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CHARACTERIZATION OF PHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF THE LEAVES OF THE FORGOTTEN MEDICINAL PLANT *BALSAMITA MAJOR* GROWN IN TUSCANY, ITALY, DURING THE GROWTH CYCLE

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Abstract

Balsamita major (Asteraceae), known as costmary, is a medicinal plant rich of ethnobotanical interest in particular in Europe and Middle East, known and used since Greek and Roman times. The aim of the present study was to characterize the phenolic pattern of *B. major* leaves cultivated in Italy, and to measure the overall anti-radical (DPPH) and antioxidant activity (ORAC) during the growth cycle of the plant. Total polyphenols and total hydroxycinnamic acid derivatives were abundant in the leaves collected at all the growth stages, only decreasing in the flowering stage; flavonoids reached the highest content in the very early growth stage. Chlorogenic acid and 3,5-O-dicaffeoylquinic acid resulted to be the main phenolic constituents in all the extracts; their concentrations were highest at the late and early growth stages, respectively. Glycosilated flavonoids were found to be the abundant in all the extracts and quercetin resulted the main flavonoid aglycone, with the highest content in the very early growth stage. Both DPPH and ORAC tests registered good antiradical and antioxidant properties for all the extracts. Thus, *B. major* is worthy of further investigation, being an interesting source of antioxidant compounds, for pharmaceutical, nutraceutical and cosmetic purposes.

Keywords: Scavenger activity, antioxidant properties, *Balsamita major*, Chlorogenic acid, Growth cycle.

Introduction

Costmary (*Balsamita major* Desf. (syn. *Chrysanthemum balsamita* L., *Tanacetum balsamita* L.) is a medicinal plant, belonging to the Asteraceae family. It is a large perennial plant of Asian origin with yellow flowers, grown in Europe and Asia since the Middle Ages (Bylaitè et al., 2000). This plant has a hairy stem, complete shiny leaves, highly branched from the base and 70-120 cm height (Hassapouraghdam et al., 2008; Mozzafarian, 2004; Nickavar et al., 2003). Leaves and flowers have a characteristic mint-like odour due to its volatile oil constituents. The main volatile compounds (above 3%) are: carvone, α-thujone, germacrene B, benzaldehyde, ethylbenzene and germacrene D. Costmary has different phytochemicals, such as oxygenated terpenes, hydrocarbon terpenes, aromatic hydrocarbons,

aromatic oxygenated and other components. It is also rich in sesquiterpene lactones and polyphenols such as phenylpropane derivatives, flavones, and tannins (Gallori et al., 2001).

In Italy, *Balsamita major* Desf. had been knowing since Greek and Roman times, but the first botanical description was found during the 9th century A.D. (Ena et al., 2009). Its use was mainly popular, and it was mainly employed in food preparations in Lombardy and Piedmont. *B. major* has been also traditionally used for its therapeutic properties, such as calming, antispasmodic, and diuretic. In the 17th century, in Tuscany, Italy, an herbal preparation containing the essential oil of *B. major*, cinnamon and other spices (*Acqua Antisterica* in italian, i.e. anti-hysterical water) was developed and it became very popular and largely used throughout Europe (Ena and Nelli, 2012). Currently, in Tuscany the water extract of this species is mostly used in cosmetic preparations (Ena and Nelli, 2012). A study performed in the district of Acquapendente (Latium, Italy) reported the ritual use of *B. major* on the eve of St. John's feast, while in north-western (Guarrera et al., 2005), while in North-Western Ligurian Alps the plant is used as an ingredient in omelette or omelettes or as stuffing for vegetable pies (Cornara et al., 2014). Ethnomedicinal studies on the plant resources of Anatolia (Turkey) have shown that *B. major* is known in traditional medicine. Infused in hot water, it is used as stimulant, antipyretic, diuretic, stomachic and to treat headache, gall bladder disorders, abscess and minor wounds (Altundag and Munir , 2011). Most published reports about *B. major* deal with its essential oil composition (Bylaitè et al., 2000; Gallori et al 2001). Other publications were focused on particular components of *B. major* extracts. For example, Tămaş et al. (1989) identified five compounds in the *o*-dihydroxyphenolic fraction of *B. major* var. *tanacetoides* (Boiss.) Moldenke, which included caffeic, chlorogenic, and ferulic acids. Todorova and Ognyanov (1989) isolated seven germacranolides from the flowers of a *B. major* population cultivated in Bulgaria and found that these compounds were different from the presently known eudesmanolides in a *B. major* population cultivated in Poland. Pukalskas et al., (2010) characterized phenolics and flavonoids from costmary growing in Lithuania. Benedec et al. (2016) identified phenolic substances in two varieties of *B. major* (var. *balsamita* and *tanacetoides*) and measured the antioxidant capacity of the extracts. Some studies from Iran and Lithuania are available on the polyphenol characterization and antioxidant activity of ethanolic extracts of *B. major* (Derakhshani *et al*., 2012; Pukalskas *et al*., 2010).

To date, however, very little information is available regarding the phytochemistry and biological properties of Italian *B. major*.

Given this framework, the aim of this work was to better investigate the polyphenolic pattern, and to focus on the antioxidant activity of Italian *B. major* at different growth stages by means of the 1,1 diphenyl-2-picryl-hydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) tests.

Materials and methods

Material: *B. major* leaves were collected at the garden of Officina Profumo Farmaceutica Santa Maria Novella (Florence, Italy) at Villa Petraia (Florence, Italy) each week from June to early July during the growth stages.. *B. major* leaves were authenticated at Botany Department, University of Florence (Italy) and by Daniele Ermini, agronomist and Florence tribunal expert. Seeds were of Italian provenance. Growth conditions followed organic procedures. Leaves were sampled at very early growing stage (VEG stage), early growing stage (EG stage), late growing stage (LG stage) and flowering stage. Fresh leaves were stored at -80 °C just after sampling and lyophilised.

Polyphenols extraction: Total polyphenolic compounds (TP) were extracted according to Luthria et al. (2006). Briefly, 100 mg of each sample of lyophilised *B. major* leaves were placed in a test tube with 10 ml of methanolic:water solution (80:20% V/V). Tubes were sonicated at room temperature for 30 s; the mixture was then centrifuged at 10000 rpm for 10 min at 10 °C and the supernatant was transferred in a 10 ml volumetric flask. The residue was re-suspended in 5 ml of the methanolic solution and extracted as before for two times; supernatants were combined and volume was adjusted to 20 ml (final concentration of dried matter was 5 mg/ml). Chlorophyll was removed from extracts by adding 20 ml of petroleum ether to sample solutions that were then agitated and centrifuged at maximum speed for 5 minutes. Upper petroleum phase containing chlorophyll was removed. Operations were repeated two times.

Total Polyphenols quantification: The total phenol assay was performed by using the Folin-Ciocalteau reagent as described by Biagi et al. (2014). 500 μl of the extracted samples (5 mg/ml) were placed in 25-ml flask and diluted with methanol:water 80:20% V/V to 15 ml. 2.5 ml of Folin-Ciocalteau reagent (10% V/V water solution) were added and mixture was shaken for 30 seconds. Afterwards, 5 ml of a saturated $NaCO_3$ water solution was added and the mixture was left at room temperature for 1 h. The absorbance of the coloured reaction product was read at 730 nm using a

Varian UV-Visible spectrophotometer Cary 50 Scan and using distilled water as blank. Results were expressed as mg of gallic acid equivalent per g of dry weight (mg GAE/gDW) using pure gallic acid as standard (Sigma-Aldrich, Milan, Italy). The total phenolic compounds quantification were performed in triplicate.

Total Flavonoids and hydroxycinnamic acids derivatives quantification: Total flavonoids were quantified by using the direct absorbance reading described for herbal extracts by Biagi et al. (2014). Briefly: 50 μl of the methanol/water solutions were diluted to 5 ml with water and absorbance was read at 360 nm. Measurements were performed in triplicate. Standard grade quercetin (Sigma-Aldrich, Milan) was used to construct the calibration curve (313 to 4.5 mg/l, R^2=0,99). Results were expressed as mg of quercetin equivalent per g of dry weight. Analyses were performed in triplicate. Using the same method, a comparison of caffeic acid derivatives was also performed. In this case absorbance was read at 330 nm and standard grade chlorogenic acid (Sigma-Aldrich, Milan, Italy) was used.

Flavan-3-ols quantification: Total flavan-3-ols were quantified by using the acid vanillin assay (Collodel et al., 2014). 100 μl of the methanol/water solutions were diluted to 500 μl with water. The following reagents were then added: 500 μl of a 1% m/V ethanolic vanillin (Sigma-Aldrich, Milan, Italy) solution and, after shaking for 30 seconds, 1 ml of concentrated HCl. Absorbance was read at 500 nm using pure (-)-epicatechin (Sigma-Aldrich, Milan, Italy) as reference standard. Measurements were performed in triplicate.

HPLC analysis

Analyses of *B. major* extracts were carried out using a Varian Multisolvent pump ProStar 210, coupled with a photodiode array detector Varian ProStar 335. A Phenomenex Kinetex Phenyl-Hexyl 100 A, 150 x 4.6 mm reverse-phase C18 column with identical pre-column operating at 25 °C was employed. The eluent was composed of (A) H_2O/CH_3COOH (99.9:0.1%) and (B) $MeOH/H_2O/CH_3COOH$ (95:4.9:0.1%). A three-step linear solvent gradient system was used starting from 5% to 99% of B for 50-min. . B reached 25% at 22 min, then 99% at 45 min, then 5% at 50 min. Flow rate was set at 1.0 ml/min The injection was 25 μl. UV–Vis spectra were recorded in the 220–700 nm range and the chromatograms were recorded at 278 nm, 330 and 365 nm.

Standard solutions, calibration curves and calculation of hydroxycinnamic acids and flavonoid content

Chlorogenic acid, 3,5-O-dicaffeoylquinic acid, hyperoside and quercetin (Sigma-Aldrich, Milan, Italy) stock solutions were prepared by dissolving the standard in ethanol 80% V/V (1 mg/ml). The stock solutions of the standards were diluted with 80% V/V methanol to obtain five different concentrations ranging from 10 to 320 μg/ml. Calibration curves were obtained for each standard with high linearity ($R^2 > 0.996$) by plotting the standard concentrations as a function of the peak area... Each analysis was performed in triplicate.

DPPH assay

The free radical scavenging activity using the 1,1 diphenyl-2-picryl-hydrazyl (DPPH) reagent was determined for *B. major* leaves samples according to Biagi et al. (2018): 0.1 ml of appropriately diluted methanolic extracts of *B. major* (2.5-0.16 mg/ml) was added to 1 ml of freshly prepared methanolic DPPH solution (0.9×10^{-4} M) and stirred. The decolorizing process was recorded at the beginning and after 20 min of reaction at 517 nm and compared with a blank control. Antiradicalic activity was calculated according to the following formula:

DPPH inhibition% = (control absorbance-sample absorbance)/control absorbance) x 100.

Values were expressed as mg/g DW of extracts necessary to inhibit 50% of DPPH (EC_{50})..

Oxygen Radical Absorbance Capacity (ORAC) Assay

The test was performed working with a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA, USA), adapting the method described by Cao & Prior (1998). Each sample was added to a free-radical generator (AAPH, 2,2'-azobis(2-aminopropane) dihydrochloride) and the inhibition of the free radical was measured. Fluorescein was used as a target for free-radical attack. Free radicals caused conformational changes in the protein structure of fluorescein, leading to dose- and time-dependent fluorescence quenching. In 1 cm path length quartz cuvette reagents were added as follows: 2738 μl fluorescein (25.5 mg/l solution, maintained at 4 °C), 37 μl phosphate buffer solution (75 mM, pH 7.4) and 150 μl Trolox standard (Sigma-Aldrich, 100 μM), blank (buffer solution) or sample solution. After incubation at 37 °C for 30 min, the addition of 75 μl AAPH solution (86.8 mg/ml in buffer solution and kept in ice) started the reaction. The excitation λ was 490 nm and the emission λ was 512 nm. Total antioxidant capacity or ORAC unit (μM) was obtained by the following formula:

$$\text{ORAC unit } (\mu M) = 20\ k\ (S_{sample} - S_{blank}) / (S_{Trolox} - S_{blank})$$

where k = dilution factor, S_{sample} = the area under curve area of the sample, S_{blank} the under curve area of the blank and S_{Trolox} the under curve area of the standard.

ORAC values were expressed as μM Trolox Equivalents (TE)/g of dry leaves.

Both DPPH and ORAC tests were repeated three times in duplicate (n=6).

Statistical analysis.

One-way analysis of variance (ANOVA) was used to evaluate the levels of statistical significance tested by a post hoc comparison test (Tukey test HSD) at $p < 0.05$ for all analysis (Statgraphics Plus, version 5.1 for Windows). All data are reported as mean of replicates ± standard deviation. Linear regression curves were calculated by Excel program by Windows.

Results

Total polyphenols concentration: Concentrations of total polyphenols in methanolic extracts of *B. major* leaves are shown in Table 1. Mean reported values were 68.04 ± 8.34 mg/g DW for extracts (Table 1). Highest polyphenol concentration was observed in VEG stage leaves, 72.25 ± 1.86 mg/g DW, with a small decrease, in the range of 2.2-4.6%, in the EG and LG stages, whereas the lowest concentration was observed at flowering stage, with 60.34 ± 2.26 mg/g DW. **Total flavonoids and hydroxycinnamic acids derivatives concentration:** Total flavonoids (table 2) resulted to be most abundant in VEG stage, 17.42 ± 1.33 mg/g DW, representing more than 25% of total polyphenols. In the other growth cycle stages, flavonoids were found in comparable concentration, from 10.72 ± 0.98 mg/g DW in FL stage to 11.51 ± 0.45 mg/g DW in EG stage.

Hydroxycinnamic acids derivatives resulted to be the most representative polyphenols subclass in *B. major* extracts, being more than 50% of total polyphenols (table 2). Hydroxycinnamic acids derivatives abundance correlated with total polyphenols concentration in extracts and EG stage resulted the richest one, with 43.20 ± 3.01 mg/g DW. Extract obtained from leaves in VEG stage was found to have comparable hydroxycinnamic acids derivatives, i.e. 40.20 ± 2.31 mg/g DW. The less rich extract was that obtained in FL stage, with 32.94 ± 1.52 mg/g DW.

Flavan-3-ols concentration: All the tested extracts were found to contain small amounts of flavan-3-ols, less than 1 mg/g DW.

HPLC analysis

Investigations on the phenolic profile of *B. major* methanolic extracts were conducted by means of HPLC-DAD analysis. The three main constituents of all the extracts resulted to be chlorogenic acid (RT = 16.33 min), 3,5-O-dicaffeoylquinic acid (RT = 22.31 min) and a flavonoid at RT = 31.18 min (figure 1, chromatogram registered at 366 nm for VEG stage, and table 3). Chlorogenic acid maintained a constant concentration along the *B. major* growth in VEG, EG and FL stage, exhibiting an increase in correspondence of LG stage. 3,5-O-dicaffeoylquinic acid followed the trend of high total polyphenols concentrations in EG-LG stages, with 9.21 and 9.07 mg/g DW, respectively.

Registered chromatograms showed that in the zone of hydroxycinnamic acid derivatives, RT between 15 and 25 minutes, no other constituents were present in high amounts.

Coherently to colorimetric essays and according to Ena et al. (2009) and Kazemzadeh et al. (2017), flavonoid glycosides and quercetin (RT = 36.30 min) were found to be the main flavonoid in *B. major* extracts. The main flavonoid found in the extracts at RT = 31.18 could not be assigned unambiguously and, altough the RT was very similar to hyperoside standard (RT = 31.56), the UV-vis profile (λ_{max} at 258 and 352 nm) of this flavonoid was slightly different to hyperoside and known glycosylated flavonoids, such as rutin and isoquercitrin, thus permitting us only to consider it a glycosylated flavonoid.

Quercetin concentration, as measured by HPLC, in different *B. major* extracts was highest at VEG and EG stage with a content equal to 3.22 and 2.86 mg/g DW, respectively, which remained constant in the LG stage and declined to 2.59 mg/g DW in FL stage.

Scavenger and Antioxidant activity

The antioxidant properties of the extracts were analysed by means of DPPH test, a radical scavenging test and ORAC test, which measures the oxidative degradation. Results from both tests are shown in Table 4.

DPPH test exploit a single-electron transfer reaction; it measure the antioxidant reducing capacity and permits to evaluate anti-radical activity of studied samples This test compares scavenging capacity (expressed as a percentage) and concentration of the sample permitting a correlation between these two terms.

ORAC test consists in a hydrogen-atom transfer reaction; it quantifies the hydrogen-atom donor capacity and gives a measure of oxidative degradation. This test is extensively used to evaluate oxidative degradation in biological samples, supplements and food.

Methanolic extracts of *B. major* exhibited an average EC_{50} equal to 0.05±0.02 mg/g DW of extract. EC_{50} of extracts resulted similar for VEG and FL, slightly but not significantly better than average value. On the other hand, the LG extract resulted to have an higher DPPH EC_{50} value. No correlation was found either between total phenolics content and DPPH values, nor with flavonoids or hydroxycinnamic acids content. However, the scarce correlation between polyphenols and antioxidant activity in our study, may be explained considering that phenolic compounds are not the only factors responsible for the antiradicalic activity, but the presence of other phytochemicals (e.g. carotenoids, terpenes, reducing carbohydrates) may influence total antioxidant activity (Craft et al., 2012).

ORAC tests displayed a significant difference between the methanolic extracts. Average value obtained resulted to be 1257.67±363.48 µMTE/g DW (Table 4). ORAC assay test measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (Cao and Prior, 1998).

Discussion

The first characterization of the phenolic profile of Italian *B. major* in different growth stages was achieved. This work highlighted that this species is rich in polyphenols, namely hydroxycinnamic and flavonoids derivatives, since the very early growth stage to the beginning of the flowering stage. Analysis of total phenolic content during vegetative growth of *B. major* indicated a constancy of high phenolic levels in *B. major* leaves, with a maximum peak of the total phenolic and hydroxycinnamic acid derivatives content recorded just before flowering stage. Only the content of total flavonoids was significantly higher in the very early growth stage. Chlorogenic acid and 3,5-O-dicaffeoylquinic acid resulted the main phenolic constituents in all the extracts, with their maximum content in late growth stage and early growth stage, respectively. Previously Pukalskas et al. (2010) yet reported that chlorogenic acid and 3,5-O-dicaffeoylquinic acid were found to be also the main constituents of *B. major* harvested in Lithuania. The highest content of quercetin was recorded in the very early growth stage. Bylaitè et al., (2000) reported that also *B. major* essential oil from leaves and flowers has seasonal variations in content and composition and they reported that the highest amount of most components was determined at the bud formation stage. Polyphenols content in the growth cycle significantly differs among different species. For example, Gai et al. (2017) studied *Perilla frutescens* L. and they found that polyphenols content was high in early vegetative stage, decreased in medium and late stage and it was highest in flowering stage. Thi and Hwang (2014) found a higher polyphenols concentration in young leaves of *Aronia melanocarpa* (Michx.) Elliott with respect to old ones..

Depending on the collecting time, *B. major* leaves extracts were found to exert interesting antiradicalic and antioxidant properties verified by means of DPPH and ORAC tests. In the latter only a weak correlation between antioxidant activity and phenolic content was found, while no correlation was found in DPPH test. These results are in accordance with those recorded in other species (Chiocchio et al., 2018) and confirm that interactions between the various components of the phytocomplex can determine the scavenger and antioxidant activities of *B. major* leaves.

Our study on Italian *B. major* lead us to mark this species worthy of further and more specific investigations since it can be considered a resource of antioxidant phytochemicals exploitable *in primis* in food as a flavouring or preservative, as dietary supplement with antioxidant claim, but also in cosmetic as antiaging products.

Disclosure statement

The authors declare that they have no conflict of interest.

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Table 1 Total polyphenolic content in 80% methanolic extracts of *Balsamita major* in the different stage of growth.

Period of sampling	Total polyphenols
	mg GAE/g DW
VEG stage	72.25±1.86
EG stage	68.91±1.98
LG stage	70.65±2.29
FL stage	60.34±2.26

Table 2 Total flavonoids and hydroxycinnamic acid derivatives in extracts of *Balsamita major*.

Period of sampling	**Total flavonoids expressed as quercetin**	**Total hydroxycinnamic acid derivatives expressed as chlorogenic acid**
	mg/g DW	**mg/g DW**
VEG stage	17.42±1.33	40.20±2.31
EG stage	11.51±0.45	43.20±3.01
LG stage	11.09±0.75	38.58±2.87
FL stage	10.72±0.98	32.94±1.52

Table 3 HPLC-DAD characterization of main constituents of methanolic extracts of *Balsamita major* in the different period of growth

Period of sampling	Chlorogenic acid	3,5-O-dicaffeoylquinic acid	Quercetin
		mg/g	
VEG stage	18.30±0.85	7.29±0.41	3.22±0.15
EG stage	17.06±0.81	9.21±0.15	2.86±0.14
LG stage	24.32±1.14	9.07±0.42	2.86±0.13
FL stage	17.74±0.83	7.67±0.35	2.68±0.14

Table 4 DPPH EC_{50} and ORAC values of extracts of *Balsamita major*.

Period of sampling	**DPPH**	**ORAC**
	EC_{50} mg/g DW	**µMTE/g DW**
VEG stage	0.04±0.01	1681±112
EG stage	0.02±0.01	1217±147
LG stage	0.07±0.02	1689±175
FL stage	0.03±0.01	1444±163

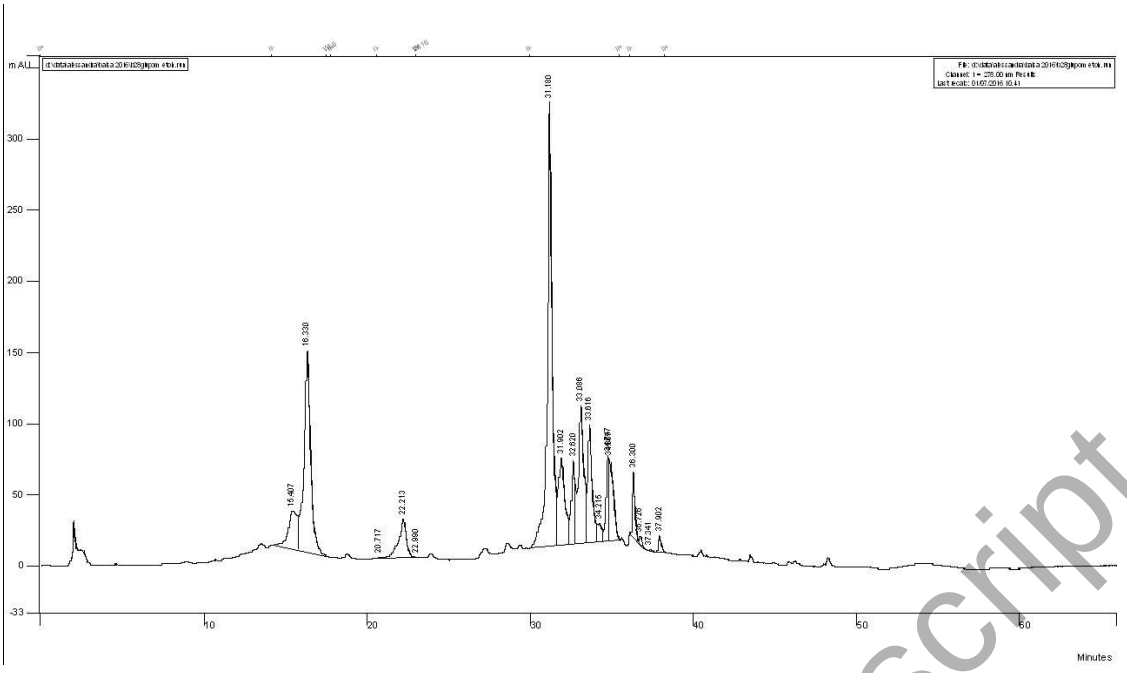

Figure 1: Chromatogram of the water-methanolic extract of *Balsamita major* in very early growth stage recorded at 366 nm. The main constituents is a glycosylated flavonoid at RT = 31.18. chlorogenic acid (RT = 16.33 min) and 3,5-O-dicaffeoylquinic acid (RT = 22.31 min) are the two main hydroxycinnamic acid derivatives in the extract.