# *Bryonia dioica*, *Tamus communis* and *Lonicera periclymenum* fruits: characterization in phenolic compounds and incorporation of their extracts in hydrogel formulations for topical application

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**ABSTRACT**

Plants are being increasingly used in dermatological formulations, since their collateral effects are lower than those caused by synthetic products. Hydrogels represent efficient formulations to incorporate plant extracts, providing good percutaneous absorption, non-greasy texture and easy application. *Bryonia dioica* Jacq. (white-bryony), *Tamus communis* L. (black-bryony) and *Lonicera periclymenum* L. (honeysuckle) fruits have important applications as topical homemade remedies. The phenolic extracts of *B. dioica*, *T. communis* and *L. periclymenum* were characterized by HPLC-DAD-ESI/MS, and further incorporated into hydrosoluble gels in order to evaluate their antioxidant potential. The hydrogels were prepared with each fruit extract at the $EC_{50}$ value (obtained herein for each ethanolic extract). The antioxidant activity of extracts and hydrogels was evaluated following different chemical and biochemical assays. Eighteen compounds were identified in honeysuckle (7 flavonols, 7 hydroxycinnamoyl derivatives and 4 anthocyanins), fourteen in white-bryony (9 flavonols and 5 flavones) and twelve in black-bryony (7 flavonols and 5 flavones). Honeysuckle showed the highest concentrations of phenolic compounds, but the antioxidant activity was similar among the three assayed species. The obtained formulations revealed antioxidant activity close to 50% for reducing power, scavenging activity and β-carotene/linoleate assays. The studied fruits might be considered as good source of phenolic compounds that can be used in formulations for topical application, benefiting their antioxidant effects.

**Keywords:** Wild fruits; Phenolic profiles; HPLC-DAD-ESI/MS; Hydrogels; Topical medicines; Antioxidant activity.

## 1. Introduction

The topical use of plant preparations is among the most common practices in traditional healthcare (Marc et al., 2008). In recent ethnobotanical surveys conducted in Portugal and Spain (Neves et al., 2009; Benitez et al., 2010; Carvalho, 2010; González et al., 2010) several plant materials were reported as having interesting application as topical homemade remedies. *Bryonia dioica* Jacq. (white-bryony), *Tamus communis* L. (black-bryony) and *Lonicera periclymenum* L. (honeysuckle) are among the most cited (Carvalho, 2010). Their fruits are generally and popularly considered toxic to humans (Carvalho, 2010), mainly due to triterpene glucosides and calcium oxalate crystals (Castroviejo et al., 2001). Therefore, they are used as topical remedies being applied directly or macerated in water, alcohol or brandy to achieve anti-inflammatory effects (Rafael et al., 2011). The phytochemical screening of white-bryony roots showed the presence of polyphenols, sterols and triterpenes, alkaloids, flavonoid *C*-heterosides, carbohydrates and saponins. However, condensed and hydrolyzable tannins, anthocyanins, coumarins, anthraquinones and flavonoid *O*-heterosides were not detected (Benarba et al., 2012). Recently, our research group reported the phenolic profiles of white-bryony and black-bryony shoots, identifying five flavones (glycosylated derivatives of luteolin and apigenin) and one flavonol (kaempferol 3,7-di-*O*-rhamnoside) in the first one, and mainly flavonols (glycosylated kaempferol and quercetin compounds) and a hydroxycinnamoyl derivative (chlorogenic acid) in the second one (Barros et al., 2011).

As far as we know, the phenolic profile of *Lonicera periclymenum* has not been yet studied in any of its botanical parts, but other related *Lonicera* species, for instance *Lonicera japonica* Thunb. are known for their high content in hydroxycinnamic acids (Zhang et al., 2008; Seo et al., 2012).

The methanolic extracts obtained from immature and ripened fruits of these three species revealed interesting antioxidant properties, being the highest activity detected in the extracts

from ripened fruits of black-bryony (Rafael et al., 2011). Considering the involvement of oxidative stress in inflammation, topical antioxidants might bring health benefits for the treatment and prevention of oxidative stress-mediated inflammatory diseases (Casagrande et al., 2006). The topical application of formulations containing compounds with free radical scavenging properties protects tissues from oxidative damage (Meenakshi et al., 2006). Medicinal plants are being progressively employed in dermatological and cosmetic products, mainly due to the lower collateral effects when compared with the effects caused by synthetic products (Rang et al., 2003).

Often, the antioxidant potential of either plant extracts with active compounds or the pure isolated compounds is evaluated, but very few reports are available on the antioxidant properties of final formulations. Gels are becoming popular due to their easy application, better percutaneous absorption (when compared with other semisolid preparations) and resistance to the physiological stress caused by skin flexion, blinking and mucociliary movement, adopting the shape of applied area (Haneefa et al., 2010).

In the present study, the phenolic profiles of white-bryony, black-bryony and honeysuckle fruits were analyzed by high performance liquid chromatography and mass spectrometry. Further, their ethanolic extracts, rich in phenolic compounds, were incorporated into hydrosoluble gels, which are easily dispersed, non-oily and can carry hydrosoluble active principles, for antioxidant potential assessment.

## 2. Material and methods

### *2.1. Samples*

Ripened fruits (fleshy and soft red fruits in late autumn) of *Bryonia dioica* Jacq. (white-bryony), *Tamus communis* L. (black-bryony) and *Lonicera periclymenum* L. (honeysuckle) were gathered in Bragança, Trás-os-Montes, north-eastern Portugal. The samples for analysis were collected

and prepared according to the main medicinal topical applications as described by informants from this Portuguese region (Carvalho, 2010). The vegetal material was gathered haphazardly from several plants inside a selected area.

Morphological key characters from the *Flora Iberica* (Castroviejo, 2001) were used for plant identification. Voucher specimens (ETBO33, ETBO36, and ETBO53) are deposited in the herbarium of the 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 analyses.

### *2.2. Standards and reagents*

Imidazolidinyl urea, triethanolamine, 1,2-propanediol and poly(acrylic acid) (carbopol 940) were purchase from Sigma (St. Louis, MO, USA); 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and trifluoroacetic acids were purchased from Prolabo (VWR International, France). The phenolic compound standards were from Extrasynthese (Genay, France). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

### *2.3. Characterization of phenolic compounds*

#### *2.3.1. Non-anthocyanin phenolic compounds*

Each sample (~1 g) was extracted by stirring with 30 mL of methanol:water 80:20 (*v/v*), at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman no. 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-µm disposable LC filter disk for high performance liquid chromatography (HPLC-DAD-MS) analysis. 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 µm (4.6 mm × 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 isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% 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 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to record full scan spectra 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. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1000. Analysis in EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) detected in

the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the samples were characterized 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 (1-100 μg/mL) of different standards compounds: luteolin-6-*C*-glucoside ($y = 508.54x - 152.82$; $R^2 = 0.997$); apigenin-7-*O*-glucoside ($y = 159.62x + 7.5025$; $R^2 = 0.999$); quercetin-3-*O*-rutinoside ($y = 281.98x - 0.3459$; $R^2 = 1.000$); quercetin-3-*O*-glucoside ($y = 253.52x - 11.615$; $R^2 = 0.999$); kaempferol-3-*O*-rutinoside ($y = 239.16x + 10.587$; $R^2 = 1.000$); kaempferol-3-*O*-glucoside ($y = 288.55x - 4.0503$; $R^2 = 1.000$); chlorogenic acid ($y = 313.03x - 58.2$; $R^2 = 0.999$); caffeic acid ($y = 611.9x - 4.5733$; $R^2 = 0.999$).

*2.3.2. Anthocyanins*

Each sample (~1 g) was extracted with 30 mL of methanol containing 0.5% TFA, and filtered through a Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 mL portions of 0.5% TFA in methanol. The combined extracts were evaporated at 35 ºC to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 15 mL of water and anthocyanin pigments were further eluted with 5 mL of methanol/water (80:20, *v/v*) containing 0.1% TFA. The methanolic extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

The extracts were analysed in the HPLC system indicated above using the conditions described elsewhere (García-Marino et al., 2010). Separation was achieved on an AQUA® (Phenomenex)

reverse phase $C_{18}$ column (5 μm, 150 mm × 4.6 mm i.d) thermostatted at 35 ºC. The solvents used were: (A) 0.1% TFA in water, and (B) 100% acetonitrile. The gradient employed was: isocratic 10% B for 3 min, from 10 to 15% B for 12 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min and from 30 to 35% for 5 min, at a flow rate of 0.5 mL/min. Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and MS using the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 ºC) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupols were set at unit resolution. The ion spray voltage was set at 5000V in the positive ion mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V and parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V.

The anthocyanins present in the samples were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (50-0.25 μg/mL) of different standards compounds: cyanidin-3-*O*-glucoside ($y = 630276x - 153.83$; $R^2 = 1.000$) and peonidin-3-*O*-glucoside ($y = 537017x - 71.469$; $R^2 = 1.000$).

*2.4. Preparation of ethanolic extracts and incorporation in hydrosoluble gels*

For the extracts preparation, a fine dried powder (20 mesh; ~0.5 g) was stirred with 30 mL of ethanol at 25 °C and 150 rpm for 1 h, and filtered through Whatman No. 4 paper. The obtained residue was re-extracted under the same conditions. The combined extracts were evaporated at 35 °C under reduced pressure, re-dissolved in ethanol at 10 mg/mL, and stored (4 °C) for further evaluation of antioxidant activity or incorporation in gels.

For the gels preparation, a semi-solid base was prepared by adding 0.5 g Carbopol 940 to 20 mL of deionised water. The gel base was allowed to stand for 1 h after which 1 mL of triethanolamine and an exact volume of each fruits extract, corresponding to a final concentration equal to the average $EC_{50}$ obtained for each species in the antioxidant activity assays (section 2.5.). Subsequently, citric acid (0.45 g), disodium EDTA (0.005 g), imidazolidinyl urea (0.1 g) and propylene glycol (7.5 g) were added for gel preparation. The final product was adjusted to 50 g by the addition of deionised water, and further submitted to evaluation of antioxidant potential. A blank formulation (negative control) was also prepared in the same conditions but without incorporation of the extract.

### *2.5. Evaluation of antioxidant activity*

#### *2.5.1. DPPH radical-scavenging activity*

This methodology was performed using an ELX800 microplate reader (Bio-Tek Instruments, Inc). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the extracts or gels (30 μL) and methanolic solution (270 μL) containing DPPH radicals ($6\times10^{-5}$ mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: $\% \text{ RSA} = [(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where $A_S$ is the absorbance of the solution when the sample extract/gel has been added at a particular level, and $A_{DPPH}$ is the absorbance of the DPPH solution. The concentration providing 50% of radicals scavenging activity ($EC_{50}$) was calculated from the graph of RSA percentage against extract concentration.

#### *2.5.2. Reducing power*

This methodology was performed using the microplate reader described above. The different concentrations of the extracts or gels (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% *w/v*, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% *w/v*, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionised water (0.8 mL) and ferric chloride (0.1% *w/v*, 0.16 mL), and the absorbance was measured at 690 nm. The concentration providing 0.5 of absorbance ($EC_{50}$) was calculated from the graph of absorbance at 690 nm against extract concentration.

*2.5.3. Inhibition of β-carotene bleaching*

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 ºC under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing solutions extract or gel with different concentrations (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (AnalytikJena 200-2004 spectrophotometer). β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The concentration providing 50% antioxidant activity ($EC_{50}$) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration.

*2.6. Statistical analysis*

For each sample three independent experiments were performed, and each of them was analysed in triplicate (spectrophotometry) or duplicate (chromatography). The results were expressed as mean values ± standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference *post hoc* test (homoscedastic distributions) or Tamhane's T2 test (heteroscedastic distributions) with $\alpha = 0.05$, coupled with Welch's statistic. All statistical tests were performed with the SPSS v.18.0 software. The normality within groups and homogeneity of variances and of variance-covariance matrices, were checked using the Kolmogorov-Smirnov with Lilliefors correction, the Levene and M-Box tests, respectively.

## 3. Results and discussion

### *3.1. Characterization of phenolic compounds*

The characterization of the phenolic compounds present in the fruits was performed by HPLC-DAD-MS analysis, and data of the retention time, $\lambda_{max}$, pseudomolecular ion, main fragment ions in $MS^2$, tentative identification and concentration of phenolic acid derivatives, flavonoids and anthocyanins are presented in **Tables 1** and **2**. The HPLC phenolic profiles of white-bryony (A), black-bryony (B) and honeysuckle (C), which was injected in a concentration 4 times lower than the other samples can be observed in **Figure 1**.

UV and mass spectra of the studied samples obtained by HPLC-DAD-ESI/MS analysis showed that the phenolic composition of white and black bryony was characterized by the presence of flavonoids, whereas flavonoids and hydroxycinnamoyl derivatives were present in the sample of honeysuckle. The analysis of the $MS^2$ fragments revealed that both *O*- and *C*-glycosides of flavonoids were present derived respectively from two flavonols (quercetin and kaempferol) and

two flavones (luteolin and apigenin). Sugar substituents consisted of hexoside, deoxyhexosides and pentosides, as deduced from the losses of 162 Da, 146 Da and 132 Da, respectively.

*3.1.1. Flavonols*

Peaks 7, 8, 9 and 10 in honeysuckle were respectively identified as quercetin 3-*O*-rutinoside, quercetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucoside, according to their retention, mass and UV-Vis characteristics by comparison with commercial standards. Peak 13 in white-bryony (10 in black-bryony) was identified as kaempferol-3,4'-*O*-dirhamnoside according to its pseudomolecular ion and fragmentation pattern as previously reported by Barros et al. (2011) and fully characterised by Shaheen et al. (2009) using NMR. Tentative identification of the remaining flavonols was made based on their molecular ions and fragmentation patterns according to the principles established by Cuyckens et al. (2001) and Ma et al. (2001) for the fragmentation of flavonoid *O*-glycosides.

Assignment of *O*-neohesperidosides and *O*-rutinosides was made assuming that greater abundance of the $Y_0$ ion (aglycone) than $Y_1$ ion (produced from the breakdown of the interglycosidic linkage) should be expected in the case of 1,2 disaccharides, while $Y_1$ should be more abundant than $Y_0$ in the case of a 1,6 linkage (Cuyckens et al., 2001). Thus, peak 5 in white-bryony (same as peaks 6 in black-bryony and honeysuckle) was tentatively assigned to quercetin 3-*O*-neohesperidose, once the signal corresponding to the $Y_0$ ion (*m/z* at 301) was greater than that of the $Y_1$ ion (*m/z* at 463). Peak 7 in white-bryony might be assigned as a quercetin *O*-rhamnosyl-pentoside owing to the lack of a fragment at *m/z* 433 (-146 u), which suggests that it corresponds to a disaccharide where the pentose was the terminal unit.

Peaks 9, 11 and 12 in white-bryony could be interpreted as kaempferol *O*-diglycosides in which each of the sugar moieties were located at different positions on the aglycone, owing to the observation of fragments derived from the loss of each sugar residue. However, they might also

be rationalised as kaempferol *O*-disaccharides, in which an irregular fragment ion is produced by an internal rearrangement in the sugar moieties following the loss of the internal dehydrated glucose/pentose and further linkage of the terminal rhamnose to the aglycone, as observed by Ma et al. (2001). Thus, peak 9 (7 in black-bryony) might correspond to kaempferol 3-*O*-neohesperidose, and peaks 11 and 12 (9 in black-bryony) could be interpreted as possible kaempferol *O*-pentosyl-rhamnosides with 1,6 and 1,2 interglycosydic linkages, respectively. Similarly, peak 10 in white-bryony (8 in black-bryony) could correspond either to a quercetin *O*-rhamnoside-*O*-rhamnoside (sugar moieties at different positions) or a quercetin *O*-rhamnosyl-rhamnoside (in which the two sugars constitute a disaccharide).

Peak 5 ($[M-H]^-$ at *m/z* 755) in honeysuckle was coherent with a quercetin bearing two rhamnosyl and one hexosyl residues. The loss of a fragment of 146 u to yield the ion at *m/z* 609 suggested different location on the aglycone of one of the rhamnosyl moieties in relation to the other two sugars; furthermore, its fragmentation pattern closer to peak 7 (quercetin-3-*O*-rutinoside) than to peak 8 (quercetin-3-*O*-neohesperidoside) might allow assigning it as quercetin-3-*O*-rutinoside-*O*-rhamnosyl. Similarly, peak 8 ($[M-H]^-$ at *m/z* 739) in white-bryony could be a kaempferol-*O*-rhamnosyl-hexoside-*O*-rhamnoside, according to its pseudomolecular ion and $MS^2$ fragment ions at *m/z* 593 ($[M-146]^-$, loss of a rhamnose moiety) and *m/z* 431 ($[M-308]^-$, loss of a rhamnosyl-hexoside moiety), although no fragment ion corresponding to kaempferol aglycone (*m/z* 285) was detected. The suggested presence of kaempferol-3-*O*-neohesperidoside (peak 9) in relevant amounts in this extract might point out that peak 8 could be a kaempferol-3-*O*-neohesperidoside-*O*-rhamnoside, although there are differences in their fragmentation patterns that might be possibly explained by the very low amounts of peak 8. Finally, peaks 14 in white-bryony and honeysuckle, and peak 12 in black-bryony would be different quercetin *O*-hexosides.

### *3.1.2. Flavones*

Flavones were only identified in white- and black-bryony as *C*-glycosylated derivatives. This type of compounds is characterized by the loss of characteristics fragments from the cleavage of the sugar pyrano ring, namely -120 u and 90 u in the case hexosides (Ferreres et al., 2003). Taking it into account, five peaks in white-bryony (1, 2, 3, 4 and 6) were assigned to different *C*-glycosyl flavones. Peak 6 ($[M-H]^-$ at *m/z* 431) in white-bryony showed characteristic fragment ions at *m/z* 341 ($[M-90]^-$), 311 ($[M-120]^-$), and 281 ($[M-150]^-$). The fragment at *m/z* 281 would correspond to the cleavage of bonds 0 and 1 $[^{0,1}X]^-$ in the sugar moiety (Ma et al., 1997). This compound was positively identified as apigenin-6-*C*-glucoside by comparison with an authentic standard. Peaks 1-4 presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 593, but differed in their $MS^2$ fragmentation. Majority peak 2 showed characteristic fragment ions at *m/z* 503 (-90 u), 473 (-120 u), 383 (-120-90 u) and 353 (-120-120 u). The ion at *m/z* 353 has been associated to apigenin aglycone bearing some sugar residues (270 + 83 u) that remained attached to it (Ferreres et al., 2003; 2004). Although the identity of the hexoside moieties cannot be established, they might be well associated as being glucose. According to these characteristics, peak 2 was tentatively identified as apigenin-6-*C*-glucoside-8-*C*-glucoside.

Peak 1 also showed the ions at *m/z* 503 (-90 u) and 473 (-120 u), but also other two minority ones at *m/z* 431 (-162 u) and 311 (-120-162 u). In this case the observation of the complete loss of a hexosyl moiety (-162 u) could be interpreted as one of the hexosides being *O*-linked, so that the compound might be assigned to an apigenin-*C*-hexoside-*O*-hexoside. The ions at *m/z* 431 and 311 were also observed in the case of peak 3, as well as a minor one at *m/z* 341, that can be explained from the loss of 90 u from the fragment at *m/z* 431, supporting also the identity of the compound as an apigenin-*C*-hexoside-*O*-hexoside, namely apigenin 6-*C*-glucoside-7-*O*-glucoside (saponarin) as previously identified by our group in white-bryony shoots (Barros et al., 2011). Peak 4 presented a fragmentation pattern similar to peak 3, suggesting that it could also be an apigenin-*C*-hexoside-*O*-hexoside.

According to their pseudomolecular ion and retention times, peaks 2-5 in black-bryony would correspond to peaks 1-4 in white-bryony, although they showed some differences in their $MS^2$ fragmentation and UV spectra. In addition, peak 1 in black-bryony ($[M-H]^-$ at *m/z* 609) can also be associated to a *C*-glycosyl flavone derived from luteolin, showing characteristic loss of 120 u (fragment at *m/z* 489), and two fragments at *m/z* 399 and 369, that might correspond to the luteolin aglycone plus the residues of the sugars that remained linked to it (luteolin + 113) and (luteolin + 83), respectively. Similar fragmentation pattern was described by Ferreres et al. (2003) for luteolin 6-*C*-glucoside-8-*C*-glucoside, so that it was assigned that identity. The same compound was also previously reported by our group in samples of *Tuberaria lignosa* (sweet) (Pinela et al., 2012).

*3.1.3. Hydroxycinnamoyl derivatives*

These compounds were only detected in the sample of honeysuckle and mostly correspond to chlorogenic acids (*i.e.*, caffeoylquinic acids) containing one or two caffeic acid moieties (peaks 1, 3, 11, 12 and 13 in **Table 1).** Peak assignments were made using the hierarchical keys previously developed (Clifford et al., 2003; 2005) and according to the recommended IUPAC numbering system (IUPAC, 1976). Peak 1 was distinguished from the other two isomers by its base peak at *m/z* 173 [quinic acid-H-$H_2O$]$^-$, accompanied by a secondary fragment ion at *m/z* 179 with approximately 78% of the abundance of the base peak, which allowed its identification as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford et al. (2003; 2005). Peak 4 was positively identified as *trans*-5-*O*-caffeoylquinic acid by comparison with an authentic standard, and also based on its MS fragmentation pattern. Peak 3 was assigned as the corresponding *cis* isomer of 5-*O*-caffeoylquinic acid, taking into account that hydroxycinnamoyl *cis* derivatives are expected to elute before the corresponding *trans* derivatives. This assumption was supported after UV irradiation (366 nm, 24 h) by the

observation of an increase in peak 3 (*cis* isomer) with respect to peak 4 (*trans* isomer) suggesting *trans*/*cis* inter-conversion. Following the same reasoning, peaks 11 and 12 were tentatively assigned as *cis*- and *trans*-3,5-*O*-dicaffeoylquinic acid ([M-H]$^-$ at m/z 515), respectively. Peak 13 ([M-H]$^-$ at *m*/*z* 515) could be assigned as 4,5-*O*-dicaffeoylquinic acid according to its fragmentation pattern and relative abundances fragment ions as described by Clifford et al. (2003; 2005). Finally, peak 2 should correspond to caffeic acid bearing a hexosyl residue, compound previously reported by Barros et al. (2012) in tomato.

*3.1.4. Anthocyanins*

In coherence with the presented pigmentations, anthocyanins were detected only in honeysuckle. The analytical characteristics, identities and concentrations of the four detected compounds are presented in **Table 2**. All of them were positively identified by comparison with standards.

*3.2. Antioxidant activity of ethanolic extracts and derived hydrogels*

The antioxidant activity of the fruits of white-bryony, black-bryony and honeysuckle had been previously assayed in methanolic extracts (Rafael et al., 2011). However, the toxicity of methanol makes it unsuitable to be included in topical formulations with dermocosmetic applications. Hence, the extracts used in this work were prepared using ethanol. The results obtained for the antioxidant activity assays (**Table 3**) were very similar to those obtained for methanolic extracts (Rafael et al., 2011), except for β-carotene bleaching inhibition effect, which was higher in the latter. The activity was not the same for all assays. This might be explained by the differences in reaction mechanisms in each assay: DPPH is based on the premise that a hydrogen donor is an antioxidant (Moon and Shibamoto, 2009), which can convert the radical to 2,2-diphenyl-1-picrylhydrazine (or an analogue substituted hydrazine) (Ferreira et al., 2007). The FRAP (ferric reducing antioxidant power) assay is characterized by the reduction of $Fe^{3+}$ to depending on the available reducing species (Antolovich et al., 2002), but not all reducing agents

with the ability to reduce $Fe^{3+}$ are antioxidants; in fact any substance able to donate electrons with a lower redox potential than $Fe^{3+}/Fe^{2+}$ might contribute to FRAP value (Karadag et al., 2009). β-carotene in turn may suffer rapid discolouration in the presence of a linoleate radical. During oxidation, an H atom is removed from the active bis-allylic methylene group on $C_{11}$. The formed pentadienyl radical attacks β-carotene to reacquire H atoms, causing its discoloration (Amarowicz et al., 2004). In this case, the extracts of honeysuckle and black-bryony behave as the best DPPH scavengers, whereas honeysuckle showed the highest reducing power, and black-bryony and white-bryony were greater inhibitors of β-carotene bleaching. To verify if the antioxidant activity of the extracts was maintained after being incorporated in the corresponding hydrosoluble gel, three independent assay gels were prepared for each extract. The color of gels varied from light green (black- and white-bryony) to pale pink (honeysuckle), showing all of them a non-greasy texture and fast skin absorption. Imidazolidinyl urea was added as antimicrobial agent, since parabens are nowadays looked at as potentially harmful compounds. Carbopol 940 was added at 1% concentration, previously determined as allowing an adequate consistency (Barreira et al., 2013). To check gel stability, pH was measured (at room temperature, employing a digital pH meter, Hanna Instruments) monthly during six months without significant alterations (pH values ranging between 5.5 and 6.5), indicating the absence of hydrolysis or decomposition processes (Queiroz et al., 2009). The extract of each plant species was incorporated in a concentration close to that providing the mean $EC_{50}$ value in the three performed assays for the antioxidant activity, expecting to obtain approximately 50% of activity in each assay. To prevent the matrix effect, a blank gel was also prepared. As it can be observed in **Table 3**, the results obtained for all antioxidant activity assays are in the expected range, *i.e.* close to 50%, indicating that the incorporated fruit extracts maintained their antioxidant potential, with the additional advantage of being a formulation designed for topical application which present interesting physical characteristics, regarding its absorption dynamics and

stability. However, future development of the suggested formulations should include viscosity, extrudability and spreadability assays, followed by the evaluation of potential irritancy by different instrumental techniques like erythema measurement, Hen's egg test-chorioallantoic membrane assay or transepidermal water loss.

## 4. Conclusions

Among the assayed species, *Lonicera periclymenum* (honeysuckle) showed the highest content in phenolic compounds, mainly due to the high amount of hydroxycinnamoyl derivatives, particularly *cis*-5-*O*-caffeoylquinic and *trans*-3,5-*O*-dicaffeolyquinic acids. Neither *Bryonia dioica* (white-bryony) nor *Tamus communis* (black-bryony) presented phenolic acids derivatives. Honeysuckle was also the species with the highest flavonoid contents, with quercetin-3-*O*-glucoside and kaempferol 3-*O*-glucoside as majority compounds. Kaempferol 3-*O*-neohesperidoside was the main flavonol in white-bryony, while quercetin-*O*-rhamnosyl-*O*-rhamnoside and kaempferol-3,4'-di-*O*-rhamnoside were the most abundant flavonols in black-bryony. Flavones were only detected in white-bryony and black-bryony, with special relevance for apigenin-6-*C*-glucoside-8-*C*-glucoside.

The phenolic compounds found in plants are often related with their bioactivity, mostly resulting from the synergistic or additive influence of the different classes of compounds present in the extract (Ramful et al., 2008). Therefore, it would have been expectable that the higher phenolic contents quantified in honeysuckle were followed by a higher antioxidant activity, both for its extracts and for the prepared hydrosoluble gels. However, the antioxidant activity was quite similar among the three assayed species, which might be probably explained by the lower purity degree of the extracts from *L. periclymenum* as indicated by the extraction yields (see **Table 3**). Hence, the assayed plants can be considered as important sources of flavonoids, phenolic acids

or anthocyanins, proving their potential to be included in new pharmaceutical formulations (mainly for topical applications).

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**Table 1.**

Retention time (Rt), wavelengths of maximum absorption ($\lambda_{max}$), mass spectral data, relative abundances of fragment ions, tentative identification and quantification of the phenolic compounds in the studied extracts.

| Peak | Rt (min) | $\lambda_{max}$ (nm) | Pseudomolecular ion $[M-H]^-$ (*m/z*) | $MS^2$ (*m/z*) | Tentative identification | Quantification (μg/g of extract) |
|---|---|---|---|---|---|---|
| | | | | *Bryonia dioica* (white-bryony) | | |
| 1 | 9.1 | 266/330 | 593 | 593(100), 503(18), 473(44), 431(4), 311(7) | Apigenin-*C*-hexoside-*O*-hexoside | 21±2 |
| 2 | 11.1 | 270/336 | 593 | 593(100), 503(10), 473(27), 383 (22), 353(45) | Apigenin-6-*C*-glucoside-8-*C*-glucoside | 708±10 |
| 3 | 14.4 | 334 | 593 | 593(100), 431(32), 341(8), 311(72) | Apigenin-6-*C*-glucoside-7-*O*-glucoside | 194±2 |
| 4 | 14.7 | 270/336 | 593 | 593(100), 473(8), 431(38), 353(10), 311(53), 297(5) | Apigenin-*C*-hexoside-*O*-hexoside | 360±16 |
| 5 | 16.7 | 354 | 609 | 609(100), 463(17), 301(27) | Quercetin-3-*O*-neohesperidoside | 407±17 |
| 6 | 18.3 | 332 | 431 | 431(100), 341(11), 311(91), 281(31) | Apigenin-6-*C*-glucoside | 20±3 |
| 7 | 18.4 | 354 | 579 | 579(100), 447(14), 301(24) | Quercetin-*O*-rhamnosyl-pentoside | 53±3 |
| 8 | 18.8 | 342 | 739 | 739(100), 593(55), 431(13) | Kaempferol-*O*-rhamnosyl-hexoside-*O*-rhamnoside | 17±1 |
| 9 | 19.3 | 348 | 593 | 593(100), 549(4), 447(57), 431(46), 327(7), 285(51) | Kaempferol-3-*O*-neohesperidoside | 629±12 |
| 10 | 19.9 | 346 | 593 | 593(100), 447(40), 301(34) | Quercetin-*O*-rhamnosyl-rhamnoside | 277±23 |
| 11 | 20.5 | 348 | 563 | 563(100), 431(17),417 (29), 285(22) | Kaempferol-*O*-pentosyl-rhamnoside | 149±6 |
| 12 | 21.2 | 348 | 563 | 563(100), 431(32), 417 (8),285(29) | Kaempferol-*O*-pentosyl-rhamnoside | 23±2 |
| 13 | 23.5 | 316/342 | 577 | 577(100), 431(80), 285(84) | Kaempferol-3,4'-di-*O*-rhamnoside | 510±32 |
| 14 | 27.8 | 316 | 463 | 301(100) | Quercetin-*O*-hexoside | 8±1 |
| | | | | | **Flavonols** | **2073±91** |
| | | | | | **Flavones** | **1304±9** |
| | | | | | **Total phenolics** | **3376±82** |
| | | | | *Tamus communis*(black-bryony) | | |
| 1 | 8.2 | 272/348 | 609 | 609(100), 489(28), 399(23), 369(18) | Luteolin-6-*C*-glucoside-8-*C*-glucoside | 67±5 |
| 2 | 9.1 | 270/324 | 593 | 593(100), 473(43), 353(2), 341(2), 311(10) | Apigenin-*C*-hexoside-*O*-hexoside | 153±8 |
| 3 | 11.1 | 270/336 | 593 | 593(100), 473(41), 383(20), 353(38), 311(1) | Apigenin-6-*C*-glucoside-8-*C*-glucoside | 1383±48 |
| 4 | 14.4 | 330 | 593 | 593(100), 473(6), 431(46), 311(31) | Apigenin-6-*C*-glucoside-7-*O*-glucoside | 208±17 |
| 5 | 14.7 | 336 | 593 | 593(100), 473(11), 431(97), 353(6), 341(6), 311(46) | Apigenin-*C*-hexoside-*O*-hexoside | 130±1 |
| 6 | 16.7 | 354 | 609 | 609(100), 463(14), 301(20) | Quercetin-3-*O*-neohesperidoside | 453±20 |
| 7 | 19.3 | 348 | 593 | 593(100), 447(35), 431(53), 285(35) | Kaempferol-3-*O*-neohesperidoside | 430.8±0.2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | **Flavones** | **1941±43** |
| | | | | | **Total phenolics** | **5041±166** |
| | | | | *Lonicera periclymenum*(honeysuckle) | | |
| 1 | 7.4 | 330 | 353 | 191(45), 179(78), 173(100), 155(9), 135(93) | 4-*O*-caffeoylquinic acid | 191±3 |
| 2 | 7.6 | 316 | 341 | 179(100), 161,(2), 135(87) | Caffeoyl-hexoside acid | 203±6 |
| 3 | 8.2 | 326 | 353 | 191(100), 179(8), 161(12), 155(2), 135(4) | *cis*-5-*O*-Caffeoylquinic acid | 7047±48 |
| 4 | 9.7 | 308 | 353 | 191(100), 179(4), 161(4), 155(1), 135(3) | *trans*-5-*O*-Caffeoylquinic acid | 801±53 |
| 5 | 15.9 | 354 | 755 | 755(100), 609(2), 301(26) | Quercetin-3-*O*-rutinoside-*O*-rhamnoside | 396±29 |
| 6 | 16.8 | 354 | 609 | 609(100), 463(27), 447(33), 301(62) | Quercetin-*3*-*O*-neohesperidoside | 353±52 |
| 7 | 18.4 | 368 | 609 | 301(100) | Quercetin-3-*O*-rutinoside | 382±29 |
| 8 | 19.3 | 354 | 463 | 301(100) | Quercetin-3-*O*-glucoside | 2126±96 |
| 9 | 19.6 | 346 | 593 | 285(100) | Kaempferol-3-*O*-rutinoside | 399±43 |
| 10 | 20.7 | 340 | 447 | 285(100) | Kaempferol-*3*-*O*-glucoside | 1911±55 |
| 11 | 21.9 | 328 | 515 | 353(100), 335(4), 191(81), 179(43), 173(6), 161(6), 155(1), 135(31) | *cis*-3,5-*O*-Dicaffeolyquinic acid | 225±15 |
| 12 | 22.4 | 328 | 515 | 353(100), 335(3), 191(99), 179(80), 173(16), 161(8), 155(1), 135(43) | *trans*-3,5-*O*-Dicaffeolyquinic acid | 4316±34 |
| 13 | 25.2 | 328 | 515 | 353(76), 335(4), 191(31), 179(65), 173(100), 155(6), 135(27) | 4,5-Dicaffeolyquinic acid | 560±23 |
| 14 | 25.7 | 360 | 463 | 301(100) | Quercetin-*O*-hexoside | 338±27 |
| | | | | | **Phenolic acids** | **13342±8** |
| | | | | | **Flavonols** | **5905±330** |
| | | | | | **Total phenolics** | **19247±323** |

**Table 2.**
Retention time (Rt), wavelengths of maximum absorption in the visible region ($\lambda_{max}$), mass spectral data, tentative identification and concentration of anthocyanins in *Lonicera periclymenum* (honeysuckle) fruit extracts.

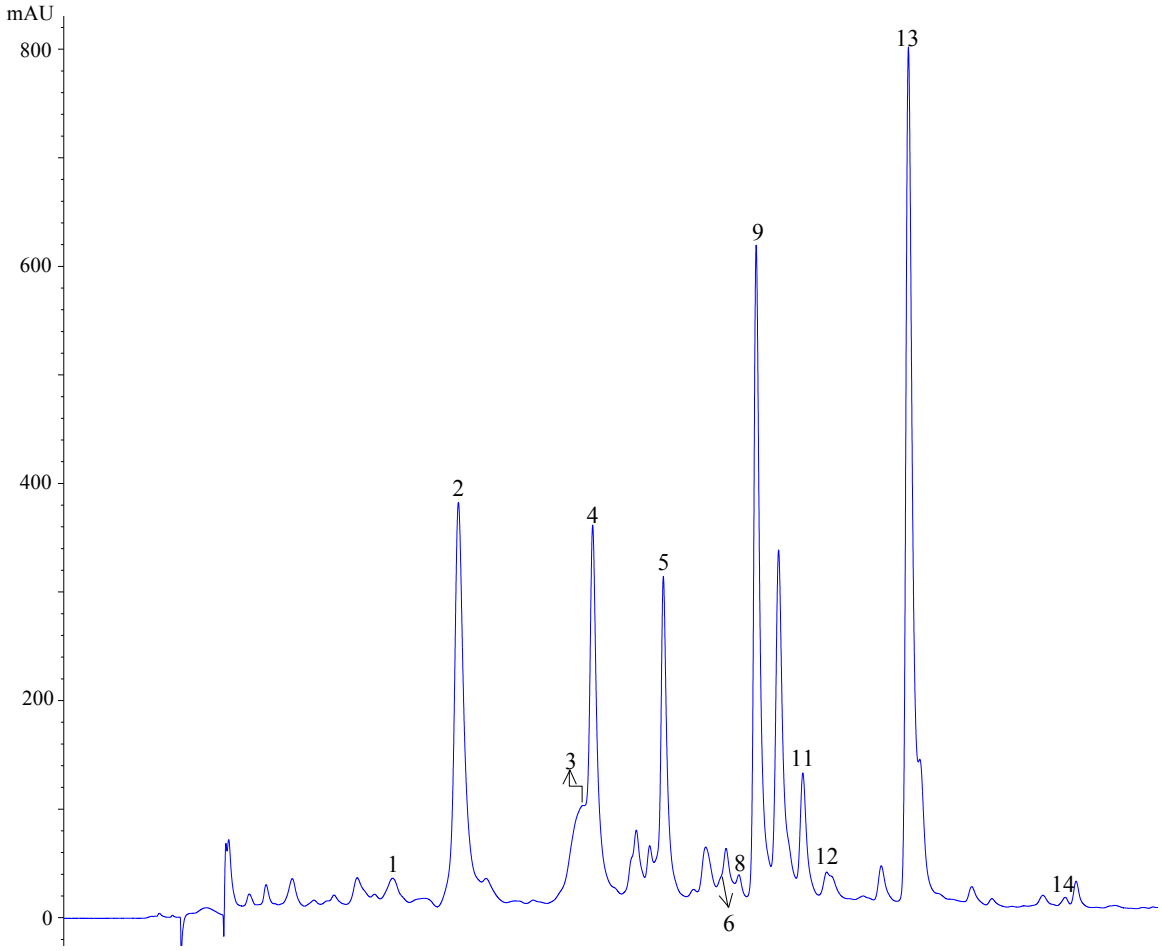
| Peak | Rt (min) | $\lambda_{max}$ (nm) | Molecular ion $[M+H]^+$ (*m/z*) | $MS^2$ (*m/z*) | Tentative identification | Quantification (μg/g of extract) |
|---|---|---|---|---|---|---|
| 1 | 14.9 | 516 | 611 | 449(36),287(100) | Cyanidin-3,5-*O*-diglucoside | 1.278±0.005 |
| 2 | 19.4 | 516 | 625 | 463(46),301(100) | Peonidin-3,5-*O*-diglucoside | 0.1914±0.0004 |
| 3 | 22.3 | 516 | 449 | 287(100) | Cyanidin-3-*O*-glucoside | 0.0422±0.0004 |
| 4 | 24.9 | 518 | 463 | - | Peonidin-3-*O*-glucoside | 0.0091±0.0001 |
| | | | | | **Total anthocyanins** | 1.52±0.01 |

**Table 3.**

Antioxidant activity of the individual ethanolic extracts ($EC_{50}$ values, mg/mL) and of hydrogels (values in activity percentage) incorporating them (mean±SD, n=9).

| | Extraction yield (%)[4] | DPPH scavenging activity[4] | Reducing power[4] | β-Carotene bleaching inhibition[4] |
|---|---|---|---|---|
| *Bryonia dioica* (white-bryony) extract | 17±1 b | 1.5±0.1 a | 0.9±0.1 a | 1.9±0.1 b |
| *Tamus communis* (black-bryony) extract | 22±2 b | 0.9±0.1 b | 0.6±0.2 b | 2.3±0.1 a |
| *Lonicera periclymenum* (honeysuckle) extract | 56±3 a | 1.0±0.1 b | 0.8±0.2 a | 1.8±0.3 b |
| Homocedasticity[1] (*p*-value) | 0.146 | 0.019 | <0.001 | <0.001 |
| Normal distribution[2] (*p*-value) | 0.048 | <0.001 | <0.001 | 0.012 |
| One-way ANOVA[3] (*p*-value) | <0.001 | <0.001 | <0.001 | <0.001 |
| *Bryonia dioica* (white-bryony) hydrogel | - | 44±6 c | 51±4 a | 49±1 a |
| *Tamus communis* (black-bryony) hydrogel | | 55±1 a | 47±5 ab | 51±1 a |
| *Lonicera periclymenum* (honeysuckle) hydrogel | | 49±3 b | 44±5 b | 47±3 b |
| Homocedasticity[1] (*p*-value) | | <0.001 | 0.228 | <0.001 |
| Normal distribution[2] (*p*-value) | | 0.001 | 0.011 | 0.003 |
| One-way ANOVA[3] (*p*-value) | | <0.001 | 0.002 | <0.001 |

[1]Homoscedasticity among samples was tested through the Levene test
[2]Normal distribution of the residuals was evaluated using Shpairo-Wilks or Kolmogorov-Smirnov with Lilliefors correction test.
[3]As $p<0.05$, the mean value of the evaluated parameter of at least one sample differs from the others and multiple comparison could be performed.
[4]Extraction yield was calculated by dividing the dry extract mass (g) by the total amount (g) of dry vegetal sample used for extraction; the obtained ratio was then multiplying by 100%.
[5]Different letters in each column indicate mean values that differ significantly ($P<0.001$). These differences were classified using Tukey's HSD (homoscedastic distributions) or Tamhane's T2 (heteroscedastic distributions) tests.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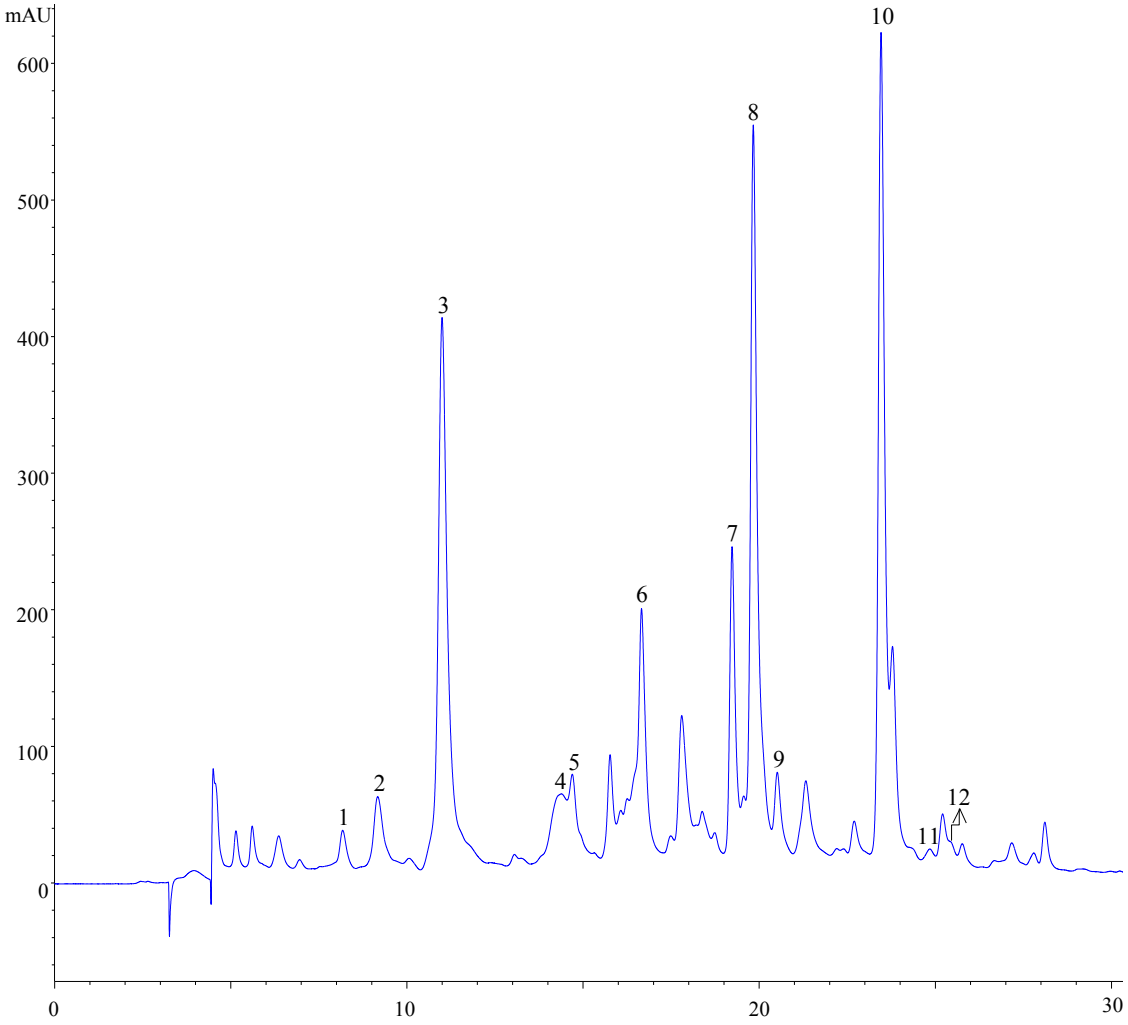


(B)

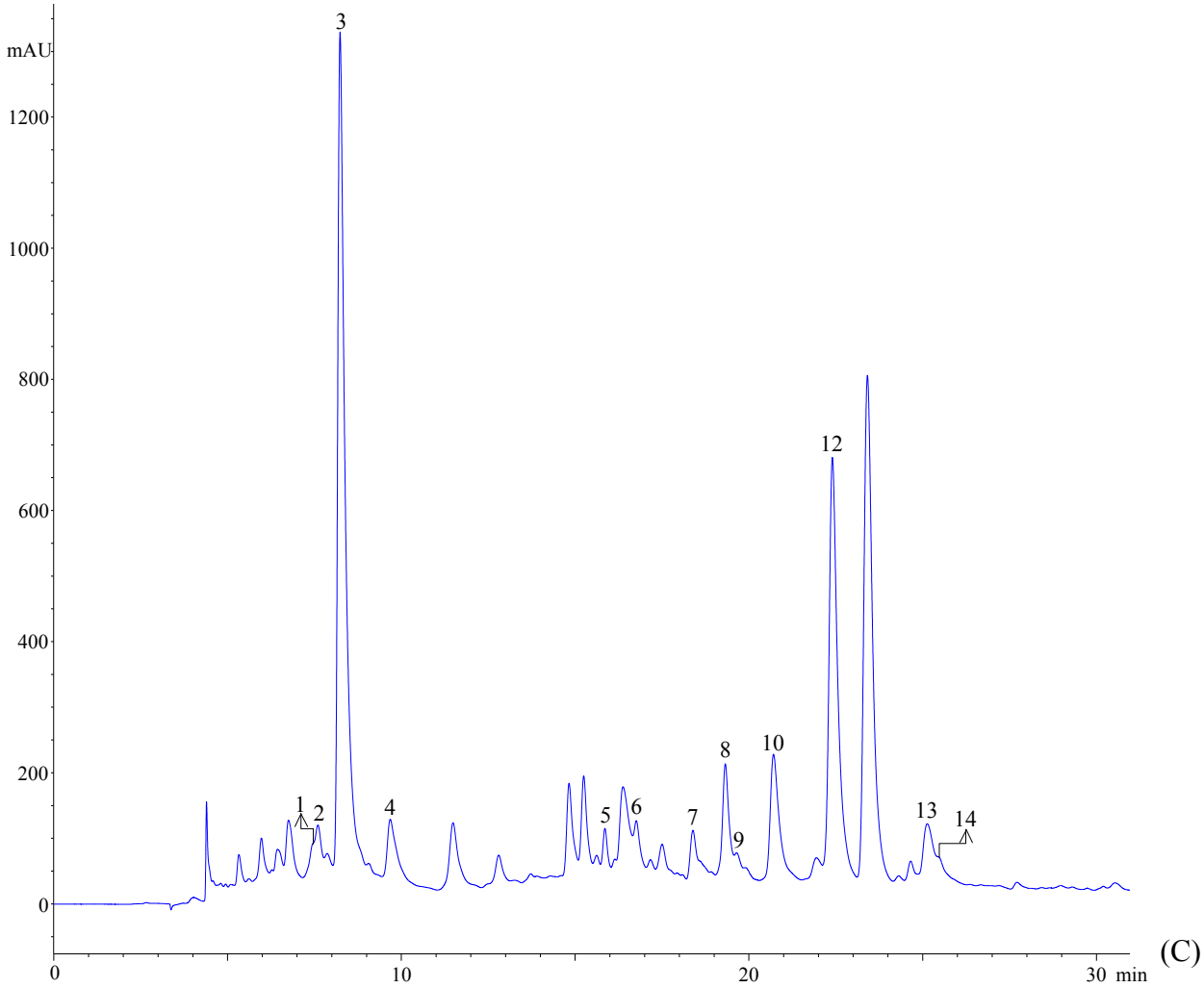


**Figure 1.** HPLC chromatogram of the phenolic compounds of *Bryonia dioica* (white-bryony) (A), *Tamus communis* (black-bryony) (B) and *Lonicera periclymenum* (honeysuckle) (C) fruits recorded at 370 nm (A and B) and 280 nm (C).