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Identification of phenolic constituents of *Cytisus multiflorus*

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ABSTRACT

The phenolic composition of the ethanolic extract obtained from the flowers of the medicinal plant *Cytisus multiflorus* has been elucidated by high performance liquid chromatography, electrospray mass spectrometry and nuclear magnetic resonance analysis. The extract was mainly composed of flavones, including the common chrysin, orientin, luteolin-5-*O*-glucoside, luteolin-7-*O*-glucoside, apigenin and apigenin-7-*O*-glucoside, which appeared as minor components. The major flavone in the extract was chrysin-7-*O*-β-D-glucopyranoside, and it also contained moderate amounts of a dihydroxyflavone isomer of chrysin, as well as of 2″-*O*-pentosyl-6-*C*-hexosyl-luteolin, 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin and 6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-apigenin, which are not commonly found in the Fabaceae family. Other novel phenolic compounds found in the ethanolic extract of *C. multiflorus* comprised the flavones 2″-*O*-pentosyl-6-*C*-hexosyl-apigenin, 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin and 6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-luteolin. The assessment of the biological activities of the main compounds of this extract are now keen, in order to determine their relevance in the beneficial properties of the plant.

1. Introduction

Cytisus Desf. (Leguminosae–Cytiseae) is a large and diversified genus including approximately 60 species, which are particularly abundant around the Mediterranean Sea, although they are found in distinct geographic regions such as the north and south of Africa, the western and central Europe, the Black Sea and Turkey to the East (Cristofolini & Conte, 2002; Cristofolini & Troia, 2006). Plants of this genus exhibit bioactive properties, including antioxidant (Raja et al., 2007; Sundararajan et al., 2006), diuretic, hypnotic, anxiolytic (Nirmal, Babu, Harisudhan, & Ramanathan, 2008; Siegel, 1976), antiparasitic (Di Giorgio et al., 2008) and antidiabetic (Castro, 1998, 2001) activities. The therapeutic properties and, in particular, the antioxidant activity of *Cytisus* is related to their high concentration of phenolic compounds (Luis, Domingues, Gil, & Duarte, 2009). In general, plants of this genus are rich in flavonoids. Namely, *Cytisus scoparius* has been described to contain the flavone 6″-*O*-acetyl-scoparin, the flavonols kaempferol, rutin, quercetin, quercitrin and isorhamnetin, and the isoflavones genistein and sarothamnoside, while the species *Cytisus nigrians* and *Cytisus albus* were shown to contain the isoflavones ononin and genistin (Hanganu, Vlase, & Olah, 2010a, 2010b; Raja et al., 2007).

Cytisus multiflorus (L'Hér.) Sweet, also known as White Spanish Broom, is a leguminous shrub native from Iberian Peninsula that is distributed in the south-west Mediterranean region (Cristofolini & Troia, 2006). This specie grows in poor and acidic soils, and frequently appears in degraded or marginal areas. It has a great number of white flowers with a valvular type pollen presentation system. The *C. multiflorus* is vastly used as an ornamental plant, as well as for animal nutrition. Other applications of this plant include the collection of their pollen for apiculture purposes and land fertilising in agriculture (Ciudad et al., 2004; Rodriguez-Riano, Ortega-Olivencia, & Devesa, 1999, 2004; Rodriguez-Riano, Valtuena, & Ortega-Olivencia, 2006).

C. multiflorus has also been used as an ethnopharmacological agent for centuries mainly due to its diuretic, anti-inflammatory, anti-hypertensor and antidiabetic properties (Gião et al., 2007). However, this specie has been far less studied than other of the same genus and, to our knowledge, its phenolic profile remains unknown. In this context, the present study intends to characterise the phenolic constituents of *C. multiflorus*, by high performance

Abbreviations: CID, collision-induced dissociation; DAD, diode array; ESI-MS, electrospray ionisation-mass spectrometry; MS^n, Tandem mass spectrometry; GAE, gallic acid equivalent; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

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liquid chromatography associated with diode array detection (HPLC-DAD), electrospray mass spectrometry (ESI-MS and MS^n) and nuclear magnetic resonance analysis (NMR) techniques.

2. Material and methods

2.1. Chemicals

The phenolic standard gallic acid was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Luteolin-8-*C*-glucoside (orientin), luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, rutin and chrysin were obtained from Extrasynthese (Genay Cedex, France). Folin–Ciocalteu reagent, Na_2CO_3, formic acid and ethanol were purchased from Panreac (Barcelona, Spain). *n*-Hexane, methanol and acetonitrile with HPLC purity were purchased from Lab-Scan (Lisbon, Portugal). DMSO-d_6 containing 0.03% of TMS was obtained from CortecNet (Paris, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Plant material

The dried flowers of *C. multiflorus* were purchased from ERVITAL (Castro de Aire, Portugal). The plants have been cultivated under an organic regime and the flowers were collected in the Spring of 2009. After collection, these were dried at 25–30 °C in a ventilated incubator for approximately 5 days.

2.3. Extraction of phenolic compounds

The flowers of *C. multiflorus* (5 g) were grounded and defatted with 150 ml *n*-hexane, for three times. The residue was extracted with 150 ml of an 80% ethanol solution (v/v) at room temperature, for 1 h and the resulting mixture was filtered. The residue was extracted in the same conditions for three more times and the filtrated solutions were combined, concentrated, frozen at −20 °C and freeze-dried. The dried extract (ethanolic extract) of *C. multiflorus* was stored in vacuum, at a desiccator in dark, for subsequent use. This procedure was performed in triplicate.

2.4. Purification of phenolic compounds

The ethanolic extract of *C. multiflorus* was purified in order to obtain a suitable sample for NMR analyses. For that, 55 mg of this extract were dissolved in 3 ml of water and eluted in a Strata SPE C18-E cartridge Sephadex (2 g, Waters, Milford, MA, USA). The cartridge was then washed with 5 ml of water, for three times, and the phenolic compounds were recovered by elution with 10 ml of methanol. Following crystallization by evaporation of the solvent to a minimum volume (approximately 1 ml), the supernatant was removed by decantation and the precipitated material was solubilised in DMSO-d_6 for NMR analysis.

2.5. Quantification of total phenolic compounds

The total concentration of phenolic compounds in the ethanolic extract of *C. multiflorus* was determined according to the adapted Folin–Ciocalteu colorimetric method (Singleton & Rossi, 1965) described by Ferreira et al. (2002). The results of the total phenolic compounds were expressed as gallic acid equivalent (mg GAE)/g dried weight of plant material using a calibration curve of gallic acid as standard (5–37.5 μg/ml). All samples were tested in triplicate.

2.6. HPLC apparatus and chromatographic conditions

The HPLC analysis was performed on a Varian 9010 separation module equipped with PDA Varian Prostar detector. The data acquisition and remote control of the HPLC system were conducted by Varian Star chromatography Workstation® (Lake Forest, CA, USA) software. The column used was a 250 mm × 4 mm id, 5 μm bead diameter, end-capped Nucleosil C18 (Macherey–Nagel), and its temperature was maintained at 30 °C.

The flow rate used was 1 ml/min and the gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% (v/v) of formic acid in water and solvent B consisted of acetonitrile, which were degassed and filtrated before use. The solvent gradient consisted in a series of linear gradients, starting from 10% to 30% of solvent B over 20 min, from 30% to 100% of solvent B over 5 min, decreasing to 10% of solvent B after 5 min followed by the return to the initial conditions. For the HPLC analysis, the samples (10 mg) were dissolved in 1 ml of methanol, filtered through a 0.2 μm Nylon membrane (Whatman) and 10 μl of each solution was injected. The UV–Vis spectra were recorded between 220 and 500 nm and the chromatographic profiles were recorded at 280 nm.

2.7. Identification of the phenolic compounds

Compounds for which standards were available were first identified by comparison of the retention times and UV–Vis spectra of the corresponding HPLC peaks. Further analysis by electrospray ionisation mass spectrometry (ESI-MS and $ESI\text{-}MS^n$) allowed the confirmation of their structure (in the case of previous identification by HPLC-DAD) or to obtain structural information on the eluting compounds. In order to have enough amount of sample to carry out this latter analysis, the peak-forming fractions from three independent runs were collected manually according to the visualisation of the UV profile and were freeze-dried. Note that as the NMR analysis requires an amount of sample of approximately 5 mg, this technique was not performed for the HPLC collected fractions (the collection procedure only resulted in micrograms quantities of sample). Still, NMR assays were performed on a purified fraction of the ethanolic extract, in order to elucidate the structure of the main compound in the extract.

2.8. Quantification of the identified phenolic compounds

Fraction 1 (2″-*O*-pentosyl-6-*C*-hexosyl-luteolin), fraction 2 (2″-*O*-pentosyl-8-*C*-hexosyl-luteolin) and fraction 3 (orientin), were quantified using orientin as the reference compound as, in accordance to their UV–Vis and MS spectra, they were mainly rich in luteolin-glucoside derivatives. In a similar approach, fraction 4 (2″-*O*-pentosyl-8-*C*-hexosyl-apigenin), fraction 5 (2″-*O*-pentosyl-6-*C*-hexosyl-apigenin), fraction 7 [6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-apigenin, quercetin-3-*O*-glucoside and luteolin-7-*O*-glucoside], fraction 8 (apigenin-7-*O*-glucoside) and fraction 11 (apigenin) were quantified using apigenin-7-*O*-glucoside as reference. Moreover, fraction 6 [rutin, luteolin-5-*O*-glucoside and 6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-luteolin] was quantified using rutin as reference, while chrysin was used as the reference for the quantification of phenolic compounds in fractions 9 (chrysin-7-*O*-glucoside), 10 (dihydroxyflavone chrysin isomer) and 12 (chrysin). Five-points calibration curves were used for each standard. In particular, for orientin, the tested range was 0.013–0.1 mg/ml and the achieved equation was $y = 6E + 07x - 286{,}681$, with R^2 value of 0.9995 ($n = 13$). The quantification limit (LQ) and detection limit (LD) of this compound were 0.0175 and 0.0058 mg/ml, respectively. For apigenin-7-*O*-glucoside, the tested range was 0.003–0.04 mg/ml, the equation was $y = 8E + 07x - 52{,}202$ with R^2 value of 0.9996 ($n = 13$). LQ and LD were 0.0131 and 0.0043 mg/ml, respectively. The calibration curves of the phenolic standards rutin and chrysin were performed for ranges of 0.018–0.14 and 0.006–0.374 mg/ml, respectively. The

respective equations were $Y = 4E + 07x - 401{,}004$ ($n = 13$) and $Y = 1E + 08x - 354{,}600$ ($n = 13$), with LQ values of 0.0625 and 0.0160 mg/ml (respectively) and LD values of 0.018 and 0.0053 mg/ml, respectively.

2.9. Mass spectrometry analysis by ESI-MS and ESI-MS^n

The HPLC fractions or the phenolic standards were dissolved in methanol and directly injected into the ESI source by means of a syringe pump, at a flow rate of 8 μl/min. ESI-MS analyses were performed in the negative ion mode within the *m/z* range 50–1000, using a Linear Ion trap LXQ instrument (ThermoFinnigan, San Jose, CA, USA) equipped with Xcalibur® software (ThermoFinnigan, San Jose, CA, USA). Typical ESI conditions were: nitrogen sheath gas 30 psi, spray voltage 4.7 kV, capillary temperature 275 °C, capillary voltage −37.0 V and tube lens voltage −81.89 V. CID-MS/MS and MS^n experiments were performed on mass-selected precursor ions using a standard isolation and excitation configuration. Full scan data acquisition was performed from *m/z* 100 to *m/z* 1000 in MS scan mode.

2.10. Nuclear magnetic resonance (NMR) studies

^{1}H and ^{13}C NMR spectra of the purified phenolic extract were recorded at 298 K on a Bruker Avance 500 spectrometer operating at 500.13 and 125.77 MHz, respectively. The phase sensitive ^{1}H-detected (^{1}H,^{13}C) *g*HSQC (heteronuclear single quantum coherence, using gradient pulses for selection) spectrum was recorded with 216 transients over 256 increments (zero-filled to 512) and 2 K data points with spectral widths of 4500 Hz in F_2 and 20 kHz in F_1. The repetition time was 1.9 s. A cosine multiplication was applied in both dimensions. The delays were adjusted according to a coupling constant ^1J(CH) of 147 Hz. The *g*HMBC (heteronuclear multiple quantum coherence, using gradient pulses for selection) spectrum was recorded with 240 transients over 256 increments (zero-filled to 1 K) and 2 K data points with spectral widths of 4500 Hz in F_2 and 25 kHz in F_1. The repetition time was 1.9 s. A sine multiplication was applied in both dimensions. The low-pass *J*-filter of the experiment was adjusted for an average coupling constant ^1J(CH) of 147 Hz and the long-range delay utilised to excite the heteronuclear multiple quantum coherence was optimised for 7 Hz. Chrysin (Sigma) was used as a reference compound for the structural elucidation of the purified ethanolic extract. According to the interpretation of its ^{1}H, ^{13}C NMR, HSQC, COSY and HMBC spectra the ^{1}H and ^{13}C NMR chemical shifts of chrysin were assigned as follow: ^{1}H NMR: $\delta = 6.22$ (d, $J = 2.1$ Hz, H-6), 6.53 (d, $J = 2.1$ Hz, H-8), 6.98 (s, H-3), 7.54–7.64 (m, H-3′,4′,5′), 8.07 (dd, $J = 1.7$ and 7.9 Hz, H-2′,6′); ^{13}C NMR: $\delta = 94.1$ (C-8), 99.0 (C-6), 104.0 (C-10), 105.2 (C-3), 126.4 (C-2′,6′), 129.2 (C-3′,5′), 130.7 (C-1′), 132.1 (C-4′), 157.5 (C-9, 161.5 (C-5), 163.2 (C-2), 164.4 (C-7), 181.9 (C-4).

3. Results and discussion

The ethanolic extract of *C. multiflorus* represented 32% of the dried plant mass and its total phenolic compounds accounted for 140 ± 12 mg GAE/g of extract (data not shown). This amount corresponds to a recovery of 44.7 ± 4.0 mg GAE/g dried plant and thus, it is higher than those values reported by Gião et al. (2007)) for extracts of *C. multiflorus* obtained by infusion or boiling (12.9 mg/g or 26.2 mg/g GAE/g dried plant, respectively).

3.1. Identification of the phenolic compounds of the ethanolic extract of C. multiflorus

In order to characterise the phenolic compounds of the ethanolic extract of *C. multiflorus*, this was further analysed by HPLC-DAD. The corresponding chromatogram, at 280 nm is shown in Fig. 1. Only four of the twelve fractions matched with the available phenolic standards, namely fractions 3, 6, 8 and 12, which corresponded to orientin, rutin, apigenin-7-*O*-glucoside and chrysin, respectively. These four assignments, as also the identification of the remaining phenolic components in the ethanolic extract of *C. multiflorus* were elucidated considering the HPLC-DAD figures, together with electrospray ionisation mass spectrometry (ESI-MS and MS^n) data. Moreover, the NMR analysis of the purified ethanolic extract provided crucial information for the assignment of the main phenolic compound in the extract (fraction 9).

Table 1 summarises the HPLC-DAD and MS data obtained for each of the analysed fractions. MS analysis was preferentially obtained in the negative mode, because of its higher sensitivity in the detection of the distinct classes of phenolic compounds (Cuyckens & Claeys, 2004) although in some cases, analysis in the positive mode was also used in order to confirm the data from the negative mode (data not shown). Together with the NMR analysis, it is possible to conclude that the ethanolic extract of *C. multiflorus* is mainly rich in flavones. Indeed, besides this class of compounds, only two derivatives of quercetin (flavonol) were found. The following sections will focus on the assignments of the structural features of these compounds.

3.1.1. Chrysin derivatives

Besides chrysin, which appeared in the HPLC-DAD profile as a minor component in fraction 12 (eluted at 23.7 min), the ethanolic extract of *C. multiflorus* contained two other chrysin derivatives, which were eluted in fractions 9 and 10. The analysis of these fractions by ESI-MS/MS, together with the analysis of a purified fraction by NMR, allowed to fully elucidate the structure of the compound in fraction 9 and to obtain some structural features on the chrysin derivative detected in fraction 10.

The ESI-MS spectrum of fraction 9 showed two distinct molecular species (at *m/z* 451 and 461), which corresponded to the ionisation of the same compound. In fact, the ESI-MS/MS spectra of those two molecular ions showed similar product ions, namely at *m/z* 415 and 253. The formation of the product ion at *m/z* 415 corresponded to the loss of 36 Da (for the molecular specie at *m/z* 451) and 46 Da (for the molecular specie at *m/z* 461), thus suggesting that they respectively correspond to the chloride adduct $[M + Cl]^-$ and formic acid adduct $[M + CH_2O_2]^-$ of the compound (MW 416 Da). Moreover, the presence of the product ion at *m/z* 253 in the ESI-MS/MS spectrum of the adducts (at *m/z* 451 and *m/z* 461) suggested that the compound of MW 416 Da was a chrysin derivative. This hypothesis was supported by the MS^3 spectrum of the ion at *m/z* 253 and also by the UV–Vis spectrum of fraction 9, which were similar to that of the reference compound. Moreover, the main product ion of the MS^3 spectrum of the $[M + Cl - HCl]^-$ at *m/z* 415 is the ion at *m/z* 253, which was formed by the loss of 162 Da. These results suggested that the main compound in fraction 9 was an hexoside derivative of chrysin.

The total structural elucidation of the phenolic compound detected in fraction 9 (MW 416 Da) was accomplished by NMR analysis. The assays were conducted on a purified sample, in order to simplify the interpretation of the spectra. Still, it must be noted that NMR experiments were also performed on the ethanolic extract (non purified) in order to assure that its main compound corresponded to that of the purified fraction (data not shown). As can be concluded from Fig. 2 and Table 2, all the NMR signals corresponded to one compound, suggesting that the purified procedure was efficient. The ^{1}H and ^{13}C NMR chemical shifts presented in Table 2 were assigned according to the analysis of its ^{1}H (Fig. 2), ^{13}C, COSY, HSQC, and HMBC NMR spectra (data not shown) and further comparison to those of chrysin and to the literature data (El Antri et al., 2004). All the signals in the spectra

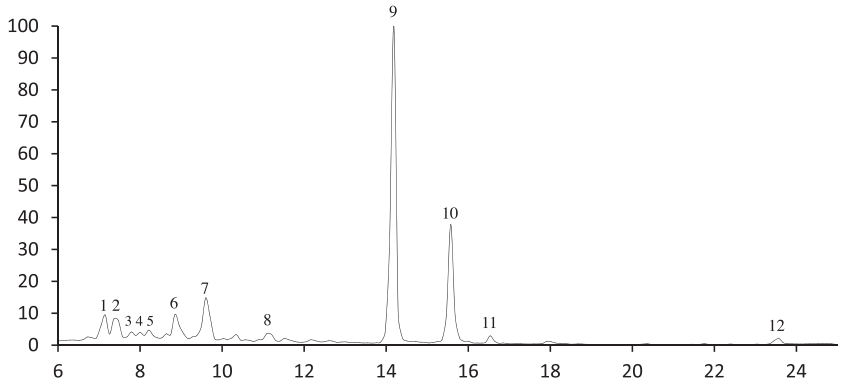


Fig. 1. Chromatographic profile of ethanolic fractions of *Cytisus multiflorus* at 280 nm. The numbers on the figure correspond to the fractions that were collected for by ESI-MS analysis.

Table 1
Identification of HPLC eluting fractions by HPLC-DAD and ESI-MS from the ethanolic extract of *Cytisus multiflorus*.

Peak	RT (min)	λ_{max}	Compound (MW)	Main fragment ESI$^-$ MS^n	Compound
1	7.2	256, 266, 347	580	MS^2 [579]: 459(50), 429(100), 357(20), 327(40), 309(5), 285(1)	2″-*O*-pentosyl-6-*C*-hexosyl-luteolin
2	7.5	257, 266, 346	580	MS^2 [579]: 459(75), 449(15), 429(100), 357(65), 327(100), 309(5), 297(<1), 285(<1); MS^3 [459]:327(100); MS^4 [327]:299(100), 284(15), 255(2); MS^5 [299]: 271(15), 255(100), 240(10), 213(25), 199(3), 175(15), 165(5), 163(1)	2″-*O*-pentosyl-8-*C*-hexosyl-luteolin
3	7.9	256, 266, 345	448	MS^2 [447]: 357(40), 327(100), 285(10); MS^3 [357]: 339(35), 297(100), 285(90)	Orientin
4	8.1	267, 338	564	MS^2 [563]: 545(<1), 473(<1), 443(2), 413(100), 341(<1), 311(<1), 293(4); MS^3 [413]: 293(100); MS^4 [293]: 275(7), 265(30), 249(100), 175(60)	2″-*O*-pentosyl-8-*C*-hexosyl-apigenin
5	8.3	267, 338	564	MS^2 [563]: 443(4), 413(100), 293(8); MS^3 [413]: 293; MS^4 [293]: 265(40), 249(100), 175(50)	2″-*O*-pentosyl-6-*C*-hexosyl-apigenin
			356	MS^2 [355]: 337(15), 199(5), 183(20), 179(100), 175(15), 161(15), 149(2), 143(15), 131(4), 113(10); MS^3 [179]: 161(45), 143(100), 119(3), 89(50)	Unknown
6	9.3	255, 352	610	MS^2 [609]: 343(7), 301(100); MS^3 [301]: 273(10), 257(10),) 179(100), 151(60); MS^4 [179]: 151(100); MS^5 [151]: 107	Rutin
			448	MS^2 [447]: 285(100); MS^3 [285]: 257(7), 241(100), 217 (45), 199 (75), 175 (60), 151 (12)	Luteolin-5-*O*-glucoside
			724	MS^2 [723]: 661(5), 621(15), 579(100), 459(15), 357 (15), 327 (15); MS^3 [579]: 459 (80), 429(90), 357 (70), 327 (100); MS^4 [459]: 327(100); MS^5[327]: 299(100), 284(20), 255(2); MS^5 [299]: 255	6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-luteolin
7	9.7	266, 342	708	MS^2 [707]: 645(7), 605(10), 563(100); MS^3 [563]: 443(5), 413(100), 293(10); MS^4 [413]: 293; MS^5 [293]: 249(100), 205(1), 175(20)	6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-apigenin
			464	MS^2 [463]: 301(100), 300(20); MS^3 [301]: 283(3), 273(15), 257(15), 229(3), 193(5), 179(100), 151(65), 107(2)	Quercetin-3-*O*-glucoside
		255, 262, 347	448	MS^2 [447]: 285; MS^3 [285]: 267(12), 257(20), 243(50), 241(100), 217(50), 201(15), 199(65), 197(10), 175(60), 151(15)	Luteolin-7-*O*- glucoside
8	11.3	266, 342	432	MS^2 [431]: 269	Apigenin-7-*O*- glucoside
9	14.3	267, 303	462	MS^2 [461]: 415 (15), 253(100); MS^3 [253]: 209(100), 181(4), 153(1)	Chrysin-7-*O*-glucoside
			452	MS^2 [451]: 415(5), 253(100); MS^3 [253]: 209(100), 181(4), 151(1)	
10	15.7	267, 303	254	MS^2 [253]: 225(5), 209(100), 167(3), 165(5), 159(5), 151(4), 113(10), 107(3); MS^3 [209]: 181(40), 167(5), 165(50), 153(15)	Chrysin isomer
11	16.7	ND	270	MS^2 [269]: 251(40), 241(25), 227(15), 225(100), 207(20), 201(40), 197(20), 183(10), 181(35), 175(30), 169(10), 151(3), 149(5)	Apigenin
12	23.7	267, 313	254	MS^2 [253]: 209	Chrysin

corroborated the presence of chrysin-7-*O*-β-D-glucopyranoside in the extract. Indeed, besides the characteristic signals of the chrysin aglycone, the spectra showed typical ^{1}H and ^{13}C chemical shifts for β-Glc*p* ($\delta_{H\text{-}1}$ = 5.1 ppm, *J* = 7.5 Hz, $\delta_{C\text{-}1}$ = 100.6 ppm). Moreover, the long-range H-1″ → C-7 correlation in the *g*HMBC spectrum, allowed to confirm that the anomeric carbon of glucose was linked to C-7 of the flavone skeleton. Thus, overall, the mass spectrometry and NMR analysis allowed the conclusion that the compound eluted in fraction 9 corresponded to the known flavonoid chrysin-7-*O*-β-D-glucopyranoside. To the best of our knowledge, this flavone was detected for the first time in the Fabaceae family.

Concerning fraction 10, its ESI-MS spectrum showed the molecular ion at *m/z* 253 (negative mode) or at *m/z* 255 (positive mode), suggesting the presence of a chrysin isomer. This hypothesis was

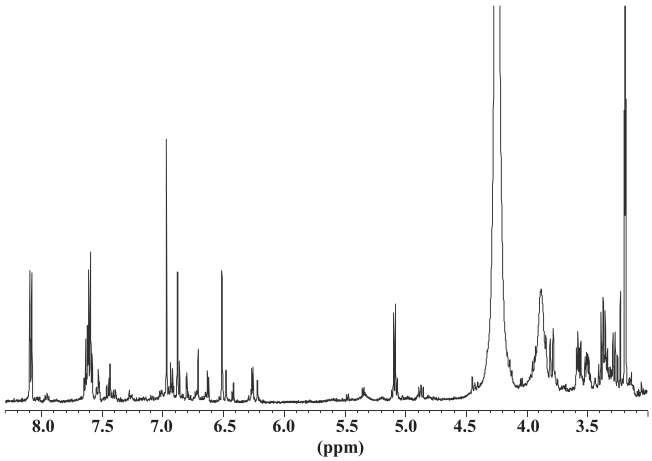


Fig. 2. ^{1}H NMR spectrum of a purified fraction from the ethanolic extract of *Cytisus multiflorus*.

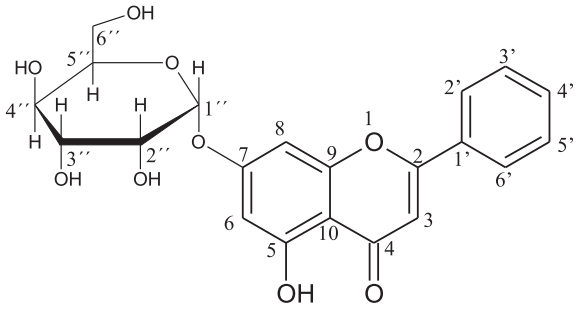
further confirmed by the UV–Vis spectrum of that fraction, which was similar to that of fractions 9 and 12. Attending to these data, we tentatively assigned the compound in fraction 10 to a dihydroxyflavone (MW 254 Da), although the position of the hydroxyl groups in ring A could not be determined.

3.1.2. Luteolin derivatives

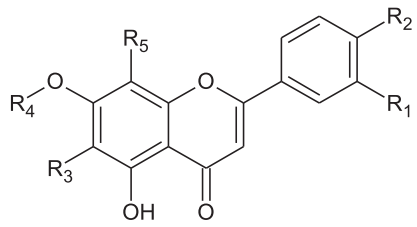
Luteolin derivatives in *C. multiflorus* were mostly *O*-glycosyl-*C*-glycosyl-flavones, with the *O*-glycosylation located on the sugar moiety of a *C*-hexosyl-flavone skeleton, as shown in Fig 3. Indeed, as observed in Table 1, the interpretation of the fragmentation pathway of the molecular ions in the HPLC-DAD fractions allowed the identification of three of these derivatives, namely in fractions 1, 2 and 6. In more detail, the analysis of the negative ESI-MS spectra of fractions 1 and 2 showed a $[M-H]^-$ ion at *m/z* 579, and its MS^2 spectrum revealed the ions at *m/z* 459, 429, 357, 327, and 285. This latter product ion, as also the UV–Vis spectra of these two fractions, supported the occurrence of luteolin derivatives. Moreover, the base peak at *m/z* 429 (−150 Da) was formed by the loss of a pentose sugar and is indicative of a *O*-pentosyl group in those compounds (Ferreres, Gil-Izquierdo, Andrade, Valentao, & Tomas-Barberan, 2007), while the ion at *m/z* 459 (−120 Da) corresponds to the intramolecular breakage of the hexose on the *C*-glycosyl-flavone unit (Cuyckens & Claeys, 2004) and is characteristic of 2″-substituted hexoses (Ferreres, Gil-Izquierdo, et al., 2007). The existence of 2″-*O*-glycosyl-*C*-glycosyl-flavones in fractions 1 and 2 was also supported by the MS analysis in the positive mode (data not shown). Indeed, the analysis of the $[M+H]^+$ ion at *m/z* 581 showed the product ions at *m/z* 431, 329 and 287, that correspond to some of the most abundant ions in the negative ion analysis. Moreover, the fragmentation pathway of the base peak at *m/z* 449 corroborated the hypothesis of a luteolin-*C*-glucoside derivative (Ioset et al., 2007) and the loss of 132 Da from the product ion at *m/z* 449 confirmed the presence of a pentose unit with a *O*-linkage (Cuyckens & Claeys, 2004; Han et al., 2008; Regos, Urbanella, & Treutter, 2009; Ye, Yan, & Guo, 2005).

Overall, the above results allowed the detection, for the first time, of two isomers of 2″-*O*-pentosyl-*C*-hexosyl-luteolin derivatives in *Cytisus*. The presence of these two isomers in contiguous fractions were confirmed by HPLC-MS analysis (results not shown). Moreover, considering that in nature the *C*-glycosyl moieties appear almost exclusively at 6 and/or 8-positions of flavones (Cuyckens & Claeys, 2004) and that the 8-*C*-glycosyl-luteolin isomer elutes before the 6-*C*-glycosyl-luteolin under reversed phase conditions (Kazuno, Yanagida, Shindo, & Murayama, 2005; Pereira, Yariwake, & McCullagh, 2005; Piccinelli et al., 2008), the phenolic compounds in fractions 1 and 2 were respectively assigned to 2″-*O*-pentosyl-6-*C*-hexosyl-luteolin and 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin. The structures of these two compounds are depicted in Fig. 3.

Table 2
Chemical shifts (δ) of chrysin-7-*O*-β-D-glucopyranoside obtained from purified ethanolic extract of *Cytisus multiflorus* (in DMSO-d_6).



Atom	^{13}C	^{1}H	Atom	^{13}C	^{1}H
2	164.6	–	*Glucose*		
3	106.0	6.97	1″	100.6	5.10 (*J* = 7.5 Hz)
4	182.8	–	2″	73.7	3.34 (m)
5	161.7	–	3″	76.9	3.37 (dd, *J* = 9.6, 7.9 Hz)
6	100.3	6.89 (d, *J* = 2.2 Hz)	4″	70.1	3.28 (t, *J* = 9.6 Hz)
7	163.9	–	5″	77.7	3.50 (m)
8	95.5	6.51 (d, *J* = 2.2 Hz)	6″	61.2	3.58 (dd, *J* = 12.0, 5.8 Hz)
					3.80 (dd, *J* = 12.0, 1.9 Hz)
9	157.9	–			
10	106.3	–			
1′	131.3	–			
2′,6′	127.0	8.09 (dd, *J* = 8.2, 1.4 Hz)			
3′,5′	129.7	7.54–7.64			
4′	132.7	7.54–7.64			



Peak	Flavones	R1	R2	R3	R4	R5
1	2″-*O*-pentosyl-6-*C*-hexosyl-luteolin	OH	OH	Hex-Pent	H	H
2	2″-*O*-pentosyl-8-*C*-hexosyl-luteolin	OH	OH	H	H	Hex-Pent
4	2″-*O*-pentosyl-8-*C*-hexosyl-apigenin	H	OH	H	H	Hex-Pent
5	2″-*O*-pentosyl-6-*C*-hexosyl-apigenin	H	OH	Hex-Pent	H	H
6	6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-8-*C*-hexosyl-luteolin	OH	OH	H	H	Hex-Pent-HMG
7	6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-8-*C*-hexosyl-apigenin	H	OH	H	H	Hex-Pent-HMG
9	Chrysin-7-*O*-glucoside	H	H	H	Glc	H

Hex- Hexose; Pent- Pentose; Glc- Glucose; HMG- 3-hydroxy-3-methylglutaroyl

Fig. 3. Proposed structures for flavones identified in the ethanolic extract of *Cytisus multiflorus*.

The luteolin derivative found in fraction 6 (molecular ion at *m/z* 723 or *m/z* 725, in negative or positive ion modes, respectively) was structurally related to the previous ones. Indeed, the MS^2 of the ion at *m/z* 723 (negative mode) showed a base peak ion at *m/z* 579, which corresponds to one of the 2″-*O*-pentosyl-*C*-hexosyl-luteolin isomers described above (fraction 1 or 2). This product ion was formed by the loss of 144 Da, and other ions in the MS^2 spectrum were formed by the loss of 62 Da (at *m/z* 661) and 102 Da (at *m/z* 621). Luteolin derivatives containing a 144 Da moiety were previously described by Ferreres, Sousa, et al. (2007) in *Passiflora* genus, although at that time, these authors have not proposed a structural feature for that unit. Yet, the same fragmentation pattern (−62 Da, −102 Da and −144 Da) has been previously assigned to 3-hydroxy-3-methylglutaroyl flavonoid glycosides in *Citrus bergamia* (Di Donna et al., 2009) and in *Oxytropis racemosa* plant of Fabaceae family (Song et al., 2010). Thus, based on that data, these results suggest the existence of a 3-hydroxy-3-methylglutaroyl derivative of 2″-*O*-pentosyl-*C*-hexosyl-luteolin in *C. multiflorus*. To our knowledge, luteolin derivative compounds containing a 3-hydroxy-3-methylglutaroyl moiety have never been reported in Fabaceae and thus, further studies are needed in order to elucidate the specific linkage position of the 3-hydroxy-3-methylglutaroyl moiety to the phenolic skeleton. In this context, the structure proposed in Fig. 3 should only be regarded as an example.

More common glycosyl-luteolin derivatives (MW 448 Da) occurred as minor components of the ethanolic extract and were detected in fractions 3, 6 and 7. The assignment of these compounds to orientin (fraction 3) (Kazuno et al., 2005), luteolin-5-*O*-glucoside (fraction 6) and luteolin-7-*O*-glucoside (fraction 7) (Rauter et al., 2009), which was based on the HPLC-DAD and MS^n data, will not be discussed in detail, as they were previously described to occur in Fabaceae.

3.1.3. Apigenin derivatives

New apigenin derivatives in *C. multiflorus* belonged to the same group as those of luteolin derivatives, i.e., the 2″-*O*-glycosyl-*C*-glycosyl-flavones. Indeed, besides apigenin (fraction 11) and apigenin-7-*O*-glucoside (fraction 8), the derivatives of this flavone detected in fractions 4, 5 and 7 had comparable fragmentation pathway to that described for 2″-*O*-glycosyl-*C*-glycosyl-luteolin derivatives. Namely, the MS^2 of the abundant molecular ion at *m/z* 563 (fractions 4 and 5) showed a base peak formed by the loss of 150 Da (ion at *m/z* 413), indicating the presence of an *O*-pentosyl group (Ferreres, Gil-Izquierdo, et al., 2007). Moreover, the detection of the product ion $[M–H–120]^-$ (ion at *m/z* 443) in the MS^2 spectrum, as also the ion at *m/z* 293 in the MS^3 spectrum (representing the apigenin aglycone +41–18 Da), corroborated the presence of 2″-*O*-pentosyl-*C*-hexosyl-apigenin isomers in fractions 4 and 5 (Ferreres, Gil-Izquierdo, et al., 2007). Overall, the MS data suggested the presence of 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin (2″-*O*-pentosyl-vitexin) and 2″-*O*-pentosyl-6-*C*-hexosyl-apigenin (2″-*O*-pentosyl-isovitexin) in those fractions (structures represented in Fig. 3). Attending that 8-*C*-glucosyl-apigenin elutes before 6-*C*-glucosyl-apigenin under HPLC reverse phase conditions (Kazuno et al., 2005; Pereira et al., 2005; Piccinelli et al., 2008), compounds of MW 564 Da in fractions 4 and 5 were respectively assigned to 2″-*O*-pentosyl-vitexin and 2″-*O*-pentosyl-isovitexin. To our knowledge, these compounds were here detected for the first time in Fabaceae family.

Similarly to that described for luteolin derivatives, the ethanolic extract of *C. multiflorus* also contained one 3-hydroxy-3-methylglutaroyl derivative of 2″-*O*-pentosyl-*C*-hexosyl-apigenin (MW 708 Da). The base peak in the MS^2 spectrum in the negative mode ($[M + H]^-$ at *m/z* 707) corresponded to one 2″-*O*-pentosyl-*C*-hexosyl-apigenin moiety (ion at *m/z* 563), and this latter ion had a similar fragmentation pattern to that of the *C*-glycosyl isomer. As described before for the luteolin derivative in fraction 6 (MW 724 Da), the presence of an 3-hydroxy-3-methylglutaric acid moiety was proposed based on literature data (Di Donna et al., 2009; Song et al., 2010). Apigenin derivatives containing a 144 Da moiety were also previously described by Ferreres, Sousa, et al. (2007) in *Passiflora* genus, although no structural feature was proposed. As for the luteolin derivative, the proposed structure of the apigenin derivative detected in fraction 7 (Fig 3) is based on the literature

Table 3
Quantification of the identified compounds in the ethanolic extract of *Cytisus multiflorus*.

Number fraction	Compound	mg/g dried plant
1	2″-*O*-pentosyl-6-*C*-hexosyl-luteolin	3.3 ± 0.5
2	2″-*O*-pentosyl-8-*C*-hexosyl-luteolin	3.5 ± 0.3
3	Orientin	0.8 ± 0.1
4	2″-*O*-pentosyl-8-*C*-hexosyl-apigenin	0.5 ± 0.1
5	2″-*O*-pentosyl-6-*C*-hexosyl-apigenin	0.9 ± 0.1
6	Rutin	4.5 ± 0.7
7	6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-apigenin	3.6 ± 0.7
8	Apigenin-7-*O*- glucoside	0.8 ± 0.1
9	Chrysin-7-*O*-β-D-glucopyranoside	15.9 ± 2.3
10	Dihydroxyflavone isomer of chrysin	7.0 ± 1.3
11	Apigenin	0.5 ± 0.1
12	Chrysin	0.5 ± 0.1
Total		41.8 ± 3.0

data reported for the Fabaceae family (Liu, Liu, Liu, Hou, & Mabry, 1994), although further studies are needed in order to confirm that hypothesis.

3.1.4. Quercetin derivatives

Besides the above described flavones, the ethanolic extract of *C. multiflorus* also contained two common derivatives of the flavonol quercetin. According to the HPLC-DAD the ESI-MS^n figures, and also the comparison to the literature data, flavonols were assigned to rutin (MW 610 Da in fraction 6) and quercetin-3-*O*-glucoside (MW 464 Da in fraction 7), which have been previously described in Fabaceae family (Raja et al., 2007).

3.2. Quantification of phenolic compounds by HPLC-DAD

The quantified phenolic compounds in the ethanolic extract of *C. multiflorus* (Table 3) accounted for 41.8 ± 3.0 mg/g dried plant, which is a close value to that obtained by the Folin–Ciocalteu method (44.7 ± 4.0 mg/g dried plant). The extract was shown to be mostly rich in chrysin derivatives, in particular the flavone chrysin-7-*O*-β-D-glucopyranoside. This latter component, together with the dihydroxyflavone (chrysin isomer in fraction 10), accounted for approximately 50% of the extract total phenolic content. Besides those two compounds, the flavones 2″-*O*-pentosyl-6-*C*-hexosyl-luteolin, 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin and 6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-apigenin, as well as flavonol rutin can also be pointed as occurring in moderate concentrations in the ethanolic extract of *C. multiflorus*.

4. Conclusions

The ethanolic extract obtained from flowers of *C. multiflorus* was here described in detail for the first time, by means of HPLC-DAD, ESI-MS and MS^n analyses and NMR assays. The main compound in the phenolic extract of the flowers of this plant was chrysin-7-*O*-β-D-glucopyranoside, but it also contained considerable amounts of rutin, a dihydroxyflavone isomer of chrysin, 2″-*O*-pentosyl-6-*C*-hexosyl-luteolin, 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin and 6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-apigenin, which are not commonly found in the Fabaceae family. Moreover, other unusual phenolic compounds found in minor amounts in the ethanolic extract of *C. multiflorus* were identified as 2″-*O*-pentosyl-6-*C*-hexosyl-apigenin, 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin and 6″-*O*-(3-hydroxy-3-methylglutaroyl)-2″-*O*-pentosyl-*C*-hexosyl-luteolin. Overall, the present work is a valuable contribution for the phenolic elucidation of the *Cytisus* genus and of the Fabaceae family.

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References

Castro, V. (1998). Chromium in a series of Portuguese plants used in the herbal treatment of diabetes. *Biological Trace Element Research, 62*, 101–106.
Castro, V. (2001). Chromium and zinc in a series of plants used in Portugal in the herbal treatment of non-insulinized diabetes. *Acta Alimentaria, 30*, 333–342.
Ciudad, A. G., Santos, B. F., de Aldana, B. R. V., Zabalgogeazcoa, I., Gutierrez, M. Y., & Criado, B. G. (2004). Use of near infrared reflectance spectroscopy to assess forage-quality of a Mediterranean shrub. *Communications in Soil Science and Plant Analysis, 35*, 665–678.
Cristofolini, G., & Conte, L. (2002). Phylogenetic patterns and endemism genesis in *Cytisus* Desf. (Leguminosae-Cytiseae) and related genera. *Israel Journal of Plant Sciences, 50*, 37–50.
Cristofolini, G., & Troia, A. (2006). A reassessment of the sections of the genus *Cytisus* Desf. (Cytiseae, Leguminosae). *Taxon, 55*, 733–746.
Cuyckens, H., & Claeys, M. (2004). Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry, 39*, 1–15.
Di Donna, L., De Luca, G., Mazzotti, F., Napoli, A., Salerno, R., Taverna, D., et al. (2009). Statin-like Principles of Bergamot Fruit (*Citrus bergamia*): Isolation of 3-Hydroxymethylglutaryl Flavonoid Glycosides. *Journal of Natural Products, 72*, 1352–1354.
Di Giorgio, C., Delmas, F., Tueni, M., Cheble, E., Khalil, T., & Balansard, G. (2008). Alternative and complementary antileishmanial treatments: Assessment of the antileishmanial activity of 27 Lebanese plants, including 11 endemic species. *Journal of Alternative and Complementary Medicine, 14*, 157–162.
El Antri, A., Messouri, I., Tlemcani, R. C., Bouktaib, M., El Alami, R., El Bali, B., et al. (2004). Flavone glycosides from *Calycotome villosa* subsp. intermedia. *Molecules, 9*, 568–573.
Ferreira, D., Guyot, S., Marnet, N., Delgadillo, I., Renard, C., & Coimbra, M. A. (2002). Composition of phenolic compounds in a Portuguese pear (*Pyrus communis* L. var. S. Bartolomeu) and changes after sun-drying. *Journal of Agricultural and Food Chemistry, 50*, 4537–4544.
Ferreres, F., Gil-Izquierdo, A., Andrade, P. B., Valentao, P., & Tomas-Barberan, F. A. (2007a). Characterization of C-glycosyl flavones O-glycosylated by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A, 1161*, 214–223.
Ferreres, F., Sousa, C., Valentao, P., Andrade, P. B., Seabra, R. M., & Gil-Izquierdo, A. (2007b). New C-deoxyhexosyl flavones and antioxidant properties of *Passiflora edulis* leaf extract. *Journal of Agricultural and Food Chemistry, 55*, 10187–10193.
Gião, M. S., Gonzalez-Sanjose, M. L., Rivero-Perez, M. D., Pereira, C. I., Pintado, M. E., & Malcata, F. X. (2007). Infusions of Portuguese medicinal plants: Dependence of final antioxidant capacity and phenol content on extraction features. *Journal of the Science of Food and Agriculture, 87*, 2638–2647.
Han, J., Ye, M., Qiao, X., Xu, M., Wang, B.-R., & Guo, D.-A. (2008). Characterization of phenolic compounds in the Chinese herbal drug *Artemisia annua* by liquid chromatography coupled to electrospray ionization mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 47*, 516–525.
Hanganu, D., Vlase, L., & Olah, N. (2010a). LC/MS analysis of isoflavones from Fabaceae species extracts. *Farmacia, 58*, 177–183.
Hanganu, D., Vlase, L., & Olah, N. (2010b). Phytochemical analysis of isoflavons from some Fabaceae species extracts. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 38*, 57–60.
Ioset, J. R., Urbaniak, B., Ndjoko-Ioset, K., Wirth, J., Martin, F., Gruissem, W., et al. (2007). Flavonoid profiling among wild type and related GM wheat varieties. *Plant Molecular Biology, 65*, 645–654.
Kazuno, S., Yanagida, M., Shindo, N., & Murayama, K. (2005). Mass spectrometric identification and quantification of glycosyl flavonoids, including dihydrochalcones with neutral loss scan mode. *Analytical Biochemistry, 347*, 182–192.
Liu, M., Liu, Q., Liu, Y. L., Hou, C. Y., & Mabry, T. J. (1994). An acylated flavone C-Glycoside from *Glycyrrhiza eurycarpa*. *Phytochemistry, 36*, 1089–1090.
Luis, A., Domingues, F., Gil, C., & Duarte, A. P. (2009). Antioxidant activity of extracts of Portuguese shrubs: *Pterospartum tridentatum*, *Cytisus scoparius* and *Erica* spp.. *Journal of Medicinal Plants Research, 3*, 886–893.
Nirmal, J., Babu, C. S., Harisudhan, T., & Ramanathan, M. (2008). Evaluation of behavioural and antioxidant activity of *Cytisus scoparius* Link in rats exposed to chronic unpredictable mild stress. *BMC Complementary and Alternative Medicine, 8*, 8.
Pereira, C. A. M., Yariwake, J. H., & McCullagh, M. (2005). Distinction of the C-glycosylflavone isomer pairs orientin/isoorientin and vitexin/isovitexin using HPLC-MS exact mass measurement and in-source CID. *Phytochemical Analysis, 16*, 295–301.

Piccinelli, A. L., Mesa, M. G., Armenteros, D. M., Alfonso, M. A., Arevalo, A. C., Campone, L., et al. (2008). HPLC-PDA-MS and NMR characterization of C-glycosyl flavones in a hydroalcoholic extract of *Citrus aurantifolia* leaves with antiplatelet activity. *Journal of Agricultural and Food Chemistry, 56*, 1574–1581.

Raja, S., Ahamed, K., Kumar, V., Mukherjee, K., Bandyopadhyay, A., & Mukherjee, P. K. (2007). Antioxidant effect of *Cytisus scoparius* against carbon tetrachloride treated liver injury in rats. *Journal of Ethnopharmacology, 109*, 41–47.

Rauter, A. P., Martins, A., Lopes, R., Ferreira, J., Serralheiro, L. M., Araujo, M. E., et al. (2009). Bioactivity studies and chemical profile of the antidiabetic plant *Genista tenera*. *Journal of Ethnopharmacology, 122*, 384–393.

Regos, I., Urbanella, A., & Treutter, D. (2009). Identification and Quantification of Phenolic Compounds from the Forage Legume Sainfoin (*Onobrychis viciifolia*). *Journal of Agricultural and Food Chemistry, 57*, 5843–5852.

Rodriguez-Riano, T., Ortega-Olivencia, A., & Devesa, J. A. (1999). Reproductive phenology in three Genisteae (Fabaceae) shrub species of the W Mediterranean Region. *Nordic Journal of Botany, 19*, 345–354.

Rodriguez-Riano, T., Ortega-Olivencia, A., & Devesa, J. A. (2004). Reproductive biology in *Cytisus multiflorus* (Fabaceae). *Annales Botanici Fennici, 41*, 179–188.

Rodriguez-Riano, T., Valtuena, F. J., & Ortega-Olivencia, A. (2006). Megasporogenesis, megagametogenesis and ontogeny of the aril in *Cytisus striatus* and *C-multiflorus* (Leguminosae: Papilionoideae). *Annals of Botany, 98*, 777–791.

Siegel, R. K. (1976). Herbal intoxication – Psychoactive effects from herbal cigarettes, tea, and capsules. *Jama-Journal of the American Medical Association, 236*, 473–476.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Song, S. A., Zheng, X. P., Liu, W. D., Du, R. F., Bi, L. F., & Zhang, P. C. (2010). 3-Hydroxymethylglutaryl Flavonol Glycosides from a Mongolian and Tibetan Medicine, *Oxytropis racemosa*. *Chemical & Pharmaceutical Bulletin, 58*, 1587–1590.

Sundararajan, R., Haja, N. A., Venkatesan, K., Mukherjee, K., Saha, B. P., Bandyopadhyay, A., et al. (2006). *Cytisus scoparius* link-a natural antioxidant. *BMC Complementary and Alternative Medicine, 6*, 8.

Ye, M., Yan, Y. N., & Guo, D. A. (2005). Characterization of phenolic compounds in the Chinese herbal drug Tu–Si–Zi by liquid chromatography coupled to electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 19*, 1469–1484.