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Original Article

Analysis of flavonoids in *Rubus erythrocladus* and *Morus nigra* leaves extracts by liquid chromatography and capillary electrophoresis

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ABSTRACT

This study uses high performance liquid chromatography and capillary electrophoresis as analytical tools to evaluate flavonoids in hydrolyzed leaves extracts of *Rubus erythrocladus* Mart., Rosaceae, and *Morus nigra* L., Moraceae. For phytochemical analysis, the extracts were prepared by acid hydrolysis and ultrasonic bath and analyzed by high performance liquid chromatography using an ultraviolet detector and by capillary electrophoresis equipped with a diode-array detector. Quercetin and kaempferol were identified in these extracts. The analytical methods developed were validated and applied. Quercetin and kaempferol were quantified in *R. erythrocladus*, with $848.43 \pm 66.68\ \mu g\,g^{-1}$ and $304.35 \pm 17.29\ \mu g\,g^{-1}$, respectively, by HPLC-UV and quercetin, $836.37 \pm 149.43\ \mu g\,g^{-1}$, by CE-DAD. In *M. nigra* the quantifications of quercetin and kaempferol were $2323.90 \pm 145.35\ \mu g\,g^{-1}$ and $1446.36 \pm 59.00\ \mu g\,g^{-1}$, respectively, by HPLC-UV and, $2552.82 \pm 275.30\ \mu g\,g^{-1}$ and $1188.67 \pm 99.21\ \mu g\,g^{-1}$, respectively, by CE-DAD. The extracts were also analyzed by ultra-performance liquid chromatography coupled with a diode-array detector and mass spectrometer (MS), UPLC-DAD/MS.

Introduction

The fruits of *Rubus* (Rosaceae) and *Morus* (Moraceae) have been extensively studied around the world because of their beneficial effects on health. *Rubus*, popularly known as the genus of raspberries and blackberries, is widely distributed around the world, both as wild and cultivated species (Deighton et al., 2000). *Morus* is known as the genus of mulberry, and is found from temperate regions to subtropical regions of the Planet (Gundogdu et al., 2011). In Brazil, plants belonging to the genus *Morus* are listed by the government as interesting to research because of their use in folk medicine, and their potential for use as phytotherapics (Portal da Saúde, 2013). However, *Rubus* and *Morus* are often mistaken in the differentiation, due to the similarity of their fruits. Also, there is a lot of information about the fruits of these species, but little information about their leaves.

Studies have shown that *Rubus* leaves demonstrate antioxidant (Venskutonis et al., 2007; Martini et al., 2009), anticancer (Durgo et al., 2012), gastrointestinal (Rojas-Vera et al., 2002), antiangiogenic (Liu et al., 2006), antithrombotic (Han et al., 2012), hypoglycemic (Jouad et al., 2002), antimicrobial (Panizzi et al., 2002; Thiem and Goslinska, 2004; Martini et al., 2009; Ostrosky et al., 2011) and anxiolytic activities (Nogueira et al., 1998; Nogueira and Vassilieff, 2000). For the leaves of *Morus*, there are studies reporting antioxidant (Katsube et al., 2010; Choi et al., 2013), anticancer (Dat et al., 2010; Skupien et al., 2008), hypoglycemic (Volpato et al., 2011; Chung et al., 2013), anti-obesity (Oh et al., 2009; Tsuduki et al., 2013), antimicrobial (Omidiran et al., 2012) and vasodilation activities (Xia et al., 2008).

Flavonoids derived from quercetin and/or kaempferol were reported in extracts of *Rubus* leaves analyzed by TLC (Tzouwara-Karayanni and Philianos, 1982), HPLC (Venskutonis et al., 2007; Martini et al., 2009; Durgo et al., 2012; Gudej and Tomczyk, 2004) and NMR (Panizzi et al., 2002; Han et al., 2012; Gudej, 2003). There are no studies about analysis of *Rubus* leaves by CE, and the species *erythrocladus* has never been studied before. In *Morus* leaves, secondary metabolites derived from quercetin and/or kaempferol have

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been identified by HPLC (Katsube et al., 2010; Thabti et al., 2012; Choi et al., 2013), and by NMR (Dat et al., 2010; Katsube et al., 2010), and there are two studies on the chemical composition of *Morus alba* by CE (Suntornsuk et al., 2003; Chu et al., 2006).

High performance liquid chromatography is a classic method used for the separation and quantification of secondary metabolites in plant extracts but sometimes it can be an expensive analytical tool (Merken and Beecher, 2000). Capillary electrophoresis is characterized by high separation efficiency and good compromise between analysis time and satisfactory characterization of phenolic compounds in plants. The low operational cost and the small residue generation make this technique an attractive option for the development of analytical methods for phytochemical analysis (Carrasco-Pancorbo et al., 2006).

The aim of this study was to develop HPLC-UV and CE-DAD analytical methods for the evaluation of quercetin and kaempferol in *Rubus erythrocladus* Mart. and *Morus nigra* L. leaves extracts. The results were statistically analyzed using validation parameters and the methods were applied to determine the amount of these flavonoids in these samples.

Experimental

Samples

Leaves of *Rubus erythrocladus* Mart., Rosaceae, were collected at Embrapa – Temperate Climate (Pelotas, Brazil) and leaves of *Morus nigra* L., Moraceae, were collected in Porto Alegre (Brazil). Vouchers have been deposited in the Herbarium – UFRGS, Federal University of Rio Grande do Sul (179856 and 176765, respectively). The samples were dried at room temperature for two weeks and kept in the dark.

Chemicals and materials

Quercetin (purity ≥ 98%) and kaempferol (purity ≥ 97%) were obtained from Sigma (USA) and methylparaben from Fluka (USA). HPLC-grade acetonitrile and methanol were purchased from Merck (Germany). Throughout the study, deionized and ultrapure water were used. All other chemicals were analytical grade. Dichloromethane and hydrochloric acid were obtained from Synth (Brazil), trifluoroacetic acid from Vetec (Brazil), and ethyl acetate, sodium hydroxide and sodium tetraborate decahydrate from Merck (Germany).

HPLC conditions

HPLC analysis were performed on a Waters Alliance 2695 (USA) chromatograph using a UV detector (UV/VIS Waters 2487, USA) and a C18 reversed-phase column (Phenomenex, 150 × 4.6 mm, 4 μm) with guard-column (C18). The Empower software was used for the data acquisition. A photodiode array detector (DAD) (UV/VIS Waters 996, USA) was also used to evaluate the specificity.

Flavonoids were eluted using a gradient system. Mobile phase A consisted of water with 0.01% (v/v) of trifluoroacetic acid and B consisted of acetonitrile with 0.08% (v/v) of trifluoroacetic acid. The gradient profile was: 0 min 50% of B, 0–2 min 60% of B, 2–4 min 80% B and 4–7 min 95% B. The flow-rate was 0.6 ml min^{-1} and detection was at 370 nm.

UPLC conditions

An Ultra Performance Liquid Chromatography (Waters Acquity UPLC, USA) equipped with a diode array detector (DAD) coupled with eletrospray ionization in the positive ion mode (ESI(+)) quadrupole time-of-flight (Q-Tof) mass spectrometry (MS) (MS Q-Tof Micro-Micromass) was used. The separation of the compounds was performed on a C18 reverse phase column (Acquity UPLC beh, 50.0 × 2.1 mm, 1.7 μm). The software program Mass Lynx v.4.1 was used for the analysis and data acquisition and the program Acquity UPLC Columns Calculator v.1.1.1 was used to adjust the HPLC method to the UPLC method.

Mobile phase A consisted of water with 0.1% (v/v) of formic acid and acetonitrile B with 0.1% (v/v) of formic acid. The gradient was 0 min 50% of B, 0–0.09 min 60% of B, 0.09–0.38 min 80% of B and 0.38–0.80 min 95% of B. Column temperature was maintained at 25 °C, flow rate was 0.294 ml min^{-1}, injection volume was 1.0 μl and detection was between 200 and 400 nm.

CE conditions

The CE assays were conducted in a capillary electrophoresis system (Agilent Technologies, model 7100, Palo Alto, CA, USA) equipped with a diode array detector, temperature-control device (maintained at 25 °C) and data acquisition and analysis software supplied by the manufacturer (HP ChemStation®). Before the first run, the capillary column was sequentially rinsed with 1 mol l^{-1} NaOH (30 min) and water (30 min). Between runs, the capillary was flushed for 1 min with background electrolyte (BGE). At the beginning of each day the capillary was conditioned by flushing with 1 mol l^{-1} NaOH (20 min), followed by a 20 min flush with deionized water and an electrolyte solution (20 min). Between runs, the capillary was reconditioned with 1 mol l^{-1} NaOH (2 min), deionized water (1 min) and electrolyte solution (2 min). At the end of each working day, the capillary was rinsed with 1 mol l^{-1} NaOH (10 min) and water (10 min).

Separations were conducted in an uncoated fused-silica capillary 48.5 cm in length (40 cm effective length × 50 μm I.D. × 375 μm O.D.). Direct UV detection was set at 210 nm, and the temperature was maintained at 25 °C. The standards and samples were introduced to the capillary with a hydrodynamic pressure of 50 mbar, for 3 s. The separation voltage applied was 30 kV under normal polarity. The optimized BGE used in the proposed method was comprised of 20 mmol l^{-1} (pH 9.2) and 10% (v/v) acetonitrile, and methylparaben was used as internal standard, diluted to obtain a final concentration of 45.46 μg ml^{-1}.

Standard preparation and calibration curves

The standard stock solutions were prepared by dissolving 1 mg of each compound in 1 ml of methanol. These solutions were stored in dark glass bottles at 4 °C. Working standard solutions were freshly prepared by dissolving a suitable amount of the above solutions with methanol before injection. Analytical curves for both quercetin and kaempferol were prepared, with nine points between 1 and 100 μg ml^{-1} for the HPLC analysis and seven points between 15 and 150 μg ml^{-1} with increments of methylparaben, 45.46 μg ml^{-1}, for the CE analysis.

Sample preparation of extracts

Boiling water (10 ml) was added to 0.1 g of dried, crushed leaves and this infusion process was maintained for 15 min. The extract was filtered and partitioned with ethyl acetate (5 × 20 ml) and the organic phase was evaporated (extract 1). The solid residue was dissolved in 1 ml of methanol and 14 ml of HCl 50% (v/v). The sample was hydrolyzed for 2 h in an ultrasound bath, then partitioned with dichloromethane (5 × 20 ml). The organic phase was evaporated and dissolved in 5 ml of methanol (extract 2). This extract was filtered through a 0.22 μm membrane (Durapore, USA), injected in HPLC-UV, HPLC-DAD, UPLC-DAD/MS and CE-DAD with no dilution. For the CE analysis, methylparaben (45.46 μg ml^{-1}) was added to the samples as internal standard.

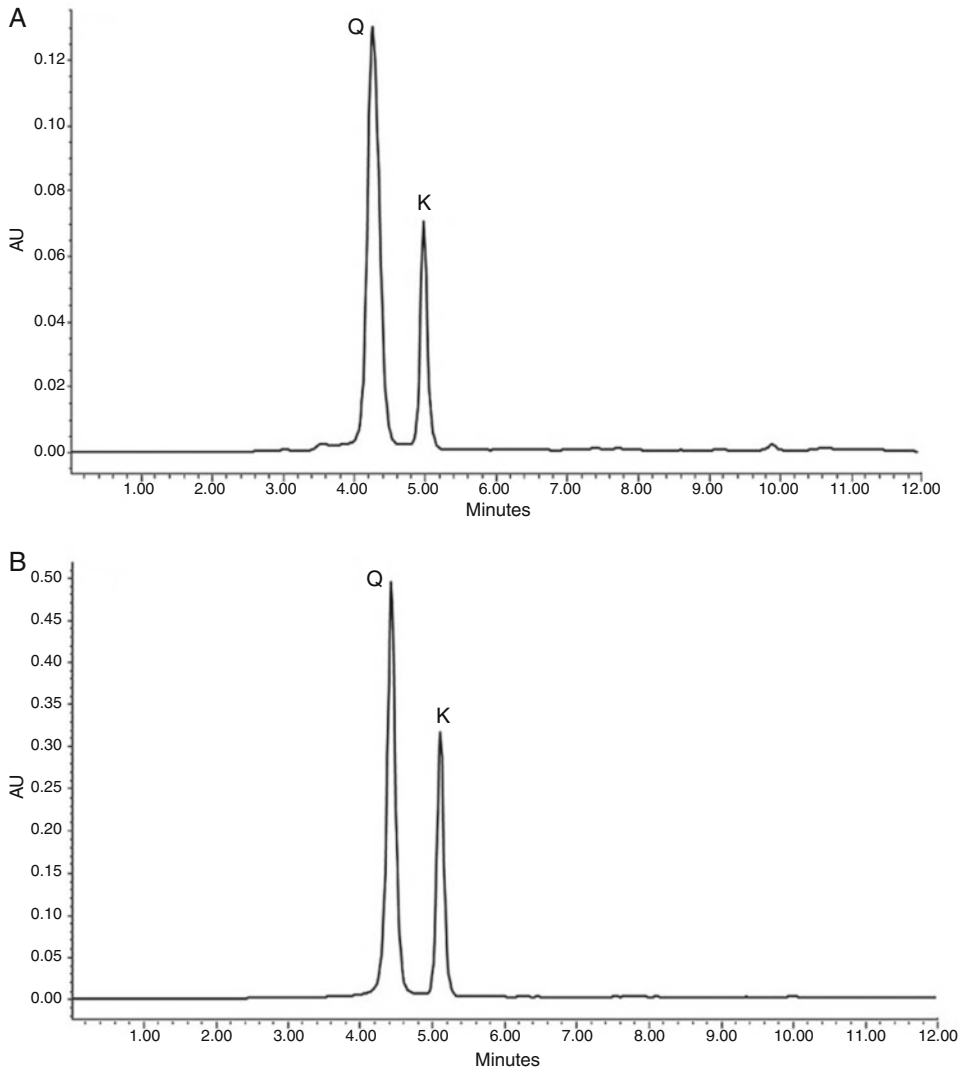

Fig. 1. Chromatograms of *Rubus erythrocladus* (A) and *Morus nigra* (B) hydrolyzed leaf extracts by HPLC-UV, 370 nm. Q = quercetin and K = kaempferol.

Validation

The HPLC and CE methods were validated for the quantitative analysis of quercetin and kaempferol present in *R. erythrocladus* and *M. nigra* leaves, in accordance with the International Conference on Harmonization guidelines, using the parameters of linearity, specificity, precision, accuracy (recovery test), limit of detection, limit of quantification and robustness (ICH, 2005). For validation in CE, we always used an internal standard (IS) – methylparaben – to give greater certainty to the results.

Specificity was determined by checking the peak purity of the quercetin and kaempferol peaks, using a photodiode-array detector by HPLC and CE. Linearity was analyzed using three standard curves obtained on three different days at nine different concentrations of quercetin and kaempferol (1–100 $\mu g\,ml^{-1}$) by HPLC and seven different concentrations of quercetin and kaempferol (15–150 $\mu g\,ml^{-1}$) by CE. For electrophoresis analysis, 45.46 $\mu g\,ml^{-1}$ of methylparaben was added in all the calibration curve points, as internal standard. Linearity was evaluated by calculating the regression line using the least squares method.

Precision was performed in two different levels: intra-day precision and inter-day precision. The intra-day precision was determined after analyses of three solutions prepared at different concentrations, in triplicate, injected in one day. The inter-day precision was studied by analyzing a solution of quercetin and kaempferol, prepared in triplicate, over three consecutive days. The accuracy (assay recovery) of the method was assessed by analyzing samples of *R. erythrocladus* and *M. nigra*, by HPLC and CE, respectively – the samples were spiked with known amounts of quercetin and kaempferol standard solutions at three different concentrations: 20, 40 and 60 $\mu g\,ml^{-1}$ of quercetin and kaempferol for chromatography analysis and 30, 60 and 90 $\mu g\,ml^{-1}$ of quercetin and kaempferol for electrophoresis analysis. Accuracy was determined by the mean concentration recovered, and the results were expressed as recovery percentage.

Detection (LOD) and quantitation (LOQ) limits were calculated directly from the standard curve. LOD was calculated by the equation $3.3\,\sigma/S$, while for LOQ, the equation $10\,\sigma/S$ was used, where σ is the SD of y intercepts of regression lines and S is the slope of the standard curve.

Robustness was available from the injection of a quercetin and kaempferol solution by normal conditions and by varying the following analytical parameters: chromatographic column batch, pH of the mobile phase and flow rate for HPLC and temperature of cassette, injection time and organic solvent of electrolyte for CE.

Results and discussion

The flavonoids quercetin and kaempferol were identified in hydrolyzed leaves extracts of *R. erythrocladus* and *M. nigra* by

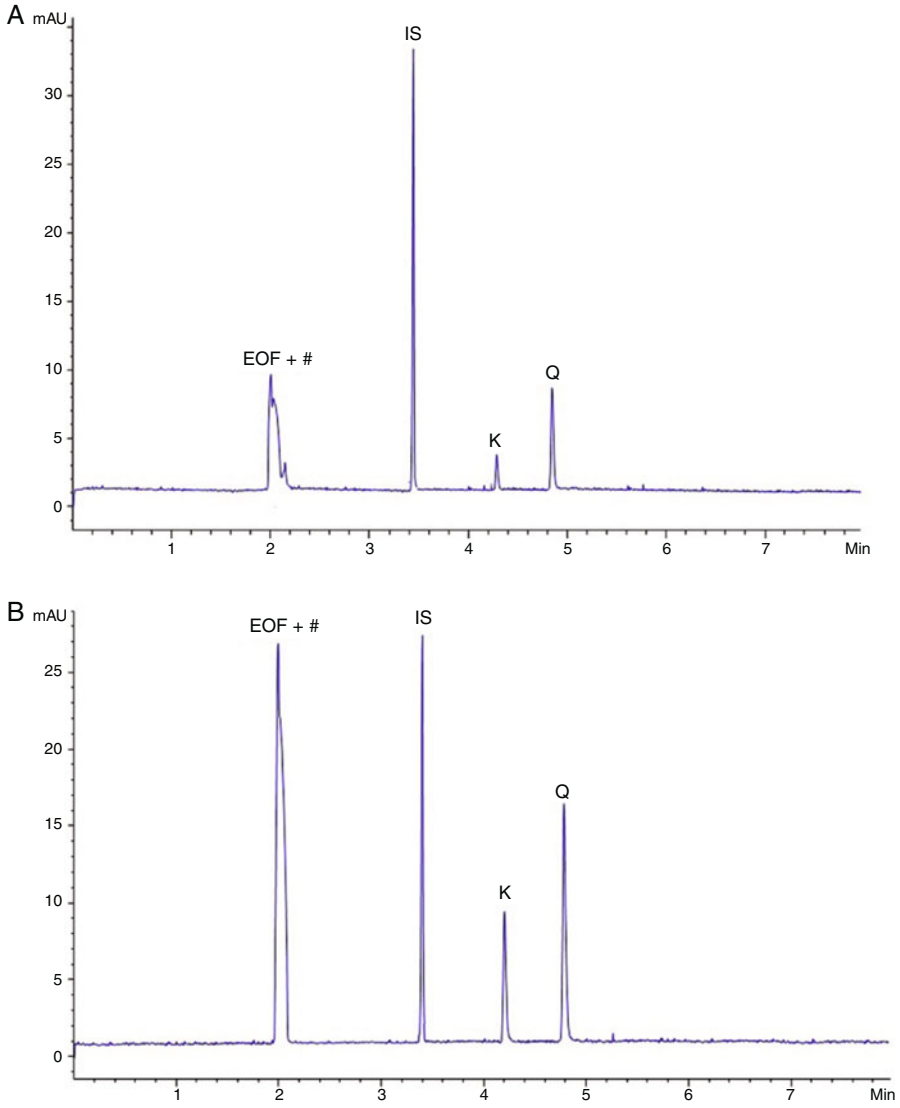

Fig. 2. Electhropherograms of *Rubus erythrocladus* (A) and *Morus nigra* (B) hydrolyzed leaves extracts by CE-DAD, 200 nm. EOF + # = electroosmotic flow + unidentified peak, IS = internal standard, K = kaempferol and Q = quercetin.

Table 1
Linear equation, linearity, limit of detection (LOD) and quantification (LOQ), intra-day and inter-day precision (values expressed in RSD%) and recovery of quercetin and kaempferol by HPLC-UV and CE-DAD.

	Flavonoid	Linear equation	R^2	LOD ($\mu g\,ml^{-1}$)	LOQ ($\mu g\,ml^{-1}$)	Precision intraday	Precision interday	Mean recovery (%) ± RSD%
HPLC	Quercetin	$y = 83082x + 42{,}829$	0.9996	0.9519	2.8844	0.30	1.69	95.35 ± 2.88
	Kaempferol	$y = 69225x + 56{,}605$	0.9995	1.0519	3.1876	0.23	2.02	95.76 ± 2.77
CE	Quercetin	$y = 0.0176x + 0.0276$	0.9979	5.3043	16.0737	2.48	7.76	104.56 ± 1.77
	Kaempferol	$y = 0.0203x + 0.005$	0.9987	4.1887	12.6931	3.68	8.44	104.31 ± 1.66

Table 2
Different parameters and results of robustness of quercetin and kaempferol in HPLC-UV and CE-DAD methods. Values expressed in mean (%) ± RSD%.

HPLC parameter	Quercetin	Kaempferol	CE Parameter	Quercetin	Kaempferol
Flow 0.55 ml min^{-1}	109.40 ± 0.31	109.40 ± 0.45	25.5 °C	97.63 ± 0.26	106.27 ± 0.07
Flow 0.65 ml min^{-1}	92.35 ± 0.26	92.38 ± 0.02	24.5 °C	98.39 ± 0.63	106.37 ± 0.71
0.005% TFA	95.89 ± 7.70	93.50 ± 11.58	2 s	95.76 ± 1.87	107.73 ± 0.14
0.015% TFA	101.57 ± 0.13	100.14 ± 0.39	4 s	97.94 ± 0.25	103.19 ± 4.12
0.04%TFA	102.96 ± 0.61	100.89 ± 0.69	5.0%	99.30 ± 3.71	98.73 ± 2.41
0.12% TFA	104.90 ± 0.16	104.28 ± 0.05	15.0%	98.77 ± 1.43	104.76 ± 0.16
DAD	94.27 ± 0.54	94.29 ± 0.71	–	–	–

Table 3
Quantification of quercetin and kaempferol in hydrolyzed leaves extracts of *Rubus erythrocladus* and *M. nigra* by HPLC-UV and CE-DAD. Values expressed as $\mu g\, g^{-1}$ of dried plant (D.P.).

	Flavonoid	*R. erythrocladus* Mean ($\mu g\, g^{-1}$) ± RSD%	*M. nigra* Mean ($\mu g\, g^{-1}$) ± RSD%
HPLC	Quercetin	848.43 ± 10.48	2323.90 ± 7.28
	Kaempferol	304.35 ± 6.83	1446.36 ± 6.72
CE	Quercetin	836.37 ± 9.36	2552.82 ± 7.96
	Kaempferol	N.Q.	1188.67 ± 9.20

N.Q. = not quantified.

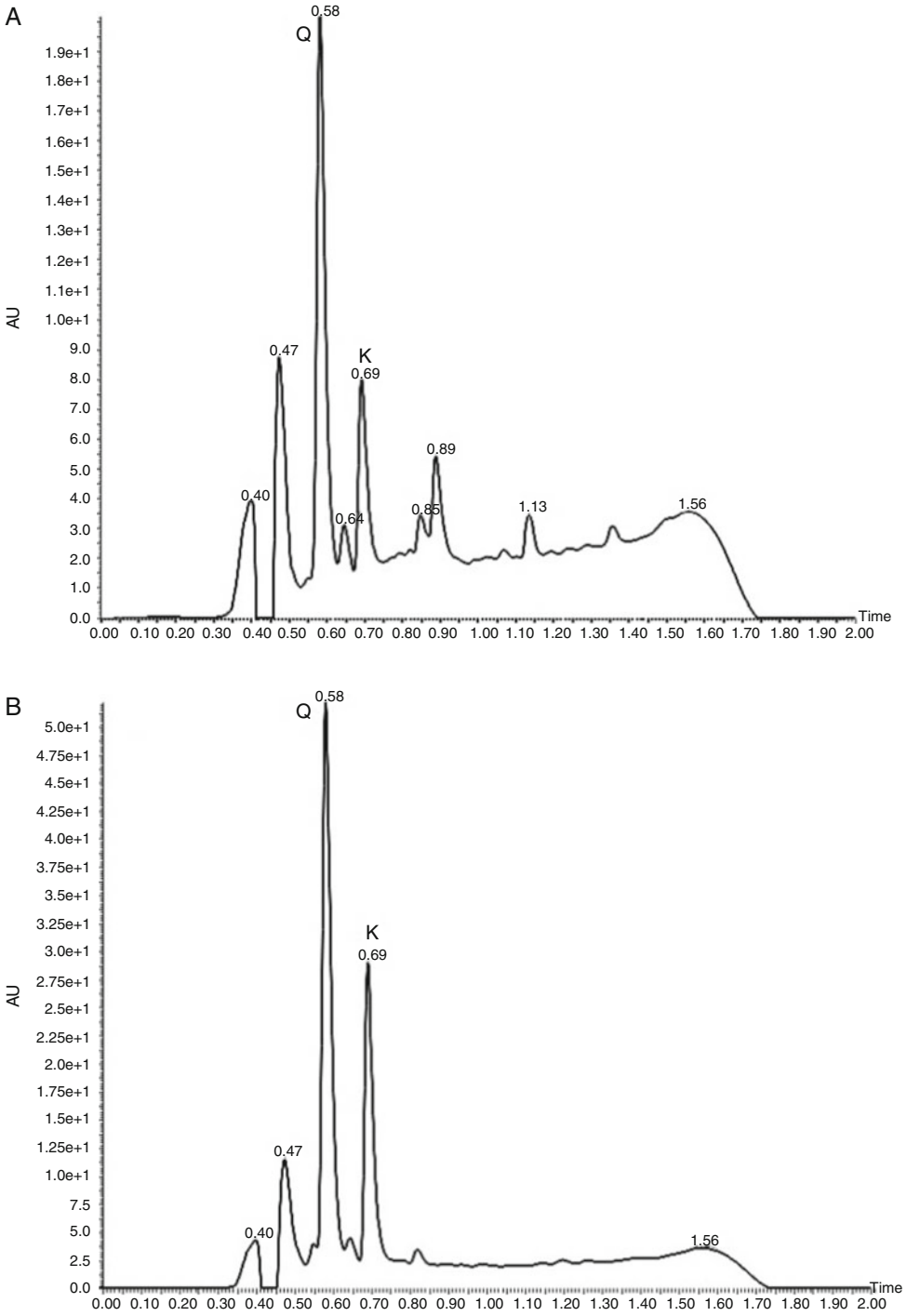

Fig. 3. Chromatograms of *Rubus erythrocladus* (A) and *Morus nigra* (B) hydrolyzed leaf extracts by UPLC-DAD/MS, 200–400 nm. Q = quercetin and K = kaempferol.

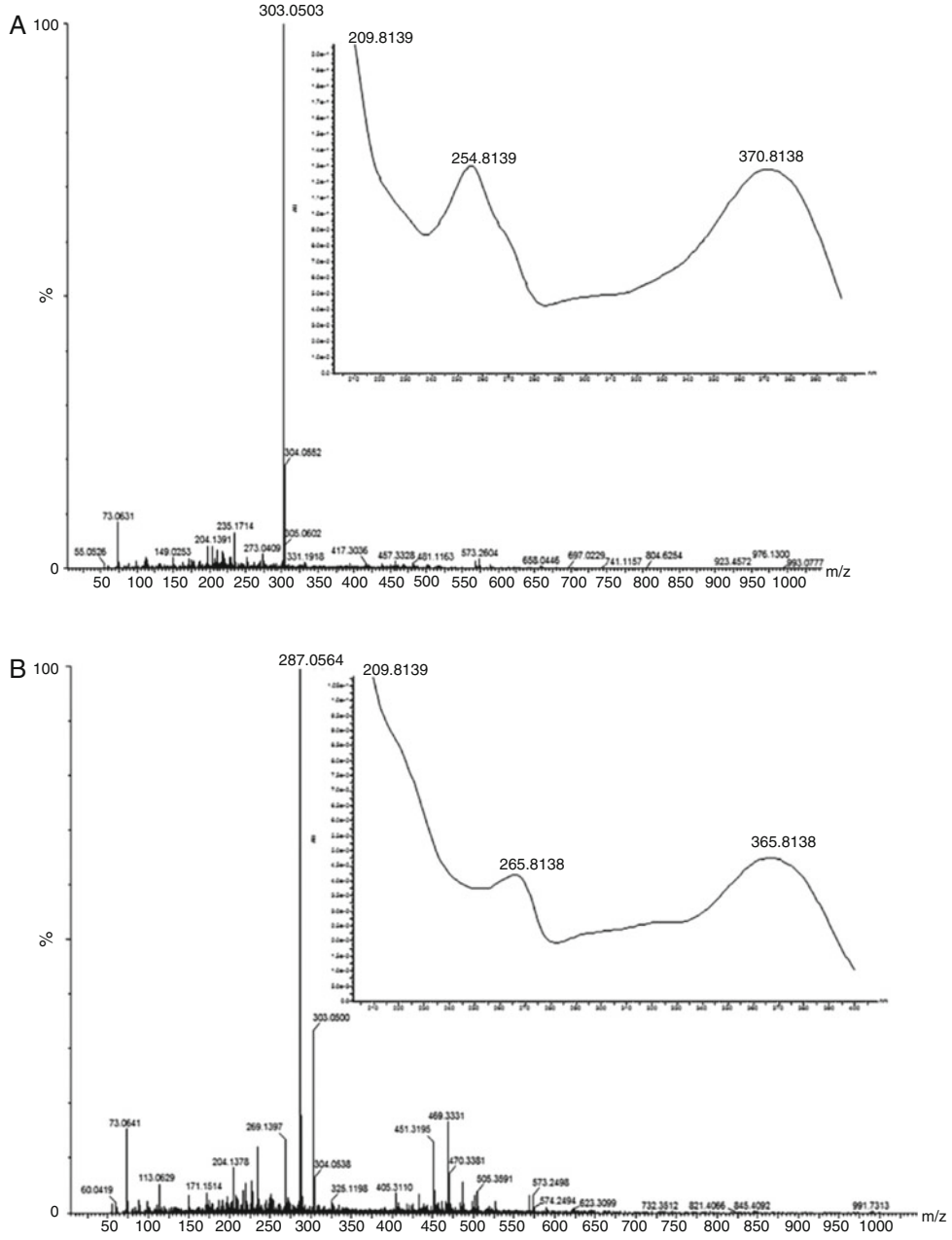

Fig. 4. Mass and UV spectra of quercetin (A) and kaempferol (B) of *Rubus erythrocladus* hydrolyzed leaf extracts by UPLC-DAD/MS.

chromatography (Fig. 1) and electrophoresis (Fig. 2). The hydrolyzation process gives us clearer chromatograms than crude extract and the identification of quercetin and kaempferol in hydrolyzed leaves extracts suggests the presence of quercetin and kaempferol derivative compounds in these leaves too.

HPLC methods are developed based on the polarity of the molecules. This differs from the CE methodology, in which both flavonoids were ionized in the electrolyte used in this work, so that the separation of quercetin and kaempferol was determined by their molecular weight. First we observed the electromigration of kaempferol (MW = 286.24) and then we observed the electromigration of quercetin (MW = 302.2).

The pH of the buffer will influence the degree of ionization of the solutes, and hence, their electrophoretic mobilities. Given that the pK_a of quercetin is 7.76 and the pK_a of kaempferol is 7.89 (Tungjai et al., 2008), sodium tetraborate (pH 9.2) was chosen as the electrolyte system to initiate the optimization procedure. At this pH, the electroosmotic flow is usually greater than the electrophoretic velocities of the anions, so quercetin and kaempferol (negatively charged), which are attracted to the positive electrode, are carried toward the cathode. In this case, anions elute in inverse sequence to their charge-to-size ratios. Initial results have shown that insufficient resolution occurred with electrolyte system containing only tetraborate, so the use of an additional modifier was required. A dramatic improvement can be obtained in selectivity, resolution, and separation efficiency when organic solvents, such as methanol, ethanol and acetonitrile or mixtures thereof, are added to the electrolyte (Baker, 1995). The acetonitrile concentration was studied over the range of 0–15% for a fixed amount of sodium tetraborate, and 10% (v/v) was selected because it represented a compromise between resolution and analysis time. The separation of quercetin, kaempferol and I.S. methylparaben under investigation at the optimized conditions is shown in Fig. 2.

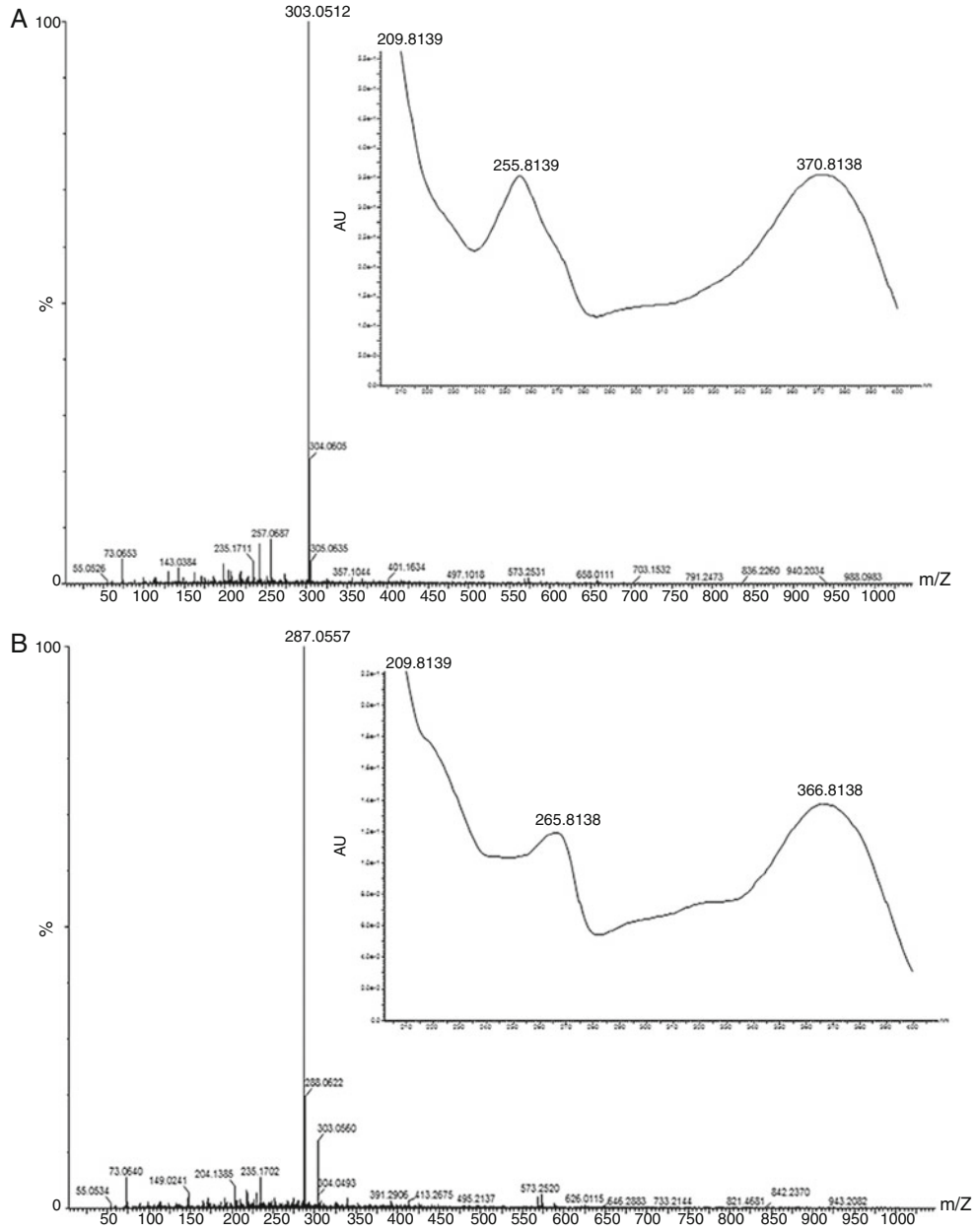

Fig. 5. Mass and UV spectra of quercetin (A) and kaempferol (B) of *Morus nigra* hydrolyzed leaf extracts by UPLC-DAD/MS.

Methods validation

All the parameters tested in the validation methods for the quantification of quercetin and kaempferol in hydrolyzed leaf extracts of *R. erythrocladus* and *M. nigra* showed satisfactory results, with RSD of less than 15% (Tables 1 and 2). The purity of the chromatographic and electrophoretic peaks obtained for quercetin and kaempferol was evaluated using a DAD detector. The results showed that other compounds did not co-elute/migrate with the standards.

Sample analysis

It was possible to apply the developed methods in *Rubus* and *Morus* samples (Table 3). By CE, kaempferol could not be quantified in *R. erythrocladus*. This result is due to the lower sensitivity of this technique, as shown in Table 1. The CE sensitivity is related to the capillary detection window, capillary diameter and light UV dispersion (Li, 1996). Less diluted samples could have provided better results. Applying the analytical tool *t*-student it was observed that no significant differences were observed in the quantities of these flavonoids between the two forms of analyses – HPLC and CE ($p > 0.05$) – Table 3.

In *R. erythrocladus*, adding the quantities of quercetin and kaempferol, we found 0.12% and 0.08% of these substances by HPLC and CE, respectively, less than has been reported for other *R.* species: 1.06% for *Rubus nessensis* and 0.27% for *Rubus fruticosus* by HPLC (Gudej and Tomczyk, 2004). Analyzing the amounts of quercetin and kaempferol quantified in *M. nigra* extract by CE (2552.82 $\mu g\,g^{-1}$ and 1188.67 $\mu g\,g^{-1}$ of dried plant, respectively), intermediate values were observed, compared to the values for leaf extracts of *M. alba* identified in the literature. Authors of other works have reported only 4.0 $\mu g\,g^{-1}$ of dried plant of quercetin in this species (Chu et al., 2006). In contrast, some studies have quantified 4520.00 $\mu g\,g^{-1}$ of dried plant of quercetin in leaves of *M. alba* (Suntornsuk et al., 2003).

Both analytical techniques proved to be fast and reliable. The HPLC method could be applied as a form of quality control of quercetin and kaempferol in hydrolyzed leaf extracts of *R. erythrocladus* and *M. nigra*. Even with the lower sensitivity of the CE method, it is possible to observe higher efficiency of separation peaks (Fig. 2). The high versatility, the low volume of reagents and sample, and the good results obtained for quercetin and kaempferol quantification by CE suggest that this analytical tool could be applied as a complementary technic for the quality control of these compounds in natural products.

Botanical and chemical analyses of plants are very important for the identification and quality control of medicinal plants (Brazilian Pharmacopoeia, 2010). By UPLC-DAD/MS, a difference was identified between the chemical qualitative profiles of the *Rubus* and *Morus* species (Fig. 3).

All peaks were fragmented, but only quercetin and kaempferol (at 0.58 and 0.69 s, respectively) were identified in hydrolyzed leaf extracts of *R. erythrocladus* and *M. nigra* by UPLC-DAD/MS (Figs. 4 and 5, respectively).

The presence of kaempferol and quercetin in *Rubus* and *Morus* chromatograms, and small unidentified peaks in the chromatogram for *R. erythrocladus* (Fig. 3) by UPLC-DAD/MS suggest that the *Rubus* leaf extract may be more complex than the *Morus* leaf extract.

Conclusions

It was possible to determine the flavonoids present in hydrolyzed leaves extracts of *R. erythrocladus* and *M. nigra* and it was showed two fast and reliable methods to quantify quercetin and kaempferol in these extracts, HPLC-UV and CE-DAD. These two methods were validated and could be applied to the quality control of these extracts. Moreover, we report chemical differences between the *Morus* and *Rubus* leaves studied by UPLC-DAD/MS and we also contribute with new information about the *Morus* genus for the Brazilian government.

Authors' contributions

LRT contributed in sample preparation, chromatography and electrophoresis analysis, analysis of data and draft of the paper. GPRP contributed in sample preparation and chromatography analysis. ACOC and MS contributed in electrophoresis analysis and draft of the paper. SALB contributed to plants collections. AF and JASZ designed the study, lead and advised the laboratory work and performed the analysis of the data and drafted the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Baker, D.R., 1995. Capillary Electrophoresis. Wiley, New York.
Brazilian Pharmacopoeia, 2010 vol. I., 5th ed. Anvisa, Brazil, 546 p.
Carrasco-Pancorbo, A., Gómez-Caravaca, A.M., Cerretani, L., Bendini, A., Segura-Carretero, A., Fernández-Gutiérrez, A., 2006. A simple and rapid electrophoretic method to characterize simple phenols, lignans, complex phenols, phenolic acids, and flavonoids in extra-virgin olive oil. J. Sep. Sci. 29, 2221–2233.
Choi, J., Kang, H.J., Kim, S.Z., Kwon, T.O., Jeong, S.-I., Jamg, S.-I., 2013. Antioxidant effect of astragalin isolated from the leaves of *M. alba* L. against free radical-induced oxidative hemolysis of human red blood cells. Arch. Pharmacal Res. 36, 912–917.
Chu, Q., Lin, M., Tian, X., Ye, J., 2006. Study on capillary electrophoresis-amperometric detection profiles of different parts of *M. alba* L. J. Chromatogr. A 1116, 286–290.
Chung, H.I., Kim, J., Kim, J.Y., Kwon, O., 2013. Acute intake of mulberry leaf aqueous extract affects postprandial glucose response after maltose loading: randomized double-blind placebo controlled pilot study. J. Funct. Foods 5, 1502–1506.
Dat, N.T., Binh, P.T.X., Quynh, L.T.P., Ninh, C.V., Houng, H.T., Lee, J.J., 2010. Cytotoxic prenylated flavonoids from *M. alba*. Fitoterapia 81, 1224–1227.
Deighton, N., Brennan, R., Finn, C., Davies, H.V., 2000. Antioxidant properties of domesticated and wild *Rubus* species. J. Sci. Food Agric. 80, 1307–1313.
Durgo, K., Belscak-Cvitanovic, A., Stancic, A., Franekic, J., Komes, D., 2012. The bioactive potential of red raspberry (*R. idaeus* L.) leaves in exhibiting cytotoxic and cytoprotective activity on human laryngeal carcinoma and colon adenocarcinoma. J. Med. Food 15, 258–268.
Gudej, Y., 2003. Kaempferol and quercetin glycosides from *R. idaeus* L. leaves. Acta Pol. Pharm. 60, 313–316.
Gudej, J., Tomczyk, M., 2004. Determination of flavonoids, tannins and ellagic acid in leaves from *Rubus* L. Species. Arch. Pharmacal Res. 27, 1114–1119.
Gundogdu, M., Muradoglu, F., Gazioglu Sensoy, R.I., Yilmaz, H., 2011. Determination of fruit chemical properties of *M. nigra* L., *M. alba* L. and *M. rubra* L. by HPLC. Sci. Hortic. 132, 37–41.
Han, N., Gu, Y., Ye, C., Cao, Y., Lie, Z., Yin, J., 2012. Antithrombotic activity of fractions and components obtained from raspberry leaves (*R. chingii*). Food Chem. 132, 181–185.
ICH, 2005. Harmonized tripartite guideline: validation of analytical procedures: text and methodology Q2 (R1).
Jouad, H., Maghrani, M., Eddouks, M., 2002. Hypoglycaemic effect of *R. fructicosis* L. and *Globularia alypum* L. in normal and streptozotocin-induced diabetic rats. J. Ethnopharmacol. 8, 351–356.
Katsube, T., Imawaka, N., Kawano, Y., Yamazaki, Y., Shiwaku, K., Yamane, Y., 2010. Antioxidant flavonol glycosides in mulberry (*M. alba* L.) leaves isolated based on LDL antioxidant activity. Food Chem. 97, 25–31.
Li, S.F.Y., 1996. Capillary Electrophoresis: Principles, Practice and Applications. Journal of Chromatography Library, Netherlands.
Liu, Z., Schwimer, J., Liu, D., Lewis, J., Greenway, F., York, D.A., Woltering, E.A., 2006. Gallic acid is partially responsible for the antiangiogenic activities of *Rubus* leaf extract. Phytother. Res. 20, 806–813.
Martini, S., D'addario, C., Colacevich, A., Focardi, S., Borghini, F., Santucci, A., Figura, N., Rossi, C., 2009. Antimicrobial activity against Helicobacter pylori strains and antioxidant properties of blackberry leaves (*R. ulmifolius*) and isolated compounds. Int. J. Antimicrob. Agents 34, 50–59.
Merken, H.M., Beecher, G.R., 2000. Measurement of food flavonoids by high-performance liquid chromatography: a review. J. Agric. Food Chem. 48, 577–585.
Nogueira, E., Rosa, G.J.M., Haraguchi, M., Vassilieff, V.S., 1998. Anxiolytic effect of *R. brasilensis* in rats and mice. J. Ethnopharmacol. 61, 111–117.
Nogueira, E., Vassilieff, V.S., 2000. Hypnotic, anticonvulsant and muscle relaxant effects of *R. brasiliensis*. Involvement of GABAA-system. J. Ethnopharmacol. 70, 275–280.
Oh, K.-S., Ryu, S.Y., Lee, S., Seo, H.W., Oh, B.K., Kim, Y.S., Lee, B.H., 2009. Melanin concentrating hormone-1 receptor antagonism and anti-obesity effects of ethanolic extract from *M. alba* leaves in diet-induced obese mice. J. Ethnopharmacol. 122, 216–220.
Omidiran, M.O., Baiyewu, R.A., Ademola, I.T., Faforede, O.C., 2012. Phytochemical analysis, nutritional composition and antimicrobial activities of white mulberry (*M. alba*). Pak. J. Nutr. 11, 456–460.
Ostrosky, E.A., Marcondes, E.M.C., Nishikawa, S., de, O., Lopes, P.S., Varca, G.H.C., Pinto, T., de, J.A., Consiglieri, T.O., Baby, A.R., Velasco, M.V.R., Kaneko, T.M., 2011. *R. rosaefolius* extract as a natural preservative candidate in topical formulations. AAPS PharmSciTech 12, 732–737.
Panizzi, L., Caponi, C., Catalano, S., Cioni, P.L., Morelli, I., 2002. *In vitro* antimicrobial activity of extracts and isolated constituents of *R. ulmifolius*. J. Ethnopharmacol. 79, 165–168.
Portal da Saúde. http://portalsaude.saude.gov.br/index.php/cidadao/principal/agencia-saude/noticias-anterioresagencia-saude/3487- (accessed October 2013).
Rojas-Vera, J., Patel, A.V., Dacke, C.G., 2002. Relaxant activity of raspberry (*R. idaeus*) leaf extract in guinea-pig ileum *in vitro*. Phytother. Res. 16, 665–668.
Skupien, K., Kostrzewa-Nowak, D., Oszmianski, J., Tarasiuk, J., 2008. *In Vitro* antileukaemic activity of extracts from chokeberry (*Aronia melanocarpa* [Michx] Elliott) and Mulberry (*M. alba* L.) leaves against sensitive and multidrug resistant HL60 cells. Phytother. Res. 22, 689–694.
Suntornsuk, L., Kasemssok, S., Wongyai, S., 2003. Quantitative analysis of aglycone quercetin in mulberry leaves (*M. alba* L.) by capillary zone electrophoresis. Electrophoresis 24, 1236–1241.
Thabti, I., Elfalleh, W., Hannachi, H., Ferchichi, A., Campos, M., da, G., 2012. Identification and quantification of phenolic acids and flavonol glycosides in tunisian *Morus* species by HPLC-DAD and HPLC–MS. J. Funct. Foods. 4, 367–374.
Thiem, B., Goslinska, O., 2004. Antimicrobial activity of *R. chamaemorus* leaves. Fitoterapia 75, 94–95.
Tsuduki, T., Kikuchi, I., Kimura, T., Nakagawa, K., Miyazawa, T., 2013. Intake of mulberry 1-deoxynojirimycin prevents diet-induced obesity through increases in adiponectin in mice. Food Chem. 139, 16–23.
Tungjai, M., Poompimon, W., Loetchutinat, C., Kothan, S., Dechsupa, N., Mankhetkorn, S., 2008. Spectrophotometric characterization of behavior and

the predominant species of flavonoids in physiological buffer: determination of solubility, lipophilicity and anticancer efficacy. Open Drug Deliv. J. 2, 10–19.
Tzouwara-Karayanni, S.M., Philianos, S.M., 1982. Isolation of quercetin and kaempferol from *R. ulmifolius* and their fluorometric assay. Microchem. J. 27, 144–161.
Venskutonis, P.R., Dvaranauskaite, A., Labokas, J., 2007. Radical scavenging activity and composition of raspberry (*R. idaeus*) leaves from different locations in Lithuania. Fitoterapia 78, 162–165.
Volpato, G.T., Calderon, I.M.P., Sinzato, S., Campos, K.E., Rudge, M.V.C., Damasceno, D.C., 2011. Effect of *M. nigra* aqueous extract treatment on the maternal–fetal outcome, oxidative stress status and lipid profile of streptozotocin-induced diabetic rats. J. Ethnopharmacol. 138, 691–696.
Xia, M., Qian, L., Zhou, X., Gao, Q., Bruce, I.C., Xia, Q., 2008. Endothelium-independent relaxation and contraction of rat aorta induced by ethyl acetate extract from leaves of *M. alba* (L.). J. Ethnopharmacol. 120, 442–446.