

Characterization of phenolic compounds in flowers of wild medicinal plants from Northeastern Portugal

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ABSTRACT

Crataegus monogyna, *Cytisus multiflorus*, *Malva sylvestris* and *Sambucus nigra* have been used as important medicinal plants in the Iberian Peninsula since a long time ago, and are claimed to have various health benefits. This study aimed to determine the phenolic profile and composition of wild medicinal flowers of those species. The analysis was performed by HPLC-DAD-ESI/MS. Flavonoids, and particularly flavonols and flavones, were the main groups in almost all the studied samples. *C. multiflorus* sample gave the highest levels of total flavonoids (54.5 mg/g dw), being a chrysin derivative the most abundant flavone found (22.3 mg/g dw). *C. monogyna* revealed the highest concentration in phenolic acids (5.5 mg/g dw) that were not found in *C. multiflorus* sample; 5-*O*-caffeoylquinic acid was the most abundant phenolic acid found in the first species, being a procyanidin trimer also found (1.4 mg/g dw). Kaempferol-3-*O*-rutinoside (0.84 mg/g dw) and quercetin-3-*O*-rutinoside (14.9 mg/g dw) were the main flavonols present in *M. sylvestris* and *S. nigra*, respectively. Due to the well established antioxidant activity of phenolic compounds, the studied wild medicinal flowers could be selected for processing extracts with health-promoting properties or to be incorporate into functional beverages or products with bioactive properties related to oxidative stress.

Keywords: MAP; Wild flowers; flavonoids; HPLC-DAD-ESI/MS.

1. Introduction

Reactive oxygen and nitrogen species (ROS and RNS) such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), hydrogen peroxide (H_2O_2), nitric oxide (NO^{\bullet}) and peroxynitrite anion ($ONOO^-$) are highly reactive species that are responsible for many cell disorders through their action on proteins, DNA, and lipid peroxidation. By modifying the oxidative balance within the cells, these ROS and RNS are important mediators of cell injuries. They are assumed to play an important role in the development of many diseases such as atherosclerosis, reperfusion injury, cataractogenesis, rheumatoid arthritis, neurodegenerative and inflammatory disorders, cardiovascular disease and cancer, besides being involved in the aging process itself (Halliwell and Gutteridge, 1999; Ghafourifar and Cadenas, 2005; Valko et al., 2007).

In the last years epidemiological studies have shown an inverse correlation between increased consumption of antioxidants such as polyphenols and risk of some of the mentioned disorders induced by oxidative stress (López-Lázaro, 2002; Youdim et al., 2002; Steinberg et al., 2003; He et al., 2006). However, information about their bioactive forms *in vivo* and the mechanisms by which they may contribute toward disease prevention is still necessary. Therefore, absorption, distribution, metabolism or excretion, or the plasma/tissue levels of the metabolites of phenolic compounds that might occur, should be considered on studies of their biological (Rechner et al., 2002).

Polyphenols are widely distributed and can exist as simple phenolic acids and flavonoids, or as complex molecules with numerous phenolic groups, e.g. acylated flavonoid glycosides, proanthocyanidins or oligomeric hydrolysable tannins (Fecka, 2009). The positive effects of polyphenols in relation to cardiovascular diseases are probably associated with their ability to increase the antioxidative capacity of the blood plasma and prevent oxidation of low density lipoprotein (LDL) and platelet aggregation.

Their potential cancer preventive effects may be due to their ability to modulate enzyme activities resulting in decreased carcinogenicity of xenobiotics and preventing the development of oxidative stress induced cancer. Finally, polyphenols may also provide anti-inflammatory properties (Kaack and Christensen, 2010). Overall, these substances have the capacity for acting as potent radical scavengers, inhibiting a variety of enzymes, and have also an antihemorrhagic activity by tightening blood vessels (Dawidowicz et al., 2003).

Recently, there has been an increased interest in wild plants rich in phenolic compounds due to their possible beneficial effects on human health. Moreover, some flowers from wild species such as *Crataegus monogyna*, *Cytisus multiflorus*, *Malva sylvestris* and *Sambucus nigra* have been traditionally used for several medicinal applications (Table 1). Our research group has already reported their powerful antioxidant properties (Barros et al., 2010; Barros et al., 2011a; Barros et al., 2011b), that are certainly related to their composition in phenolic compounds.

In this study we performed an exhaustive characterization of phenolic compounds present in those medicinal flowers. There are available studies in literature that described phenolic composition in *M. sylvestris* leaves (Nawwar and Buddrust, 1981; Billeter et al., 1991), but not in its flowers. Regarding *C. multiflorus*, there is a recent report on the phenolic composition of non-wild samples (Pereira et al., 2012). *C. monogyna* (Froehlicher et al., 2009) and *S. nigra* (Dawidowicz et al., 2003; Rieger et al., 2008; Christensen et al., 2008; Kaack and Christensen, 2010) flowers were studied, but with plant material from other European countries, i.e not growing under Mediterranean particular climatic and soil conditions.

2. Materials and methods

2.1. Samples

All plant materials (the parts most cited for medicinal use by key informants during ethnobotanical surveys) were collected in early and late spring of 2009 (depending on each species phenology), in the Natural Park of Montesinho territory, Trás-os-Montes, North-eastern Portugal, considering the Portuguese folk pharmacopoeia, local medicinal criteria of use and each plant growth pattern. *Crataegus monogyna*, the flowers during anthesis plus few expanded leaves attached at the base of the inflorescence peduncle; *Cytisus multiflorus* and *Sambucus nigra*, the inflorescences with flowers fully open and functional; *Malva sylvestris* the flowers' buds and flowers on the upper stems.

Morphological key characters from the Flora Ibérica (Castroviejo 2001, 2005 and 2007) were used for plant identification. Voucher specimens are kept at the Herbário da Escola Superior Agrária de Bragança (BRESA). Each sample was lyophilized (Ly-8-FM-ULE, Snijders, Holland) and stored in the deep-freezer at -20°C for subsequent analysis.

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compounds standards (apigenin-6-*C*-glucoside, catechin, chrysin, caffeic acid, chlorogenic acid (5-*O*-caffeoylquinic acid), gallic acid, isorhamnetin, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, luteolin-6-*C*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside) were from Extrasynthese (Genay, France). All other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Phenolic compounds extraction

Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (v:v) at room temperature, 150 rpm, for 1h. The extract was filtered through Whatman n° 4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (v:v). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210) to remove methanol. The aqueous phase was lyophilized and re-dissolved in 20% aqueous methanol at 5 mg/mL and filtered through a 0.22- μ m disposable LC filter disk for High Performance Liquid Chromatography (HPLC) analysis.

C. monogyna sample and standards 5-*O*-caffeoylquinic acid, *p*-coumaric acid and caffeic acid were also analysed after submitting it to UV irradiation at 366 nm for 24h so as to check inter-conversion between *trans* and *cis* isomers.

2.4. HPLC-DAD-ESI/MS analyses for phenolic compounds identification and quantification

The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C18, 3 μ m (4.6 mm \times 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 ml/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between m/z 100 and 1000.

The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times compared with commercial standards when available. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (2.5-100 $\mu\text{g/mL}$) of different standards compounds: apigenin-6-*C*-glucoside ($y=246.05x-309.66$; $R^2=0.9994$); catechin ($y=132.76x-59.658$; $R^2=0.9997$); chrysin ($y=43.957x-620.02$; $R^2=0.9988$); caffeic acid ($y=617.91x-691.51$; $R^2=0.9991$); chlorogenic acid ($y=600.27x-763.62$; $R^2=0.9998$); gallic acid ($y=556.94x-738.37$; $R^2=0.9988$); isorhamnetin ($y=629.14x-2323.4$; $R^2=0.9967$); isorhamnetin-3-*O*-glucoside ($y=262.31x-9.8958$; $R^2=1.000$); kaempferol-3-*O*-glucoside ($y=190.75x-36.158$; $R^2=1.000$); kaempferol-3-*O*-rutinoside ($y=175.02x-43.877$; $R^2=0.9999$); luteolin-6-*C*-glucoside ($y=365.93x-17.836$; $R^2=0.9997$); quercetin-

3-*O*-glucoside ($y=316.48x-2.9142$; $R^2=1.000$); quercetin-3-*O*-rutinoside ($y=222.79x-243.11$; $R^2=0.9998$). The results were expressed in mg per g of dry weight (dw), as mean \pm standard deviation of three independent analyses.

3. Results

3.1. Quantification of the identified phenolic compounds

Figure 1 shows the profiles of different classes of phenolic compounds determined in *C. monogyna*, *C. multiflorus*, *M. sylvestris* and *S. nigra* flowers, which include phenolic acids and flavonoids (flavonols, flavones and procyanidins). Flavonoids, and particularly flavonols and flavones, were the main groups in almost all the studied samples. *C. multiflorus* sample gave the highest levels of total flavonoids (54.5 mg/g dw), being flavones (41.5 mg/g dw) the main compounds found, and therefore the highest levels of total phenolic compounds (**Figure 1**). *S. nigrans* presented the highest levels of flavonols (20.5 mg/g dw); otherwise *C. monogyna* presented the highest concentration in phenolic acids (5.5 mg/g dw), which were not found in *C. multiflorus* sample, whereas procyanidins (1.4 mg/g dw) were only found in minor levels in *C. monogyna* (**Figure 1**). As an example, the HPLC phenolic profiles of *C. monogyna* flowers recorded at 280 nm and 370 nm can be observed in **Figure 2**.

Table 2 presents the data obtained from HPLC-DAD-MS analysis (retention time, λ_{\max} in the visible region, mass spectral data) used for the identification and quantification of phenolic compounds in the four medicinal flowers.

3.2. Phenolic acids and derivatives

Phenolic acids and derivatives were found in all the medicinal flowers with exception of *C. multiflorus*, which did not present these compounds. Diverse hydroxycinnamic acid

derivatives were found in *C. monogyna*, *M. sylvestris* and *S. nigra* samples. Chlorogenic acids are a family of esters formed between certain *trans* cinnamic acids, most commonly caffeic, *p*-coumaric and ferulic and quinic acid (IUPAC, 1976, Clifford et al., 2003, 2005, 2006, 2007). Using a more general definition, all esters of quinic acids and their diastereomers can be considered as chlorogenic acids. In the IUPAC system, (-)-quinic acid is defined as 11-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid (IUPAC, 1976), but Eliel and Ramirez (1997) propose 1 α ,3R,4 α ,5R-tetrahydroxycyclohexane carboxylic acid. The detected compounds showed UV spectra with a λ_{max} at 312, 326 and 330 nm and pseudo molecular ions [M-H]⁻ at *m/z* 353 or *m/z* 337, all of them yielding a product ion at *m/z* 191, due to the deprotonated quinic acid, so that they could be clearly identified as quinic acid derivatives containing one caffeic acid or *p*-coumaric acid moiety, respectively (**Table 2**). Free caffeic acid was only found in *M. sylvestris* and was identified by comparison of its UV spectrum and retention time with a commercial standard.

Peak assignments of the different caffeoylquinic acid isomers found in the extracts from *C. monogyna* and *S. nigra* were made using the recommended IUPAC numbering system (IUPAC,1976) as also the hierarchical keys previously developed by Clifford et al. (2003, 2005), and specimen structures are presented in **Figure 3**. According to Clifford et al. (2003, 2005, 2006, 2007; Lin and Harnly, 2008), many plants produce different caffeoylquinic acids in which esterification occurs at positions 3, 4 and 5 of the quinic acid moiety.

Peaks 4 and 3 in *C. monogyna* and *S. nigra*, respectively, were positively identified 5-*O*-caffeoylquinic acid by comparison with an authentic standard. Peak 2 in *C. monogyna*, identified as 3-*O*-caffeoylquinic acid, yielded the base peak at *m/z* 191 (deprotonated quinic acid) and also gave an ion at *m/z* 179 [caffeic acid-H]⁻, with an

intensity >50% base peak. Similar fragmentation pattern was already reported by [Clifford, et al. \(2003, 2005\)](#) and used to distinguish 3-acylchlorogenic acids.

Peak 1 in *C. monogyna* and *S. nigra* showed similar fragmentation pattern and fragment abundances as those obtained for 3-*O*-caffeoylquinic acid, pointing out to they could be the corresponding *cis* isomer. With the aim of obtaining further support to this assumption, the *Crataegus* sample was UV-irradiated so as to try and induce the partial conversion of the *trans* isomer to the corresponding *cis* isomer. However, no relevant decrease in the size of peak 2 was observed nor an increase in the size of peak 1, which indeed did not support the *cis* nature of peak 1. Furthermore, commercial standards of chlorogenic acid (i.e., *trans* 5-*O*-caffeoylquinic acid), (*trans*) caffeic acid and (*trans*) *p*-coumaric acid were also submitted to UV irradiation in the same conditions as used for the *Crataegus* sample (i.e., 366 nm, 24h). Only partial transformation of *p*-coumaric acid with appearance of a new peak at earlier retention time in the HPLC chromatogram, attributed to the corresponding *cis* isomer was found, whereas no changes were observed in the chromatograms obtained after irradiation of the chlorogenic and caffeic acids. In these circumstances, it might be assumed that the UV irradiation conditions used were sufficient for the transformation of the *p*-coumaric acid but not the caffeic acid moiety to the corresponding *cis* isomer. Therefore, it might be possible that conversion from peak 2 to 1 following irradiation was not produced or occurred in a small extent, insufficient to produce a sizeable modification in the peak areas. All in all, although the actual identity of peak 1 remains unclear, based on its fragmentation pattern and small levels it was tentatively assigned as *cis*-3-*O*-caffeoylquinic acid.

Peak 3 in *C. monogyna* was easily distinguished from the other two isomers by its base peak at m/z 173 [quinic acid-H-H₂O]⁻, accompanied by a secondary fragment ion at m/z

179 with approximately 70% abundance of base peak, which allowed identifying as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford et al. (2003, 2005). A signal at m/z 515 was observed in *C. monogyna* (peak 15) and *S. nigra* (peak 11), which would correspond to a dicaffeoylquinic acid and was tentatively assigned as 3,5-*O*-dicaffeoylquinic acid based on its fragmentation pattern and abundances described by Clifford et al. (2005). MS² base peak was at m/z 353, produced to the loss of one of the caffeoyl moieties [M-H-caffeoyl]⁻, and subsequent fragmentation of this ion yielded the same fragments as a 5-*O*-caffeoylquinic acid at m/z 191 and 179 and 135. The weak signal (5% of base peak) of the ion at m/z 173 could indicate the absence of a C4 substituent.

Various signals at m/z 337 were detected in *C. monogyna* (peaks 5, 6, 8 and 9) and *S. nigra* (peaks 2, 5 and 6) samples. These compounds were tentatively identified according to their MS² fragmentation as different isomers of *p*-coumaroyl quinic acid. Identities were assigned based on the patterns reported for the caffeoylquinic acid isomers. Thus, fragmentation of both peak 5 and 6 (*C. monogyna*), yielding a majority MS² product ion at m/z 173 was coherent with 4-*O-p*-coumaroylquinic acid. Peak 6 with higher areas was associated to the *trans* isomer, whereas the much smaller peak 5 with similar mass spectral characteristics was assigned to *cis* 4-*O-p*-coumaroylquinic acid. This assumption was supported in the analysis of the *Crataegus* sample after UV irradiation. In this case, contrary to what happened for peak 2, the irradiation led to a decrease in the area of peak 6 and a concomitant increase in that of peak 5 suggesting inter-conversion and supporting their *trans/cis* nature. As above indicated, the *p*-coumaric moiety seemed to be more sensible to isomerization than caffeic acid. It should also be noted that in our HPLC chromatograms the *cis* isomer elutes before the corresponding *trans*, contrary to what was reported by Clifford et al. (2003, 2006) in

samples of cider and *Aster ageratoides*. The particular HPLC conditions used by Clifford's and the closer retention times between both isomers may explain this difference in the HPLC elution order.

Peaks 9 (*C. monogyna*) and 6 (*S. nigra*) were identified as *trans* 5-*O-p*-coumaroylquinic acid due to the base peak at m/z 191, accompanied by a weak fragment at m/z 163 corresponding to [*p*-coumaric acid-H]⁻. Peaks 8 in *C. monogyna* and 5 in *S. nigra* with UV spectra and MS² fragmentation patterns and abundances identical to that of the usual *trans* 5-*O-p*-coumaroylquinic (peak 9 in *C. monogyna* and peak 6 in *S. nigra*) were tentatively identified as the corresponding *cis* isomers.

3.3. Flavonols

Flavonols were the main flavonoids found in all the studied samples, being quercetin derivatives (λ_{max} around 354 nm, and MS² fragment at m/z 301), particularly abundant. Quercetin-3-*O*-rutinoside was found in all the studied medicinal flowers, while quercetin-3-*O*-glucoside was only found in *C. monogyna* and *S. nigra* samples. Both compounds were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Other detected quercetin glycosides were peak 14 in *C. monogyna*, peaks 1 and 2 in *C. multiflorus* and peak 7 in *S. nigra*, which were respectively assigned to a quercetin acetyl hexoside ([M-H]⁻ at m/z 505), peaks 1 ([M-H]⁻ at m/z 505), quercetin dihexoside ([M-H]⁻ at m/z 625), quercetin acetyl dihexoside ([M-H]⁻ at m/z 667), and quercetin hexoside (pseudomolecular ion [M-H]⁻ at m/z 463), according to their pseudomolecular ions and MS² spectra, releasing fragments corresponding to the losses of hexosyl moieties (-162 amu) and, in the respective cases, the acetyl residue (-42 amu). In none of them the identity of the sugar and positions of location of the substituents could be established.

Kaempferol derivatives (λ_{\max} around 346 nm, and MS² fragment at m/z 285) were also found in all the samples, being kaempferol-3-*O*-rutinoside present in all the analysed flowers but *C. multiflorus*. Another undefined kaempferol hexoside was found in this latter sample (peak 7) as well as in *M. sylvestris* (peak 6), for which the nature of the hexosyl residue could not be established. A kaempferol acetyl hexoside was also detected in *C. multiflorus* (peak 8) releasing MS² fragments at m/z 327 ([M-162]⁻, loss of a hexose moiety) and at m/z 285 ([M-42]⁻, loss of an acetyl moiety).

Another group of detected flavonols were isorhamnetin derivatives according to their UV-vis and mass spectra (all of them released an MS² product ion at m/z 315); they were found in *C. monogyna* (peak 17), *M. sylvestris* (peak 8) and *S. nigra* (peak 12). The two latter peaks were positively identified as isorhamnetin-3-*O*-rutinoside and isorhamnetin-3-*O*-glucoside by comparison with standards, whilst peak 17 in *C. monogyna* was assigned to an undefined isorhamnetin acetylhexoside from the loss of -204 amu (-162-42 amu, corresponding to hexosyl + acetyl residues).

Finally, laricitrin and syringetin derivatives were tentatively in *M. sylvestris* sample, presenting UV spectra with λ_{\max} at 354 nm, and showing pseudomolecular ions [M-H]⁻ at m/z 639 and 653, respectively, each of them releasing a unique MS² fragment at m/z 331 (laricitrin) and 345 (syringetin), respectively. The loss of -308 amu in both peaks was coherent with the presence of a rutinoside moiety.

3.4. Flavones

Flavones were also found in all the medicinal flowers, with exception of *S. nigra*. The main flavones found were *C*-glycosylated, as revealed from their fragmentation pattern different to that of the *O*-glycosides (Ferrerres et al., 2003; Ferreres et al., 2004; Ferreres et al., 2007; Barros et al., 2011c; Barros et al., 2011d; Ferreres et al., 2011). Different

peaks could be associated to apigenin derivatives according to their UV and mass spectral characteristics. Peak 10 in *C. monogyna* showed a pseudomolecular ion $[M-H]^-$ at m/z 577, releasing five MS^2 fragments ions. The loss of -120 amu (ion at m/z 457 ($[M-120]^-$)) is characteristic of *C*-hexosyl flavones (Ferrerres et al., 2003), whereas the loss of 164 amu, leading to the fragment at m/z 413 ($[M-146-18]^-$) is characteristic of an *O*-glycosylation on the hydroxyl group at position 2 of the *C*-glycosylation sugar (Ferrerres et al., 2007). The other three ions at m/z 341 ($[aglycone+71]^-$), m/z 311 ($[aglycone+41]^-$) and m/z 293 ($[aglycone+41-18]^-$) are usual in mono-*C*-glycosyl derivatives *O*-glycosylated on 2'' position (Ferrerres et al., 2007). Thus, this peak could be tentatively identified as 2''-*O*-rhamnose-*C*-hexoside apigenin. This assumption was also supported by the observations of Ferrerres et al. (2007, 2011) that found similar fragmentation patterns and abundances for *O*-glycosyl-*C*-glycosyl flavones with *O*-glycosylation on the phenolic hydroxyl or on the *C*-glycosyl residue or combination of both forms. Similar reasoning was applied for the assignment of peak 16 ($[M-H]^-$ at m/z 619) in *C. monogyna* as an acetyl derivative of peak 10, as both of them presented similar fragmentation pattern and UV spectra with the difference that peak 16 was 42 amu (acetyl residue) greater than peak 10.

Peaks 4 and 5 in *C. multiflorus* presented pseudomolecular ions $[M-H]^-$ at m/z 579 and 563, respectively, that yielded MS^2 fragment ions at m/z 459 and 443, ($[M-120]^-$, characteristic of *C*-hexosyl flavones), and m/z 429 and 413 ($[M-150]^-$) from the loss of 150 amu (132+18), typical of a pentosyl moiety from *O*-glycosylation in a phenolic hydroxyl group. Other three fragment ions released from both peaks corresponded to $[aglycone+71]^-$, $[aglycone+41]^-$ and $[aglycone+41-18]^-$ associated to mono-*C*-glycosyl derivatives *O*-glycosylated on 2'' position, which allowed identifying them as 2''-*O*-pentosyl-8-*C*-hexosides of luteolin and apigenin, respectively. The fragmentation

pattern and relative abundance of fragments indicated sugar substitution at C-8 according to Ferreres et al. (2007, 2011).

Peak 11 in *M. sylvestris* was identified as apigenin 7-*O*-glucoside, according to its retention, UV spectrum and mass characteristics compared with an authentic standard. Peaks 12 in the same sample presented similar UV spectra with λ_{\max} around 336 nm and the same pseudomolecular ion $[M-H]^-$ at m/z 431 as peak 11. MS² yielded two fragment ions at m/z 311 (loss of 120 amu, characteristic of C-hexosyl flavones), and at m/z 269 (-162 amu, loss of an hexosyl moiety). The loss of 120 amu but not 90 amu suggested that the sugar was located at position 8-*C*, and thus the compound was identified as apigenin 8-*C*-hexoside.

Peaks 9 ($[M-H]^-$ at m/z 877) and 10 ($[M-H]^-$ at m/z 973) in *C. multiflorus* showed identical UV spectra with λ_{\max} at 268-280 nm, and their parent ions released a major MS² fragment at m/z 253, which allowed tentatively assigning them as chrysin derivatives, although they could not be fully identified.

The MS² analysis of peak 9 yielded five fragments at m/z 831 $[M-46]^-$, (loss of formic acid), m/z 669 $[M-46-162]^-$, (loss of formic acid and hexoside moiety). The successive losses of the formic acid molecule and sugar moiety could point out that the sugar linked to chrysin, could be esterified with formic acid. The other three fragments at m/z 461, 415 and 253 presented the same fragmentation pattern; the loss of 208 amu (46+162), which suggested the loss of two hexosides moieties and formic acid molecules, leading to an tentatively suggestion that the second hydroxyl group of the aglycone could be linked to disaccharide that could be esterified with two formic acid molecules. Peak 10 has a similar fragmentation pattern to peak 9, also having a similar chemical structure to this compound, it was identified as a chrysin derivative, but no more conclusions could be taken in the identification of this peak.

3.5. Procyanidins

A peak that could correspond to a procyanidin trimer (peak 7) was also detected in the *C. monogyna* sample. This identity was assigned based on its a UV spectrum, with λ_{\max} at 280 nm, and pseudomolecular ion $[M-H]^-$ at m/z 865.

4. Concluding remarks

As far as we know, there is no information on the phenolic composition of *M. sylvestris* flowers, although previous studies have been published dealing *M. sylvestris* leaves (Nawwar and Buddrust, 1981; Billeter et al., 1991). Otherwise, for *C. monogyna* and *S. nigra* flowers there are a few publications on their phenolic composition but from other countries, such as France (Froehlicher et al., 2009) for *C. monogyna*, Austria (Rieger et al., 2008), Denmark (Christensen et al., 2008; Kaack and Christensen, 2010) and Poland (Dawidowicz et al., 2003) for *S. nigra*. Froehlicher et al. (2009) only described the most abundant phenolic compounds present in *C. monogyna* flowers, being phenolic acids (chlorogenic and caffeic acids), and some flavonoids such as epicatechin and quercetin-3-*O*-galactoside the majority compounds found. In the present study up to 17 compounds have been identified, including some of those previously reported, but also other flavonols, flavones and chlorogenic and *p*-coumaric acid derivatives.

For *S. nigra*, all the previous studies reported the presence of quercetin-3-*O*-glucoside (isoquercitrin) and quercetin-3-*O*-rutinoside (rutin), as well as different caffeoylquinic acids, as also observed in the present study (Christensen et al., 2008; Rieger et al. 2008).

In a sample analysed by Kaack and Christensen (2008) not only quercetin but also other flavonols (kaempferol-3-*O*-rutinoside isorhamnetin acetylhexoside) were detected as for the Portuguese sample here studied.

Regarding *C. multiflorus* flowers, there is a recent report on the phenolic composition in cultivated samples (Pereira et al., 2012), which showed some similarities with the herein studied wild flowers, such as the presence of flavone-C-glycosides and flavonols, namely quercetin derivatives, although the variety of flavones and flavonols detected in our study was greater.

Overall, the present study represents a contribution to the chemical characterization of phenolic extracts from wild flowers with reported antioxidant activity and traditionally used for several medicinal applications.

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Table 1. Medicinal uses of flowers from these medicinal species reported in some European ethnobotanical surveys (Carvalho, 2010; Barros et al., 2010; Barros et al., 2011a; Barros et al., 2011b).

Species	English name	Local name	Preparations	Therapeutics	Country
<i>Crataegus monogyna</i> Jacq	Common hawthorn	Espinheiro, escaramunheiro	Infusion, gargles	Cardiovascular disease, atherosclerosis, arthritis, and hypertension, respiratory system affections, tranquilizer, obesity and menopause disturbances	Portugal, Albania, Bulgaria, France, Italy, Spain
<i>Cytisus multiflorus</i> L.	White Spanish broom	Giesta branca	Infusion, decoction	Metabolic and endocrine system disorders, hypertension, rheumatism and headache. Panacea	Portugal
<i>Malva sylvestris</i> L.	Common mallow	Malva	Decoctions, baths, gargles, lotions, vapour baths, syrups	Acne, skin condition and injuries, eyes, throat pain, cough, stomachic, mild laxative. Panacea	Portugal, Albania, Bulgaria, France, Italy, Spain
<i>Sambucus nigra</i> L.	Elder	Sabugueiro	Infusion, poultices, ointments	Respiratory system affections, skin injuries, wounds diabetes, blood cleanser, stomachic, diuretic. Panacea	Portugal, Albania, Bulgaria, France, Italy, Spain

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, relative abundances of fragment ions, tentative identification and quantification of the phenolic compounds in the studied medicinal flowers.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H] ⁻ (m/z)	MS ² (m/z)	Tentative identification	Quantification (mg/g, dw)
<i>Crataegus monogyna</i>						
1	7.1	326	353	191(100),179(84),173(7),135(55)	<i>cis</i> 3- <i>O</i> -Caffeoylquinic acid	0.02 ± 0.00
2	8.7	328	353	191(100),179(63),173(11),135(74)	<i>trans</i> 3- <i>O</i> -Caffeoylquinic acid	0.16 ± 0.02
3	10.2	326	353	191(49),179(70),173(100),135(61)	4- <i>O</i> -Caffeoylquinic acid	0.21 ± 0.01
4	10.7	326	353	191(100),179(23),173(25),135(18)	5- <i>O</i> -Caffeoylquinic acid	2.33 ± 0.10
5	11.4	312	337	191(19),173(100),163(24),155(4),137(7)	<i>cis</i> 4- <i>O-p</i> -Coumaroylquinic acid	0.22 ± 0.01
6	12.5	312	337	191(6),173(100),163(70),155(16),137(23)	<i>trans</i> 4- <i>O-p</i> -Coumaroylquinic acid	1.44 ± 0.08
7	13.2	280	865	451(44),425(59),407(97), 289(65)	Procyanidin trimer	1.38 ± 0.01
8	13.4	312	337	191(100),173(23),163(15),155(3),137(2)	<i>cis</i> 5- <i>O-p</i> -Coumaroylquinic acid	0.39 ± 0.00
9	13.6	312	337	191(100),173(25),163(15),155(3),137(2)	<i>trans</i> 5- <i>O-p</i> -Coumaroylquinic acid	0.22 ± 0.02
10	15.0	339	577	457(16),413(100),341(6), 311(23),293(91)	2''- <i>O</i> -Rhamnose- <i>C</i> -hexoside apigenin	0.75 ± 0.00
11	15.6	354	609	301(100)	Quercetin 3- <i>O</i> -rutinoside	0.88 ± 0.01
12	16.2	354	463	301(100)	Quercetin 3- <i>O</i> -glucoside	4.66 ± 0.27
13	16.9	346	593	285(100)	Kaempferol-3- <i>O</i> -rutinoside	0.64 ± 0.03
14	17.1	354	505	463(9),301(63)	Quercetin acetyl hexoside	0.45 ± 0.01
15	17.3	328	515	353(100),191(81),179(57),173(5),135(29)	3,5-Di- <i>O</i> -caffeoylquinic acid	0.55 ± 0.04
16	18.1	340	619	499(12),457(2),413(100),341(3),311(15),293(91)	2''- <i>O</i> -Rhamnose- <i>C</i> -acetyl-hexoside apigenin	2.00 ± 0.04
17	18.3	336	519	315(100)	Isorhamnetin acetylhexoside	0.82 ± 0.03
<i>Cytisus multiflorus</i>						
1	11.3	354	625	463(100),301(50)	Quercetin dihexoside	0.43 ± 0.05
2	13.3	354	667	505(62),463(75), 301(50)	Quercetin acetyldihexoside	0.14 ± 0.02

3	14.0	348	693	459(41),429(100),357(34),327(81),309(34)	Luteolin derivative	1.72 ± 0.03
4	14.2	348	579	459(23),429(73),357(71),327(100),309(66),	2''-O-Pentoxide-8-C-hexoside luteolin	2.31 ± 0.01
5	15.1	336	563	443(4),413(100),323(2),311(9),293(84)	2''-O-Pentoxide-8-C-hexoside apigenin	2.40 ± 0.02
6	15.6	354	609	301(100)	Quercetin-3-O-rutinoside	4.06 ± 0.34
7	16.5	346	447	285(100)	Kaempferol hexoside	5.74 ± 0.70
8	18.5	346	489	489(100),327(1),285(61)	Kaempferol acetylhexoside	2.61 ± 0.25
9	23.5	268,308sh	877	831(9),669(12),461(42),415(10),253(100)	Chrysin derivative	12.82 ± 0.11
10	25.8	270,306sh	973	669(3),598(18),461(28),339(32),253(100)	Chrysin derivative	22.29 ± 0.05

Malva sylvestris

1	12.1	328	353	191(27),179(13),173(44),135(6)	Caffeoylquinic acid	0.15 ± 0.01
2	13.3	328	179	135(100)	Caffeic acid	0.63 ± 0.01
3	14.4	292,334sh	463	301(100)	Hesperetin hexoside	0.25 ± 0.02
4	15.6	354	609	301(100)	Quercetin-3-O-rutinoside	0.20 ± 0.00
5	15.8	354	639	331(100)	Laricitrin rutinoside	0.09 ± 0.01
6	16.4	345	447	285(100)	Kaempferol hexoside	0.31 ± 0.02
7	16.9	346	593	285(100)	Kaempferol-3-O-rutinoside	0.84 ± 0.03
8	17.2	348	623	315(100)	Isorhamnetin rutinoside	0.05 ± 0.00
9	17.4	354	653	345(100)	Syringetin rutinoside	0.07 ± 0.01
10	17.9	350	447	447(100), 285(49)	Kaempferol-3-O-glucoside	0.12 ± 0.00
11	18.6	337	431	431(100), 269(65)	Apigenin-7-O-glucoside	0.26 ± 0.02
12	18.9	336	431	431(100), 311(17),269(13)	Apigenin-8-C-hexoside	0.87 ± 0.01

Sambucus nigra

1	7.2	326	353	191(100),179(82),173(8),135(68)	<i>cis</i> 3-O-Caffeoylquinic acid	0.23 ± 0.03
2	9.4	312	337	191(20),173(12),163(100),155(6)	3-O- <i>p</i> -Coumaroylquinic acid	0.15 ± 0.01
3	10.7	326	353	191(100),179(8),173(10),135(4)	5-O-Caffeoylquinic acid	3.38 ± 0.05
4	12.0	326	353	191(100)	Caffeoylquinic acid	0.03 ± 0.00
5	13.4	312	337	191(100),173(10),163(4)	<i>cis</i> 5-O- <i>p</i> -Coumaroylquinic acid	0.33 ± 0.02

6	13.8	312	337	191(100),173(4),163(6)	<i>trans</i> 5- <i>O-p</i> -Coumaroylquinic acid	0.23 ± 0.01
7	14.9	354	463	301(100)	Quercetin hexoside	0.07 ± 0.00
8	15.6	354	609	301(100)	Quercetin 3- <i>O</i> -rutinoside	14.93 ± 0.18
9	16.3	354	463	301(100)	Quercetin 3- <i>O</i> -glucoside	2.39 ± 0.02
10	16.9	346	593	285(100)	Kaempferol 3- <i>O</i> -rutinoside	1.57 ± 0.07
11	17.3	330	515	353(100),191(54),179(44),173(4),135(15)	3,5-Di- <i>O</i> -caffeoylquinic acid	1.06 ± 0.17
12	18.1	354	477	315(100)	Isorhamnetin 3- <i>O</i> -glucoside	1.58 ± 0.03

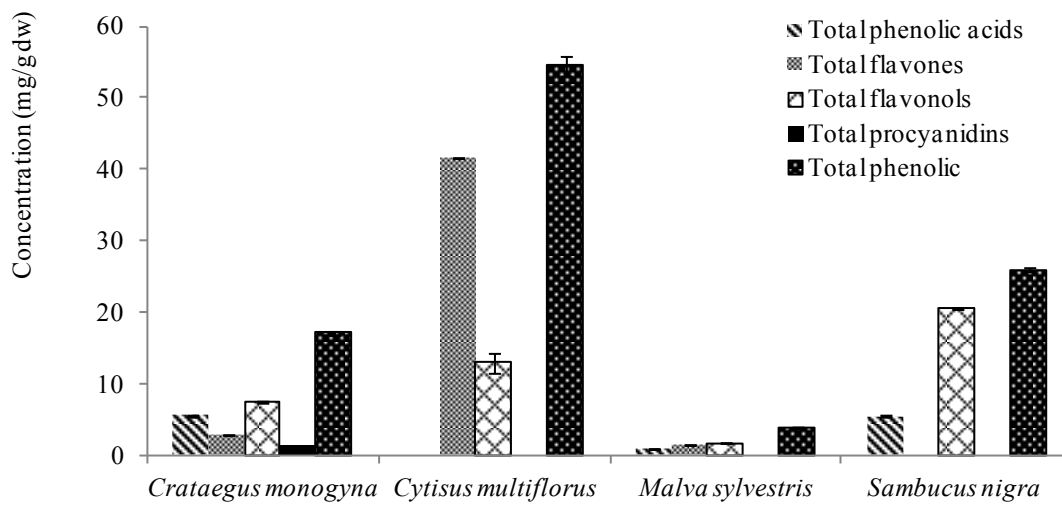


Figure 1. Concentrations of the different classes of phenolic compounds found in the studied wild flowers.

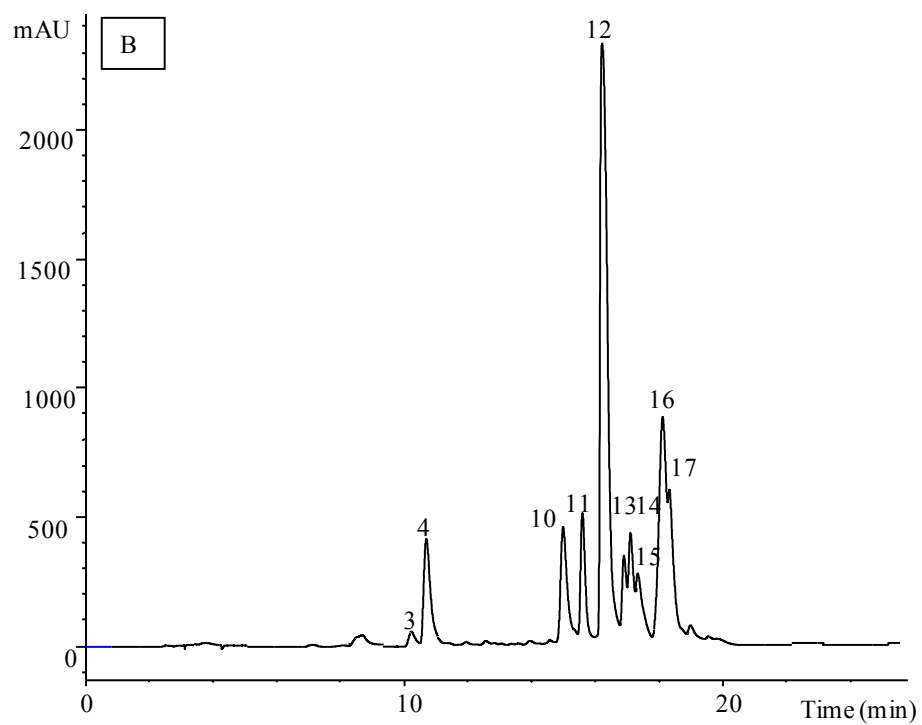
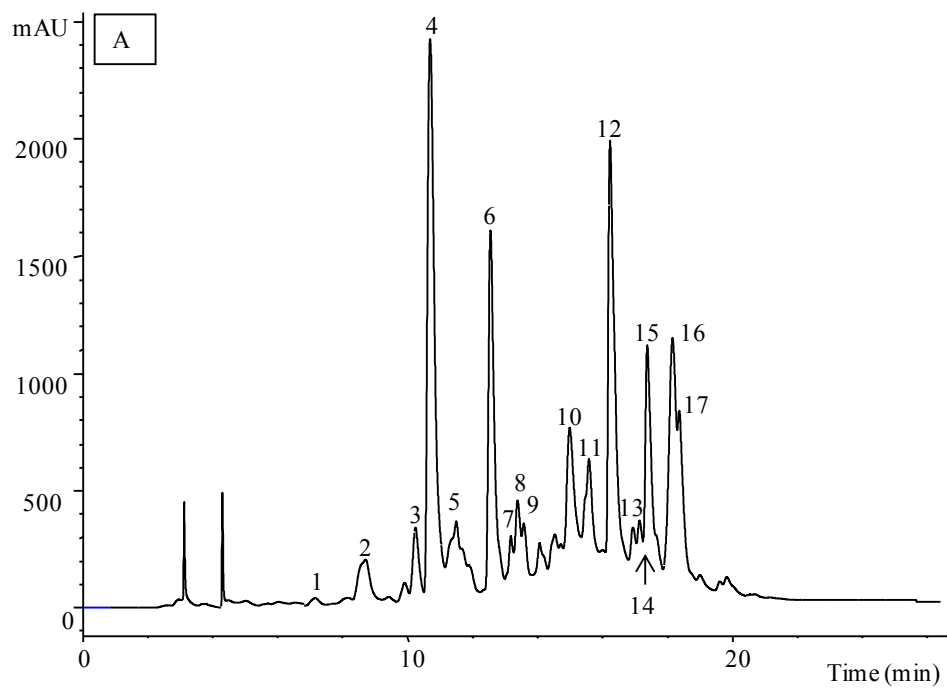
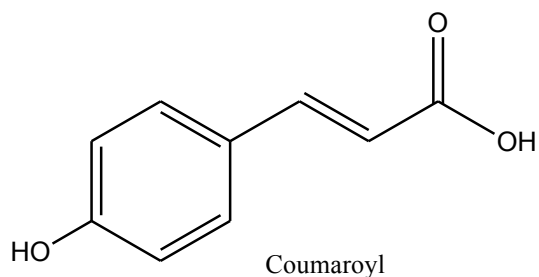
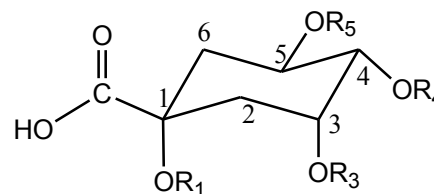
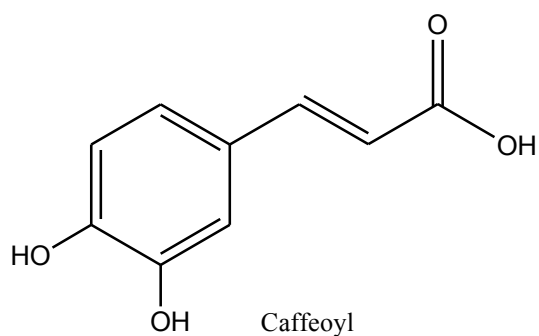


Figure 2. HPLC chromatogram of the phenolic extract of *Crataegus monogyna* flowers recorded at 280 nm (A) and 370 nm (B).



1-*O*-Caffeoylquinic acid, $R_1 = \text{caffeoyl}$, $R_3 = R_4 = R_5 = \text{OH}$

3-*O*-Caffeoylquinic acid, $R_3 = \text{caffeoyl}$, $R_1 = R_4 = R_5 = \text{OH}$

4-*O*-Caffeoylquinic acid, $R_4 = \text{caffeoyl}$, $R_1 = R_3 = R_5 = \text{OH}$

5-*O*-Caffeoylquinic acid, $R_5 = \text{caffeoyl}$, $R_1 = R_3 = R_4 = \text{OH}$

3,5-Di-*O*-caffeoylquinic acid, $R_3 = R_5 = \text{caffeoyl}$, $R_1 = R_4 = \text{OH}$

3-*O-p*-Coumaroylquinic acid, $R_3 = p\text{-coumaroyl}$, $R_1 = R_4 = R_5 = \text{OH}$

4-*O-p*-Coumaroylquinic acid, $R_4 = p\text{-coumaroyl}$, $R_1 = R_3 = R_5 = \text{OH}$

5-*O-p*-Coumaroylquinic acid, $R_5 = p\text{-coumaroyl}$, $R_1 = R_3 = R_4 = \text{OH}$

Figure 3. Structures of the different caffeoylquinic acids and coumaroylquinic acids identified in the studied wild flowers (IUPAC numbering) (IUPAC, 1976).