

Flavonoid Derivatives from the Aerial Parts of *Trifolium trichocephalum* M. Bieb. and Their Antioxidant and Cytotoxic Activity

Gülin Renda^{1*}, Ufuk Özgen¹, Zeynep Ünal², Suna Sabuncuoğlu²,
Erhan Palaska³ and İlkay Erdoğan Orhan⁴

¹Karadeniz Technical University, Faculty of Pharmacy, Department of Pharmacognosy, Trabzon, Türkiye

²Hacettepe University, Faculty of Pharmacy, Department of Toxicology, Ankara, Türkiye

³Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Ankara, Türkiye

⁴Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Türkiye

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Abstract: *Trifolium* L. species with a rich isoflavone content have been used as expectorant, analgesic, antiseptic, tonic, and wound-healer in folk medicine. The aim of the study is to evaluate pharmacological properties of the extracts and isolated compounds of *T. trichocephalum*. Phytochemical investigation of the aerial parts of *T. trichocephalum* led to the isolation of daidzein, genistein, quercetin, and daidzein 4'-*O*- β -glucoside for the first time from this species. Isolated compounds along with the methanol extract, water, ethyl acetate and chloroform subextracts were tested for their radical scavenging and cytotoxic activity which was evaluated by MTT assay. According to the results of activity tests, extracts showed a concentration-dependent radical scavenging activity as well as cytotoxic effect on HepG2 cells at 400 μ g/mL, whereas the compounds did not exert any obvious cytotoxic effect at tested concentrations.

Keywords: Antioxidant activity; cytotoxicity; Fabaceae; isoflavonoids; *Trifolium trichocephalum*. © 2017 ACG Publications. All rights reserved.

1. Plant Source

The genus *Trifolium* L. (Fabaceae) is represented by 300 species around the world and 103 species in Turkish flora [1]. The aerial parts of *T. trichocephalum* were collected from İkizdere town (Rize Province, Turkey) in June, 2011 and identified by one of authors; Dr. Gülin Renda. A voucher specimen (coded as HUEF 09335) was deposited at the Herbarium of Faculty of Pharmacy, Hacettepe University (Ankara, Turkey).

2. Previous Studies

In Turkish folk medicine, *Trifolium* species have been recorded to be used as expectorant, antiseptic, wound-healer, sedative, anticancer, antidiabetic and for the treatment of rheumatism pain,

* Corresponding author: E- Mail: grenda@ktu.edu.tr

menopausal or premenstrual symptoms [2-4]. Over ground parts of some *Trifolium* species are consumed as snack [5]. *Trifolium* species are also forage crops that have economic value; especially *Trifolium pratense* L. is largely cultivated [6]. Up to date, many phytochemical investigations were performed on *Trifolium* species from which mainly isoflavonoids, saponins, flavonoids, and megastigmane glycosides have been isolated [4, 7-11]. *Trifolium* species, known as clover, have been patented as commercially available extracts on the market all over the world [4].

Trifolium trichocephalum M. Bieb. is a perennial species growing at eastern regions of Turkey [1]. Luteolin 3'-*O*-D-glucoside, luteolin 7'-*O*-D-glucoside, and luteolin were reported to be isolated from *T. trichocephalum* previously [12]. Since chemical content of plant species grown in different geographic locations may show variability, phytochemical properties of *T. trichocephalum* have been decided to be re-investigated in the current study. This is the first study that reports some pharmacological properties of the extracts and isolated compounds of *T. trichocephalum*.

3. Present Study

The dried aerial parts of *T. trichocephalum* (260 g) were extracted with methanol (MeOH, 1500 mL × 4) at 40°C. The MeOH extract (51.0 g) was dissolved in H₂O-MeOH (9:1) mixture and partitioned with chloroform and then ethyl acetate, which were concentrated under reduced pressure to give the sub-extracts (10.4 and 3.2 g, respectively). The remaining aqueous sub-extract was 36.2 g.

0.9 g of ethyl acetate sub-extract was subjected to a gel chromatography (Sephadex LH-20) column and eluted with MeOH; 17 fractions were collected. Fraction 4 (25 mg) gave compound **1** (20 mg). The fractions 5-17 (0.8 g) were combined and further applied to silica gel column chromatography using CHCl₃:MeOH:H₂O (80:20:2; 70:30:3) solvent system. The sub-fractions 3-4 (0.2 g) of silica gel column chromatography were combined and subjected to gel chromatography (Sephadex LH-20) eluting with MeOH and preparative TLC, respectively, that yielded compound **2** (22 mg). The sub-fractions 5-7 (0.19 g) eluted from silica gel column were also combined and purified by gel chromatography (Sephadex LH-20) eluting with MeOH to give compound **3** (30 mg). 32 g of aqueous sub-extract was subjected to reversed phase silica gel column chromatography using 0-100% aqueous MeOH as solvent systems. Fraction 9 gave compound **4** (25 mg).

Compound 1: ¹H NMR (200 MHz, CD₃OD) δ: 8.28 (1H, H-2, s), 7.95 (1H, H-5, d, *J*= 9.0 Hz), 7.36 (2H, H-2',6', d, *J*= 8.2 Hz), 6.91 (1H, H-6, d, *J*= 8.6 Hz), 6.85 (1H, H-8, s), 6.79 (2H, H-3',5', d, *J*= 8.6 Hz); ¹³C NMR (50 MHz, CD₃OD) δ: 176.2 (C-4), 164.0 (C-7), 158.9 (C-9), 158.7 (C-4'), 154.4 (C-2), 131.6 (x2C) (C-2',6'), 128.8 (C-5), 125.0 (C-1'), 124.0 (C-3), 118.1 (C-10), 116.6 (C-6), 116.4 (x2C) (C-3',5'). Data agree with the data given in the literature for daidzein [13] (Figure 1).

Compound 2: ¹H NMR (200 MHz, CD₃OD): δ 8.10 (1H, H-2, s), 7.38 (2H, H-2',6', d, *J*= 8.2 Hz), 6.86 (2H, H-3',5', d, *J*=8.2 Hz), 6.34 (1H, H-8, s), 6.22 (1H, H-6, s); ¹³C NMR (50 MHz, CD₃OD): δ 179.4 (C-4), 169.2 (C-7), 161.0 (C-5), 156.9 (C-9), 156.0 (C-4'), 152.0 (C-2), 128.6 (x2C) (C-2',6'), 121.9 (C-3), 120.5 (C-1'), 113.4 (x2C) (C-3', 5'), 101.3 (C-10), 97.3 (C-6), 92.0 (C-8). ¹H NMR and ¹³C NMR data agree with the data given in the literature for genistein [13,14] (Figure 1).

Compound 3: C₁₅H₁₀O₇ (mol. wt. 302); ESI-MS: *m/z*: 303.04 [M+H]⁺; ¹H NMR (600 MHz, DMSO-*d*₆) δ: 7.61 (1H, H-2', d, *J*=2.4 Hz), 7.50 (1H, H-6', dd, *J*=2.4, 8.8 Hz), 6.85 (1H, H-5', d, *J*= 8.8 Hz), 6.38 (1H, H-8, d, *J*= 1.8 Hz), 6.15(1H, H-6, d, *J*= 2.3 Hz); ¹³C NMR (125 MHz, DMSO *d*₆): δ 176.2 (C-4), 164.3 (C-7), 161.1 (C-5), 156.6 (C-9), 148.1 (C-4'), 147.2 (C-2), 145.5 (C-3'), 136.1 (C-3), 122.4 (C-1'), 120.5 (C-6'), 116.0 (C-5'), 115.4 (C-2'), 103.4 (C-10), 98.6 (C-6), 93.8 (C-8) ¹H NMR and ¹³C NMR data agree with the data given in the literature for quercetin [15] (Figure 1).

Compound 4: ¹H NMR (600 MHz, DMSO-*d*₆): δ 8.38 (1H, H-2, s), 8.04 (1H, H-5, d), 7.40 (2H, H-2',6', d), 7.22 (1H, H-8, s), 7.14 (1H, H-6, d), 6.81 (2H, H-3',5', d), 5.11 (1H, H-1'', d), 3.68-3.18 (6H, m, sugar protons); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 180.3 (C-4), 166.8 (C-7), 162.7 (C-9), 162.5 (C-4'),

158.3 (C-2), 135.6 (x2C) (C-2',6'), 132.4 (C-5), 129.1 (C-1'), 127.7 (C-3), 123.9 (C-10), 121.1 (C-6), 120.4 (x2C) (C-3', 5'), 108.8 (C-8), 105.3 (C-1''), 82.6 (C-5''), 81.8 (C-2''), 78.5 (C-3''), 75.0 (C-4''), 66.0 (C-6''). Data agree with the literature for daidzein 4'-*O*- β -glucoside [16] (Figure 1).

The correlation between phenolic compounds and strong antioxidant activity has been showed with many studies up to date [17]. Also it has been known that isoflavonoids show antioxidant activity and some of the beneficial effects of them are reported to be connected with this activity [18]. By this point of view, the ethyl -acetate and water subextracts of the MeOH extract were separated using chromatographic methods to obtain four compounds in total. The structures of the isolated compounds, elucidated by ^1H and ^{13}C NMR experiments, were identified as daidzein, genistein, quercetin, and daidzein 4'-*O*-D-glucoside by comparison of their spectroscopic data (UV, 1D- and 2D-NMR, and MS) with previously published data [13-16].

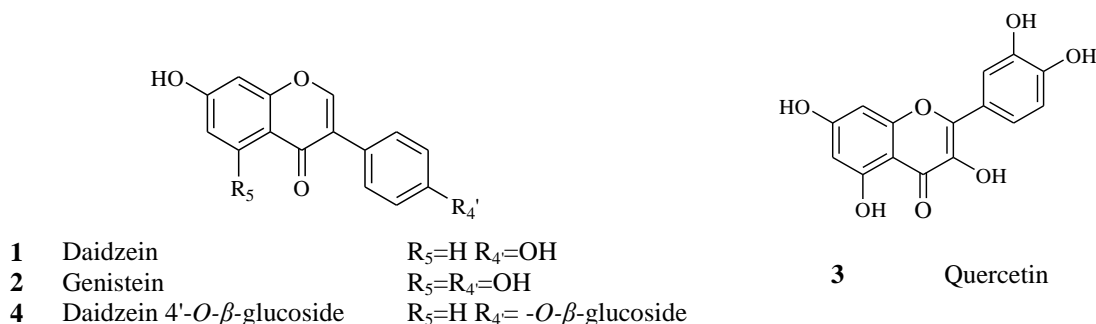


Figure 1. The isolated compounds 1-4.

Furthermore, the radical scavenging effect and cytotoxicity of the methanol extract and water, ethyl acetate, and chloroform subextracts of *T. trichocephalum* as well as four compounds isolated from this plant were examined.

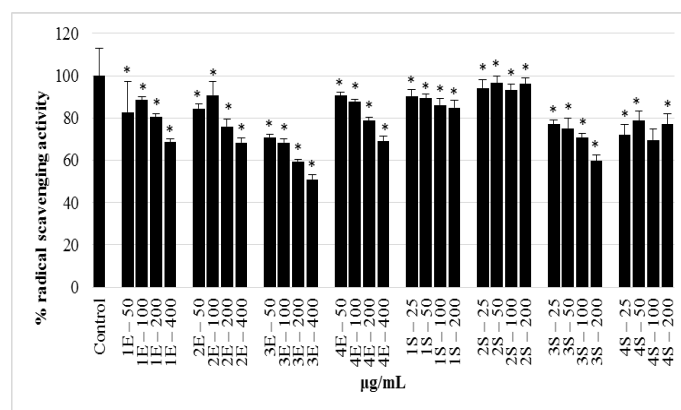


Figure 2. DPPH radical scavenging activity of the extracts and compounds.

1E: MeOH extract, 2E: H₂O subextract, 3E: EtOAc subextract, 4E: CHCl₃ subextract, 1S: Quercetin, 2S: Daidzein-4'-*O*- β -glucoside, 3S: Genistein, 4S: Daidzein.* significantly different from control ($p < 0.05$). Results are expressed as mean \pm SD values of three observations.

DPPH radical scavenging effect: DPPH radical scavenging effect of the extracts and compounds was assessed through the decolorization of the DPPH solution [19]. The samples at various concentrations (50, 100, 200, and 400 $\mu\text{g/mL}$ for extracts; 25, 50, 100 and 200 $\mu\text{g/mL}$ for the compounds) were added to DPPH solution. After incubating the remaining DPPH was determined by spectrophotometry at 517 nm. The radical scavenging activity of each sample was expressed as % \pm standard deviation (SD) compared to blank. The experiment was conducted as triplicate.

SO₂^{•-} radical scavenging effect by alkaline DMSO method: The method of Kunchandy and Rao [20] was used for the detection of SO₂^{•-} scavenging activity of the extract with slight modification [21]. The absorbance was measured at 560 nm using microplate reader (Spectramax). The radical scavenging activity was given as % activity ± SD compared to blank. The experiment was conducted as duplicate.

NO scavenging effect: Nitric oxide (NO) radical scavenging activity of extracts was determined by the method of Tsai et al. [22]. The absorbance was measured at 577 nm. The radical scavenging activity of each sample was expressed as % ± SD compare to blank.

MTT assay for cytotoxicity: Cell viability was evaluated by the reduction of MTT [23, 24]. Briefly, HepG2 cells (10000 cells/well) were treated with the samples at various concentrations (50, 100, 200 and 400 µg/mL for extracts; 25, 50, 100 and 200 µg/mL for compounds). After the incubation with MTT solution, the cells were lysed in DMSO and the MTT formosan was qualified by determining the absorbance at 570 nm. Cell viability was expressed as a percent of the control culture value.

Statistical analysis: The results obtained *in vitro* were statistically processed using Microsoft Excel program. Student's *t*-test was applied and *p* < 0.05 was accepted for statistical significance.

All tested extracts possessed a potent DPPH radical scavenging effect and the maximum effect was observed at 400 µg/mL concentration for each extract. Water extract exhibited 90.84% scavenging effect on DPPH radical at the concentration of 100 µg/mL. All compounds exhibited scavenging effect, among which daidzein 4'-*O*-β-glucoside was the most active one with the inhibition ratio of 96.48% at the concentration of 50 µg/mL.

According to the results obtained from the SO method as seen in Figure. 3, all of the samples showed significant activity. Ethyl acetate subextract and daidzein exhibited the best activity with inhibition ratio of 202.43% (50 µg/mL) and 100.49% (200 µg/mL), respectively.

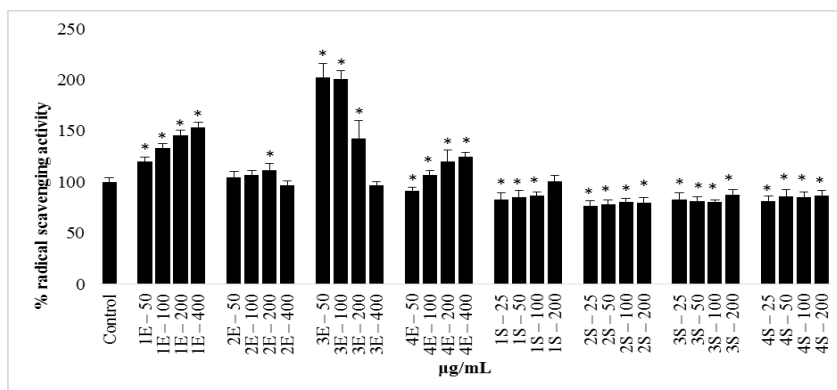


Figure 3. SO radical scavenging activities of the extracts and compounds

1E: Methanol extract, 2E: H₂O subextract, 3E: EtOAc subextract, 4E: CHCl₃ subextract, 1S: Quercetin, 2S: Daidzein-4'-*O*-β-glucoside, 3S: Genistein, 4S: Daidzein. * significantly different from control (*p* < 0.05). Results are expressed as mean ± SD values of three observations.

Application of the NO method revealed the best property for chloroform subextract with an inhibition ratio of 310.69% at 400 µg/mL concentration and, among the compounds, daidzein was the most active one with an inhibition ratio of 168.59% at 50 µg/mL concentration (Figure 4).

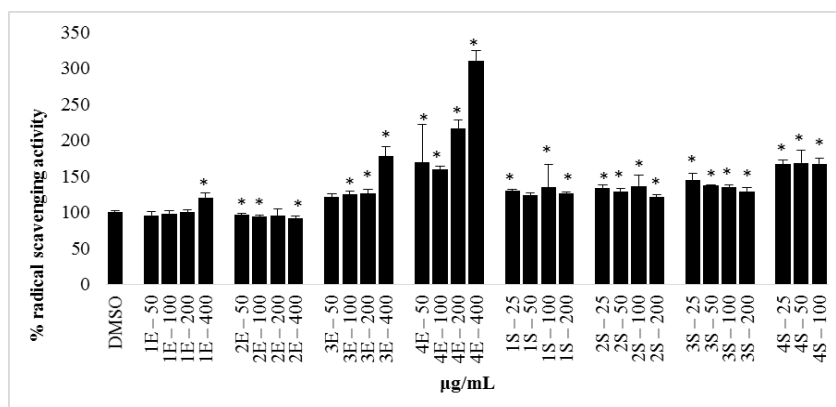


Figure 4. NO radical scavenging activities of the extracts and compounds

1E: Methanol extract, 2E: H₂O subextract, 3E: EtOAc subextract, 4E: CHCl₃ subextract, 1S: Quercetin, 2S: Daidzein-4'-O-β-glucoside, 3S: Genistein, 4S: Daidzein. * significantly different from control (p<0.05). Results are expressed as mean ± SD values of three observations.

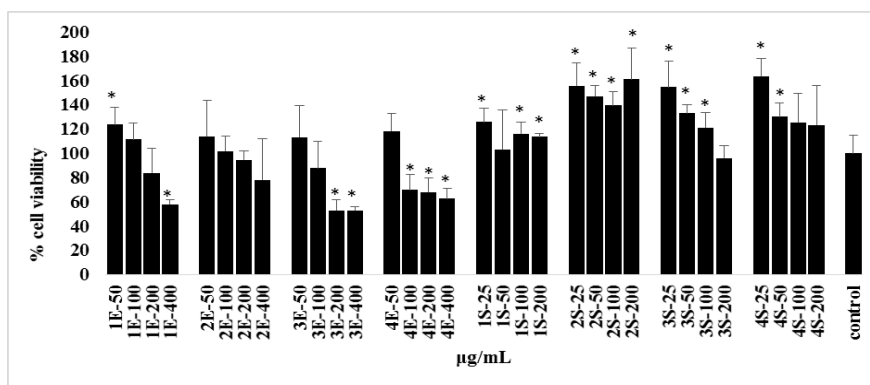


Figure 5. Cytotoxic effect of the extracts and compounds.

1E: Methanol extract, 2E: H₂O subextract, 3E: EtOAc subextract, 4E: CHCl₃ subextract, 1S: Quercetin, 2S: Daidzein-4'-O-β-glucoside, 3S: Genistein, 4S: Daidzein. * significantly different from control (p<0.05). Results are expressed as mean ± SD values of three observations.

Our findings revealed that extracts have higher cytotoxic effect when compared with the compounds which did not show any cytotoxic effect at tested concentrations (Figure. 5). The most active extract was ethyl acetate subextract that exhibited cytotoxic effect at 400 µg/mL. However, the cytotoxic potential of the compounds and extracts were not distinctive. Besides, the cell viability did not drop till 50% at the concentration used.

As a conclusion, in our study, we investigated the radical scavenging and cytotoxic activity of the extracts and compounds obtained from *T. trichocephalum*. Our literature search displayed that no any biological activity studies on *T. trichocephalum* is available up to date. Additionally, daidzein, genistein, quercetin, and daidzein 4'-O-β-glucoside have been isolated for the first time from this species in our study.

In furtherance, the antioxidant capacity of the *T. trichocephalum* extracts presented herein can be attributed possibly to its flavonoid and isoflavonoid constituents. According to the results of our study, especially the isolated compounds can be considered as potential antioxidant agents. Further research on the isolated compounds in terms of therapeutic efficacy and toxicity seems to be useful.

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Supporting Information

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

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