Comparative polyphenolic content and antioxidant activities of *Genista tinctoria* L. and *Genistella sagittalis* (L.) Gams (Fabaceae)

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Abstract: The aim of this study was focused on the polyphenolic composition and antioxidant capacity of *Genista tinctoria* L. and *Genistella sagittalis* (L.) Gams. A qualitative and quantitative characterization of the main phenolic compounds from the extracts were carried out using a HPLC-MS method. The total polyphenolic and flavonoid content was spectrophotometrically determined. The antioxidant activity towards variousradicalsgeneratedindifferent systems was evaluated usingDPPH bleaching method, Trolox equivalent antioxidant capacity assay (TEAC) and Oxygen radical absorbance capacity (ORAC), and all indicated that *G. tinctoria* extract was more antioxidant than *G. sagittalis* extract.That was in good agreement with the total polyphenolic and flavonoidic content.Chlorogenic acid, *p*-coumaric acid, isoquercitrin and apigenin were identified in bothspecies. Caffeic acid, ferulic acid, hyperoside, rutin, quercitrin and luteolin were found only in *G. tinctoria,* while quercetin was determined in *G. sagittalis*.

Keywords: *Genista, Genistella,* polyphenols, antioxidant capacity.

INTRODUCTION

Fabaceae Lindl. *(Leguminosae Adans.)*is the third largest family within flowering plants and is constituted of 650 genera that include about 18 thousand species (Hakki et al., 2010). In the Romanian Flora, this family is represented with 44 genera and 236 species (Ciocarlan, 2009). Genista tinctoria and Genistella sagittalis, two morphologically similar species, are someof the most importantspeciesof Fabaceae family, both in terms of its medical and tictorial values respectively the chemical composition. These specieshave been reclassified and theycurrentlybelongto twodifferent genres: Genista L. and Genistella Ortega (Ciocarlan, 2009).

Genista tinctoria L. (Dyer's greenwood) is a leguminous perennial shrub, widespread most of Europa from Estonia southwards and eastward to the Urals, Caucasus and Asia Minor. It grows to 60 cm in height and has glabrous with erect, ridged, branched slender, spineless stems. The leaves are simple, elliptic or elliptic-lanceolate, glabrous or with scattered hairs, acute at the apex, narrowed at the base, entire, shining; stipules none; up to 30mm in length. Flowers are crowded at the tip in racemose nearly sessile, about 15 mm long ; bracts leaf-like, pedicels 1-3 mm; bracteole of 1 mm, at middle of pedicels; calyx 3-7 mm, glabrous to dense pubescent; corolla yellow, 8-15 mm, with short clawed petals. The pod (25-30 mm) is glabrous, flat, tapering and obtuse at both ends. The ellipsoidal seeds are dark brown, shining, of 2.5-2 mm (Clapham *et al*., 1987; Clapham *et al*., 1981). By phytochemical analysis alkaloids (cytosine, anagyrine), flavonoids (luteolin, apigenin) and isoflavonoids (daidzin, daidzein, genistein, prunetin, isoprunetin, biochanin) were observed in its herb (Tero-Vescan *et al*., 2009). *G. tinctoria* shows different activities due to the presence of isoflavones, such as phytoestrogenic, antioxidant and cytotoxic activities against different human cancer cell lines, and effects on the thyroid gland (Rigano *et al*., 2009).

Genistella sagittalis (L.) Gams. (syn. *Genista sagittalis* L.) is a perennial plant with procumbent, woody, mat-forming stems, and usually erect, herbaceous, simple or little branched flowering stems, of 10-50 cm long; wings constricted at the nodes, but entire, without teeth or lobes. The leaves, of 5-20mm long, are elliptical, glabrous or subglabrous above, pubescent beneath. The calyx is sericeous, of 5-8 mm. The corolla (10-12mm) is usually glabrous and yellow, in short dense racemes. The pod (14-20 x 4-5mm) is pubescent, with estrophiolate seed.*This species* is widely distributed across Europe, the species range extends from southern Spain to Ukraine (Tutin *et al*., 1968; Bojnansky and Fargasova, 2007). Untill now, through phytochemical analysis, only the presence of some isoflavones (daidzin, genistin, genistein, ononin, daidzein, formononetin) was reported (Tero-Vescan *et al*., 2009; Hanganu *et al*., 2010). Polyphenolic

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compounds are secondary plant metabolites and constitute the largest group of health-promoting phytochemicals. These compounds act as primary antioxidants or free radical scavengers (Meriane *et al*., 2014). *Genista* species are sources of antioxidant phenolic compounds such as flavonoids, isoflavonoids. Recent studies have investigated the potential of different *Genista* species as antioxidants against various free radicals: *G. saharae* Coss (Bouchouka *et al*., 2012), *G. sandrasica*Hartvig & Strid., *G. vuralii* A. Duran & Dural (Orhan *et al*., 2011), *G. quadriflora* Munby (Boubekri *et al*., 2014), *G. tinctoria* L (Popa *et al*., 2014).

Our scientific literature survey showed that thepolyphenolicchemistry of the two species has been poorly investigated; especially, chemicaland pharmacological data of *Genistella sagitallis* are very limited (Hanganu *et al*., 2010).

The aim of this comparative study was to determine the polyphenolic composition of extracts from the aerial parts of *two Romanian species: Genista tinctoria* and *Genistella sagitallis*and to evaluate their *in vitro* antioxidant properties, for a better characterization and exploitation of these medicinal plants.

EXPERIMENTAL

Plant material and extraction procedure

Plant materials (aerial parts) from the two species were collected in 2013, during the blooming period (June-July) from the Province of Transylvania (NW of Romania). Identification of plant species was made by PhD. Paltineanu R. from the Department of Pharmaceutical Botany. Voucher specimens (No. 919, 920) were deposited in the Herbarium of the Department of Pharmacognosy of the Faculty of Pharmacy, Cluj-Napoca, Romania. The vegetal material was air dried at room temperature in shade, separated and grinded to fine powder (300µm). Two grams of the material were extracted with 20ml of 70% ethanol (Merck, Darmstadt, Germany), for 30min on a water bath, at 60°C. The samples were then cooled down and centrifuged at 4.500 rpm for 15min and the supernatant was recovered (Benedec *et al*., 2012; Vlase *et al*., 2013).

Chemicals

Ferulic acid, sinapic acid, gentisic acid, gallic acid, patuletin, luteolin were purchased from Roth (Karlsruhe, Germany), cichoric acid, caftaric acid from Dalton (Toronto, ON, Canada), chlorogenic acid, *p*-coumaric acid, caffeic acid, rutin, apigenin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetol, fisetin from Sigma (St. Louis, MO, USA). HPLC grade methanol, analytical grade *orto*-phosphoric acid, hydrochloric acid and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany), hydrogen peroxide, ABTS (2,2'-azinobis-3-ethylbenzotiazoline-6-sulphonic acid) from Sigma (Germany); aluminum chloride, sodium acetate, sodium carbonate, ethanol (Merck, Darmstadt, Germany), DPPH (2,2-diphenyl-1-picrylhydrazyl), BHT (butyl-hydroxy-toluene) were obtained from Alfa-Aesar (Germany). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), fluorescein, potassium persulfate were purchased from Sigma–Aldrich (Germany).

HPLC/MS analysis, apparatus and chromatographic conditions

Qualitative and quantitative analysis of polyphenols was achieved by high-performance liquid chromatographic (HPLC) coupled with mass spectroscopy (MS) (Meda *et al*., 2011; Benedec *et al*., 2012; Benedec *et al*., 2013). The experiment was carried out using an Agilent Technologies 1100 HPLC Series system (Agilent, Santa Clara, CA, USA) equipped with G1322A degasser, G13311A binary gradient pump, column thermostat, G1313A auto sampler and G1316A UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18 100 × 3.0 mm i.d., 3.5µm particle); the work temperature was 48°C. The detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5min, then at 370nm. The MS system was operated using an electrospray ion source, in negative mode. The chromatographic data were processed using Chem Station and Data Analysis software from Agilent. Stock standard solutions for HPLC were prepared by accurately weighing 10mg of chlorogenic, *p*-coumaric, caffeic, cichoric, caftaric, ferulic, sinapic, gentisic and gallic acids, rutin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetol, fisetin, patuletin, apigenin and luteolin, reference standards into separate 10ml volumetric flasks and dissolving in methanol.

The mobile phase was a binary gradient: methanol and acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; then 42% methanol for the next 3 minutes (Meda *et al*., 2011; Benedec *et al*., 2012; Benedec *et al*., 2013). The flow rate was 1 $mLmin^{-1}$ and the injection volume was 5 µL.

The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol. The MS spectra obtained from a standard solution of polyphenols were integrated in a mass spectra library. Later, the MS traces/spectra of the analysed samples were compared to spectra from library, which allows positive identification of compounds, based on spectral match. The UV trace was used for quantification of identified

compounds from MS detection. Using the chromatographic conditions described above, the polyphenols were eluted in less than 40min. Four polyphenols cannot be quantified in current chromatographic conditions due overlapping (caftaric acid with gentisic acid and caffeic acid with chlorogenic acid). However, all four compounds can be selectively identified in MS detection (qualitative analysis) based on differences between their molecular mass and MS spectra. For all compounds, the limit of quantification was 0.5 µg/mL and the limit of detection was 0.1µg/mL. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. The detection limits were in the range of 18 to 92ng/mL. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5-50µg/mL range with good linearity (R^2> 0.999) for a five point plot were used to determine the concentration of polyphenols in plant samples (Benedec *et al*., 2012; Benedec *et al*., 2013, Vlase *et al*., 2013). For all compounds, the accuracy was between 94.1.3% and 105.3%. Accuracy was checked by spiking samples with a solution containing each polyphenol in a 10µg/mL concentration. In all analyzed samples the compounds were identified by comparison of their retention times and recorded electrospray mass spectra with those of standards in the same chromatographic conditions.

Determination of total polyphenols and flavonoids content

The total phenolic content (TPC) of these extracts was determined by the Folin-Ciocalteau method with some modifications (Benedec *et al*., 2013; Singleton *et al*., 1999; Cai *et al*., 2004; Li *et al*., 2008; Gan *et al*., 2008; Ph. Eur., 2005; Romanian Pharmacopoeia, 1993). The absorbance was measured at 725nm using a Biotek Synergy HT spectrophotometer and the results were expressed in gallic acid equivalent (mg GAE/g sample) using a gallic acid standard curve. Gallic acid was plotted at 0.02, 0.04, 0.06, 0.08, and 0.10mg/mL, prepared in methanol: water (50:50, v/v). Additional dilution was done if the absorbance value measured was over the linear range of the standard curve. TPC values were calculated using a first-degree equation obtained from the calibration curve of gallic acid (R^2=0.995). Two milliliters of each ethanolic extract diluted 25 times were mixed with 1.0 mL of Folin-Ciocalteu reagent, 10.0mL of distilled water and diluted to 25.0mL with a 290g/L solution of sodium carbonate. The samples were incubated in the dark for 30 min. The quantitative determination of flavonoids was performed using the spectrophotometric aluminum chloride method. Five milliliters of each extract were mixed with 5.0mL of sodium acetate 100g/L, 3.0mL of aluminum chloride 25g/L and filled up to 25mL by methanol in a calibrated flask. The absorbance was measured at 430nm (Benedec *et al*., 2013; FR, 1993; Huang *et al*., 2005; Zia *et al*., 1999). Total flavonoids content values was determined using an equation obtained from calibration curve of the rutin (R^2=0.999). The spectrophotometric data were acquired using a Jasco V-530 UV-Vis spectrophotometer (Jasco International Co., Ltd., Japan).

DPPH radical scavenging assay

The antioxidant potential of the ethanol extracts was quantified using the stable DPPH radical method. The DPPH$^{\bullet}$ solution (0.1g/L) was prepared in methanol and 4.0ml of this solution was added to 4.0ml of extract solution (or standard) in methanol at different concentrations (10-50µg/mL). After 30 minutes of incubation at40°Cin a thermostatic bath, the decrease in the absorbance (n=3) was measured at 517 nm. The percent of DPPH$^{\bullet}$ scavenging ability was calculated as: DPPH$^{\bullet}$ scavenging ability = $(A_{control} - A_{sample})/A_{control} \times 100$, where $A_{control}$ is the absorbance of DPPH radical + methanol (containing all reagents except the sample) and A_{sample} is the absorbance of DPPH radical + sample extract or standard. The antiradical activity (three replicates per treatment) was expressed as IC_{50}, the concentration (µg/mL) of the test extract or compound required to cause a 50% DPPH$^{\bullet}$ inhibition (Singleton *et al*., 1999; Simirgiotis *et al*., 2013; Nimmi and George *et al*., 2012). The positive controls were those using the standard solution of quercetin and butyl-hydroxy-toluene (BHT). The spectrophotometric data were acquired using a Jasco V-530 UV-Vis spectrophotometer (Jasco International Co., Ltd., Japan). The DPPH radical-scavenging activity was also determined using the method proposed by Brand-Williams and DPPH assay values were expressed in mM Trolox/g sample, using freshly made standard curves and the spectrophotometric data were acquired using aBiotek Synergy HT spectrophotometer (Brand-Williams *et al*., 1995; Benedec *et al*., 2015; Mocanu *et al*., 2015).

Trolox equivalent antioxidant capacity (TEAC) assay

The ABTS assay is based on the scavenging ability of antioxidants to the long-life radical cation ABTS$^{\bullet+}$. A stable stock solution of ABTS$^{\bullet+}$ was produced by treating a 7mmol/L aqueous solution of ABTS with 2.45mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. At the beginning of the analysis day, an ABTS$^{\bullet+}$ working solution was obtained by the dilution in ethanol of the stock solution to an absorbance of 0.70 ± 0.02AU at 734nm, verified by Biotek Synergy HT spectrophotometer. For ABTS assay, the followed procedure was the method of Arnao et al. with some modifications. The content of the generated ABTS$^{\bullet+}$ radical was measured at 734nm after 600s reaction time and was converted in Trolox equivalents by the use of a calibration curve (R^2=0.9987) constructed with 0, 2, 4, 6, 8, 10mg/L Trolox standard (Arnao *et al*., 2001; Obon *et al*., 2005; Prior *et al*., 2005).

Oxygen radical absorbance capacity (ORAC)

The ORAC assay depends on the free radical damage to a fluorescent sample through the change in its fluorescence intensity. The change of fluorescence intensity is an index of the degree of free radical damage. In the presence of antioxidant, the inhibition of free radical damage is reflected in the protection against the change of sample fluorescence. The improved ORAC assay only measures the antioxidant activity against peroxyl radical and hydroxyl radical. Data are expressed as mM Trolox equivalents (TE) per gram of sample. Results have been obtained using a fluorescence microplate reader with 96 well format. Fluorescence intensity ($\lambda_{excitation}$=485nm, $\lambda_{emission}$ = 525 nm) was measuredusing Biotek Synergy HT micro plate reader for 35 min, pH=7.4, 37ºC (Prior *et al*., 2005).

STATISTICAL ANALYSIS

Data were presented as means and standard deviation (SD). All the samples were analyzed in triplicate; the average and the relative SD were calculated using the Excel software package.

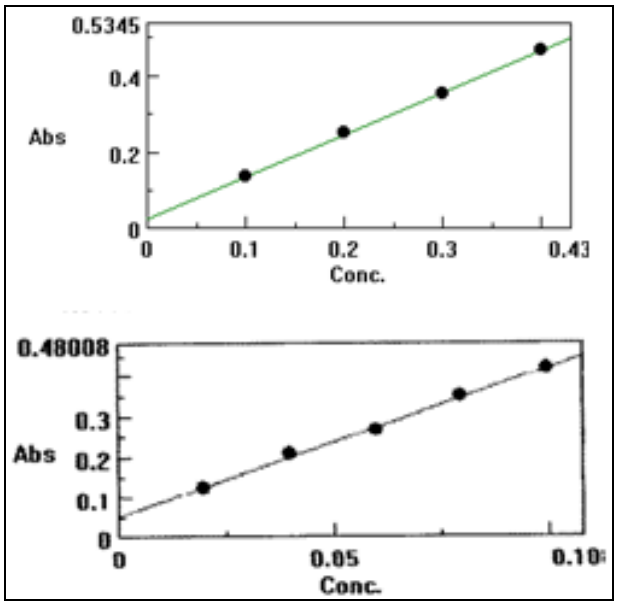
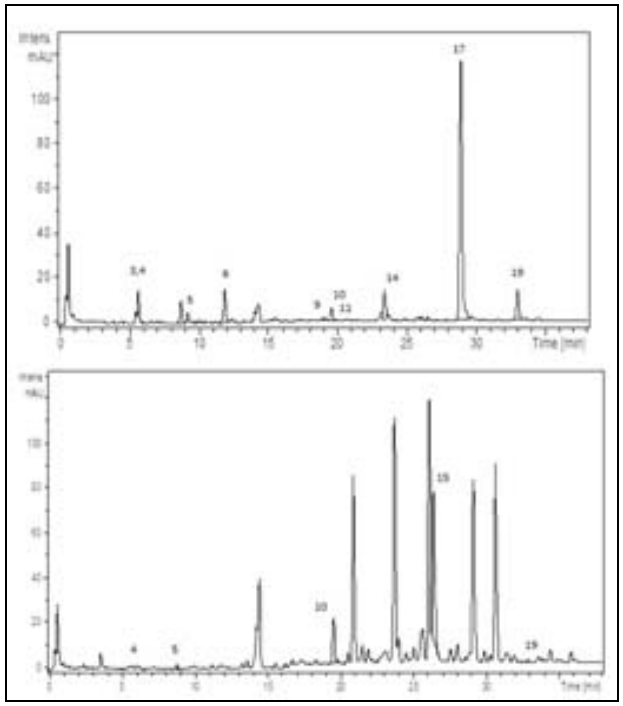


Fig. 1: Calibration curve of rutin (left) and gallic acid (right)

RESULTS

The HPLC-MS method has been developed for the determination of 19 phenolic compounds (eight phenolic acids, four quercetin glycosides and seven flavonol and flavone aglycones) from plant materials. The applicability of the proposed analytical method and the qualitative and quantitative determination of the standard phenolic compounds have already been verified (Benedec *et al*., 2012; Benedec *et al*., 2013, Meda *et al*., 2011). The quantitative determination was performed using the external standard method. The concentrations of identified polyphenolic compounds in both analyzed samples are shown in table 1 and organized in the order of their retention time. The HPLC chromatograms of these samples are shown in fig. 2.



Notes: 3.Caffeic acid, 4.Chlorogenic acid, 5.*p*-Coumaric acid, 6. Ferulic acid 9.Hyperoside, 10.Isoquercitrin, 11.Rutin, 14. Quercitrin, 15. Quercetin, 17.Luteolin, 19. Apigenin

Fig. 2: HPLC chromatogram of *G. tinctoria* (left) and *G. sagittalis* (right)

The chromatographic profiles of these two species revealed differences of their chemical composition. HPLC profiles of polyphenolic compounds allowed the possibility of identification of ten polyphenolic compounds in *G. tinctoria* and only five compounds in *G. sagittalis* (fig. 2, table 1).

The total phenolic contents (TPC) for *G. tinctoria* and *G. sagittalis* extracts expressed as mg GAE/g sample and total flavonoid content presented as mg RE/g sample are presented in table 2.

The antioxidant capacity of the ethanol extracts of *G. tinctoria* and *G. sagittalis* was determined by several methods: DPPH bleaching assay, the Trolox equivalent antioxidant capacity (TEAC), Oxygen radical absorbance capacity (ORAC) (table 3).

DISCUSSION

In the ethanol extract of *Genista tinctoria*, four phenolic acids, namely caffeic, chlorogenic, ferulic and *p*-coumaric acids were identified but only *p*-coumaric and ferulic

Table 1: Polyphenolic compounds content (µg/g plant material) in *G. tinctoria* and *G. sagittalis* extracts

Polyphenolic compounds	*m/z* value	$t_R \pm SD$ /min	*G. tinctoria*	*G. sagittalis*
Caffeic acid	179	5.60±0.04	<0.2	NF
Chlorogenic acid	353	5.62±0.05	<0.2	<0.2
p-Coumaric acid	163	9.08±0.08	54.97±0.12	4.91±0.04
Ferulic acid	193	11.8±0.10	79.89±0.18	NF
Hyperoside	463	19.00±0,12	4.04±0.12	NF
Isoquercitrin	463	19.60±0.10	95.97±0.45	163.56±0.25
Rutin	609	20.70±0.15	<0.2	NF
Quercitrin	447	23.64±0.13	276.61±0.55	NF
Quercetin	301	26.80±0.15	NF	183.10±0.03
Luteolin	285	29.10±0.19	1119.89±0.45	NF
Apigenin	279	33.00±0.15	169.49±0.65	7.31±0.05

Note*:* NF - not found, below limit of detection. Values are the mean ± SD (n = 3).

Table 2: The content of total polyphenols and flavonoids in *G. tinctoria* and *G. sagittalis*

Samples	TPC (mg GAE/g)	Flavonoids (mg RE/g)
G. tinctoria	33.52±0.21	16.06±0.4
G. sagittalis	15.71±0.35	10.72±1.3

Note: Each value is the mean ± SD of three independent measurements.

Table 3: Antioxidant activity parameters

Samples	DPPH (IC_{50} µg/mL)	DPPH (mmol Trolox/g)	TEAC (mmolTrolox/mg)	ORAC (mmol Trolox/ mg)
G. tinctoria	65.45±0.75	237.37±7.84	94.45±5.09	88.12±1.17
G. sagittalis	304.05±0.65	24.76±0.94	40.49±4.88	67.14±2.04
Quercetin	5.40±0.32	-		
BHT	15.6±0.44	-		

Note: Each value is the mean ± SD of three independent measurements.

acids were quantified; caffeic and chlorogenic acids were in too low concentration to be quantified (table 1). Four flavonoid glycosides, hyperoside, isoquercitrin, rutin were identified and quantified (except rutin), considering the used flavonoid standards (table 1). Quercitrin was the compound found in the largest amount followed by isoquercitrin and hyperoside. Two free flavonoid aglycons, *i.e.*, luteolin and apigenin, were identified and quantified. Luteolin was found in the largest quantities followed by apigenin.

In the ethanol extract of *Genistella sagittalis,* two phenolic acids were identified: chlorogenic and *p*-coumaric acids and only *p*-coumaric acid was quantified. Isoquercitrin was the only identified and quantified flavonoid glycoside. There were identified and quantified only two flavonoid aglycons: Quercetin and apigenin.

Some flavonoid compounds, i. g. hyperoside, rutin, quercitrin and luteolin were detected only in *Genista tinctoria* and quercetin was found only in *Genistella sagittalis.*

Considering the 19 standard compounds used in this study, some other peaks were not identified. Thus, this comparative study showed large qualitative and quantitative differences between the analyzed extracts.

The difference between the separated compounds from these two taxa could serve as a differentiation method to detect the adulterations of these species.

Scientific data on the polyphenolic compounds of these species are difficult to compare because of the lack of studies.

The highest amount of polyphenols (table 2) was determined for *G. tinctoria* followed by *G.sagittalis*. Concerning the content of flavonoids, the extract of*G. Tinctoria* was richer inflavonoids, than the extract of *G. sagittalis*. The seobservationsarein agreement with the content of isoflavones determined on the same Romanian samples and the content of flavonoids and total polyphenols was found smaller than other species of the genus (Tero-Vescan *et al*., 2009; Hanganu *et al*., 2010; Meriane *et al*., 2014).

The antioxidant activity of the extracts was further assessed by the DPPH radical bleaching method. The DPPH scavenging ability of the extract obtained from *G. tinctoria* was larger than that of *G. sagittalis* (table 3). This is in good agreement with the TPC and total flavonoides values listed in table 2. Compared to the reference compounds, quercetin and BHT, the extracts of *G. tinctoria* showed lower antioxidant capacity. The DPPH scavenging ability of the extract obtained from *G. tinctoria* is very close to other reports (Boubekri *et al*., 2014).

According to TEAC method, the extract of *G. tinctoria* has significantly higher antioxidant capacity than the extract of *G. sagittalis,* in agreement with DPPH results and TPC values. In the TEAC assay, the stable radical is dissolved in an aqueous solution, thus expecting a different mechanism of interaction between antioxidants molecule and the radical since TEAC assesses the more hydrophilic components while DPPH describes all components. This can be observed by the final TEAC values (table 3), even though the antioxidant power was significantly higher for *G. tinctoria* extract, the difference in absolute value was not as great as in the case of DPPH method. The ORAC results reveal a higher antioxid ant potential for *G. tinctoria* than *G. sagittalis* (table 3). This could be explained by the high era mounts of antioxidant compounds inhibitors of peroxyl and hydroxyl radicalsin *G. tinctoria*. Even than there are more unidentified compounds into *G. sagittalis* extract, these seem to not influence its antioxidant activity relative to the *G. tinctoria* extract.

CONCLUSIONS

There were determined the polyphenolic profile and the antioxidant activity for two Romanian species of Fabaceae family, *Genista tinctoria* and *Genistella sagittalis* -two very similar species of the botanical point of view. The simultaneous determination of a wide range of polyphenolic compounds was performed using a rapid, highly accurate and sensitive HPLC method assisted by UV-Vis and mass spectrometry detection. The antioxidant activity was evaluated by several methods: DPPH, TEAC and ORAC. The results revealed qualitative and quantitative differences of polyphenolic compounds that could avoid adulterations among these two taxa, and there were completed the lack of phytochemical literature data with new information concerning polyphenolic compounds of these species and their bioactivity. Therefore, it can be concluded that the *G. tinctoria* and *G. sagittalis* extracts could be valuable free-radical-scavengers and they represent a potential natural source of phenolic antioxidants. The importance of this research consists in its novelty as it comes with new scientific information on mainly *Genistella sagittalis* and it offers novel perspectives for pharmaceutical and industrial applications of these species.

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