may be that sugar group addition increase the polarity of the molecule and thus the polar antioxidants can retard lipid peroxidation by scavenging water soluble oxygen species more effectively.

Acknowledgments

The authors would like to thank Prof. İhsan Çalış and Atatürk University Research Funding for financial support (Grant No. 2003/32).

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