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Original Article

Gas chromatography–mass spectrometry analysis of phenolic compounds from *Carica papaya* L. leaf

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Abstract

Phenolic compounds are important components in vegetable foods, infusions and teas for their beneficial effects on human health. The presence of such compounds, evidenced for the first time in *Carica papaya* leaves, could partially explain the pharmacological properties of this plant and demonstrates its importance in alimentation and daily intake. *C. papaya* leaves were extracted with methanol in a Soxhlet apparatus and later with a liquid–liquid extraction (LLE) with the aim of identifying and quantifying secondary metabolites from this plant, using gas chromatography–mass spectrometry (GC–MS) in the selected ion-monitoring (SIM) mode. Derivatization procedure of the extract was necessary to analyze the polar compounds in GC–MS. 5,7-Dimethoxycoumarin and polar molecules such as protocatechuic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, kaempferol and quercetin were detected and identified in qualitative analysis. The quantitative analysis has shown the presence of phenolic acids as the main compound, while chlorogenic acid was found in trace amounts, compared to the flavonoids and coumarin compounds. The quantities detected were 0.25 mg/g (dry leaf) for caffeic acid, 0.33 mg/g for *p*-coumaric acid and 0.11 mg/g for protocatechuic acid. Kaempferol and quercetin were 0.03 and 0.04 mg/g, respectively, while that for 5,7-dimethoxycoumarin was 0.14 mg/g.
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Keywords: *Carica papaya*; 5,7-Dimethoxycoumarin; Protocatechuic acid; *p*-Coumaric acid; Caffeic acid; Chlorogenic acid; Kaempferol; Quercetin; Gas chromatography–mass spectrometry

1. Introduction

Carica papaya (papaya) is a tree-like herbaceous plant, a member of the small family Caricaceae and widely cultivated for its edible fruits. It originates in the lowlands of eastern Central America, from Mexico to Panama, and can be found in all tropical countries and many sub-tropical regions of the world. Parts of the plant are used in tropical diets as a fruit or vegetable; it is sometime used as a therapeutic remedy for several of its medicinal properties. Papaya fruit is thought to contain some immuno-stimulating and anti-oxidant agents (Aruoma et al., 2006; Mehdipour et al., 2006): immature fruits and roots are used for their abortifacient activity (Cherian, 2000; Sarma and Mahanta, 2000); the seeds are now being used as a potential post-testicular anti-fertility drug (Lohiya et al., 2000); the pulp is used by African hospitals for treating wounds and burns (Starley et al., 1999); the latex and the seeds are used in the care of gastrointestinal nematode infections and they have shown anthelmintic activity (Stepek et al., 2004); and the seeds and immature fruit have shown bacteriostatic activity against the human enteric pathogens (Osato et al., 1993). The leaves are used to relieve the symptoms of asthma and as a vermifuge, in the treatment of gastric problems, fever and amoebic dysentery. Methanolic leaf extract demonstrated vasodilatatory and anti-oxidant effects, both implicated in the

Abbreviations: BSTFA, *N*, *O*-Bis(trimethylsilyl)trifluoroacetamide; GC, gas chromatography; IS, internal standard; LLE, liquid–liquid extraction; MS, mass spectrometry; *m*/*z*, fragment mass/charge ratio; r^2, correlation coefficients; rt, retention time; SIM, selected ion monitoring; TIC, total ions chromatogram; TMCS, trimethylchlorosilane

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reduction of cardiovascular risks (Runnie et al., 2004). The aqueous extract showed beneficial effects for the acceleration of wound healing processes in rats (Mahmood et al., 2005).

Very little has been published on the chemical composition or biochemistry of *C. papaya*. Chemical characterization of the metabolites extracted from the plant has shown the presence of active compounds in the plant tissues, such as cysteine endopeptidases, a class-II and class-III chitinase, and glutaminyl cyclase in the latex (Azarkan et al., 2005), the linalool in fruit pulp (Winterhalter et al., 1986), the alkaloids carpaine, pseudocarpaine, dehydrocarpaine I and II in the leaves (Khuzhaev and Aripova, 2000), kaempferol and quercetin in the shoots (Miean and Mohamed, 2001), the cyanogenic compounds in leaves and roots and benzylglucosinolate and its breakdown product benzylisothiocyanate in all the tissues (Olafsdottir et al., 2002).

The aim of this work was to carry out a phytochemical analysis of *C. papaya* leaf extracts. Our analytical attention was focused on secondary metabolites, particularly phenolic compounds from papaya leaves, which have never before been reported in the literature. These molecules play several roles in plant physiological processes, such as protection from UV, defense against pathogens and phytophagous, pollination and dissemination, symbiosis and allelopathic interactions (Buchanan et al., 2000). Biological activity is the basis for traditional medicine, which uses the pharmacological efficacy of natural compounds present in herbal preparations for treating human diseases. Furthermore, papaya leaves are used in tropical alimentation cooked as a vegetable and in preparation of teas and infusions. In connection with this, epidemiological evidence has supported the role that antioxidants, including phenolic compounds, play in the prevention of several chronic diseases such as cardiovascular disease, cancer, diabetes, bacterial and parasitic infections (Murakami et al., 1994; Sherman and Billing, 1999). These reasons explain the importance of papaya plant for tropical and also non-tropical populations and the scientific interest in knowing the chemical composition of the extracts.

Gas chromatography–mass spectrometry (GC–MS) was selected as the method of chemical analysis to identify and quantify the metabolites in leaf extracts obtained using liquid–liquid extraction (LLE). This extraction method made it possible to clean up the extracted sample before derivatization reaction and chromatographic separation and identification. Such a method is extremely useful for the purification of the samples resulting from the interferences due to the biological matrix. An analytical comparison was even carried out between nonhydrolyzed and hydrolyzed extracts, to quantify free and bound phenolic compounds. In fact, the flavonoids occur in nature as their glycoside derivatives in which one or more sugar groups are attached to the phenolic groups (Croft, 1998; Zuo et al., 2002; Chen and Zuo, 2007). Such derivatives can be found especially for the phenolic acids, which occur mostly esterified, glycosylated or polymerized (Naczk and Shahidi, 2004). The extraction recovery of bound phenolics was evaluated using alkaline hydrolysis.

2. Materials and methods

2.1. Reagents

N,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), pyridine, methanol and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard reference compounds were 5,7-dimethoxycoumarin, bergapten (5-methoxypsoralen), protocatechuic acid (3,4-dihydroxybenzoic acid), *p*-coumaric acid (*trans*-4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), chlorogenic acid [1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)], kaempferol (3,4′,5,7-tetrahydroxyflavone), quercetin (3,3′,4′,5,7-pentahydroxyflavone) and genistein (4′,5,7-trihydroxyisoflavone) and they were also purchased from Sigma-Aldrich. All solvents and reagents used in this study were of analytical grade purity.

2.2. Plant material

Samples of *Carica papaya* L. (Caricaceae) leaves (300 g) were collected in West Cameroon (Africa) in March 2006 and identified by one of us (A.C.). A voucher specimen of this raw material is deposited at Herbarium of University of Rome "Tor Vergata".

2.3. Extraction of phenolic compounds

The extraction of phenolic compounds from *C. papaya* leaf was carried out as follows. Plant material (10 g) was reduced to a fine powder and extracted in a Soxhlet apparatus (90 °C for 24 h) with 200 ml of 70% aqueous methanol (v/v) acidified to pH 2 with some drops of concentrated HCl.

A part of the filtered extract was used for LLE of free phenolic compounds. After methanol evaporation in a rotary evaporator, the resulting aqueous solution was extracted three times with 30 ml of diethyl ether in a separatory funnel and the organic solutions were combined and evaporated to dryness. Before GC injection, the dried sample was suspended in BSTFA + TMCS 1% for derivatization of the polar compounds. The remaining solution was evaporated to dryness and then submitted to alkaline hydrolysis by adding NaOH (5 M, 30 mL) under nitrogen at room temperature for 4 h, after which the sample was concentrated and the solution was acidified with HCl 12 M (Germanò et al., 2006). The released phenolic compounds were extracted with diethyl ether and dried for derivatization as reported above.

Table 1
Secondary metabolites detected using GC/SIM–MS conditions, with relative retention time (rt) and characteristic ions (m/z) in the mass spectra of silylated derivatives and not in standard solutions and *C. papaya* extracts

Compound	Molecular weight	TMS groups	TMS derivatized molecular weight	rt (min)	m/z[a] (relative intensity)
2. Protocatechuic acid	154	3	370	11.02	370(100),355(9),193(2),73(1)
3. *p*-Coumaric acid	164	2	308	11.78	308(100),293(10),249(3),219(6)
4. 5,7-Dimethoxycoumarin	206	0	—	11.89	206(100),191(23),178(40),163(28)
5. Caffeic acid	180	3	396	12.97	396(100),381(2),219(4),73(1)
6. Kaempferol	286	4	574	19.53	574(15),559(100),487(30),272(3)
7. Quercetin	302	5	662	20.77	662(10),647(100),574(63),559(14)
8. Chlorogenic acid	354	6	786	20.07	786(100),419(7),397(11),345(28)

[a]Quantitation ions are underlined.

2.4. Derivatization procedure

For GC–MS analysis of polar compounds, standards and leaf extracts were derivatized in order to obtain the alkyl silylated derivatives. The dried extracts were treated by adding BSTFA + TMCS 1%/anhydrous pyridine (1.5 mL, 1:1). The mixture was heated at 90 °C for 1 h and derivatized sample submitted to GC–MS analysis.

2.5. Preparation of the standard solutions

Solutions of 5,7-dimethoxycoumarin, bergapten, caffeic acid, *p*-coumaric acid, chlorogenic acid, protocathecuic acid, kaempferol, quercetin and genistein were prepared for qualitative and quantitative analysis of leaf extracts. For the polar compounds, stock solutions were prepared adding the compounds to BSTFA + TMCS 1%/pyridine (50 μL, 1:1): the mixture was heated at 90 °C for 1 h and standard solutions at different concentration were prepared. For the apolar compounds, the derivatization was not necessary. In GC–MS analysis, 3 μL of each standard solution was injected into a capillary column.

2.6. Analytical protocol

GC–MS analyses were performed using a VG Quattro Mass spectrometer (VG Micromass, UK), equipped with a Supelco SPB-5 capillary column (length: 30 m; ID: 250 μm; film thickness: 1.4 μm). The helium carrier gas was set to a column head pressure of $P = 100$ kPa. The inlet temperature was set at 280 °C while the oven temperature was initially at 100 °C (held for 3 min) and then increased to 315 °C at 20 °C/min. The mass spectrometer was operated in the positive electron impact mode with an ionization energy of 70 eV. The ion source temperature was 190 °C at a pressure of 10E-4 Torr. Detection was performed in selective ion-monitoring (SIM) mode and peaks were identified and quantitated using target ions.

2.7. GC–MS analysis

GC–MS analysis of *C. papaya* extracts were carried out in SIM mode. The identification of each peak was achieved by comparing the retention time (rt) and mass spectra of the compounds in the extract to that of the standards, matching the area ratios of characteristic ions with those of respective standards.

In quantitative analysis, a series of solutions containing the standard molecules and relative internal standard (IS) in known concentrations was analyzed. Bergapten was selected as IS for 5,7-dimethoxycoumarin due to its chemical similarity with it and, for the same reason, genistein for flavonoids and phenolic acids. The linear calibration curves were traced to quantify the identified compounds in the different extracts: each standard solution was analyzed to calculate the peak area ratio of molecules compared to IS, then the response ratios (y) and the relative molecule concentrations (x) were used for the construction of the calibration curve. Finally, each extract with IS in the same concentration of standard solutions was analyzed, and the peak area ratio was used to calculate the concentration of the identified compounds by using the curve equation. Three-microliter aliquots of each standard solution and of the extracts were used for GC–MS analysis and triplicate injections were made for each sample. Ions with the mass/charge (m/z) ratio shown in Table 1 were used in the SIM mode for quantification. For the quantitative study, three independent leaf samples were extracted and analyzed independently and each sample was analyzed three times. Results are expressed as the mean ± standard deviation of three independent experiments and were evaluated by one-way analysis of variance in Microsoft Excel® 2003.

3. Results and discussion

In Table 1, the rt, obtained in GC–MS system used in this work, and the m/z ratio characteristic for each ion of all the analyzed standards are shown. The peak identification was obtained by GC–MS analysis of *C. papaya*

extracts: the results were compared with those obtained for the standard solutions, as reported in the experimental section. GC–MS analysis of papaya extract detected molecular peaks, with typical rt of analyzed standards, which are shown in total ions chromatogram (TIC) obtained from SIM analysis (Fig. 2). Peak 2 (rt 11.02 min) represents the protocatechuic acid, peak 3 (rt 11.78 min) the *p*-coumaric acid, peak 4 (rt 11.89 min) the 5,7-dimethoxycoumarin and peak 5 (rt 12.97 min) the caffeic acid. The other components were detected by selecting ion currents at particular *m*/*z* values. For *m*/*z* 559, peak 6 with a rt of 19.53 min, is equal to that of kaempferol standard; for *m*/*z* 647 the peak 7 (20.77 min) corresponds to quercetin; and for *m*/*z* 785, peak 8 (20.07 min) corresponds to chlorogenic acid (see Fig. 3). The relative mass spectra analysis confirms the presence of all these molecules in papaya extract. In the mass spectrum corresponding to each chromatographic peak there are the fragmentations of the mentioned compounds (see Table 1). Moreover, in TIC there is a significative peak (1) at a rt of 10.81 min, with a fragmentation typical of the *p*-coumaric acid. Probably, the peak represents an isomer of the phenolic acid, *m*-coumaric acid or *o*-coumaric acid (3b and 3c in Fig. 1), but standard GC–MS analysis was not carried out (Figs. 1–3).

Under the experimental conditions described above, linear calibration curves were obtained over the entire range of the concentration studied. Regression analysis of the peak area ratios (y) vs. concentrations (x) were carried out; the calibration curve demonstrated acceptable linearity with correlation coefficients $r^2 > 0.995$ except for caffeic acid ($r^2 = 0.991$). Then, the identified compounds present in the *C. papaya* extracts, before and after alkaline hydrolysis, were calculated using the corresponding linear calibration curves and they are shown in Table 2. Total phenolic content in methanolic extract of some medicinal plants were reported in the literature and estimated spectrophotometrically: a very high phenolic content was

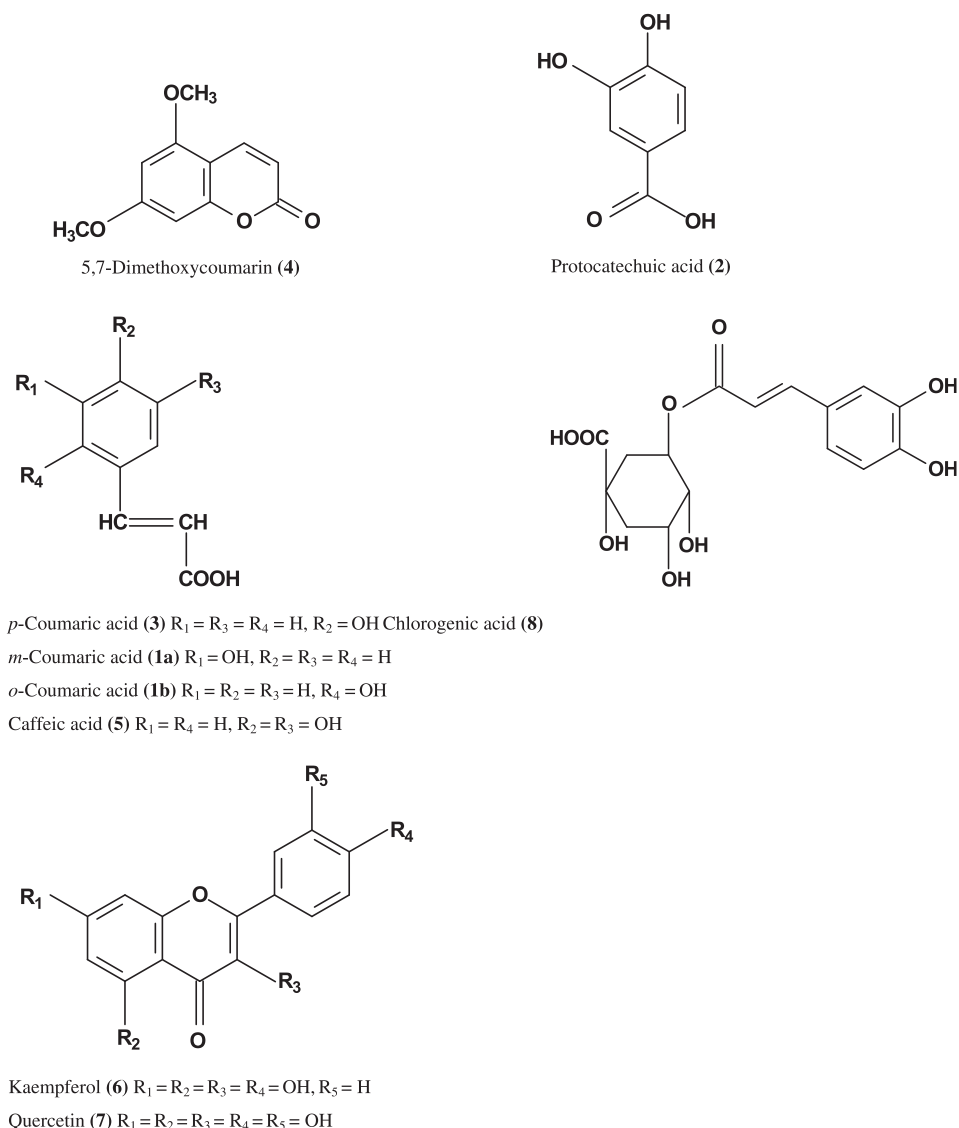

Fig. 1. Molecular structure of secondary metabolites analyzed in *Carica papaya* leaf extract.

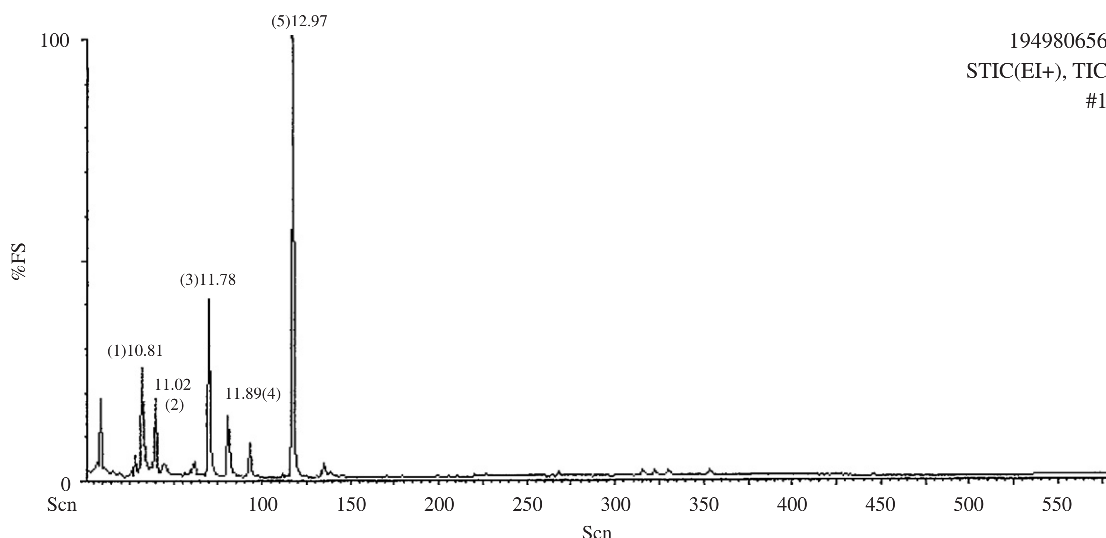

Fig. 2. TIC of *Carica papaya* extract from GC–MS analysis in SIM mode. Peak 2 (rt 11.02 min) corresponds to protocatechuic acid, peak 3 (rt 11.78 min) to *p*-coumaric acid, peak 4 (rt 11.89 min) to 5,7-dimethoxycoumarin, peak 5 (rt 12.97 min) to caffeic acid. The rt and mass spectra were compared with that of the standard compounds. Peak 1 corresponds to a compound with mass spectrum equal to *p*-coumaric acid, but with different rt: it could be an isomer of the phenolic acid.

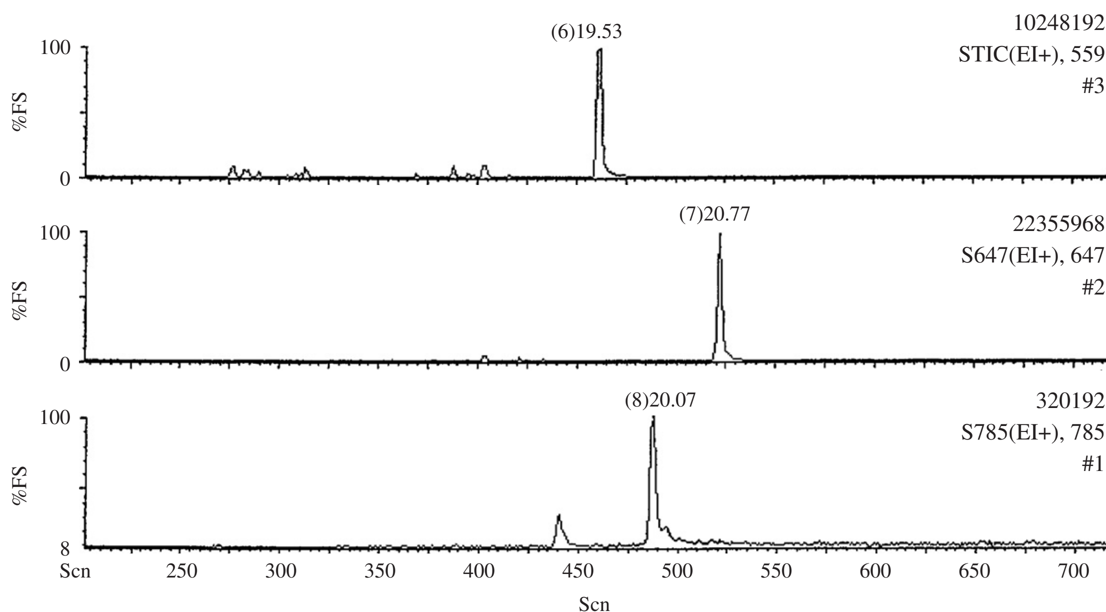

Fig. 3. Chromatogramm of *Carica papaya* extract from GC–MS analysis in SIM mode, where ionic currents of kaempferol (*m*/*z* 559), chlorogenic acid (*m*/*z* 785) and quercetin (*m*/*z* 647) are selected. Peak 6 (rt 19.53) corresponds to kaempferol, peak 7 (rt 20.77) corresponds to quercetin, peak 8 (rt 20.07) corresponds to chlorogenic acid.

observed for methanolic extract of *C. papaya* leaf (Runnie et al., 2004) and the results of GC–MS quantitative analysis confirms the spectrophotometric studies. In fact, in the nonhydrolyzed extract, phenolic acids, except chlorogenic acid, and 5,7-dimethoxycoumarin were detected at high concentrations. On the contrary, the concentrations of kaempferol and quercetin were lower. In the sample submitted to acid hydrolysis, the concentrations of protocatechuic acid, *p*-coumaric acid and caffeic acid increased and this point demonstrates that such compounds are present in vegetable tissues mostly as esters and glycosides. Caffeic acid is the most abundant of all the identified compounds in this work and this result is comparable with those reported in other papers. This is the most common representative of the family of the hydroxycinnamic acids and a fundamental metabolite of vegetable biochemistry (Germanò et al., 2006). On the contrary, the chlorogenic acid content of hydrolyzed

Table 2
Quantities[a] of phenolic compounds in *Carica papaya* extract before and after hydrolysis (mg/g of dry leaf) and RSD

Compound	mg/g ± S.D. (before hydrolysis)	RSD (%)	mg/g ± S.D. (after hydrolysis)	RSD (%)
2. Protocatechuic acid	0.11 ± 0.003	2.71	0.57 ± 0.019	3.45
3. *p*-Coumaric acid	0.33 ± 0.019	5.87	3.80 ± 0.18	4.79
4. 5,7-Dimethoxycoumarin	$0.14 \pm 0.05 \times 10^{-3}$	0.04	—	—
5. Caffeic acid	0.25 ± 0.008	3.30	9.53 ± 0.32	3.36
6. Kaempferol	0.03 ± 0.001	3.06	$0.06 \pm 0.02 \times 10^{-3}$	0.03
7. Quercetin	0.04 ± 0.001	2.35	0.11 ± 0.001	1.03
8. Chlorogenic acid	Trace[b]	3.09	n.d.[c]	—

[a]All data are means of three independent assays.
[b]Trace <0.001 mg/g.
[c]n.d. = not detected.

extract was lower than that of the nonhydrolyzed sample, considering that this compound is present in the form of caffeic acid ester which decreases with hydrolysis reaction. The increase in hydrolyzed extract was less significant for flavonoids, but it should be considered that the majority of the free phenolics are flavonols, such as kaempferol and quercetin, whereas the bound phenolics are mainly phenolic acids. In the literature, there are many contributions about the biological activity of the two identified flavonols and few reports on the phenolic acids. Kaempferol and quercetin are compounds found abundantly in many edible plants including leafy vegetables, fruits and beverages (Zhang and Zuo, 2004); they demonstrated anti-oxidant (Chopra et al., 2000) and anti-carcinogenic (Pereira et al., 1996) activities.

Furthermore, identified phenolic acids are present in appreciable concentrations in a large number of vegetable foods and they have potent anti-oxidant properties (Chen et al., 2001; Zhang and Zuo, 2004), which explain their role in the prevention of some degenerative diseases, including artherosclerosis and cancer (Kampa et al., 2004). 5,7-Dimethoxycoumarin is identified in a small group of vegetable species, it is much less studied and few data are available on it. This last compound is a member of a very large class of molecules widely distributed in nature (Egan et al., 1990). The biological activity of coumarin and more complex related derivatives appears to be based on the coumarin nucleus (Jimenez-Orozco et al., 1999; Finn et al., 2001): biological effects observed include anti-bacterial (Laurin et al., 1999), anti-platelet (Roma et al., 2003) and vasodilatatory (Campos-Toimil et al., 2002), anti-ashmatic (Fang et al., 2003), anti-mutagenic (Pillai et al., 1999), anti-inflammatory (Paya et al., 1992), lipoxygenase and cyclo-oxygenase inhibition (Hoffmanova et al., 1998), protecting against oxidative damage by acting as quencher of reactive oxygen species, and anti-tumourigenic (Egan et al., 1997; Hayes et al., 1998).

This work partially explains the medicinal activity of this plant, by identification of some phenolic compounds, but further studies are necessary to identify other active principles and to demonstrate their biological activity.

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