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Two New Flavonoids and Other Phytochemicals from Endemic *Phryna ortegioides* (Fish. & C.A.Mey.) Pax & K. Hoffm and Their Antioxidant Potentials

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Abstract: Two new flavonoids namely 7,5′-dimethoxyisoetin (**1**) and homoorientin-6″-4-*O*-methyl-*myo*-inositol (2) together with six known compounds were isolated and identified as isoorientin (**3**)**,** genkwanin (**4**), dihydrokaempferol,7,4′-dimethylether (**5**), eriodictyol 7,4′-dimethylether (**6**), D-(+)-pinitol (**7**) and 3-*O*-*β*-D-glucopyranosyl spinasterol (**8**) from the aerial parts of endemic *Phryna ortegioides* for the first time. Their chemical structures were determined by 1D (^{1}H, ^{13}C and DEPT) and 2D (COSY, HSQC, ROESY, NOESY, TOCSY and HMBC) NMR and mass spectrometry. The antioxidant properties of pure secondary metabolites were analyzed on the basis of ferric thiocyanate method. Compound **1** displayed the highest antioxidant activity with an inhibition rate of 98.09% at a concentration of 100 µg/mL.

Keywords: *Phryna ortegioides*; antioxidant activity; caryophyllaceae; flavone; inositol.

1. Introduction

Turkey has a rich flora in terms of endemic and medicinal aromatic plants and still unknown and unexplored biodiversity, especially in Eastern Anatolia Region. However, the potential uses of the species of this flora, such as pharmacological, medicinal, nutritional, along with their bioactive and phytochemical properties are still unknown. Bingöl is one of the main cities of Eastern Anatolia Region with a wide variety of plant species and *Phryna ortegioides* is an endemic plant to this region [1,2]. Higher plant species with medicinal value contain a large number of natural antioxidant compounds. These active compounds are capable of inhibiting the oxidation, reducing the concentration of free radicals and thus prevent and/or reduce the risk of diseases such as cancer, diabetes, cardiovascular diseases, arthritis, cataracts, premature aging, Alzheimer and Parkinson [3-5].

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P. ortegioides belonging to Caryophyllaceae family was previously taxonomically classified within different genera (*Gypsophila, Saponaria, Tunica*) and accepted as their member. However, Pax & K. Hoffm had evaluated *P. ortegioides* as a separate genus in 1934 (as it has some characteristic different from those of *Gypsophila, Saponaria, Tunica*). The plants named the new genus as *Phryna* and identified its type species as *P. ortegioides.* The *Phryna* genus has just a single species (*Phryna ortegioides*) and this species is distributed merely in Turkey [6,7]. A phytochemical investigation of *P. ortegioides* in Turkey was revealed as a rich source of triterpenoids [8] and the essential oil composition of *P. ortegioides* has also been investigated [9]. A study on lipophilic fractions with antioxidant activity of *P. ortegioides* has also been reported [10]. The study was focused on determining bioactive volatile sub-fractions of *P. ortegioides* with phytochemical and bioanalytical test tecniques before isolation process. As part of our ongoing researches on the new and bioactive secondary metabolites from endemic medicinal plant we chose to investigate of the aerial parts of *P. ortegioides* species, which is an endemic species to province of Bingol in the eastern Anatolian. As a result of biological antioxidant activity-guided isolation studies we purified from dichloromethane fraction two new compounds **(1, 2),** together with six known compounds called isoorientin **(3)**, genkwanin **(4)** dihydrokaempferol 7,4′-dimethylether **(5)**, eriodictyol 7,4′-dimethylether **(6)**, D-(+)-pinitol (**7**) and 3-*O*-*β*-D-glucopyranosyl spinasterol **(8).**

2. Materials and Methods

2.1. General

^{1}H, ^{13}C, and 2D NMR spectra were recorded on Agilent 600 MHz (^{1}H: 600 MHz, ^{13}C: 150 MHz) NMR spectrometer (DMSO-d_6) and standard pulse programs were used for the measurement of 2D NMR spectra (^{1}H-^{1}H COSY, HSQC, HMBC). Mass spectra were recorded on an Agilent Technologies 1260 Infinity HRESIMS System and Agilent Poroshell 120 EC-C18 (2.7 μm, 4.0 x 50 mm) column. TLC was carried out on Merck silica gel plates (Kieselgel 60 F_{254}, layer thickness 0.2 mm), and spots were detected UV lamp and by spraying with acidified ceric sulphate followed by heating. Column chromatography separations and purifications were performed on silica gel 60 (70–230 or 230–400 mesh) from Merck using 30 g of silica gel per 1 g of the material to be separated or purified.

2.2. Chemicals

All solvents used were of analytical grade. Methanol (MeOH), butanol (BuOH), n-hexane, ethyl acetate (EtOAc), and dichloromethane (CH_2Cl_2) were obtained from Sigma-Aldrich Co. Silica gel (70–230 mesh) and thin layer chromatography (TLC) plate (silica gel 60F_{254}) was purchased from Merck Co. (Germany).

2.3. Plant material

The aerial parts of *P. ortegioides* were collected during flowering period from Bingol, Turkey, in July, 2012. The materials were identified by co-authors Prof. Dr. Lütfi Behçet (Bingöl University Department of Biology). A voucher specimen (BIN-7415) has been deposited in Bingöl University Herbarium of (BIN).

2.4. Extraction and isolation

The air-dried and ground aerial parts of *P. ortegioides* (4 kg) were macerated with 1:1 MeOH: CH_2Cl_2 (MDE, v/v; 20 L x 4; 72 h each) in laboratory conditions. After filtration, it was evaporated

under reduced pressure to afford crude MeOH:CH_2Cl_2 extract. The MDE extract (287.8 g) was dissolved in 2000 mL of H_2O to form a suspension and then extracted successively with CH_2Cl_2 (FD), EtOAc (FE) and n-BuOH (FB). These three main fractions were obtained as fraction FD (227.8 g), fraction FE (40.6 g) and fraction FB (19.4 g). The fraction FD (220.0 g) was subjected to column chromatography using silica gel (150x12 cm, silica gel 200–300 mesh, 2.400 g) and eluted with n-hexane: EtOAc (from 100:0 to 0:100, v/v) and EtOAc:MeOH (from 100:0 to 0:100, v/v). The collected fractions were combined according to their thin layer chromatography (TLC) to give 10 sub-fractions (Fr.1–Fr.10). Fr.2 (32.8 g) was chromatographed over a silica gel column using CH_2Cl_2: MeOH (from 9:1 to 0:1, v/v) as eluent to obtain 7,5′-dimethoxyisoetin (**1**) (17.3 mg, **1**), dihydrokaempferol 7,4′-dimethylether (13.8 mg, **5**), genkwanin (28.7 mg, **4**) and eriodictyol 7,4′-dimethylether (11.5 mg, **6**). Fr.4 (17.6 g) was subjected to repeated silica gel column chromatography steps with eluting system of EtOAc:MeOH (from 9:2 to 0:1, v/v) to afford compounds homoorientin-6″-4-*O*-methyl-*myo*-inositol (20.4 mg, **2**), D-(+)-pinitol (123 mg, **7**) and isoorientin (54.5 mg, **3**). Fr.6 (2.7 g) was purified by preparative thin layer chromatography (silica gel 60, 20x20 cm glass plate) with a solvent system of n-hexane–EtOAc (2:7, v/v) to afford compound 3-*O*-*β*-D-glucopyranosyl spinasterol (14.1 mg, **8**).

2.5. Total antioxidant activity by FTC method

Inhibition of lipid peroxidation of samples was determined according to the ferric thiocyanate (FTC) method as reported by Osawa and Namiki [11] with some modifications. Each test tube contained 2 mL of samples at 100 µg/mL concentrations, 2 mL an emulsion of linoleic acid in ethanol (2.51%; v/v), 4 mL of phosphate buffer (0.05M; pH 7.0) and 2 mL of distilled water. The solution (10 mL) was mixed and incubated in an oven at 40 ºC in dark for 10 day. The same solution, without any additives was taken as control samples. At regular intervals during incubation, 0.1 ml aliquot of the mixture was diluted with 9.7 ml of ethanol (75%) followed by the addition of 0.2 mL of ammonium thiocyanate (30%) and 0.1 ml of ferrous chloride (20 mM) in hydrochloric acid (3.5%; v/v). The peroxide level of each sample was determined by reading absorbance at 500 nm spectrophotometrically. These steps are repeated every 24 h until the control sample reached its maximum. The low absorbance value indicates the efficiency of the test samples to inhibit lipid oxidation. Percent inhibition of lipid peroxidation is calculated by equation: % Inhibition of lipid peroxidation= $[(C_{abs}\text{-}S_{abs})/C_{abs}]$ x100 where C_{Abs} is the absorbance of the control on the day when the absorbance of the control is maximum and S_{Abs} is the absorbance of the samples and positive control.

2.6. Statistical analysis

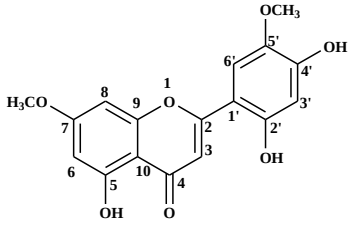
All experiments were carried out in triplicate. Data were expressed as means ± SD (standard deviation). A one-way analysis of variance (ANOVA) followed by Duncan's post-test was used for comparison between the pure compounds, fractions and extract. A difference was considered statistically significant when $p < 0.05$. All the statistical analyses were accomplished using the computer software SPSS 16 for Windows.

3. Results and Discussion

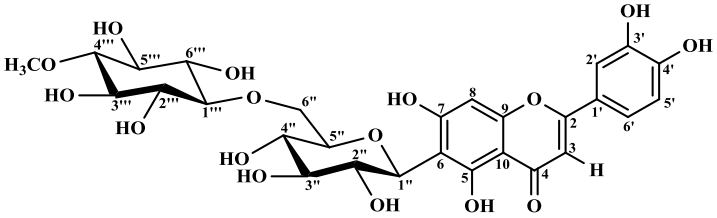
3.1. Structure elucidation

In this study, MDE (MeOH:CH_2Cl_2 (1:1; v/v)) extract of *P. ortegioides* aerial parts was divided into main-fractions soluble in FD (main fraction dichloromethane), FE (main fraction ethyl acetate), and FB (main fraction buthanol) and these main-fractions were evaluated for their antioxidant activity on ferric thiocyanate method. The FD soluble fraction displayed better antioxidant activity compared with other main functions. Bioassay-guided fractionation of this fraction via silica gel column chromatography gave sub-fractions of SF-2, SF-4 and SF-6 with the highest antioxidant

activities. From these bioactive sub-fractions, we isolated two new compounds together with six known by using further chromatographic techniques (Figure 1).



(1)



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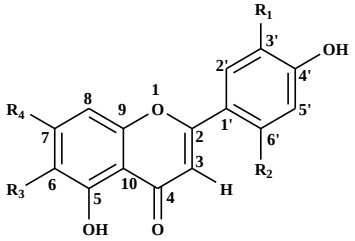
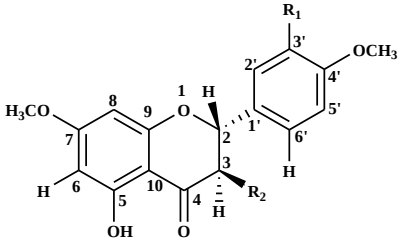
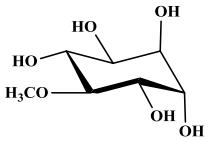


	R1	R2	R3	R4
(3)	OH	H	C-Glc	OH
(4)	H	H	H	OCH_3



	R1	R2
(5)	H	OH
(6)	OH	H



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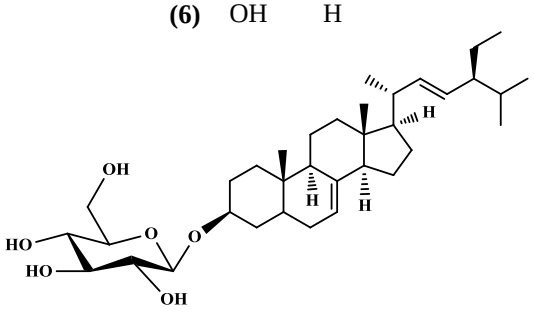

(8)

Figure 1. Chemical structures of isolated compounds from *P. ortegioides.*

Compound **1** was obtained as an orangey amorphous. In the negative HRESIMS spectrum of **1** was detected a pseudo molecular ion peak $[M-H]^-$ at *m/z* 329.0678 (Calc. 329.0667) compatible with the molecular formula $C_{17}H_{14}O_7$. The FT-IR spectrum of **1** displayed absorption bands at 3412 cm^{-1} (hydroxyl group), 1735 cm^{-1} (ketone) and 1603 cm^{-1} (olefin). The UV-VIS maximum absorption bands observed at λ_{max} (MeOH) 264, 289 and 371 nm. These bands are characteristic for a flavone skeleton. In the ^1H NMR spectrum of **1** was observed two aromatic doublets at H-6 and H-8 which resonated at δ_H 6.32 ppm (1H, J = 2.1 Hz) and 6.79 ppm (1H, J = 2.1 Hz), respectively, a ring-specific of flavone skeleton. Singlet signals of B ring resonated at δ_H 7.42 ppm (H-6′, 1H, s), 10.48 (OH, s), 6.53 (H-3′, 1H, s) and 10.05 (OH, s). The ^{13}C NMR and DEPT spectra of **1** displayed seventeen carbon signals including two methoxy, five methines and ten quaternary carbons (Table 1). Specific carbon signals in flavone skeleton belonging to one -C=O and two -OCH_3 groups in the ^{13}C NMR spectrum resonated at δ_C 182.4 ppm (C-4), 56.5 (7-OCH_3) and 57.1 (5′-OCH_3). The substitute groups at A ring were confirmed with interactions in HMBC spectrum between H-6 and C-5 (δ_C 161.5 ppm), C-7 (δ_C 165.4

ppm) and C-10 (δ_C 104.9 ppm) and H-8 with C-6 (δ_C 98.2 ppm), C-7 (δ_C 165.4 ppm), C-9 (δ_C 157.6 ppm) and C-10 (δ_C 104.9 ppm). B ring was determined by HMBC spectrum with interactions of H-6′ with C-1′ (δ_C 107.2 ppm), C-5′ (δ_C 142.0 ppm) C-4′ (δ_C 152.4 ppm) and C-2 (δ_C 162.4 ppm); H-3′ with C-4′ (δ_C 152.4 ppm), C-2′ (δ_C 153.6 ppm). Moreover, the H-3 proton at C ring was confirmed with interactions of C-4 (δ_C 182.40 ppm), C-10 (δ_C 104.91 ppm) C-1′ (δ_C 107.2 ppm) and C-2 (δ_C 162.4 ppm) (Figure 2). On the basis of the above evidences, the structure of **1** was identified as 7,5′-dimethoxyisoetin. To our knowledge, this is a new flavone from this plant. A different derivative of isoetin has been isolated by Rahman & Moon [12]. This reference study present a isoetin derivative which is substitute to 7 position a hydroxyl group in flavon skeleton, whereas in the present study was substituted to the same position a methoxy groups.

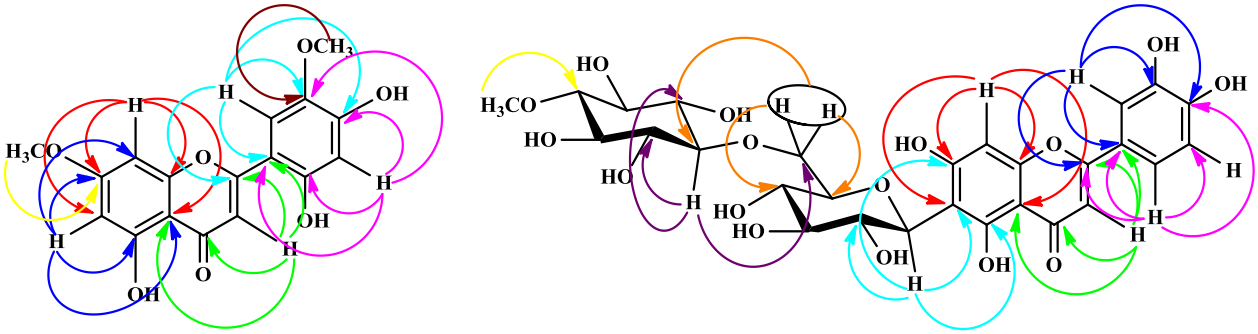

Figure 2. Key HMBC correlations of compounds **1** and **2.**

Compound **2** was obtained as pale yellowish solid. HRESIMS showed the $[M-H]^-$ ion peak at *m/z* 623.1602 (Calc. 623.1618) corresponding to the molecular formula $C_{28}H_{32}O_{16}$. The IR spectrum of **2** displayed a broad band of O–H stretching at 3284 cm^{-1} and a sharp band of C=O stretching at 1603cm^{-1}. The UV spectrum showed absorption maxima at λ_{max} (MeOH) 256 nm, 271 nm and 349 nm which are typical of a flavonoid skeleton in the molecule. The ^{13}C-NMR spectrum showed twenty eight carbon signals. The ^{13}C NMR spectrum of **2** showed a C=O group at δ_C 182.3 ppm (C-4), and quaternary carbons bonded to alcohols at δ_C 161.1 ppm (C-5), 163.7 (C-7), 146.2 (C-3′) and 150.1 (C-4′). A signal at δ_C 60.1 ppm showed the presence of a methoxy group. (Table 1). Two-dimensional specific resonances for H-3 confirmed by long-range correlations of δ_C 182.3 ppm (C-4), 164.1 (C-2), 103.9 (C-10), and 121.9 (C-1′) in the HMBC spectrum (Figure 2) and also further confirmation has been done by heteronuclear single quantum correlation of C-3 with chemical shift value of δ_C 103.2 ppm in the HSQC spectrum. HMBC interactions firstly from H-2′ (δ_H 7.37 ppm) to C-2 (δ_C 164.1 ppm), C-1′ (δ_C 121.9 ppm), C-2′ (δ_C 113.7 ppm) and C-3′ (δ_C 146.2 ppm), secondly from H-8 (δ_H 6.46 ppm) to C-6 (δ_C 109.3 ppm), C-7 (δ_C 163.7 ppm), C-9 (δ_C 156.6 ppm) and C-10 (δ_C 103.9 ppm) from H-1″ (δ_H 4.56 ppm; d, J = 9.6 Hz) to C-2″ (δ_C 70.6 ppm), C-5 (δ_C 161.1 ppm), C-6 (δ_C 109.3 ppm) and C-7 (δ_C 163.7 ppm) and thus, this substituent was assigned as glucopyranosyl moiety. Finally, HMBC correlations of H-1‴ (δ_H 3.15 ppm; d, J = 6.0 Hz) with C-2‴ (δ_C 72.9 ppm) and C-6‴ (δ_C 72.4 ppm) were detected and determined the second substituent in flavone skeleton. Another HMBC interaction from - OCH_3 (δ_H 3.42 ppm) to C-4‴ (δ_C 84.2 ppm) observed that the methoxy groups were connected to C-4‴. The HSQC showed all ^{1}H-^{13}C correlations and thus confirmed the assignment of all signals arising from the methine and methylene groups (Figure. S11).

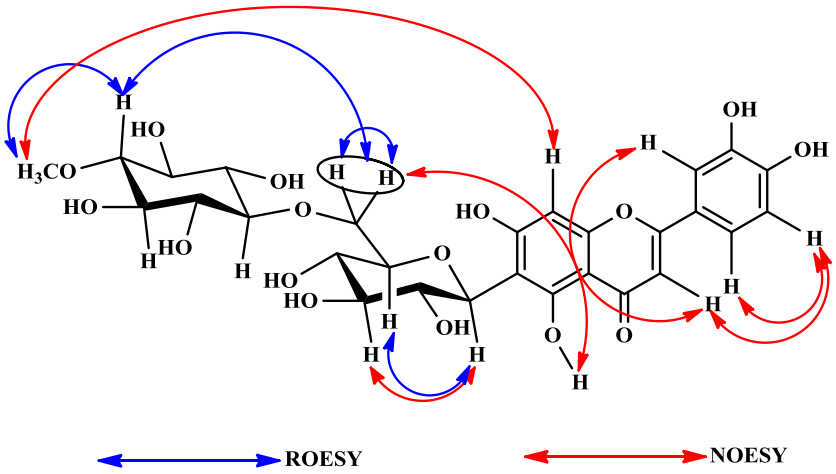

Figure 3. Key ROESY and NOESY correlations of compound **2.**

The COSY spectrum of compound **2** revealed all possible ^{1}H-^{1}H couplings and helped assigning the all proton resonances, especially of the glucopyranosyl and inositol moieties (Figure S13). The locations of the sugar and inositol residues in compound **2** were established by the presence of the ^{1}H-^{1}H NOESY (Nuclear Overhauser Enhancement SpectroscopY) cross peak between H-6″ and H-5 (Fig. 3 and Fig. S15), and the ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation) between H-1″ and C-6 (Fig. 2 and Fig. S15), and a methoxy signal at δ 3.42 (s) which displayed ^{1}H-^{1}H NOESY correlations with H-8 and the ^{1}H-^{1}H ROESY correlations with H-4‴ was deduced to be located at C-4‴ (Fig. 3 and Fig. S16). Three ^{1}H-^{1}H NOESY cross peaks were determined between H-1″ and H-3″, H-3 and H-5′ and finally H-5′ and H-6′. In addition to spatial interactions, in ROESY experiments geminal and long-range interactions were also observed as H_a-6″/H_b-6″ and H-5″/H-2″, respectively. Additionally, the ^{1}H-^{1}H NOESY correlation between H-2′ and H-5′, confirmed the structure of compound **2**. Thus, compound **2** was determined as homoorientin-6″-4-*O*-methyl-*myo*-inositol. To our knowledge, this is a new compound contained inositol substituent as seen in figure 2. This structure has 3D chemical structure with minimum energy as seen in fig. S21. Compound 2 has a distinctive feature in terms of inclusion inositol moiety. Generally, individual structure of inositol compounds have been found in plants rather than attached a substiuent on flavon skeleton. This inositol compounds found in a wide variety of plants and they have nine different isomers such as *myo*-, D-*chiro*-, L-chiro-, *scyllo*-, *muco*-, and *neo*-inositols as well as *O*-methyl-inositols [13,14]. Examples for this class of compounds are D-*chiro*-inositol and methylated at the 3-position OH (pinitol) that this compound induvidially we isolated in actual presenting study as the compound **7**. Likewise example of *myo*-inositol is methylated at the 4-position OH (ononitol) that this compound was isolated in compound **2.** The known compounds were identified as isoorientin [15] genkwanin [16], dihydrokaempferol 7,4′-dimethylether [17], eriodictyol 7,4′-dimethylether [18], D-(+)-pinitol [19] and 3-*O*-*β*-D-glucopyranosyl spinasterol [20] from ^{1}H and ^{13}C NMR data comparison with those reported in the literature.

Table 1. ^{1}H, ^{13}C and 2D NMR (in DMSO-d_6, ^{1}H: 600 MHz, ^{13}C: 150 MHz) spectral data of the isolated two new (**1→2**) compounds from *P. ortegioides*.

	Compound 1			Compound 2		
No	**δ_H (*J*, Hz)**	**δ_C**	**HMBC (C→H)**	**δ_H (*J*, Hz)**	**δ_C**	**HMBC (C→H)**
2	-	162.4	H-3, H-6′, H-3′	-	164.1	H-3, H-2′,H-6′
3	7.08 (s)	107.5		6.65 (s)	103.2	
4	-	182.4	H-3	-	182.3	H-3
5	13.04 (s, OH)	161.5	H-6, OH-5	13.54 (s, OH)	161.1	H-1″, OH-5
6	6.32 (d, *J* = 2.1 Hz)	98.2	H-8, OH-5	-	109.3	H-8, H-1", OH-5
7	-	165.4	H-8, H-6, OCH_3-5, OH-5	-	163.7	H-8, H-1"
8	6.79 (d, *J* = 2.1 Hz)	93.0	H-6	6.46 (s)	93.9	
9	-	157.6	H-8	-	156.6	H-8
10	-	104.9	H-3, H-6, H-8, OH-5	-	103.9	H-3, H-8, OH-5
OCH_3	3.85 (s)	56.5			-	
1′	-	107.2	H-3′ , H-6′, H-3	-	121.9	H-3, H-2′,H-5′, H-6′
2′	10.48 (s, OH)	153.6	H-3′, H-6′	7.37 (m)	113.7	H-6′
3′	6.53 (s)	104.8		-	146.2	H-2′, H-5′
4′	10.05 (s, OH)	152.4	H-3′, H-6′	-	150.1	H-2′, H-5′, H-6′
5′	-	142.0	H-6′, H-3′	7.39 (d, *J* = 8.0)	116.5	H-6′
6′	7.42 (s)	112.3		6.86 (dd, *J* = 8.0, 2.03)	119.4	H-5′, H-2′,H-3
OCH_3	3.78 (s)	57.1			-	
1″				4.56 (d, *J* = 9. 6)	73.5	H-2″, H-3″
2″				4.02	70.6	H-1″, H-3″
3″				3.18	79.5	H-1″, H-4″
4″				3.10	71.1	H-3″, H-5″
5″				3.14	81.1	H-4″, H-6″
6″				H_a-3.65, H_b-3.38	61.9	H-4″, H-5″, H-1‴
1‴				3.15 (d, *J* = 6.0)	82.0	H-6″, H-2‴
2‴				3.60	72.9	H-1‴, H-3‴
3‴				3.32	73.0	H-2‴, H-4‴
4‴				2.98	84.2	H-3‴, H-4‴,OCH_3
OCH_3				3.42 (s)	60.1	H-4‴
5‴				3.49	70.5	H-4‴, H-6‴
6‴				3.16	72.4	H-5‴, H-1‴

3.2. Antioxidant activity

Figure 4 shows the inhibitory effect of extract (MDE), main fractions (FD, FE and FB), sub fractions (SF-2, SF-4 and SF-6) and pure compounds (1-8) isolated from aerial parts of endemic *P. ortegioides* against linoleic acid peroxidation. Target-guided isolation process led to purification of new and effective compounds in terms of antioxidant activity. Main fractions of MDE, FE and FB were exhibited moderate activity with inhibition values of 79.97, 66.93 and 48.57% at concentration of 100 µg/mL, while sub-fraction SF-4 has a considerable activity with inhibition value of 89.24 at the same concentration. On the other hand, main fraction FD, sub-fractions SF-2, SF-6 and compound **1** showed the highest inhibition against lipid peroxidation at the end of the eighth day with inhibition values of 94.02, 94.78, 90.19 and 98.09%, respectively, at concentrations of 100 µg/mL. The antioxidant activity ranking among other examples were followed SF > (4) > (2) > MDE > (3) > (5) > FE > (4) > (7) > (8) > (6) > FB.

In results of bio-guided isolation studies, eight compounds including two new flavones were isolated from endemic *P. ortegioides* growing in Turkey for the first time. Compounds **1** and **2** were not isolated from any natural resources until now, despite isolating and identifying over 8000 flavone compounds from plants [21]. Also compound **2** representing flavone skeleton containing inositol and glucopyranosyl moieties has more elitism. Because, according to our extensive literature research on flavone compounds that containing inositol moiety isolating from plant there is only one study until this day [22]. The chemical structures of these compounds were elucidated by advanced spectroscopic techniques. Furthermore, the total antioxidant activities were evaluated by comparing these pure eight compounds, extracts, main fractions and sub-fractions. The results indicate that new flavone **1** significantly inhibited linoleic acid peroxidation in test media with a value of 98.09%.

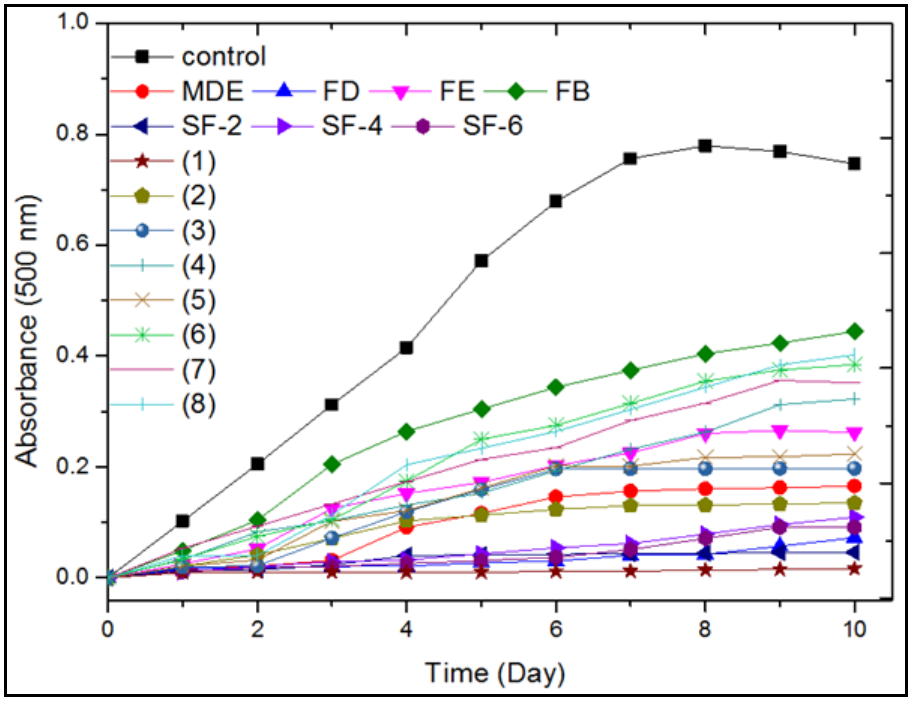

Figure 4. Comparative total antioxidant activities of all samples at concentrations of 100 µg/mL. A low absorbance value represents a high level of antioxidant activity. MDE methanol:dichloromethane (1:1;v/v) extract obtained from *P. ortegioides*. FD, FE and FB obtained by fractionation using separating funnel with dichloromethane, ethyl acetate and n-buthanol solvents, respectively. SF-2, SF-4 and SF-6 obtained by further column chromatography process and isolated compounds **1**-**8**.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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