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Secondary metabolites from *Scabiosa atropurpurea* and their antioxidant and xanthine oxidase inhibitory activities

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ABSTRACT: Two secoiridoid glycosides, lonicejaposide I (**1**) and secologanin dimethyl acetal (**2**), six flavonoids, isoorientin (**3**) hesperidin (**4**), apigenin 7-*O*-neohesperidoside (**5**), luteolin 7-*O*-β-D-glucopyranoside (**6**), kaempferol 3-*O*-(3'',6''-di-(*E*)-*p*-coumaroyl)-β-D-glucopyranoside (**7**), and kaempferol 3-*O*-(3''-*O*-acetyl, 6''-*O*-(*E*)-*p*-coumaroyl)-β-D-glycopyranoside (**8**), two caffeoylquinic acid derivatives, chlorogenic acid (**9**) and 3,5-dicaffeoylquinic acid (**10**) were isolated from the EtOAc and H_2O subextracts of the crude MeOH extract prepared from the aerial parts of *Scabiosa atropurpurea*. Their structures were identified by extensive 1D and 2D NMR experiments as well as ESI-MS analysis. Xanthine oxidase (XO) inhibitory and antioxidant activities of the isolated compounds were evaluated by *in vitro* tests. Compounds, **3**, **5**, **6**, **9** and **10** showed mild-to-moderate inhibitory effects on XO enzyme. The highest antioxidant activity was found for compound **10** according to results of DPPH, FRAP and CUPRAC assays. This is the first study on the XO inhibitory activities for compounds **1**, **2**, **5**, **7** and **8**.

KEYWORDS: *Scabiosa atropurpurea*; secoiridoids; flavonoids; caffeoylquinic acid derivatives; xanthine oxidase inhibitory activity; antioxidant activity

1. INTRODUCTION

The genus *Scabiosa* belongs to Caprifoliaceae family (formerly Dipsacaceae) and comprises approximately 100 species worldwide, mainly distributed in the Mediterranean region [1,2]. Various species belonging to this genus have been utilized in different folk medicines for their potential benefits on human health particularly against pulmonary and cutaneous diseases [3]. For instance, *Scabiosa succisa* is recommended for the treatment of diphtheria and respiratory infections including bronchitis, influenza, bronchial pneumonia as well as asthma. It is also employed as a remedy for skin disorders such as ulcer and certain types of dermatoses like scabies or herpes ringworm [4-6]. The genus *Scabiosa* is represented by 30 species in the flora of Türkiye [7]. *S. atropurpurea*L., a biennial or perennial plant growing wild in Türkiye, is known as "Moruyuzotu" [8]. This species is orally consumed to regulate menstrual cycle in Northern Peru while it is used to treat measles and furuncles in Catalonia. A herbal tea obtained from the aerial parts of *S. atropurpurea* is indicated as a veterinary diuretic in Iberian Peninsula, whereas an infusion of its flowers is applied externally for acne [1,8-10]. Besides, it is preferred as an ornamental plant due to its bluish-lilac flowers [11].

Biological studies on *S. atropurpurea* extracts showed different pharmacological effects including antimicrobial, antioxidant, antihyperglycemic and hepatoprotective [1,10-12]. Previous phytochemical investigations on this species revealed the presence of iridoids and phenolic compounds [11-13]. However, there is no detailed study on the isolation of its secondary metabolites and their *in vitro* xanthine oxidase (XO) inhibitory and antioxidant activities.

The excess production of uric acid in the serum leads to hyperuricemia which further increases the risk of gout and related metabolic disorders [14,15]. Xanthine oxidase is a critical enzyme in uric acid synthesis that catalyses the oxidation of hypoxanthine to xanthine and further, xanthine to uric acid with reactive oxygen species (ROS) production [16]. Therefore, the inhibition of XO activity will help reduce the development of hyperuricemia and gout by interrupting the transformation of xanthine to uric acid and thereby reducing uric acid levels in the bloodstream as well as contributing to antioxidant activity by decreasing ROS generation [17,18]. Although allopurinol, a well-known synthetic XO inhibitor, is clinically used for the management of hyperuricemia and gout, it may cause some side effects like gastrointestinal distress, renal toxicity or allergic

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reactions. Hence, it is important to develop a more safer XO inhibitor for the treatment of gout and hyperuricemia [19,20].

In present study, we aimed to isolate the secondary metabolites from the aerial parts of *S. atropurpurea* L. by using chromatographic techniques. Further, antioxidant and xanthine oxidase inhibitory activities of the isolates were evaluated by *in vitro* methods as some flavonoids were previously reported to inhibit XO enzyme significantly [21,22].

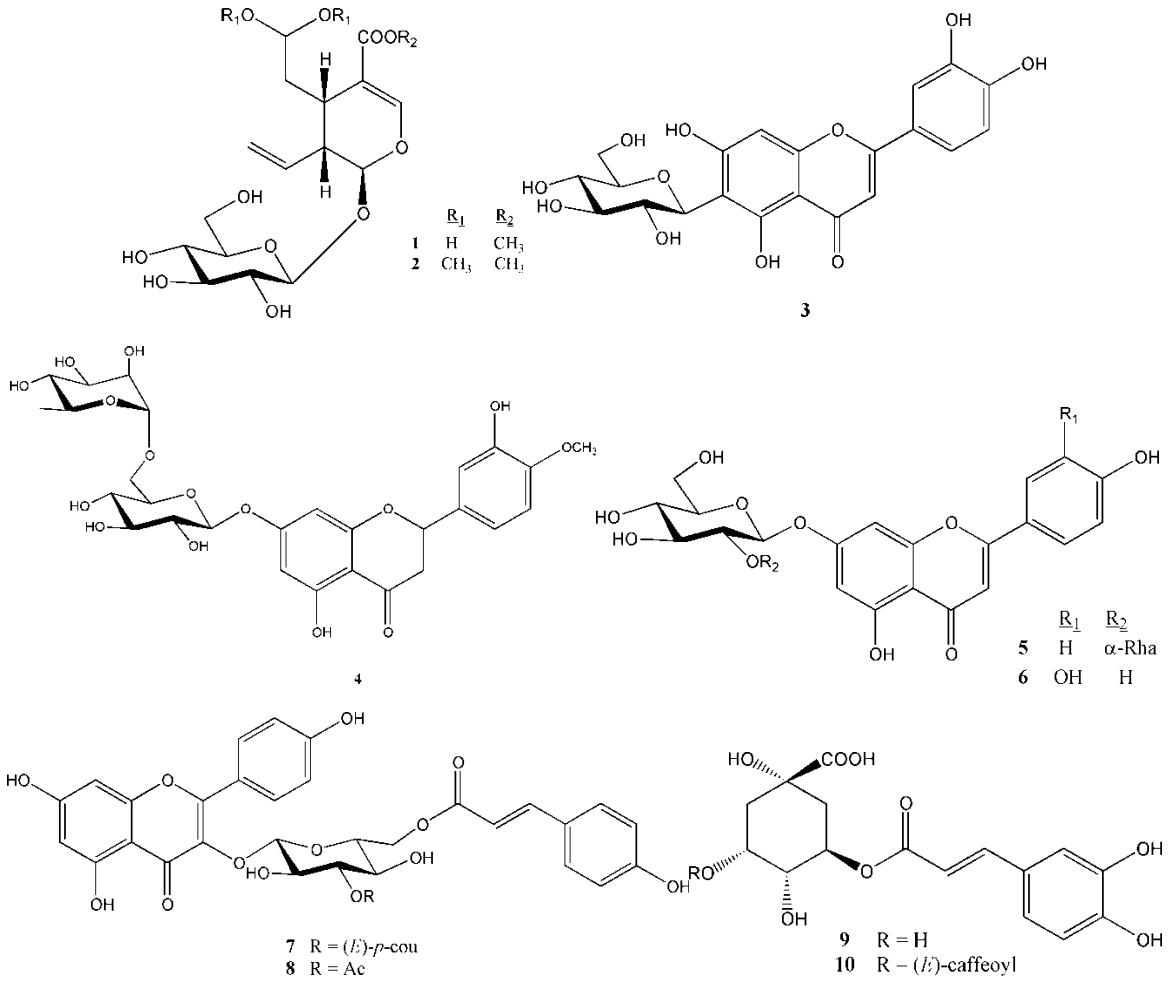

Figure 1. Chemical structures of compounds **1–10** isolated from *S. atropurpurea*.

2. RESULTS

2.1. Structure elucidation of the isolates

The air-dried and powdered aerial parts of *S. atropurpurea* were extracted with MeOH. The crude MeOH extract was suspended in H_2O and submitted to liquid-liquid extraction with $CHCl_3$ and EtOAc, respectively. Then organic solvents were evaporated to dryness to yield $CHCl_3$, EtOAc and remaining H_2O subextracts. EtOAc and H_2O subextracts were subjected to various chromatographic separations to afford ten secondary metabolites including two secoiridoid glycosides (**1** and **2**), six flavonoids (**3**-**8**) and two caffeoylquinic acid derivatives (**9** and **10**) (Figure 1). The isolates were identified as lonicejaposide I (**1**) [23], secologanin dimethyl acetal (**2**) [24], isoorientin (**3**) [25], hesperidin (**4**) [26], apigenin 7-*O*-neohesperidoside (**5**) [27], luteolin 7-*O*-β-D-glucopyranoside (**6**) [28], kaempferol 3-*O*-(3'',6''-di-(*E*)-*p*-coumaroyl)-β-D-glucopyranoside (**7**) [29], kaempferol 3-*O*-(3''-*O*-acetyl, 6''-*O*-(*E*)-*p*-coumaroyl)-β-D-glucopyranoside (**8**) [30], chlorogenic acid (**9**) and 3,5-dicaffeoylquinic acid (**10**) [31] by comparing their spectroscopic data with literature values. In previous studies, compounds **6** and **10** were characterized by LC-MS analyses from the extracts of *S. atropurpurea*while compounds **5** and **9** were recently detected in this species [10,11,32]. Previous studies on different *Scabiosa* species revealed the presence of apigenin, luteolin and kaempferol derivatives as the main flavonoids which is similar with our results [1]. Among the flavonoid glycosides herein reported, compounds **3**, **4**, **7** and **8** are

reported from *S. atropurpurea* for the first time. Iridoids and secoiridoids are widely distributed in Caprifoliaceae family, especially in the genus *Scabiosa* [33]. The occurenceof secoiridoids were previously reported in *S. atropurpurea*, but compounds **1** and **2** are being reported for the first time from this species [13]. Moreover, compounds **1**, **2**, and **4** are new for the genus*Scabiosa*. Kaempferol 3-*O*-(3''-*O*-acetyl, 6''-*O*-(*E*)-*p*-coumaroyl)-β-D-glucopyranoside (**8**) is an acetylated kaempferol derivative first isolated from *Scabiosahymettia.* Structurally, it is similar to*trans*-tiliroside, an another kaempferol derivative with a 6-*O*-(*p*-coumaroyl)-β-D-glucopyranosyl moiety [30]. Its chemical structure was elucidated on the basis of detailed 1D and 2D NMR techniques (Figure 2-5) as well as MS analysis. To our knowledge, it is being reported for the third time from the genus *Scabiosa* after *S. hymettia* and *S. stellata.* Moreover, it was only shown to possess*in vitro* antimicrobial activity [30,34].

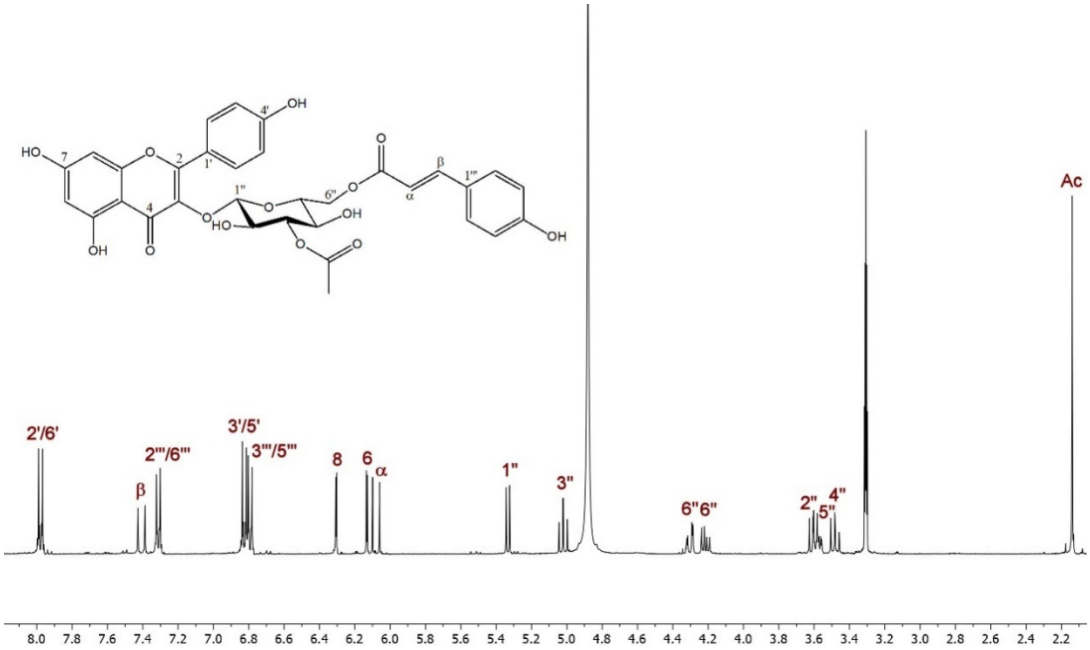

Figure 2. [1]H NMR Spectrum (400 MHz, CD_3OD) of **8**.

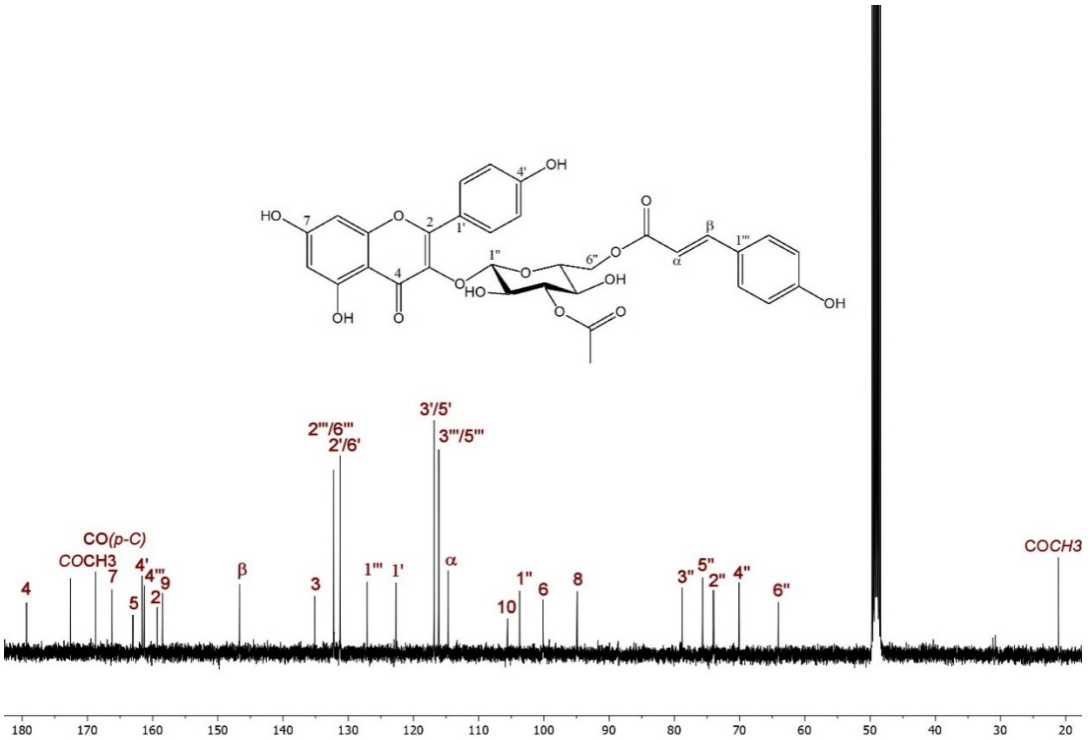

Figure 3. [13]C NMR Spectrum (100 MHz, CD_3OD) of **8**.

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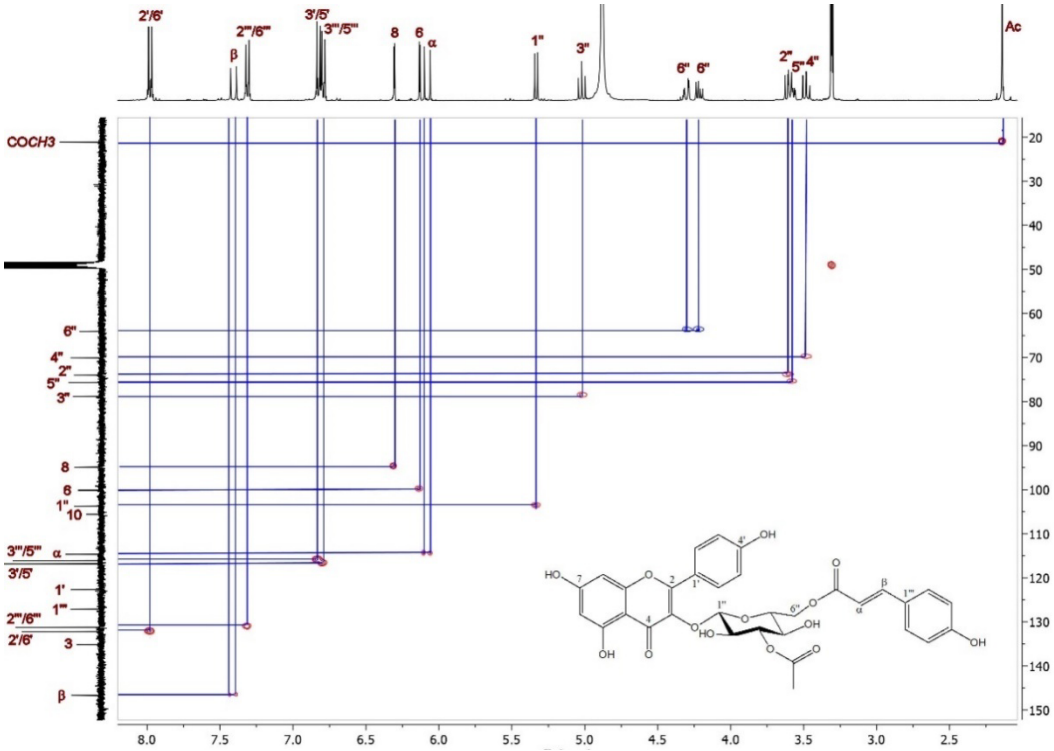

Figure 4. HSQC Spectrum of **8**.

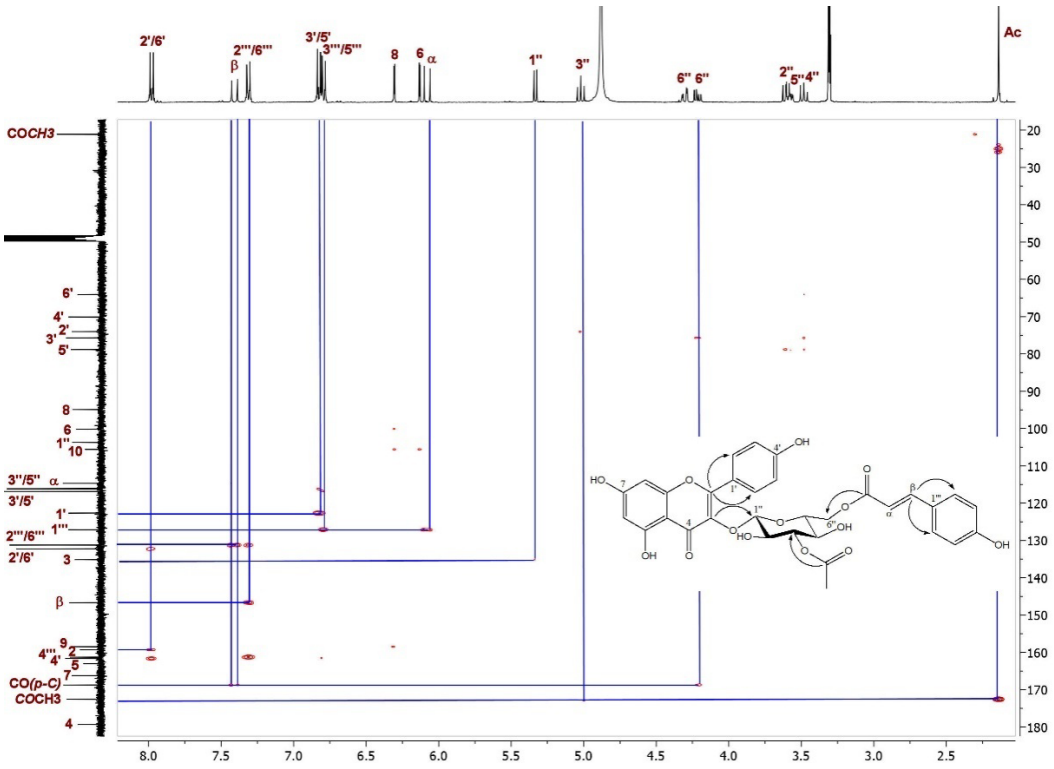

Figure 5. HMBC Spectrum of **8**.

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2.2. Antioxidant activity

Antioxidant effects of compounds (**1**-**10**) were assessed *in vitro* by DPPH, FRAP and CUPRAC methods (Table 1). The results of antioxidant tests were expressed as mg of Trolox equivalents (TE) per gram of isolated compounds (mg TE/g isolated compound). Amongst the tested isolates, 3,5-dicaffeoylquinic acid (**10**) showed the highest antioxidant activity in DPPH, FRAP and CUPRAC assays. Lonicejaposide I (**1**), a secoiridoid derivative, displayed the lowest antioxidant activity in FRAP and CUPRAC methods while possessed no activity in DPPH test.

Generally, the number of free phenolic hydroxyl groups on the chemical structures of natural compounds are directly proportional with their antioxidant activities [35].A number of researchers have reported thatcaffeoylquinic acid derivatives possess a high antioxidanteffects. Indeed, the antioxidant capacity of caffeoylquinic acid derivatives depend on thepresence of caffeoyl units in the molecule [36,37]. Thus, compound **10** (dicaffeoylquinic acid derivative) exhibited higher antioxidant activity than compound **9** (monocaffeoylquinic acid derivative). Regarding flavonoids, the glycosylation on their phenolic hydroxyl groups diminishes their antioxidant activity. In our study, isoorientin (**3**) displayed better antioxidant activity than hesperidin (**4**), apigenin 7-*O*-neohesperidoside (**5**) and luteolin 7-*O*-β-D-glucopyranoside (**7**) whose C-7 (OH) groups were substituted with sugar units.In contrast to flavonoids, caffeoylquinic acids are not glycosylated at their phenolic hydroxyl groups [38]. According to our results, flavonoid glycosides (**3**-**8**) showed lower antioxidant effect than caffeoylquinic acid derivatives (**9** and **10**) possibly due to their glycosylation at C-3 or C-7 positions. Previous studies demonstrated that iridoids and secoiridoids did not exhibit a strong antioxidant activity as similar with our findings. This is probably owing to their non-phenolic structures [39,40].

Table 1. Antioxidant activity results of compounds **1**-**10**in DPPH, FRAP, CUPRAC assays.

Compound	DPPH	FRAP	CUPRAC
		mg TE/g	
1	N.A.	24.62 ± 0.44^{g}	34.16 ± 0.81^{h}
2	N.A.	116.93 ± 2.69^{d}	25.31 ± 0.95^{h}
3	718.94 ± 1.20^{b}	896.09 ± 17.34^{b}	330.28 ± 19.52^{c}
4	12.84 ± 1.53^{c}	402.51 ± 6.36^{c}	201.83 ± 0.58^{d}
5	N.A.	46.05 ± 1.06^{fg}	78.84 ± 2.28^{g}
6	10.34 ± 2.87^{c}	66.99 ± 0.59^{ef}	140.69 ± 2.00^{f}
7	N.A.	123.40 ± 14.99^{d}	185.13 ± 0.85^{de}
8	N.A.	93.04 ± 7.98^{de}	165.17 ± 1.03^{e}
9	720.39 ± 3.02^{b}	879.84 ± 3.54^{b}	362.39 ± 12.14^{b}
10	748.20 ± 7.24^{a}	1038.49 ± 27.14^{a}	640.59 ± 7.75^{a}

DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric-reducing antioxidant power; CUPRAC: Cupric-reducing antioxidant capacity; N.A.: Not active. All experiments were done in thrice and results were expressed as means ± standard deviation (SD). The means with different superscript letters in each column ($^{a-h}$) demonstrate no significant differences ($p< 0.05$).

2.3. Xanthine oxidase inhibitory activity

Xanthine oxidase plays a key role in the formation of uric acid, the end product of purine metabolism. This enzyme catalyses the oxidative hydroxylation of hypoxanthine and xanthine to produce uric acid. The high concentration of uric acid in serum results in hyperuricemia which is a risk factor for gout [41,42]. Therefore, inhibitors of XO decrease serum uric acid levels and are potentially useful for the treatment of gout associated with hyperuricemia [43]. In our study, compounds (**1**-**10**) isolated from the *S. atropurpurea*were evaluated for their XO inhibitory activities (Table 2).

Among the tested compounds, isoorientin (**3**), apigenin 7-*O*-neohesperidoside (**5**), luteolin 7-*O*-β-D-glucopyranoside (**6**), chlorogenic acid (**9**) and 3,5-dicaffeoylquinic acid (**10**)displayed mild-to-moderate XO inhibitory activities, with IC_{50} values ranging from 9.58 to 48.67 µg/mL.In particular, luteolin 7-*O*-β-D-glucopyranoside (**6**) showed the highest XO inhibitory activity (IC_{50}: 9.58 ± 1.98µg/mL) which is comparable to positive control, allopurinol (IC_{50}: 2.13 ± 0.10µg/mL).

Previously, luteolin 7-*O*-β-D-glucopyranoside (**6**) was reported to have mild-to-moderate XO inhibitory effect with an IC_{50} value of 26.55 µM, while allopurinol showed activity with IC_{50}: 0.92 µM[44]. On the other hand, Sarawek et al. found out that **6** possessed moderate XO inhibitory activity (IC_{50}: 19.90 µM) which was five times less potent than reference drug, allopurinol (IC_{50}: 3.65 µM) [45]. Similar results for luteolin 7-*O*-β-D-glucopyranoside (IC_{50}: 9.58 ± 1.98 µg/mL) were observed in our study (allopurinol, IC_{50}: 2.13 ± 0.10 µg/mL).

Isoorientin displayed weak XO inhibitory effect (IC_{50}: 117.2 ± 13.5 µM) compared to positive control, quercetin (IC_{50}: 7.7 ± 0.3 µM) [46]. An et al. also reported that isoorientin exerted 96% inhibition of XO enzyme at concentration of 667 µmol/L which is similar to allopurinol at 80 µmol/L concentration with 97% inhibitory ratio [47]. This study also proved that isoorientin showed weak inhibitory activity against XO which seems to be compatible with our results. Chlorogenic acid (**9**) and 3,5-dicaffeoylquinic acid (**10**) were found to inhibit XO enzyme with an IC_{50} value of 28.29 µM and 43.86 µM, respectively (allopurinol, IC_{50}: 2.49 µM) [48]. However, in our study, **10** showed higher XO inhibitory activity than **9**. Previous *in vitro* experiment demonstrated that hesperidin (**4**) exhibited relatively very weak inhibitory effect (IC_{50}: >2000 mg/L) against XO which is line with our finding (did not show activity in the tested concentration ranges, 50-400 µg/mL) [49]. The lack of the activity of the tested kaempferol derivatives (**7** and **8**) may be related to their glycosidic structures, since their aglycone, kaempferol, was found to significantly inhibit the XO enzyme [50]. Yuan et al. also reported that the glycosylation of some flavonoids, such as kaempferol, might cause to decrease in their inhibition toward XO enzyme [51]. This is the first study to evaluate XO inhibitory activities of compounds **1**, **2**, **5**, **7** and **8**.

Table 2. *In vitro* xanthine oxidase (XO) inhibitory activities of compounds (**1-10**).

Compound	XO Inhibitory Activity (IC_{50}, µg/mL ± SD)
1	N.A.
2	N.A.
3	48.67 ± 6.46^{c}
4	N.A.
5	42.58 ± 3.29^{c}
6	9.58 ± 1.98^{a}
7	N.A.
8	N.A.
9	27.32 ± 2.40^{b}
10	22.38 ± 2.42^{b}
Allopurinol	2.13 ± 0.10

N.A.: Not active. The IC_{50} values of each sample were measured in triplicate. Results were stated as means ± SD. The means with lowercase letters ($^{a-c}$) indicate no significant differences ($p < 0.05$).

3. CONCLUSION

Phytochemical studies on the EtOAc and H_2O subextracts of *S. atropurpurea* yielded 10 secondary metabolites belonging to secoiridoid glycosides (**1** and **2**), flavonoids (**3-8**) and caffeoylquinic acid derivatives (**9** and **10**). Their structures were determined based on the spectroscopic methods. To our best knowledge, compounds **1**, **2**, and **4** are being purified and identified for the first time from the genus *Scabiosa*. Compounds **3**, **5**, **6**, **9** and **10** showed mild-to-moderate inhibitory activity on the XO enzyme, compared to the positive control. In particular, luteolin 7-*O*-β-D-glucopyranoside (**6**) displayed the highest inhibition against XO enzymeand 3,5-dicaffeoylquinic acid possessed the highest antioxidant activity among all tested compounds.

4. MATERIALS AND METHODS

4.1. General experimental procedures

Column chromatography (CC) was performed on silica gel 60 (Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) and Polyamide (Fluka Analytical, Sigma-Aldrich, USA). Medium-Pressure Liquid Chromatography (MPLC) was performed with Sepacore® Flash Systems X10/X50 (BuchiLabortechnik AG, Flawil, Switzerland) on RediSep columns (LiChroprep C_{18}: 150 and 100 g; SiO_2: 12 g, Teledyne Isco, Lincoln, Nebraska, USA). Thin Layer Chromatography (TLC) analyses were carried out on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), visualization was accomplished by spraying with 1% vanillin/H_2SO_4 followed by heating at 105 °C for 2-3 min, and detected with UV lights (254 and 365 nm). Analytical grade solvents (CH_2Cl_2 and MeOH) were used for chromatographic separations. 1D (^{1}H: 400 MHz, ^{13}C: 100 MHz) and 2D NMR (COSY, HSQC, HMBC and ROESY) spectra were recorded by using a Varian Mercury FT spectrometer (Palo Alto, USA) in CD_3OD or DMSO-$_{d6}$. TMS was used as an internal standard. The chemical shift values (δ) were expressed in ppm while coupling constants (*J*) were in Hz. ESI-MS data was recorded on Agilent G6530B TOF/Q-YTOF mass spectrometer (Agilent Technologies, USA) in positive ion mode.

4.2. Plant material

The aerial parts of *Scabiosa atropurpurea* L. were collected from Yeditepe University, Kayışdağı campus, İstanbul province of Türkiye in July 2021. The plant material was identified by Prof. Dr. Hasan Kırmızıbekmez. A voucher specimen (YEF 21005) has been stored in the Herbarium of the Department of Pharmacognosy, Facult of Pharmacy, Yeditepe University, İstanbul, Türkiye.

4.3. Extraction and isolation

The air-dried and powdered aerial parts of *S. atropurpurea* (250 g) were extracted two times with MeOH (2.5 L) at 45 °C for 2 h to obtain crude MeOH extract (40 g, yield: 16%) after removal of solvent under vacuum. An aliquot of MeOH extract (39.5 g) was dispersed in H_2O (80 mL) and then partitioned with $CHCl_3$ (3 x 80 mL) and EtOAc (3 x 80 mL), respectively to yield $CHCl_3$ (9.69 g), EtOAc (1.57 g), and rH_2O (25.42 g) subextracts. EtOAcsubextract (1.57 g) was applied to C_{18}-MPLC (150 g) with a gradient of H_2O-MeOH (0-100 % MeOH) to give 16 fractions (Frs. A-P). Fr. N (42 mg) was further separated by silica gel (SiO_2) (8 g) CC using CH_2Cl_2-MeOH as a solvent system (98:2 → 90:10) to obtain **2** (12 mg). Purification of **7** (10 mg) was accomplished by separation of fr. P (37 mg) on SiO_2 (6 g) column with CH_2Cl_2-MeOH mixture (95:5 → 90:10). Fr. O (21 mg) was subjected to SiO_2 (5 g) CC with CH_2Cl_2-MeOH as the eluent (96:4 → 90:10) to yield **8** (3 mg). Fr. J (84 mg) was chromatographed over SiO_2 (15 g) CC by elution of CH_2Cl_2-MeOH-H_2O mixture (95:5:0 → 80:20:2) to give six subfractions, frs. J_1-J_6. Fr. J_5 (20 mg) was further purified by Sephadex LH-20 (22 g) CC using MeOH to afford **6** (6 mg). Fr. Compound **10** (9 mg) was isolated from fr. G (105 mg) by SiO_2 (15 g) CC with CH_2Cl_2-MeOH-H_2O as the eluting solvent (97:3:0 → 70:30:3). H_2O subextract (25.42 g) was fractionated over polyamide column (100 g) eluting with stepwise gradient of MeOH in H_2O (0-100%, in steps of 20%, each 200 mL) to obtain eight fractions (Frs. 1-8). Fr. 2 (5.841 g) was further fractionated on SiO_2 (120 g) column using CH_2Cl_2-MeOH-H_2O (90:10:1 → 61:32:7) as mobile phase to give eight subfractions, frs. 2a-h. Fr. 2d (565 mg) was implemented to Sephadex LH-20 (95 g) CC eluted with MeOH to give four fractions, frs. $2d_{1\text{-}4}$. Purification of **4** (3 mg) was carried out from fr. $2d_4$ (27 mg) via SiO_2-MPLC (12 g) by gradient elution of MeOH in CH_2Cl_2 (95:5 to 80:20). Fr. $2d_3$ (444 mg) was applied to C_{18}-MPLC (100 g) with a gradient of H_2O-MeOH mixture (20 to 60% MeOH) and obtained **1** (16 mg). Fr. 6 (556 mg) was subjected to C_{18}-MPLC (150 g) using H_2O-MeOH (linear gradient 10-50%) as the eluting solvent to give **9** (49 mg) and together with seven fractions, frs. 6a-g. Among these fractions, fr. 6c (12 mg) was separated over Sephadex LH-20 (6 g) CC eluted with MeOH to yield **3** (6 mg). Purification of fr. 6g (39 mg) over SiO_2 (8 g) CC using CH_2Cl_2-MeOH-H_2O (95:5:0 → 85:15:1) mixtures afforded **5** (3 mg). Compound **3** (15 mg) was purified from fr. 6d (103 mg) by SiO_2 (15 g) CC eluting with CH_2Cl_2-MeOH-H_2O (95:5:0 → 80:20:2). Fr. 8 (50 mg) was chromatographed on Sephadex LH-20 (22 g) using MeOH as the mobile phase and obtained **9** (14 mg) together with 1 fraction, fr. 8a. Fr. 8a (22 mg) was further loaded in column that contains 4 g silica gel eluted with CH_2Cl_2-MeOH (90:10 → 85:15) to give **6** (12 mg).

4.4. Antioxidant activity

4.4.1. Preparation of standard solution for antioxidant assays

The stock standard solution of Trolox (Sigma-Aldrich, Steinheim, Germany) was prepared in MeOH and further diluted to prepare working standard solutions in the range of from 200 µg/mL to 3.125 µg/mL before DPPH, FRAP and CUPRAC analyses.

4.4.2. DPPH method

DPPH assay was performed according to the method of Degirmencioglu et al. [52] with some modifications. 20 µL of working concentrations of Trolox standard solutions, sample test solution or or blank (MeOH) were added into a 96-well plate, separately. Thereafter, 280 µL of 0.1 mM methanolic DPPH solution (abs. ~0.7, Sigma-Aldrich, Steinheim, Germany) was inserted into each well resulting the final volume of 300 µL. The plate was incubated at room temperature for 30 min in the dark conditions. Lastly, the absorbance was measured at 520 nm using the plate reader Varioskan™ LUX (Thermo Fisher Scientific, USA). Results were expressed as mg of Trolox equivalent (TE)/g.

4.4.3. FRAP method

Firstly, FRAP solution was freshly prepared by mixing 1 volume of iron (III) chloride solution ($2x10^{-2}$ M, Riedel-de Haen, Germany), 1 volume of TPTZ solution ($1x10^{-2}$ M), and 10 volumes of sodium acetate buffer (pH 3.6) solution. Then, 280 µL of FRAP solution and 20 µl of sample test solution or Trolox standard solution or blank (water) were added into a 96-well microplate. After 6 minutes, the absorbance was read spectrophotometrically at 593 nm [53]. Outcomes of the analysis were stated as mg TE/g.

4.4.4. CUPRAC method

Evaluation of the antioxidant activites of the compounds by the CUPRAC method were carried out according to study of Apak et al. [54]. Initially, 85 µL of 10 mM Copper (II) sulfate pentahydrate (Sigma-Aldrich, Steinheim, Germany) was mixed with 85 µL of 7.5 mM neocuproine, 85 µL ammonium acetate buffer solution (pH 7) and 51 µL of distilled water in wells of a 96-well microplate. Then, either 43 µL of sample test solution or Trolox standard solution or blank (water) were inserted in each well. After 30 min of kept of the plate at room temperature, the absorbance was detected at 450 nm. Findings were expressed as mg TE/g.

4.5. Xanthine oxidase inhibitory activity

The inhibitory effect on xanthine oxidase was conducted according to the procedure of Azmi et al. [16] with minor changes. 75 µL of 50 mM sodium phosphate buffer (pH 7.5), 25 µL of sample solution dissolved in water or DMSO, 25 µL of freshly prepared enzyme solution (0.2 U/mL of XO in buffer solution) and 25 µL of distilled water were added into a 96-well microplate, respectively. The reaction mixture was pre-incubated at 37°C for 15 min and followed by addition of 50 µL substrate solution (0.15 mM xanthine, Alfa Aesar, Haverhill, Massachusetts, USA) into the mixture. Then, the mixture was incubated at 37°C for 30 min, and the reaction was terminated by adding 50 µL of 0.5 M HCl. Finally, the absorbance was read at 290 nm against a blank, containing all reagents except enzyme solution. Allopurinol (Acros Organics, Geel, Belgium) used as a positive control which was prepared at different concentrations in range of from 15 to 200 µg/mL. The xanthine oxidase inhibition (%) was calculated by using the following formula:

$$\text{XO inhibition (\%)} = [1-(\text{Sample}_{abs}-\text{Sample blank}_{abs})/(\text{Control}_{abs}-\text{Control blank}_{abs})]x100$$

4.6. Statistical analysis

All experiments were performed in triplicate. Experimental results and statistical significance of the results were determined by using Minitab 17 software with one-way analysis of variance (ANOVA). Comparison of significance was ascertained by Tukey post hoc-test with 95% confidence. Results were expressed as means ± SD and significance levels were defined as $p < 0.05$.

Author contributions: Concept – C.Ö., H.K.; Design – C.Ö., H.K.; Supervision – H.K.; Resources – C.Ö., H.K.; Materials – C.Ö., H.K.; Data Collection and/or Processing – C.Ö., H.K., N.B.Ş., E.G.; Analysis and/or Interpretation – C.Ö., H.K., N.B.Ş., E.G.; Literature Search – C.Ö., H.K., N.B.Ş., E.G.; Writing – C.Ö., H.K., N.B.Ş, E.G.; Critical Reviews – C.Ö., H.K., N.B.Ş., E.G.

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