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Two new phenolic compounds and some biological activities of *Scorzonera pygmaea* Sibth. & Sm. subaerial parts

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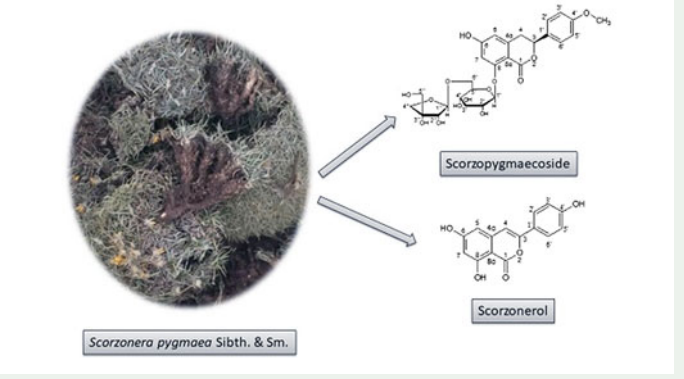
ABSTRACT

Phytochemical composition of ethyl acetate fraction and total phenolic content, in vitro antioxidant, anti-inflammatory, antimicrobial activities of petroleum ether, chloroform, ethyl acetate and *n*-butanol fractions of the ethanol extract obtained from the subaerial parts of *Scorzonera pygmaea* Sibth. & Sm. (Asteraceae) were investigated. Nine compounds; scorzopygmaecoside (**1**), scorzonerol (**2**), cudrabibenzyl A (**3**), thunberginol C (**4**), scorzocreticoside I (**5**) and II (**6**), chlorogenic acid (**7**), chlorogenic acid methyl ester (**8**), 3,5-di-O-caffeoylquinic acid (**9**) were isolated and identified using spectroscopic methods. All substances were isolated for the first time from this species. Compounds **1** and **2** are new. The fractions showed high antioxidant capacity correlated with their phenolic content and no significant antimicrobial activity against tested bacteria and fungi. COX inhibition test was used to evaluate the anti-inflammatory activity and all the fractions showed low inhibition in comparison with indomethacin.



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1. Introduction

The genus *Scorzonera* L. is a member of the family Asteraceae, subfamily Liguliflorae, tribe Lactuceae and it is represented by around 160 species in the world. There are 52 species in Turkey and 31 of them are endemic (Coşkunçelebi et al. 2015).

Scorzonera L. species are consumed as a vegetable and used in traditional medicine as analgesic, antirheumatic, anthelmintic, diuretic, wound healer, in the treatment of infertility, gout, pulmonary oedema, diarrhoea, gastric ulcer, malign gastric cancer and hypertension (Sarı et al. 2009; Tsevegsuren et al. 2007).

According to the previous phytochemical studies; dihydroisocoumarins, benzyl phthalides, flavonoids, lignans, neolignans, bibenzyl derivates, phenolic acid derivates, kavalactones, sesquiterpenes and triterpenes were found in *Scorzonera* L. species (Granica et al. 2015; Sarı et al. 2009).

Scorzonera pygmaea Sibth. & Sm. is a dwarf cushion forming, perennial herb with 1.5 – 11 cm height and pale yellow flowers (Chamberlain 1975). It is an endemic species growing in North West and South West Anatolia (Koyuncu et al. 2014). There is no record of the secondary metabolite profile and biological activities of the plant.

2. Results and discussion

Subaerial parts of *S. pygmaea* were investigated and nine compounds; four dihydroisocoumarins (scorzopygmaecoside, thunberginol C, scorzocreticoside I and II), one isocoumarin (scorzonerol), one bibenzyl (cudrabibenzyl A), three phenolic acid derivates (chlorogenic acid, chlorogenic acid methyl ester, 3,5-di-O-caffeoylquinic acid) were isolated and identified using spectroscopic methods. All substances were isolated for the first time from this species. Cudrabibenzyl A is new for the family Asteraceae.

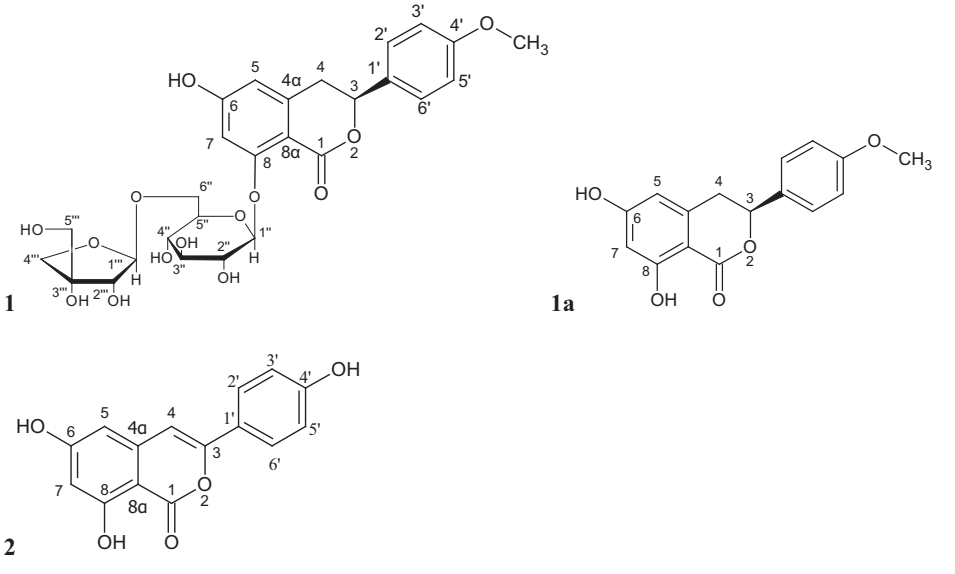

Figure 1. Structures of compounds **1, 1a** and **2**.

Thunberginol C was found for the first time in the genus *Scorzonera* L. Scorzopygmaecoside and scorzonerol are new compounds (Figure 1).

Compound **1**, was isolated as a yellowish, optically active powder and showed absorption maxima at 224, 267, 293sh nm in UV spectrum (MeOH). A protonated molecule ion $[M + H]^+$ at *m/z* 581.1863 was observed in the HRESIMSMS spectrum of compound **1** corresponding to $C_{27}H_{32}O_{14}$ (calculated 581.1865), in addition to fragment ions at *m/z* 449.1425 and *m/z* 287.0904 suggesting the presence of an apiose group (as terminal sugar) and a glucose group in the molecule. Its ^{1}H-NMR spectrum in deuterated methanol indicated to a 1,4-disubstituted aromatic ring [δ_H 7.42 (2H, d, J = 8.8 Hz), δ_H 6.97 (2H, d, J = 8.8 Hz)], a tetra substituted aromatic ring [δ_H 6.50 (1H, d, J = 2.0 Hz), δ_H 6.80 (1H, d, J = 2.0 Hz)], a methine group [δ_H 5.40 (1H, dd, J = 2.4, 12.2 Hz)], a methylene group [δ_H 3.00 (1H, dd, J = 2.4, 16.6 Hz), δ_H 3.24 (1H, dd, J = 12.2, 16.6 Hz)] and a methoxy group [δ_H 3.82 (3H, s)]. Furthermore, the ^{1}H-NMR spectrum showed two anomeric proton signals assignable to H-1 of glucosyl moiety at δ_H 4.83 (d, J = 7.3 Hz) and H-1 of apiosyl moiety at δ_H 5.05 (d, J = 2.4 Hz). The ^{13}C-NMR spectrum of compound **1** had twenty-seven carbon signals. Two anomeric carbon signals were detected at δ_C 103.5 and δ_C 109.5 attached to the anomeric protons at δ_H 4.83 and δ_H 5.05 respectively in the HSQC spectrum of compound **1**. The coupling constant of the anomeric proton at δ_H 4.83 (d, J = 7.3 Hz) and the coupling pattern of the other sugar resonances indicated a β-glucopyranosyl unit (Gurst 1991). The identification of β-apiofuranosyl unit was deduced from the ^{1}H-NMR and ^{13}C-NMR data which were in good agreement with those reported for β-apiofuranosides (Ishii and Yanagisawa 1998; Jayasinghe et al. 1995; Voutquenne et al. 2003). Acid hydrolysis of compound **1** provided aglycone (**1a**, scorzocreticin), D-Glucose and D-Apiose. The attachment sites of the sugars and other key information were determined with the HMBC spectrum of compound **1** that showed correlations between H-1″ – C-8, H-1‴ – C-6″, H-4 – C-5, H-2′,6′ – C-3, methoxy protons – C-4′. NOESY spectrum of compound **1** which showed cross peaks between H-2′,6′ – H-3, H-4a – H-3, H-4b – H-5 gave information about relative stereochemistry of the compound, however the S configuration at C-3 position was deduced from comparing specific rotations of **1a** ($[\alpha]^{20}_D$ = +22.1 (c = 0.2, MeOH) and scorzocreticin ($[\alpha]^{20}_D$ = +23.3 c = 0.2, MeOH) (Paraschos et al. 2001). After the interpretation of all data, it was deduced that compound **1** was very similar to scorzocreticoside II (a dihydroisocoumarin isolated from *Scorzonera cretica*) except for apiose instead of rhamnose as second sugar (Paraschos et al. 2001). Thus, compound **1** was named as scorzopygmaecoside.

Compound **2** was obtained as a light-yellow powder. Its UV spectrum in MeOH showed absorption maxima at 229, 268, 307sh, 319, 348 nm suggesting an isocoumarin moiety (Yoshikawa et al. 1992). A protonated molecule ion $[M + H]^+$ at *m/z* 271.0604 was observed in the HRESIMSMS spectrum of compound **2** corresponding to $C_{15}H_{10}O_5$ (calculated 271.0604) along with fragment ions at *m/z* 243.0643, *m/z* 177.0152 and *m/z* 121.0225 which were consistent with the general fragmentation pattern of isocoumarins (Hussain et al. 2001; Rama et al. 2005) . In the ^{1}H NMR spectrum of compound **2** signals were observed that ascribable to a 1,4-disubstituted benzene ring [δ_H 7.63 (2H, d, J = 8.8 Hz), δ_H 6.78 (2H, d, J = 8.8 Hz)], a tetra substituted benzene ring [δ_H 6.22 (1H, d, J = 2.0 Hz), δ_H 6.32 (1H, d, J = 2.0 Hz)] and an olefinic proton

[δ_H 6.83 (1H, s)]. All chemical shifts of the carbons were deduced from the ^{13}C NMR, HMBC spectrums and literature data (Yoshikawa et al. 1992). ^3J correlations in the HMBC spectrum, between olefinic proton H-4/C-8α and H-7/C-8α confirmed the isocoumarin moiety. Furthermore, the connectivity of the 1,4-disubstituted benzene ring to the isocoumarin skeleton was indicated from the HMBC spectrum by a cross peak between H-2′,6′ and C-3. After evaluation of the all spectroscopic findings and comparison with the literature data, compound **2** was found to be very similar to Thunberginol B except for lack of a hydroxyl group at C-3′ position and named as scorzonerol.

The known compounds; cudrabibenzyl A (**3**), thunberginol C (**4**), scorzocreticoside I (**5**) and II (**6**) were identified based on their UV, ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC and ESI-MS spectrums (Hiep et al. 2017; Nguyen et al. 2014; Paraschos et al. 2001; Toshikawa et al. 1992). The other known compounds; chlorogenic acid (**7**), chlorogenic acid methyl ester (**8**) and 3,5-di-O-caffeoylquinic acid (**9**) were determined by their UV, ^{1}H-NMR spectrums and comparing Rf values with standards (Pauli et al. 1998; Sarı 2010, 2012; Zhu et al. 2005).

The highest amount of total phenolic content of the fractions expressed as gallic acid equivalents was observed in the ethyl acetate fraction (96.36 ± 4.50 mg/g fraction) followed by the chloroform fraction (41.58 ± 5.78 mg/g fraction) and the *n*-butanol fraction (10.03 ± 1.64 mg/g fraction). No phenolic content was determined in the petroleum ether fraction. Values are the means of three replicates ± standard deviation. The greater efficiency of the ethyl acetate fraction in extracting phenolic compounds resulted in a higher antioxidant capacity of the fraction (Table 1). Phenolic compounds possess several biological activities and have been associated with the beneficial effects of fruits and vegetables. These beneficial effects have been ascribed to antioxidant potential of their phenolic content (Balasundram et al. 2006; Rice-Evans et al. 1997). Antioxidant activity studies carried out on pure compounds/extracts of *Scorzonera* L. species revealed high antioxidant activity/capacity referring to phenolic compounds (particularly dihydroisocoumarins and phenolic acids) as responsible substances (Athmouni et al. 2015; Milella et al. 2014; Nasseri et al. 2015; Tsevegsuren et al. 2007; Wang et al. 2012; Yang et al. 2013). Considering the isolated and identified

Table 1. Results of antioxidant activity studies of the fractions of the ethanol extract obtained from the subaerial parts of *Scorzonera pygmaea*.

	EC_{50} (mg/ml)[A]				TEAC[B]	FRAP[C]
	Anti-LPO	SO	DPPH	ABTS	(mmol/L)*	(mmol Fe^{2+}/L)*
PE	–	–	–	–	–	–
$CHCl_3$	3.45 ± 0.47^a	0.48 ± 0.02^a	3.49 ± 0.29^a	3.83 ± 0.32^a	0.872 ± 0.05^a	1.147 ± 0.039^a
AcOEt	1.58 ± 0.01^b	0.49 ± 0.02^a	1.21 ± 0.05^b	2.12 ± 0.23^b	1.365 ± 0.06^b	1.769 ± 0.040^b
BuOH	18.23 ± 0.50^c	1.57 ± 0.11^b	16.07 ± 0.28^c	19.22 ± 0.21^c	0.375 ± 0.02^c	0.590 ± 0.015^c
Rutin	0.71 ± 0.01^d	0.54 ± 0.02^a	0.140 ± 0.01^d	0.60 ± 0.04^d	$2.11 \pm 0.05^{d**}$	$2.884 \pm 0.040^{d**}$

[A]EC_{50} value: The effective concentration at which the LPO inhibitory activity was 50%; SOD, DPPH and ABTS radicals were scavenged by 50%.
[B]Expressed as mM Trolox equivalents.
[C]Expressed as mM ferrous ions equivalents.
[D]Values were the means of three replicates ± standard deviation. Values with different letters (a,b,c,d) in the same column were significantly ($p < 0.05$) different.
*Determined at 2.5 mg/ml.
**Determined at 1.25 mg/ml.

compounds from *S. pygmaea*, the high antioxidant potential of the fractions might be derived from the presence of a combination of these phenolic compounds.

The fractions showed no significant antimicrobial activity against tested bacteria and fungi (Table S5).

Anti-inflammatory activities of the fractions were evaluated by COX inhibition since COX-2 is considered as a target enzyme for the nonsteroidal anti-inflammatory drugs without gastric side effects. The percentage COX inhibition of the fractions (Table S6) showed that the fractions have low inhibitory activity against both COX-1 and COX-2 in comparison with indomethacin. Previous anti-inflammatory activity studies on *Scorzonera* L. genus showed promising results about inhibitory activities on TNF-α, IL-1β (both are proinflammatory cytokines) production and NF-κB nuclear translocation in THP-1 macrophages (Bahadır Acıkara et al. 2015). Thus, the investigation of anti-inflammatory activity of *S. pygmaea* by inhibition of proinflammatory cytokines that induce COX enzymes too might be considered.

3. Experimental

3.1. Plant material

The subaerial parts of *S. pygmaea* were collected from Sivrihisar/Eskişehir in July 2015. Voucher specimens are deposited at Herbarium of Eskişehir Osmangazi University (ESK 18397).

3.2. Extraction and isolation

The air-dried and powdered subaerial parts (1.4 kg) of *S. pygmaea* were macerated with ethanol and concentrated under reduced pressure using a rotary evaporator (45 °C). The ethanol extract was dissolved in MeOH/H_2O (1:2) and then successively extracted with petroleum ether (PE), chloroform ($CHCl_3$), ethyl acetate (AcOEt) and *n*-butanol (BuOH). The ethyl acetate soluble part (15g) was subjected to CC (silica gel; $CHCl_3$/MeOH 100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 90:10, 88:12, 85:15, 80:20, 75:25, 70:30, 50:50, 0:100) and gave 212 fractions. Fr 12 was purified by CC (Sephadex LH-20; MeOH) to afford **2** (1.2 mg). Fr 19-23 was subjected to prep. TLC (silica gel; toluene/AcOEt/HCOOH 5:4:1) and gave **4** (6.6 mg). Fr 56-65 was further separated by prep. TLC (silica gel; toluene/AcOEt/HCOOH 1:7:1) to provide pure **5** (20 mg) and **8** (14 mg). Fr 81-89 was subjected to CC (Sephadex LH-20; MeOH) to afford **1** (20 mg). Fr 112-115 was purified by prep. TLC (silica gel; $CHCl_3$/MeOH 75:25) to yield pure **3** (17 mg) and **6** (30 mg). Fr 143-157 was further separated by TLC (silica gel; toluene/AcOEt/HCOOH 8:2:0.5) to provide pure **7** (4.4 mg) and **9** (4.5 mg).

Acid hydrolysis of compound **1**: A solution of compound **1** (20 mg) in HCl (2 N) was refluxed for 25 min. After cooling the reaction mixture was extracted with AcOEt to obtain aglycone **1a** (8 mg). The aqueous layer was neutralized with NaOH (2 N) and evaporated to dryness. The residue was extracted with warmed MeOH to give monosaccharide fraction. The fraction was subjected to prep TLC (silica gel; CH_2Cl_2/MeOH/H_2O 70:27:30) to provide D-Glucose [1.5 mg, $[\alpha]^{20}_D = +38$ (c = 0.07, H_2O)] and D-apiose [1.0 mg, $[\alpha]^{20}_D = +7$ (c = 0.05, H_2O)].

Scorzopygmaecoside (**1**). $[\alpha]^{20}_{D}$ = –49.9 (c = 0.6, MeOH). UV (MeOH): 224, 267, 293sh nm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H **3.00** (1H, dd, *J* 2.4/16.6 Hz, H-4a), **3.24** (1H, dd, *J* 12.2/16.6 Hz, H-4b), **3.42** (1H, d, *J* 9Hz, H-4″) **3.52** (1H, dd, *J* 7.0/9.5 Hz, H- 2″), **3.53** (1H, t, *J* 9.5 Hz, H-3″), **3.63** (1H, m, H-5″), **3.63** (2H, s, H-5‴), **3.70** (1H, dd, *J* 2.1/11.3 Hz, H-6″a), **3.79** (1H, t, *J* 9.8 Hz, H-4‴a), **3.82** (3H, s, H-4′-OCH_3), **3.98** (1H, d, *J* 2.4 Hz, H-2‴), **4.03** (1H, d, *J* 9.8 Hz, 4‴b), **4.08** (1H, dd, *J* 6.5/11.3 Hz, H-6″b), **4.83** (1H, d, *J* 7.3 Hz, H-1″), **5.05** (1H, d, *J* 2.4 Hz, H-1‴), **5.40** (1H, dd, *J* 2.4/12.2 Hz, H-3), **6.50** (1H, d, *J* 2 Hz, H-5), **6.80** (1H, d, *J* 2 Hz, H-7), **6.97** (2H, d, *J* 8.8 Hz, H-3′,5′), **7.42** (2H, d, *J* 8.8 Hz, H-2′,6′). ^{13}C-NMR (125 MHz, CD_3OD): δ_C **35.8** (C-4), **54.3** (C-4′-OCH_3), **64.1** (C-5‴), **67.2** (C-6″), **69.9** (C-4″), **73.5** (C-3″), **73.6** (C-4‴), **75.7** (C-2″), **76.1** (C-5″), **76.6** (C-2‴), **79.0** (C-3‴), **79.4** (C-3), **103.5** (C-1″), **104.0** (C-7), **105.9** (C-8α), **108.9** (C-5), **109.5** (C-1‴), **113.5** (C-3′,5′), **127.6** (C-2′,6′), **130.5** (C-1′), **144.1** (C-4α), **160.0** (C-4′), **161.6** (C-8) **163.9** (C-6), **164.9** (C-1). HRESIMSMS: *m/z* 581.1863 $[M + H]^+$ (calculated 581.1865), 449.1425 $[M + H\text{-}132]^+$, 287.0904 $[M + H\text{-}132\text{-}162]^+$.

Scorzonerol (**2**). UV (MeOH): 229, 268, 307sh, 319, 348 nm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H **6.22** (1H, d, *J* 2 Hz, H-5), **6.32** (1H, d, *J* 2 Hz, H-7), **6.78** (2H, d, *J* 8.8 Hz, H-3′,5′), **6.83** (1H, s, H-4), **7.63** (2H, d, *J* 8.8 Hz, H-2′,6′). ^{13}C-NMR (125 MHz, CD_3OD): δ_C **98.0** (C-8α), **100.3** (C-4), **101.2** (C-5), **102.9** (C-7), **115.3** (C-3′,5′), **123.0** (C-1′), **126.4** (C-2′,6′), **153.4** (C-3), **159.3** (C-4′), **166.0** (C-1). HRESIMSMS: *m/z* 271.0604 $[M + H]^+$ (calculated 271.0601), 243.0643 $[M + H\text{-}28]^+$, 177.0512 $[M + H\text{-}94]^+$, 121.0225 $[M + H\text{-}150]^+$.

3.3. Antioxidant activity and TPC studies

Antioxidant activity of PE, $CHCl_3$, AcOEt and BuOH soluble parts were evaluated by ferric *reducing/*antioxidant power assay (FRAP) (Benzie and Strain 1996), inhibition of lipid peroxidation (Duh et al. 1999), scavenging activity of DPPH (Brand-Williams et al. 1995), superoxide (Nishikimi et al. 1972) and ABTS (Re et al. 1999) radicals. Total phenolic contents of the fractions were determined by using a colourimetric method described by Slinkard and Singleton (1977).

3.4. Antimicrobial activity

PE, $CHCl_3$, AcOEt and BuOH soluble parts were tested against *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Pseudomonas aeruginosa* ATCC 1539, *Enterococcus feacalis* ATCC 29212 and *Candida albicans* ATCC 10231 using microbroth dilution technique (CLSI 2000, 2006).

3.5. Anti-inflammatory activity

A COX inhibitor screening assay kit (Cayman 560131) was employed to determine the inhibition of COX enzymes by the fractions. The assay was performed according to the kit protocol.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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