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Two Acylated Isoscutellarein Glucosides with Anti-Inflammatory and Antioxidant Activities Isolated from Endemic *Stachys Subnuda* Montbret & Aucher ex Benth

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Abstract

In this study, we report anti-inflammatory and antioxidant activities of two acylated isoscutellarein glucosides isolated from ethyl acetate extract of *Stachys subnuda* aerial part. 4′-*O*-methylisoscutellarein-7-*O*-2′′-*O*-(6′′′-*O*-acetyl-*β*-D-allopyranosyl)-*β*-D-glucopyranoside (SS1) and isoscutellarein-7-*O*-2′′-*O*-(6′′′-*O*-acetyl-β-D-allopyranosyl)-*β*-D-glucopyranoside (SS2) were isolated as major compounds from ethyl acetate extract (SSEA). Also, 2 hydroxycinnamic acid derivatives, and 5 isoscutellarein glucoside derivatives in the SSEA were identified using LC-MS/MS. SS1 with IC_{50} values of 2.35 and 1.98 μg/mL and SS2 with IC_{50} values 13.94 and 12.76 μg/mL showed fairly strong antioxidant activity against DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radicals, respectively. SS1 and SS2 inhibited 5-lipoxygenase (5-LOX) activity with IC_{50} values of 47.23 and 41.60 μg/mL, respectively. The results demonstrated that SS1 and SS2 have significant anti-inflammatory and antioxidant potential. Acylated flavonoid glycosides have been first reported for *Stachys subnuda*. Also, the activities of SS1 and SS2 have been investigated for the first time in this study.

Keywords: *Stachys subnuda*; acylated flavonoids; anti-inflammatory activity; antioxidant activity; LC-MS/MS

1. Introduction

The *Stachys* genus is one of the largest species of the Lamiaceae family and is represented by about 300 species worldwide.[1] Also, Turkey is one of the richest countries in terms of variety of *Stachys* species and this genus is represented by 91 species with an endemism rate of 48%. *Stachys* species known as “Deli sage” or “Mountain tea” in Anatolia are used in the skin diseases, ulcers, cancer, respiratory diseases and kidney diseases by the people because of their antibacterial, anti-inflammatory, antipyretic, antioxidant and cytotoxic effects.[2] In many countries, especially in the Mediterranean regions, *Stachys* species are consumed as herbal tea (Mountain Tea), food and herbal remedies.[3]

There is only one report on the chemical composition and biological activity of the essential oil of *Stachys subnuda* in the literature.[4] However, no study on isolation of flavonoids from this species and their biological activities have yet been reported. In the studies on other *Stachys* species, it has revealed that secondary metabolites are generally iridoids, flavone glycosides, diterpenes and essential oils.[2,5–7] Also, it has been reported that various extracts of *Stachys* species have antioxidant, anti-proliferative, anti-inflammatory, antiulcer, antinociceptive, antimicrobial activities.[8–10] There is no scientific information on chemical composition of *Stachys subnuda* ethyl acetate extract and anti-inflammatory and antioxidant activities of its active major compounds. Therefore, the aim of this study was to test anti-inflammatory and antioxidant activities of major compounds isolated from ethyl acetate extract of aerial parts of endemic *Stachys subnuda* Montbret & Aucher Ex Benth.

2. Experimental

2. 1. Plant Material

Aerial parts of plant were collected in the flowering period from the Tunceli province of Turkey in 2015 and identified by Dr. Ahmet Dogan, a botanist of the Faculty of Pharmacy, University of Marmara. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Marmara University (MARE No: 17720).

2. 2. Extraction

Dried and ground aerial parts of *Stachys subnuda* (10 g) for activities were extracted with CH_3OH (3 × 100 mL), using an ultrasonic bath. After filtration and evaporation, the residue (SSM) was dissolved in 50 mL 50% aqueous methanol, and subjected to solvent-solvent partition between n-hexane (3 × 50 mL), chloroform (3 × 50 mL) and ethyl acetate (3 × 50 mL). The n-hexane, chloroform, ethyl acetate and aqueous methanol extracts were coded as SSH, SSC, SSEA and SSAM, respectively. Also, about 140 g of the plant was weighed for isolation and similar extraction procedures described above were carried out. All extracts were stored under refrigeration for further analysis.

2. 3. *In vitro* Anti-Inflammatory Activity

The anti-inflammatory activity was evaluated according to the method described by Phosrithong et al.[11] An aliquot of 500 μL at different concentrations of isolated compounds was added to 250 μL of 0.1 M borate buffer pH 9.0, followed by addition of 250 μL of type V soybean lipoxygenase solution in buffer (20.000 U/mL). After the mixture was incubated at 25 °C for 5 min, 1000 μL of 0.6 mM linoleic acid solution was added, mixed well and the change in absorbance at 234 nm was recorded for 6 min. Indomethacin was used as a reference standard. The percent inhibition was calculated from the following equation:

$$\% \text{ inhibition} = [(A_{control} - A_{sample}) / Ac_{ontrol}] \times 100 \quad (1)$$

A dose-response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum anti-inflammatory activity. All tests and analyses were performed in triplicates.

2. 4. DPPH Radical Scavenging Activity

Free radical scavenging capacity of isolated compounds and extracts were evaluated according to the previously reported procedure using the stable DPPH.[12] Briefly, 10 μL of sample in DMSO at different concentrations (125–0.24 μg/mL) were added to 190 μL methanol solution of DPPH (0.1 mM) in a well of 96-well plate. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 517 nm. The percent radical scavenging activity of extracts and standard against DPPH were calculated according to the following:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (2)$$

where A_0 is the absorbance of the control (containing all reagents except the test extracts or compounds), and A_1 is the absorbance of the extracts/standard. Extracts or compounds concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extracts concentration. Tests were carried out in triplicate. BHA, Ascorbic acid and Trolox were used as positive control.

2. 5. ABTS Radical-Scavenging Activity

Free radical scavenging capacity of isolated compounds and extracts was evaluated according to the previously reported procedure.[12] ABTS radical cations were prepared by mixing equal volume of ABTS (7 mM in H_2O) and potassium persulfate (4,9 mM in H_2O), allowing them to react for 12–16 h at room temperature in the dark. Then, ABTS radical solution was diluted with 96% ethanol to an absorbance of about 0.7 at 734 nm. 10 μL of sample in DMSO at different concentrations (125–0.24 μg/mL) were added to 190 μL of ABTS radical solution in a well of 96-well plate. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 734 nm. The percent radical scavenging activity of extracts and standards against ABTS were calculated according to the following:

$$\text{ABTS radical-scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (3)$$

where A_0 is the absorbance of the control (containing all reagents except the test extracts or compounds), and A_1 is the absorbance of the extracts/standards. Extracts or compounds concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extracts concentration. Tests were carried out in triplicate. BHA, ascorbic acid and trolox were used as positive control.

2. 6. Quantitative Determination of the Total Phenolic Contents of *Stachys Subnuda* Extracts

Total phenolic compound content of extracts was determined according to Gao et al.[13] The assay was adapted to the 96 well microplate format. 10 μL of extracts in various concentrations were mixed with 20 μL Folin-Ciocalteu reagent (Sigma), 200 μL of H_2O, and 100 μL of 15% Na_2CO_3 and the absorbance was measured at 765 nm after 2 h incu-

bation at room temperature. Gallic acid was used as a standard and the total phenolic contents of extracts were expressed as mg/g gallic acid equivalents (GAE)

2. 7. Quantitative Determination of the Total Flavonoid Contents of *Stachys Subnuda* Extracts

Total flavonoid compound content of extracts was determined according to Zhang et al.[14] The assay was adapted to the 96 well microplate format. 25 µL of extracts in various concentrations were mixed with 125 µL of H_2O, and 7.5 µL of 5% $NaNO_2$. After 6 min, 15 µL of 10% $AlCl_3$ solution was added and incubated for 5 min, followed by the addition of 50 µL of 1 M NaOH solution. Distilled water was added to bring the total volume to 250 µL, and the absorbance was immediately measured at 510 nm using a Shimadzu UV-1800 spectrometer. Catechin was used as a standard and total flavonoid content was expressed as mg/g catechin equivalents (CE)

2. 8. Isolation of Active Compounds

In antioxidant activity experiments, ethyl acetate extract showed the best antioxidant activity among all extracts. Therefore, ethyl acetate extract was chosen for isolation. The ethyl acetate extract (2.02 g) was fractionated by CC on silica gel, using a gradient system of $CHCl_3$/EtOAc/CH_3OH to yield twenty five fractions. Fractions showing similar TLC profiles were combined to give six sub-fractions (F7-F8: 1.00 g, F9-F10: 0.46 g, F11-F13: 0.20 g, F14-F18: 0.15 g, F19-F22: 0.06 g, F23-F25: 0,04 g). DPPH activity test was performed on these fractions. It has been continued the isolation with F7-F8, having the highest DPPH radical scavenging activity and the most intense compound content on TLC among all fractions [DPPH radical inhibition rate at concentration of 10 µg/mL: 47% (F7-F8), 22% (F9-F10), 16% (F11-F13), 44% (F14-F18), 32% (F19-F22), 31% (F23-F25)]. F7-F8 (1,0 g) was repeatedly chromatographed on a Sephadex LH-20 column, eluted with CH_3OH and then combined sub-fractions was re-chromatographed by preparative TLC with $CHCl_3$: CH_3OH:H_2O (4:1:2 drops) to give SS1 (32 mg) and SS2 (43.6 mg) (Figure 1, Detailed spectral data is included in supporting information).

2. 9. LC-MS/MS Analysis

LC-MS/MS analysis was carried out using an Absciex 3200 Q trap MS/MS dedector. Experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC- MS/MS instrument equipped with an ESI source operating in negative ion mode. For the chromatographic separation, ODS 150 × 4.6 mm, i.d., 3 µm particle size, octadecyl silica gel analytical column operating at 40 °C has been used. The solvent flow rate was maintained at 0.5 mL/min. Detection was carried out with PDA detector. The elution gradient consisted of mobile phases (A) acetonitrile:water:formic acid (10:89:1, v/v/v) and (B) acetonitrile:water:formic acid (89:10:1, v/v/v). The composition of B was increased from 10% to 100% in 40 min. LC-ESI-MS/MS data were collected and processed by Analyst 1.6 software. For enhanced mass scan (EMS), the MS was operated at mass range of 100–1000 amu. Enhanced product ion spectra were measured from m/z 100 up to *m/z* 1000. Nitrogen was used as the collision gas, and the collision energy was set at 30. The parameters were as follows: Collusion Energy Spread (CES)-0, Declustiring Potential (DP)-20, Enterance Potential (EP)-10, Curtain gas (CUR)-20, Gas Source 1 (GS1)-50, Gas Source 2 (GS2)-50, CAD- medium, Ihe- on and Temperature (TEM)-600. For the IDA experiment, the criteria were arranged for ions greater than 100.000 *m/z* and smaller than 1000 *m/z*, and excluded former target ions after 3.0 occurrence(s) for 3.000 seconds.

2. 10. Statistical Analysis

The data were given as means±standard deviations and analysed by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison tests using GraphPad Prism 5. Differences between means at $p<0.05$ levels were considered significant.

3. Results and Discussion

In the present study, it was evaluated anti-inflammatory and antioxidant activities of major compounds isolated from ethyl acetate extract of aerial parts of endemic *Stachys subnuda*. Two acylated flavone glycosides, SS1 and

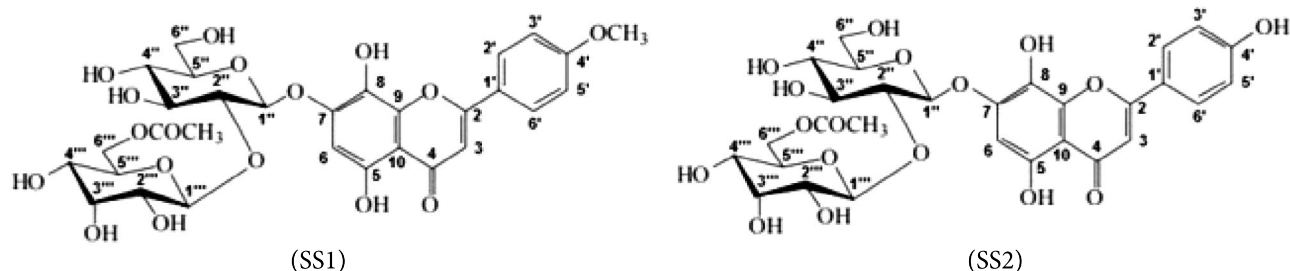

Figure 1. Chemical structures of major compounds isolated from *S. subnuda*

SS2, were isolated as major compounds from active F7-F8 sub-fraction of ethyl acetate extract (SSEA) of *S. subnuda* aerial part (Figure 1). All isolated compounds were analyzed by spectroscopic methods (^{1}H NMR and ^{13}C NMR-APT) and their data were compared with those reported in the literature.[15,16] To the best of our knowledge, there is no study on flavonoids of *S. subnuda*. However, these compounds have been previously isolated from *Stachys recta*.[17] In previous studies, it has been reported that different acetylated flavonoids have been isolated from other *Stachys* species.[5,6,9,17–19]

Seven phenolic compounds including quinic acid (SS3), 5-caffeoylquinic acid (SS4), isoscutellarein 7-*O*-allosyl(1–2)glucoside (SS5), isoscutellarein 7-*O*-[6‴-*O*-acetyl] allosyl(1→2)glucoside (SS2), 4′-*O*-methylisoscutellarein 7-*O*-allosyl(1→2)glucoside (SS6), 4′-*O*-methylisoscutellarein 7-*O*-allosyl(1→2)-[6‴-*O*-acetyl]-glucoside (SS1), 4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1–2)-[6″-*O*-acetyl]-glucoside (SS7)", were detected by LC/MS-MS (Figure 2) (Table 1).

The results of this analysis showed that the main phenolic compounds of the *S. subnuda* ethyl acetate extract are SS1 and SS2. Also, these two compounds were isolated from the ethyl acetate extract of *S. subnuda* in the present study. SS3 showed a pseudo molecular ion peak at *m/z* 191 that fragmented to several ions at *m/z* 173 and *m/z* 127. This fragmentation pattern is characteristic for quinic acid.[23] SS4 presented the molecular ion peak at *m/z* 353 [M-H]$^-$ which fragmented to *m/z* 191 (base peak) *m/z* 179, *m/z* 161 and *m/z* 135. According to Clifford and colleagues, this compound must be caffeoylquinic acids. Moreover, less amount of ion at *m/z* 179 indicates that the caffeic acid and quinic acid are linked at 3 positions. So, SS4 was identified as 3-caffeoylquinic acid.[23] SS5 and SS2 presented pseudo molecular ion peaks at *m/z* 609 and *m/z* 651, respectively. Both compounds showed the same aglycon ion at *m/z* 285. Luteolin, kaempferol, isoscutellarein etc. show the same molecular ion peak at *m/z* 285 [M-H]$^-$. Several studies published previously about *Stachys* species, indicate that isoscutellarein is dominant flavonoid aglycon for *Stachys*

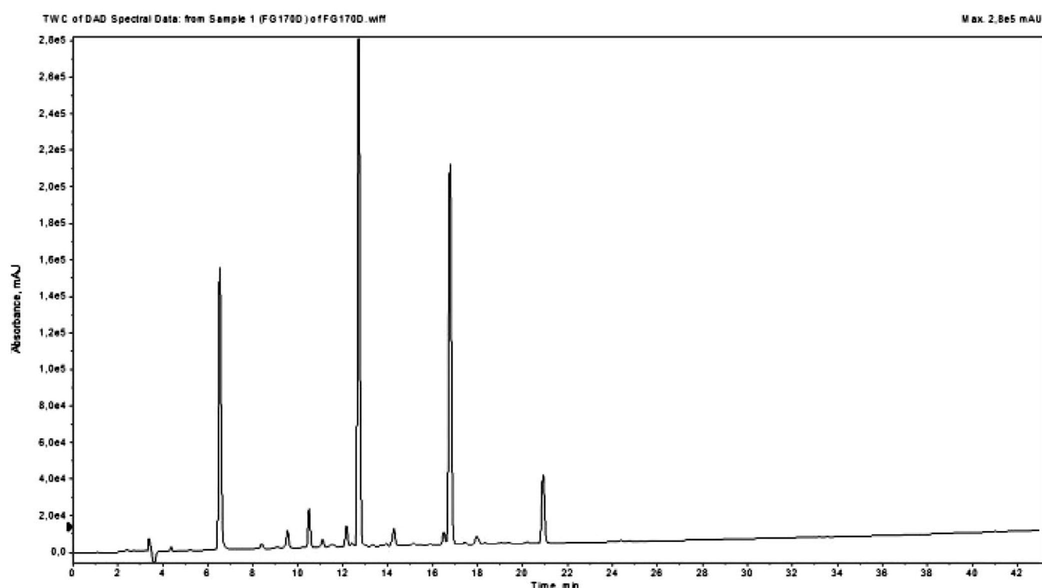

Figure 2. LC-MS/MS chromatogram of ethyl acetate extract of *S. subnuda*

Table 1. Characterization of phenolic compounds in the ethyl acetate extract of *S. subnuda*

No	Rt min	[M-H]$^-$ *m/z*	MS^2	Identified as	References
SS3	3.3	191	173, 127	Quinic acid	[20]
SS4	6.7	353	191, 179, 161, 135	5-Caffeoylquinic acid	[21]
SS5	10.7	609	429, 285	Isoscutellarein 7-*O*-allosyl(1–2)glucoside	[21]
SS2	12.9	651	609, 591, 447, 429, 285	Isoscutellarein 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside (Main compound)	[20]
SS6	14.5	623	461, 299, 284	4′-*O*-methylisoscutellarein 7-*O*-allosyl(1→2)glucoside	[20]
SS1	17.0	665	299,284	4′-*O*-methylisoscutellarein 7-*O*-allosyl(1→2)-[6‴-*O*-acetyl] -glucoside	[20,22]
SS7	21.2	707	665, 647, 503, 485, 443, 351, 299, 284	4′-*O*-Methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl (1-2)-[6"-*O*-acetyl]-glucoside	[20]

genus.[24,25] Therefore, isoscutellarein was determined as the aglycon of SS5 and SS2. (NMR spectra also supported that the isoscutellarein is an aglycon of this plant material). 324 amu difference between molecular ion peak and aglycon was indicated that isoscutellareain are linked with two-sugar unit. Moreover, the ion at *m/z* 429 ([M-H]$^-$–180) indicates that glycosylation position of the sugars is 1→2.[22,25] So, according to literature data and MS spectrums, these compounds were identified as Isoscutellarein 7-*O*-allosyl (1–2) glucoside (SS5) and Isoscutellarein 7-*O*-[6′′′-*O*-acetyl] allosyl (1→2) glucoside (SS2). According to Petreska et al., there are two substances with the same molecular weight with SS2 which presents similar fragmentation characteristics. The difference between these two compounds is that the acetylation positions of the sugar are different from each other.[22] NMR spectrum showed that sugar united acetylated at 6′′′ position for SS2 which was also isolated from the plant material. SS6, SS1 and SS7 showed a molecular ion peak at *m/z* 623 [M-H]$^-$, 665 [M-H]$^-$ and 707 [M-H]$^-$, respectively. These compounds presented the same base peak. Ion at m/z 299 that fragmented at *m/z* 284 is due to the loss of a methyl unit. Methylisoscutellerain is a flavonoid, which is found in many *Stachys* species.[24] Loss of 324 amu between SS6 and its aglycone indicates that SS6 is dihexoside of methylisoscutellerain. Loss of 366 amu (dihexose+acetyl) from molecular ion peak indicates that SS1 is acetyldihexose of methyl isoscutellerain. The difference 408 amu between SS7 and its aglycon indicates that SS7 is diacetyldihexose of methyl isoscutellerain. According to this findings and literature data survey, SS6, SS1 and SS7 identified as 4′-*O*-methylisoscutellarein 7-*O*-allosyl (1→2) glucoside, 4′-*O*-methylisoscutellarein 7-*O*-allosyl (1→2)-[6′′′-*O*-acetyl]-glucoside and 4′-*O*-Methylisoscutellarein 7-*O*-[6′′′ -*O*-acetyl]-allosyl (1–2)-[6′′-*O*-acetyl]-glucoside, respectively.

All of the extracts except for the SSH extract were found to have a significant antioxidant activity. SSEA extract showed the highest antioxidant activity in DPPH and ABTS assays with IC_{50} values of 3.7 and 5.3 μg/mL, respectively. Also, the highest total phenol and total flavonoid content were found in the SSEA (219.4 and 78.3 mg/g, respectively) (Table 2). There are no reports on antioxidant activity of extracts and isolated flavonoids from *Stachys subnuda* but a large number of studies are available on different *Stachys* species in the literature. In these studies, *Stachys* species have been reported to exhibit a significant antioxidant activity. In one of these studies, Erdemoglu et al.[26] reported that water extract of *Stachys byzantine* was active against the DPPH radical with an IC_{50} value of 640 μg/mL. In another study, the radical scavenging activity of *Stachys glutinosa* ethanol extract was found to be 280 μg/mL and 320 μg/mL of IC_{50} values in DPPH and ABTS experiments, respectively.[8] Ghasemi et al.[27] reported that methanol extract of *Stachys lavandulifolia* against DPPH and ABTS radicals had antioxidant activity with the IC_{50} values of 2320 and 3770 μg/mL, respectively. In the same study, it had been suggested that total phenolic and flavonoid contents of this species were 99 mg/g and 9.05 mg/g, respectively. Also, Šliumpaitė et al.[28] reported that total phenol content of methanol extracts of *Stachys officinalis* was 61.2 mg/g, expressed as gallic acid equivalent. When compared these results with our current study, it can be seen that *Stachys subnuda* has a better antioxidant activity and a higher total phenolic and flavonoid content. Phenolic compounds present in plants are known to be powerful antioxidants.[29] Therefore, these compounds may be responsible for the antioxidant activity of the plant.

Isolated two acylated flavone glycosides were tested for their DPPH and ABTS radical scavenging activity. SS1 showed the highest antioxidant activity in DPPH and ABTS assays with IC_{50} values of 2.35 and 1.98 μg/mL, followed by SS2 (13.94 and 12.76 μg/mL), respectively. Also, SS1 showed better antioxidant activity compared to standards (Table 3). Delazar et al.[18] investigated antioxidant activity of two flavonoid glycosides, chrysoeriol 7-*O*-[6-*O*-acetyl-*β*-D-allopyranosyl]-(1′2)-*β*-D-glucopyranoside and apigenin 7-*O*-*β*-D-(6-*p*-coumaroyl)-glucopyranoside, isolated from *Stachys bombycina* and these compounds have been demonstrated to possess strong antioxidant activity with of IC_{50} values of 12.5 and 0.77 μg/

Table 2. Antioxidant activities, total phenolic and flavonoid contents of various extracts from aerial parts *S. subnuda*

Extracts*	DPPH activity IC_{50} (μgmL^{-1})**	ABTS activity IC_{50} (μgmL^{-1})	ABTS activity mM TE/g extract***	TPC**** (mg GAE/g extract)[c]	TFC***** (mg CE/g extract)[d]
SSH	180.1 ± 0.26[e]	97.0 ± 1.37[c]	5.1 ± 0.79[a]	33.6 ± 1.54[a]	10.0 ± 0.82[a]
SSC	13.4 ± 0.00[b]	8.2 ± 0.35[a]	62.4 ± 0.92[d]	112.3 ± 3.29[c]	41.5 ± 1.92[b]
SSEA	**3.7 ± 1.00[a]**	**5.3 ± 0.20[a]**	**99.2 ± 2.02[e]**	**219.4 ± 2.18[d]**	**78.3 ± 3.13[d]**
SSM	24.4 ± 0.45[b]	16.0 ± 0.46[b]	31.2 ± 0.19[c]	112.6 ± 1.69[c]	57.4 ± 1.63[c]
SSAM	39.5 ± 1.03[d]	21.8 ± 1.21[b]	20.9 ± 1.65[b]	90.36 ± 2.83[b]	63.5 ± 1.42[c]

*The methanol, *n*-hexane, chloroform, ethyl acetate and aqueous methanol extracts were coded as SSM, SSH, SSC, SSEA and SSAM, respectively.
**Values corresponding to the amount of extract required to scavenge 50% of radicals present in the reaction mixture.
***ABTS radical scavenging activity were expressed as trolox equivalent (TE)
****Total phenolic content (TPC) was expressed as gallic acid equivalent (GAE).
*****Total flavonoid content (TFC) was expressed as catechin equivalent (CE). Each value in the table is represented as mean ± SD (n = 3). Different letter superscripts in the same column indicate significant differences ($p < 0.05$).

Table 3. Antioxidant and anti-inflammatory activities of compounds isolated from *Stachys subnuda* and standards

Compounds/ Standards	DPPH activity	ABTS activity	Anti-inflammatory activity
		IC_{50} (μgmL^{-1})	
SS1	2.35 ± 0,00^{a}	1.98 ± 0,01^{a}	47.23 ± 1.95^{b}
SS2	13.94 ± 1,65^{b}	12.76 ± 0,00^{b}	41.60 ± 2.09^{b}
Ascorbic acid	17.60 ± 0.37^{b}	14.50 ± 0.32^{b}	
Trolox	14.54 ± 0.18^{b}	13.00 ± 0.21^{b}	
Butylated hydroxyanisole	57.15 ± 0.09^{c}	17.06 ± 0.58^{b}	
Indomethacin			22.39 ± 0.26^{a}

Each value in the table is represented as mean ± SD (n = 3). Different letter superscripts in the same column indicate significant differences ($p < 0.05$).

mL, respectively. Similarly, two active acylated flavone glycosides isolated in present study, especially SS1, showed strong antioxidant activity. It has also been found that the SS1 has very good antioxidant activity when compared to the standards. When the results of the antioxidant activity of the ethyl acetate extract are compared with the isolated compounds, it might be considered that these compounds are responsible for the activity of the ethyl acetate extract. Also, flavonoids are generally known to be compounds with antioxidant activity.[30] For this reason, it can be assumed that these groups of compounds are responsible for the activity of the extract.

Each value in the table is represented as mean ± SD (n = 3). Different letter superscripts in the same column indicate significant differences ($p < 0.05$).

SS1 and SS2 displayed 5-lipoxygenase (5-LOX) inhibitory activity with IC_{50} values of 47.23 and 41.60 μg/mL (Table 3). As far as we know, there is no report on anti-inflammatory activity of these compounds. However, in a previous study, Alcaraz et al.[31] reported that isoscutellarein, a flavonoid aglycone, inhibited 15-lipoxygenase (15-LOX) activity. Also, Yoshimoto et al.[32] have shown that flavonoids are potent inhibitors of 5-lipoxygenase. Similarly, SS1 and SS2, the flavonoids isolated from ethyl acetate extract of *S. subnuda* aerial part in our current study, showed good anti-lipoxygenase activity and these results are compatible with previous findings.

4. Conclusion

These results indicate that SS1 and SS2 with other compounds present in ethyl acetate extract of *Stachys subnuda* may be responsible for the anti-inflammatory and antioxidant activity of the extract. Also, these results show that SS1 and SS2 isolated from *Stachys subnuda* have strong antioxidant and good anti-inflammatory activity. These isolated compounds can be used as a natural antioxidant source in food, pharmaceutical and cosmetic industries. However, further studies, such as *in vivo* tests, are needed to clarify the antioxidant and anti-inflammatory effects of these compounds.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary Data

Spectral data of compounds isolated from ethyl acetate extract of aerial parts of Endemic *Stachys subnuda* can be found in Appendix.

List of Abbreviations

5-LOX: 5-lipoxygenase
ABTS: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
BHA: Butylated hydroxyanisole
CE: Catechin equivalent
DMSO: Dimethyl Sulfoxide
DPPH: 1,1-diphenyl-2-picrylhydrazyl
EtOAc: Ethyl acetate
F7-F8: Fraction 7- Fraction 8
F9-F10: Fraction 9- Fraction 10
F11-F13: Fraction 11- Fraction 13
F14-F18: Fraction 14- Fraction 18
F19-F22: Fraction 19- Fraction 22
F23-F25: Fraction 23- Fraction 25
GAE: Gallic acid equivalent
HPLC: High Performance Liquid Chromatography
IC_{50}: The concentration of extract that sweeps 50% of the radical or inhibits the activity of the enzyme by 50%
LC-MS/MS: Liquid chromatography linked to tandem mass spectrometry
MARE: Herbarium of the Faculty of Pharmacy, Marmara University
ODS: Octadecyl-silica
SS1: 4′-*O*-methylisoscutellarein-7-*O*-2′′-*O*-(6′′′-*O*-acetyl-*β*-D-allopyranosyl)-*β*-D glucopyranoside

SS2: İsoscutellarein-7-*O*-2′′-*O*-(6′′′-*O*-acetyl-β-D-allopyranosyl)-*β*-D-glucopyranoside
SS3: Quinic acid
SS4: 5-Caffeoylquinic acid
SS5: Isoscutellarein 7-O-allosyl(1 -2)glucoside
SS6: 4′-O-methylisoscutellarein 7-O-allosyl(1→2) glucoside
SS7: 4′-O-Methylisoscutellarein 7-O-[6′′′-O-acetyl]-allosyl(1-2)-[6′′-O-acetyl]-glucoside
SSM: Methanol extract of *Stachys subnuda*
SSH: Hexane fraction of methanol extract of *Stachys subnuda*
SSC: Chloroform fraction of methanol extract of *Stachys subnuda*
SSEA: Ethyl acetate fraction of methanol extract of *Stachys subnuda*
SSAM: Aqueous methanol fraction of methanol extract of *Stachys subnuda*
TE: Trolox equivalent
TFC: Total flavonoid content
TLC: Thin layer chromatography
TPC: Total phenolic content

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Povzetek

V tej študiji, smo poročali o protivnetni in antioksidativni dejavnosti dveh aciliranih izoscutelarinskih glukozidov, izoliranih iz etil acetatnega ekstrakta Stachys subnuda it dela zraka. 4′-o-metilisoscutelaren-7-O-2′,-O-(6‴-O-acetil-β-D-alopiranosil)-β-D-glucopiranosid (1) in izooscutelaren-7-O-2″-O-(6 ‴-O-acetil-β-D-alopiranosl)-β-D-gluopiranoside (2) so bile izolirane kot glavne spojine iz etil acetatnega izvleček (EAE). Tudi 2 derivata hidroksicinične kisline, in 5 isoscutelaren glukozidni derivati v EAE so bili najdeni z uporabo LC-MS/MS. Spojina 1 z IC50 vrednostjo 2,35 ve 1,98 μg/mL in spojina 2, z IC50 vrednosti 13,94 ve 12,76 μg/mL, je pokazala dokaj močno antioksidantno aktivnost proti radikalom DPPH in ABTS. Spojini 1 in 2 sta inhibirali 5-lipooksigenazno (5-LOX) aktivnost z IC50 vrednostmi približno 47,23 in 41,60 μg/mL. Rezultati so pokazali, da imajo izolirane spojine pomembno protivnetno in antioksidativno zmožnost. Acetilirane flavonoidni glikozidi so bili prvič poročani za Stachys subnuda. Tudi njihove dejavnosti so bile prvič preiskane v tej študiji.

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