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# Quantification of genistein and daidzein in two endemic *Genista* species and their antioxidant activity

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*Abstract*: In the current research, the total and free genistein and daidzein contents were determined in two endemic *Genista* species (*G. sandrasica* and *G. vuralii*) by an HPLC method. The highest amount of total genistein and total daidzein was found in *G. sandrasica*, 0.582 and 0.113 %, respectively, whereas only the free daidzein content of *G. sandrasica* was higher than that of *G. vuralii*. The antioxidant activity of the crude methanol and hydrolyzed extracts of these species was evaluated by three *in vitro* methods; namely DPPH free radical scavenging, ferrous ion-chelating and ferric-reducing antioxidant power (FRAP) tests at 0.25, 0.50, and 1.0 mg $ml^{-1}$. The hydrolyzed extracts of both species displayed greater antioxidant activity than the crude methanol extracts in all tests. Total phenol and flavonoid contents in the extracts were determined *via* the Folin–Ciocalteau and $AlCl_3$ reagents, respectively. *G. vuralii* was richer in terms of total phenol and flavonoid contents compared to *G. sandrasica*.

*Keywords*: *Genista* L.; Fabaceae; isoflavones; daidzein; genistein; HPLC; antioxidant.

## INTRODUCTION

Daidzein (7,4'-dihydroxyisoflavone) and genistein (5,7,4'-trihydroxyisoflavone) are known as phytoestrogens because they are plant compounds possessing estrogen-like activity. Phytoestrogens are considered to play an important role in the prevention of cancers, cardiovascular diseases, menopausal symptoms and osteoporosis.[1,2] Isoflavones are found in highest amounts in soybeans[3] and soy foods[4,5]; they are also present in other beans and legumes.[6] Dietary supplements containing isoflavones mainly from soybean and red clover were brought

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on the market as natural alternatives to conventional hormone replacement therapy.[7]

The genus *Genista* L. (Leguminosae) is represented by thirteen species in the Turkish flora, among these species, *G. involucrata*, *G. aucheri*, *G. burdurensis*, *G. vuralii* and *G. sandrasica* are endemic.[8–10] In previous studies, the aerial parts of eleven *Genista* species growing in Turkey were analyzed for their total and free genistein and daidzein contents.[11,12] The highest amount of total and free genistein was found in *G. tinctoria*, *i.e.*, 1.05 and 0.27 %, respectively,[13] whilst the highest amount of total and free daidzein was found in *G. sessilifolia* and *G. lydia* var. *antiochia*, *i.e.*, 0.0056 and 0.0009 %, respectively.[14] Continuing the investigations on the Turkish *Genista* species,[11–14] the genistein and daidzein contents in *G. vuralii* A. Duran & H. Dural and *G. sandrasica* Hartwig & Strid (Fabaceae), which have not been investigated before, are reported herein. In addition, their antioxidant activities were measured by three *in vitro* methods: 2,2--diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, ferrous ion-chelating, and ferric-reducing antioxidant power (FRAP) tests at 0.25, 0.50, and 1.0 mg $ml^{-1}$ concentrations. The total phenol and flavonoid contents in the extracts were determined spectrophotometrically *via* the Folin-Ciocalteau and $AlCl_3$ reagents, respectively.

## EXPERIMENTAL

### *Plant materials*

*G. vuralii* (GV) and *G. sandrasica* (GS) were collected at the flowering stage from the Ilgaz Mountain in the Çankırı Province in July 2004 and from the Sandras Mountain in the Muğla Province in July 2006 in Turkey, respectively. The plant materials were identified by one of us (Prof. Dr. Ahmet Duran). Voucher specimens (A. Duran 6735 and A. Duran 7312 & M. Dinç) are kept at the Herbarium in the Department of Biology Education, Faculty of Education, Selçuk University, Konya, Turkey.

### *Chemicals*

Genistein and daidzein standards were purchased from Sigma. (St. Louis, MO, USA). HPLC grade methanol (Merck, Darmstadt, Germany) and deionized water (Millipore) was used for chromatographic and antioxidant activity studies.

### *Preparation of standards*

Five working solutions were prepared for each standard in methanol containing genistein in 1, 2, 3, 4, and 8 mg $l^{-1}$ and daidzein in 4, 8, 10, 12, and 16 mg $l^{-1}$ concentrations.

### *Extract preparation for HPLC analysis and the antioxidant activity tests*

Air-dried and powdered aerial parts of each plant species were extracted following a previously reported method[13,14] for analyzing free and total isoflavones. Each plant was milled homogenously and 1 g was precisely weighed on a digital balancer. The powdered materials were extracted with methanol under ultrasonic vibration. The extracts were analyzed for their free isoflavones. One gram of precisely weighed aerial parts of the plant materials was hydrolyzed by heating with a mixture of an equal volume of methanol and 2 M HCl for 1 h under reflux. After filtering the mixture, 1 ml filtrate was diluted with 9 ml water and loaded

on to a Sep-Pak C18 cartridge (Waters). The isoflavones were retained on the Sep-Pak C18 cartridge, which was then washed twice with 10 ml of water and eluted with 70 % methanol. These extracts were analyzed for their total isoflavones.

*HPLC Analysis*

The HPLC analyses were realized on a HP 1050 Liquid Chromatograph System equipped with a Hypersil 120-S ODS column (250 mm×4.6 mm, 10 μm) and a UV detector. The mobile phase was methanol:water (70:30, v/v) at a flow rate of 0.7 ml $min^{-1}$. The temperature of the column was 25 °C. Data were collected at 261 nm for genistein and at 248 nm for daidzein. Each extract and five solutions of each standard were injected three times.

*Antioxidant activity*

*DPPH free radical-scavenging assay*. The hydrogen atom or electron donation capacity of the corresponding extracts was computed from the bleaching property of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The stable DPPH radical scavenging activity of the extracts was determined by the Blois method.[15] The samples and reference dissolved in ethanol (75 %) were mixed with DPPH solution ($1.5\times10^{-4}$ M). The remaining amount of DPPH was measured at 520 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA). The results were compared to that of gallic acid employed as the reference. The inhibition of DPPH (*I*) in percent was calculated as follows:

$$I\ \% = 100(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}} \quad (1)$$

where $A_{\text{blank}}$ is the absorbance of the control reaction (containing all reagents except the test sample) and $A_{\text{sample}}$ is the absorbance of the extracts/reference. The experiments were run in triplicates and the results are conveyed as average values with the *SEM*. (Standard error mean).

*$Fe^{2+}$–ferrozine test system for iron chelating.* The ferrous ion-chelating effect of the extracts by the $Fe^{2+}$–ferrozine test system was estimated by the method of Chua *et al*.[16] Accordingly, 740 μl of ethanol and the samples were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 40 μl of ferrozine solution into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine–$Fe^{2+}$ complex formation was calculated by Eq. (1).

The control contained only $FeCl_2$ and ferrozine. The analyses were run in triplicates and the results are expressed as average values with the *SEM*.

*The ferric-reducing antioxidant power (FRAP).* The ferric-reducing antioxidant power (FRAP) of the extracts and reference was tested using the assay of Oyaizu.[17] One ml of different concentrations of the extracts, as well as chlorogenic acid as the reference for comparative purposes, was added to 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide. Subsequently, the mixture was incubated at 50 °C for 20 min and then trichloroacetic acid (10 %) was added. After vigorous shaking, this solution was mixed with distilled water and $FeCl_3$ (0.1 %, w/v). After 30 min incubation, the absorbance was read at 700 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA). The analyses were realized in triplicate. An increase in the absorbance of the reaction mixture indicates an increase in the reducing power of the extracts.

*Determination of the total phenol and total flavonoid contents of the extracts*

Phenolic compounds were determined using Folin-Ciocalteau reagent according to the Singleton and Rossi method.[18] In brief; the samples were mixed with 750 μl of Folin-Cio-

calteau reagent and 600 µl of sodium carbonate in test tubes. The tubes were then vortexed and incubated at 40 °C for 30 min. Subsequently, absorption was measured at 760 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA). The total flavonoid content was determined by the aluminum chloride colorimetric method.[19] In short, a number of dilutions of quercetin were obtained to prepare a calibration curve. Then, the same dilutions from the sample were also prepared and separately mixed with 95 % ethanol, 10 % aluminum chloride, 1 M sodium acetate as well as 2.8 ml of distilled water. Following incubation for 30 minutes at room temperature, the absorbance of the reaction mixture was measured at 415 nm with a Unico 4802 UV–visible double beam spectrophotometer (USA). The total phenolic and flavonoid contents of the extracts are expressed as gallic acid and quercetin equivalents (mg $g^{-1}$ extract), respectively.

## RESULTS AND DISCUSSION

The total and free genistein and daidzein amounts found in *G. vuralii* (GV) and *G. sandrasica* (GS) are given in Table I. The amounts of genistein were calculated from the regression equation obtained from a standard curve which was linear within the concentration range 1–8 mg $l^{-1}$. The amounts of the daidzein were calculated from the regression equation obtained from daidzein standard curve which was linear within the concentration range of 4–16 mg $l^{-1}$. The regression equation and correlation coefficient determined for genistein and daidzein were $y = 154.8x - 0.12$ ($r^2 = 0.9997$) and $y = 153.2x - 3.90$ ($r^2 = 0.9981$), respectively. The amount of total and free genistein was found in GS and GV to be 0.58 and 0.05 %, respectively (Table I). The total and free daidzein contents of the aerial parts of GS were higher than those of GV and twelve other Turkish *Genista* species studied previously, *i.e.*, *G. acanthoclada*, *G. albida*, *G. anatolica*, *G. aucheri*, *G. burdurensis*, *G. carinalis*, *G. involucrata*, *G. libanotica*, *G. lydia* var. *antiochia*, *G. lydia* var. *lydia*, *G. sessilifolia*, and *G. tinctoria*.[12] On the other hand, the free daidzein content (0.068 %) was much higher than the free genistein content (0.013 %) in GS. In addition, the total genistein content of GS (0.582 %) was higher than those of GV and other *Genista* species previously studied, except for *G. tinctoria* (1.05 %).[11] Considering the formerly studied species, total daidzein contents ranged between 0.0003–0.0056 %, while the total genistein contents were in the range 0.01–1.05 %.[11,12]

TABLE I. Genistein and daidzein contents (mass %) of *G. vuralii* (GV) and *G. sandrasica* (GS)

| Species | Total genistein | Free genistein | Total daidzein | Free daidzein |
|---|---|---|---|---|
| GV | 0.363 | 0.050 | 0.048 | 0.016 |
| GS | 0.582 | 0.013 | 0.113 | 0.068 |

The methanol extracts of GS and GV exerted a similar scavenging effect against the DPPH radical at the tested concentrations (Table II), whereas the hydrolyzed extracts of these two species were found to have higher effects in this test. The same tendency for the extracts was also observed in the ferric-reducing

antioxidant power test, in which the hydrolyzed extract of *G. vuralii* (GVH) was much more active than the main methanol extracts (Table III). The hydrolyzed extract of *G. sandrasica* (GSH) could not be tested due to its inadequate amount. In the ferrous ion-chelating capacity test, the extracts did not show a significant effect (Table III).

TABLE II. Total phenol (mg $g^{-1}$ extract as gallic acid equivalent) and total flavonoid (mg $g^{-1}$ extract as quercetin equivalent) contents and DPPH radical scavenging activities of the methanol extracts of *G. vuralii* (GV) and *G. sandrasica* (GS) and their hydrolyzed extracts as well as daidzein and genistein

| Extract | Total phenol content±*SEM*[a] | Total flavonoid content±*SEM* | Inhibition±*SEM*, %, against DPPH radical | | |
|---|---|---|---|---|---|
| | | | *c* / μg $ml^{-1}$ | | |
| | | | 250 | 500 | 1000 |
| GV | 212.24±2.62 | 203.82±4.08 | 16.85±1.25 | 29.91±0.01 | 50.70±3.87 |
| GS | 166.94±3.13 | 158.06±0.13 | 15.90±0.42 | 24.77±3.43 | 46.16±1.09 |
| GVH[b] | NT[c] | NT | 69.80±3.27 | 85.05±0.25 | 86.12±0.42 |
| GSH[d] | NT | NT | 69.51±2.85 | 85.64±0.59 | 87.18±0.08 |
| Daidzein | – | – | –[e] | 2.52±0.76 | 6.99±3.56 |
| Genistein | – | – | – | – | – |
| Gallic acid (reference) | – | – | 87.65±0.28 | 91.61±0.06 | 92.57±0.10 |

[a]Standard error mean; [b]the hydrolyzed extract of GV; [c]not tested; [d]the hydrolyzed extract of GS; [e]no activity

TABLE III. Ferrous ion-chelating capacity (inhibition±*SEM*, %), and ferric-reducing antioxidant power (absorbance at 700 nm±*SEM*) of the methanol extracts of *G. vuralii* (GV) and *G. sandrasica* (GS) and their hydrolyzed extracts, as well as daidzein and genistein

| Extracts | Ferrous ion-chelating capacity (inhibition±*SEM*, %) | | | Ferric-reducing antioxidant power (absorbance at 700 nm±*SEM*) | | |
|---|---|---|---|---|---|---|
| | | | | *c* / μg $ml^{-1}$ | | |
| | 250 | 500 | 1000 | 250 | 500 | 1000 |
| GV | 3.28±1.24 | 7.47±0.11 | 10.21±1.78 | 0.468±0.007 | 0.627±0.021 | 1.005±0.018 |
| GS | 6.89±0.88 | 8.29±0.71 | 8.21±0.42 | 0.458±0.023 | 0.652±0.044 | 0.860±0.046 |
| GVH | 8.83±0.89 | 8.95±0.65 | 13.90±0.91 | 1.289±0.016 | 2.478±0.053 | 2.829±0.031 |
| GSH | NT[a] | NT | NT | NT | NT | NT |
| Daidzein | –[b] | – | – | 0.206±0.021 | 0.211±0.023 | 0.292±0.007 |
| Genistein | – | – | – | 0.192±0.005 | 0.216±0.005 | 0.340±0.021 |
| Butylated hydroxyanisol (reference for ferric ion-chelating capacity) | NT | 21.71±1.10 | 26.94±1.48 | | | |
| Chlorogenic acid (reference for FRAP) | | | | 2.955±0.09 | 3.547±0.06 | 3.618±0.01 |

[a]Not tested; [b]no activity

For total phenol content, the calibration equation was calculated as $y$ = = 0.9051$x$ + 0.0549 ($r^2$ = 0.9943) (gallic acid equivalent) and as $y$ = 5.5398$x$ + + 0.1209 ($r^2$ = 0.9988) (quercetin equivalent) for the total flavonoid content in the methanol extracts (Table II). The total phenol and flavonoid amounts of GSH and GVH could not be established due to their small amounts. The methanol extracts were found to contain total phenol and flavonoid in similar quantities.

On the other hand, there have been a few reports on the antioxidant activity of *Genista* species. For instance; various extracts of *G. tenera* of Portuguese origin were tested for their scavenging activity against the DPPH radical and the ethyl acetate extract was shown to cause the best scavenging activity of 48.7 % at a concentration of 139.1 μg ml$^{-1}$.[20] In another study, the methanol extracts from the aerial parts of *G. sessilifolia* and *G. tinctoria* were tested on pBR322 DNA cleavage induced by hydroxyl radicals ($^{\bullet}$OH), generated from UV-photolysis of hydrogen peroxide ($H_2O_2$) and by nitric oxide (NO) and they displayed a protective effect on UV light and nitric oxide-mediated plasmid DNA damage.[21]

In the present antioxidant assays, genistein and daidzein were not active in the DPPH radical scavenging and ferrous ion-chelating capacity tests; whereas they exhibited mild FRAP (Tables II and III). Genistein and daidzein, the well--known isoflavone-type of phytoestrogens, have been studied up to date for their antioxidant activities by various methods and they were reported to be active in some assays. However, no data on the effect of these two isoflavones in the ferrous ion-chelating capacity test were encountered, while one study proved that metabolites of these isoflavones were more active than genistein and daidzein in FRAP.[22] In one recent study,[23] the antioxidant activities of some isoflavonoids, *i.e.*, puerarin and daidzein as well as *Pueraria lobata* and *P. miristica* extracts, were determined using the DPPH radical scavenging test and daidzein was shown to display the same level of antioxidant activity as $\alpha$-tocopherol. This discrepancy might result from differences in the method used in the present study and in the literature publication. However, in another report,[24] daidzein was concluded to up-regulate the antioxidant enzyme catalase but showed only little antioxidant capacity *per se*. Genistein and daidzein were also found to show strong antioxidant effects against the oxygen radical.[25,26] Arteaga *et al.* suggested that phytoestrogens including genistein and daidzein showed weak antioxidant activity *in vitro* in the low density lipoprotein (LDL) oxidation test.[27]

## CONCLUSIONS

To the best of our knowledge, the current study reports the first data on the antioxidant activity of and the genistein and daidzein contents in *G. vuralii* and *G. sandrasica*. The results indicated that the hydrolyzed extracts had higher antioxidant activities. *G. sandrasica* was also shown to contain larger amount of free and total daidzein than *G. vuralii*, which was richer in total phenol and fla-

vonoid than *G. sandrasica*, as well as the other *Genista* species studied previously. However, the daidzein and genistein were found to have no or little effects in the antioxidant assays applied herein.

И З В О Д

## ОДРЕЂИВАЊЕ ГЕНИСТЕИНА И ДАИДЗЕИНА У ДВЕ ЕНДЕМСКЕ ВРСТЕ *Genista* И МЕРЕЊЕ ЊИХОВЕ АНТИОКСИДАТИВНЕ АКТИВНОСТИ

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У овом истраживању одређиван је садржај укупног и слободног генистеина и даидзеина у две ендемске *Genista* врсте (*G. sandrasica* и *G. vuralii*) методом HPLC. Највећа количина укупног генистеина и даидзеина је нађена у *G. sandrasica*, 0,582 % односно 0,113 %, док је количина слободног даидзеина у *G. sandrasica* била већа од укупног у *G. vuralii*. Антиоксидативна активност сировог метанолног и хидролизованог екстракта ових врста је одређивана применом три *in vitro* методе, методом DPPH за мерење слободних радикала и тестовима хелатирања феро- и фери-јона (FRAP) при концентрацијама 0,25, 0,50 и 1,00 mg $ml^{-1}$. Хидролизовани екстракти обе врсте су испољили већу антиоксидативну активност у свим тестовима. Садржај укупних фенола и флавоноида је одређиван Folin–Ciocalteau и $AlCl_3$ реагенсом. Врста *G. vuralii* је имала више фенола и флавоноида од *G. sandrasica*.

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