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Myricitrin as the main constituent of two species of *Myrcia*

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ABSTRACT

Myricitrin was isolated as the major constituent from *Myrcia splendens* (2 g %) and *Myrcia palustris* (0.5g %) of their fractions soluble in ethyl acetate. The presence of this compound was confirmed by chromatographic analyses using HPLC-DAD. The antioxidant activity was measured using reducing power in relation to the reduction of iron (III) to iron (II) ions and DPPH radical scavenging assay. The ethyl acetate and butanol fractions showed the highest antioxidant activity for both species. In relation to the ability of the sample to scavenge DPPH radicals the EC_{50} values were 8.44 and 9.35 µg/ mL for *M. splendens* and 17.83 and 15.34 µg/ mL for M. palustris for the ethyl acetate and butanol fractions, respectively. The antioxidant activity may be related to the myricitrin in these extracts that showed an EC_{50} = 13.93 µg/mL.

INTRODUCTION

The family Myrtaceae is one of the most characteristic in Brazil, presenting significant potential economic benefits. The species of this large family are used as medicinal plants, ornamental plants, fruit trees and to obtain wood and paper. The family belongs to the order Myrtiflorae (Myrtales) and comprises around 150 genera with an estimated 5500 species predominantly distributed in tropical America, South Australia and Southeast Asia (Cronquist, 1981; Barroso, 1991; Wilson, 2011). *Myrcia* is one of the largest genera of American Myrtaceae, with more than 300 species distributed from Mexico to southern Brazil. Seventeen of these species are found in southern Brazil (Marchiori, 1997). *Myrcia* spp. are popularly known as "insulin plants", notably *M. multiflora* (pedra-ume-caá), which is used as a hypoglycemic agent in the form of an infusion or decoction (Brito & Lanetti, 1996; Batista *et al*., 2011). *M. splendens* (Sw.) DC., known in Brazil as "guamirim" or "folha-miúda", has been widely observed from Mexico to southern Brazil. There are several botanical names for this species, including *M. acutata* DC., *M. splendens* DC., *M. communis* Berg & *M..fallax* (Rich.) DC. (Oliveira-Filho, 2006; Morais & Lombardi, 2006). *Myrcia palustris* DC., known in Brazil as "pitangueira-do-mato", "murta-do-brejo" or "baga-de-sabiá", is a native species found mainly in the south and southeast of Brazil. This species is commonly reported in the literature with the names *M. banisteriifolia* (DC.), *M. garopabensis* Cambess., *Gomidesia palustris* (DC.) Kausel, *G. banisteriifolia* (DC.) O.Berg, *G. gardneriana* O.Berg, *G. garopabensis* (Cambess.) D. Legrand (Sobral *et al*., 2010). The plants of the *Myrcia* genus contain triterpenes and steroids (Gottlieb *et al*., 1972) as well as flavanones, acetophenones and flavonoid glycosides (Yoshikawa *et al*., 1998; Ferreira *et al*., 2006) and these compounds show different activities, notably inhibition of aldose reductase and alpha-glucosidase (Matsuda *et al*., 2002a,b). Studies on essential oils conducted on these species have demonstrated a high content of cyclic sesquiterpenes and varying amounts of monoterpene and aliphatic compounds (Zoghbi *et al*., 2003; Cole *et al*., 2008; de Cerqueira *et al*., 2009; Nakamura *et al*., 2010). The aim of this study was to test the crude hydroalcoholic extract and n-hexane, ethyl acetate, n-butanol and insoluble fractions of *M. splendens* and *M. palustris* in order to investigate their antioxidant activity using DPPH free radical scavenging and reducing potential assays. Also, the major compounds in the most active fractions were identified by HPLC-DAD.

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MATERIALS AND METHODS

Chemicals and reagents

All reagents and solvents were analytical or HPLC grade (J.T. Baker, USA). The water used in all experiments was purified in-house using a Milli-Q system (Millipore, Florianópolis, SC, Brazil) with a conductivity of 0.60 μS/cm. Rutin (Sigma, USA) of the highest grade (purity > 98.0%) and myricitrin (as isolated compound) were used as the external standards.

Plant Material

Plant samples of *Myrcia splendens* and *Myrcia palustris* (Myrtaceae), including stems and leaves, were collected in April 2011 in Daniela beach, Florianópolis, Santa Catarina State, Brazil, at geographic coordinates 27.4506 °S, 48.5216 °W, and identified by the botanist Daniel de Barcellos Falkenberg. The voucher specimens - FLOR 16.182 (*M. splendens* (Sw.) DC.) and FLOR 17.892 (*M. palustris* DC.) - were deposited at the Botanical Herbarium of the Federal University of Santa Catarina State (UFSC), Florianópolis, Santa Catarina State, Brazil.

Preparation of Vegetal Material

The plant samples were dried and macerated with 96% ethanol for seven days. After filtration, the extracts were concentrated to dryness in order to obtain the crude extracts. The crude extracts were dissolved in 30% EtOH and left in a refrigerator for one day and an insoluble fraction was precipitated. The remaining filtrates were sequentially extracted with solvents of increasing polarity, yielding n-hexane, ethyl acetate, butanol and aqueous fractions. The crude extracts and fractions were subjected to antioxidant assays.

Determination of total phenolics

The total phenolic content of the samples was determined using the Folin-Ciocalteu reagent. In this method, the test solution was composed of 0.5 mL of sample (crude extracts and fractions, diluted in methanol at a concentration of 1.0 $mg.mL^{-1}$), 5.0 mL of distilled water and 0.5 mL of the Folin-Ciocalteu reagent. After a period of 3 min, 1.0 mL of saturated sodium carbonate solution was added. These mixtures were shaken and allowed to stand for 1 h. The absorbance was measured at 725 nm against a blank (solution without sample). The mean of three readings was used and expressed as gallic acid (GA) equivalents (mg gallic acid/g dry extract).

Determination of flavonoid content

The flavonoid content was determined spectrophotometrically according to Lamaison and Carnat (Quettier-Deleu *et al*., 2000) with modifications. Briefly, 0.5 mL of 2% aluminum chloride ($AlCl_3$) in ethanol was mixed with the same volume of sample (1.0 $mg.mL^{-1}$). Absorption readings at 415 nm were taken after 1 h against a blank (ethanol). The mean of three readings was used and expressed as quercetin equivalent (QE); (mg quercetin/g of dry extract).

Determination of reducing power

In this assay, 100 μL of sample (1.0 $mg.mL^{-1}$) was added to 8.5 mL of deionized water followed by 1.0 mL of 0.1 M $FeCl_3$ in 0.1 M HCl and after 3 min 1.0 mL of 0.008 M potassium ferricyanide was added. After 15 min the absorbance was measured at 720 nm. A solution prepared as described above but without the addition of $FeCl_3$ solution was used as the blank. The analysis was performed in triplicate and the results were expressed as ascorbic acid (AA) equivalents (mg ascorbic acid/g dry extract).

DPPH radical scavenging assay

The free radical scavenging activity of the samples was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. DPPH is a stable free radical that accepts an electron or hydrogen atom to form a stable molecule. A change of color from purple to yellow indicates a decline in the absorbance of DPPH radicals at 517 nm caused by the reaction between the antioxidants present in sample and the free radical. For this assay 1 mL of sample (5-200 $\mu g/mL^{-1}$) was added to 2 mL of a solution of DPPH in methanol (0.004%). The mixture was shaken vigorously and allowed to stand for 30 min at room temperature. In order to subtract the absorbance promoted by the staining of the sample, a solution of ethanol (2.0 mL) and the sample (1.0 mL) was used as the blank (Abs_{blank}). A solution of DPPH (2.0 mL) and ethanol (1.0 mL) was used as the control ($Abs_{control}$) which is regarded as 100% of DPPH. The absorbance of the resulting solution (Abs_{sample}) was measured at 517 nm and converted into percentage of antioxidant activity (AA) using the following formula: $AA\% = 100-\{[(Abs_{sample}- Abs_{blank})\times100]/Abs_{control}\}$. Ascorbic acid, quercetin and myricitrin were used as standards. The radical scavenging activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}). The EC_{50} value for each sample was determined graphically by plotting the percentage disappearance of DPPH as a function of the sample concentration.

Statistical analysis

The results were subjected to analysis of variance (ANOVA) and the Fisher's test for multiple comparisons, considering statistical significance as $p<0.05$.

HPLC analysis

In the HPLC analysis, a Shimadzu HPLC system consisting of a SCL-10AV system controller, LC-10AT pump, CTO-10AS column oven and SPD-10AV DAD was used. The column was a Varian C18 column (250 mm × 4.6 mm; 5μm) and the analysis was carried out at 25 °C. The mobile phase was methanol (A) and water (B); both acidified with 1.0 % formic acid (FA). The optimized gradient profile began at time t = 0 min with the solvents A (35%) and B (65%), changing after 10 min to eluent B (55%), 25 min to B (45%), 30 min to B (40%) and 32 min to B (0%). The flow-rate was 1.0 mL/min, injection volume was 10 μL and the UV-Vis diode array detector (DAD) reading was acquired at 354 nm. Data acquisition was performed using the Shimadzu

Class VP Software. The samples (ethyl acetate and butanol fractions) were injected without pre-treatment at a concentration of 200 µg/mL^{-1} in MeOH/H_2O (2:8).

Isolation of compound (1)

The ethyl acetate fraction of *M. splendens* (10.0 g) chromatographed on silica gel using EtOAc/EtOH mixtures with increasing polarity yielded 30 fractions (100 mL), which were combined in sub-fractions according to TLC analysis. The sub-fraction 7-8 (EtOAc/EtOH 95% ; mp 190.7 - 191.6 ºC) analyzed by TLC (Rf 0.69, $CHCl_3$/MeOH 70%), yielded a yellow amorphous powder, and after successive recristallizations under methanol yield 201.6 mg of myricitrin (**1**).

The same compound was obtained from the ethyl acetate fraction of *M. palustris* after the process of liquid-liquid partitioning in the form of a precipitate. Was obtained 166.0 mg of the compound from 30.3 g of the ethyl acetate fraction which corresponds to approximately 0.5%.

RESULTS AND DISCUSSION

The crude hydroalcoholic extract and the fractions of the liquid-liquid partition (insoluble fraction, n-hexane, ethyl acetate, butanol and aqueous fractions) were subjected to four different assays: determination of the content of total phenolics and flavonoids, reducing power and DPPH radical scavenging. According to Table 1, the highest concentrations of flavonoids and phenolic compounds were observed in the ethyl acetate and butanol fractions of *M. splendens* and *M. palustris*. Likewise, in relation to the antioxidant assays, for the reducing power and DPPH radical scavenging the best results were also obtained for the ethyl acetate and butanol fractions. The EC_{50} values for the ethyl acetate and butanol fractions of *M. splendens* were very similar to those of the positive controls. These results are consistent with those obtained in studies on the chemotaxonomy of the *Myrcia* genera, highlighting a large number of flavonoid glycosides (Gottlieb *et al*., 1972, Matsuda *et al*., 2002a, Ferreira *et al*., 2006; Imatomi *et al*., 2013) and tannins (Yoshikawa *et al*., 1998) mainly in the more polar fractions obtained from liquid-liquid partition. The results for the determination of phenolic and flavonoid contents and the antioxidant assays indicated that the flavonoids in the more polar fractions of the two plant species, that is, the ethyl acetate and butanol fractions, merit further investigation. Therefore, a methodology for the analysis of flavonoids by HPLC was developed for the ethyl acetate and butanol fractions of *M. splendens* and *M. palustris*.

The chromatographic profile of the fractions can be seen in Fig.1. The ethyl acetate fraction of *M. splendens* (Fig. 1-a) showed five major peaks at 10.8 min (11.3 %), 11.8 min (6.4 %), 12.8 min (35.7 %), 14.3 min (6.9 %) and 14.4 min (7.2%), which together represent almost 80 % of the total area. The chromatogram showed a more intense peak at 12.8 min and its UV spectrum, obtained in the HPLC analysis, showed maximum absorption bands at 265 nm and 356 nm. This result suggests the presence of a flavonoid containing, as the aglycone core, a flavonol which has two characteristic absorption bands: band II (250-280 nm) and band I (330-360 nm for substituted flavonol 3-OH or 350-385 nm for unsubstituted flavonol 3-OH) (Markham, 1982). The butanol fraction provided a different profile (Fig. 1-b), with 10 peaks and two major retention times of 11.1 min (35.4 %) and 13.0 min (24.6 %), which together represent 60% of the total area. Furthermore, this fraction contained a low concentration (24.5%) of flavonoids in relation to the total phenolics. In contrast to the other fractions, the flavonoids in the ethyl acetate fraction of *M. splendens* represented 86.8 % of the total phenolics and in the ethyl acetate and butanol fractions of *M. palustris* they represent 97.1% and 74.0% of the total phenolics, respectively (Table 1). The chromatographic profiles for *Myrcia palustris* (Fig. 1-c and 1-d) were similar to that for the ethyl acetate fraction of *M. splendens*. The ethyl acetate and butanol fractions of *M. palustris* showed an intense peak with the same retention time of 12.8 min, corresponding to 77.8 % and 71.7 %, respectively.

The HPLC analysis was performed with two standards of flavonol heterosides, myricitrin and rutin, using the analysis conditions applied for the fractions, in order to determine the major compounds observed in the fractions of the two species.

Table. 1: Antioxidant activity of crude extract and fractions of *M. splendens* and *M. palustris*.

	Extract/ fraction	Phenolics mgGA/g	Flavonoids mgQE/g	Reducing potential mgAA/g	DPPH EC_{50} µg/mL
M. splendens	crude extract	77.61±2.32[a]	16.43±0.69	315.6±8.1	10.14
	insoluble	60.01±0.83	12.89±0.31	114.6±0.3	70.32
	hexane	51.16±0.46	11.55±0.98	69.9±0.1	117.47
	ethyl acetate	85.75±0.15	74.47±1.08	656.4±10.2[b]	8.44
	butanol	78.87±2.32[a]	19.33±0.24	652.2±17.8[b]	9.35
	aqueous	68.05±0.83	1.41±0.16	375.3±8.7	16.99
M. palustris	crude extract	72.22±1.95	59.17±0.53	187.2±8.8[c]	29.81
	insoluble	58.76±1.26	24.79±0.14	217.1±8.3	31.38
	hexane	4.51±0.41	9.18±0.24	6.8±0.7	>200
	ethyl acetate	110.83±0.76	107.64±0.05	363.3±3.9	17.83
	butanol	185.18±1.90	137.06±1.22	595.5±14.4	15.34
	aqueous	63.17±0.90	1.90±0.05	186.5±2.9[c]	34.04
Standards	ascorbic acid	-	-	-	9.27
	quercetin	-	-	-	6.33
	myricitrin	-	-	-	13.93

All means, except those marked by ([a],[b],[c]), showed significant differences in the Fisher test ($p<0.05$).

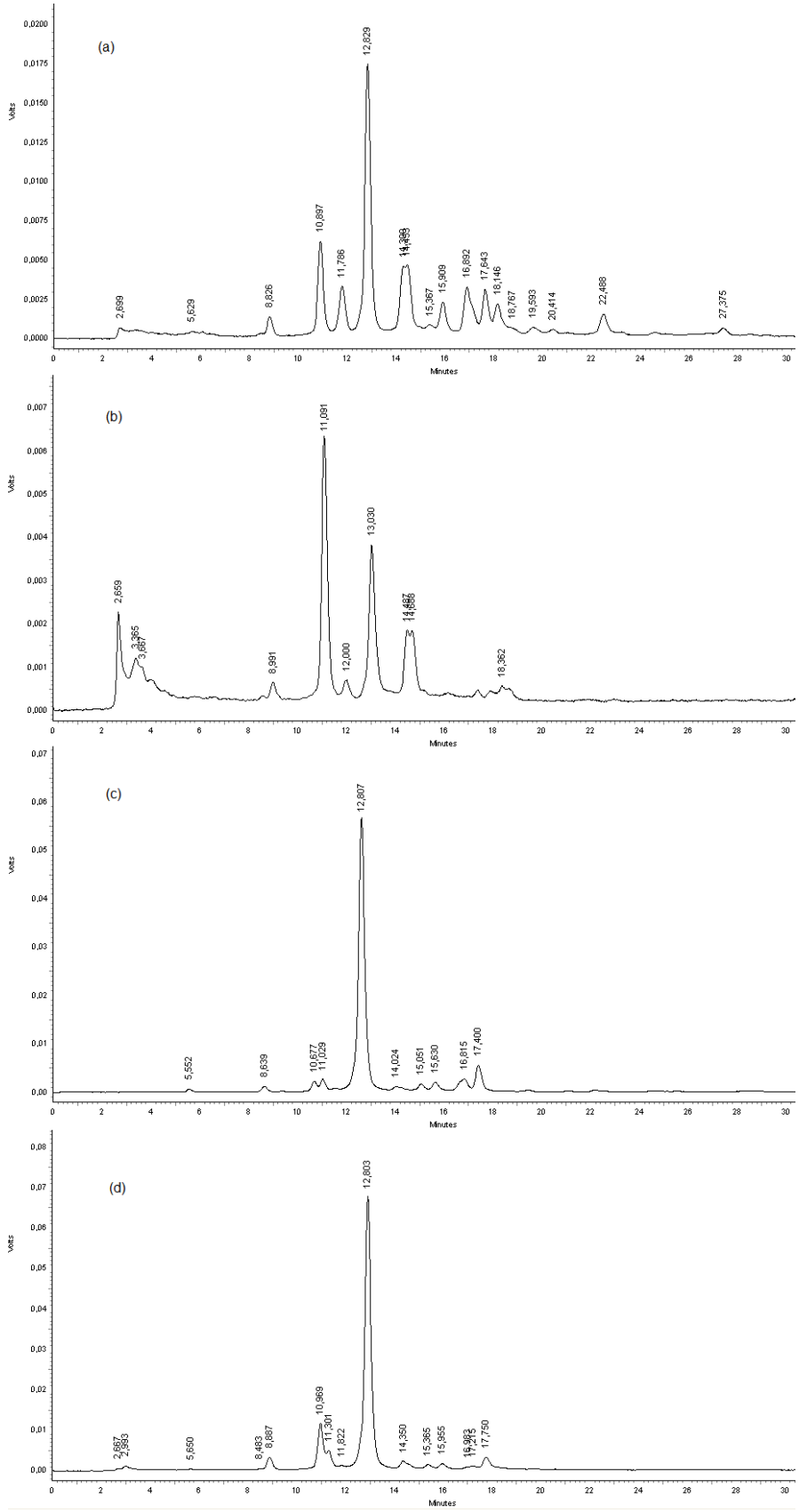

Fig. 1: HPLC-DAD chromatograms for fractions of *M. splendens* and *M. palustris* recorded at 354 nm: (a) ethyl acetate fraction of *M. splendens* , (b) butanol fraction of *M. splendens* , (c) ethyl acetate fraction of *M. palustris*, (d) butanol fraction of *M. palustris.*

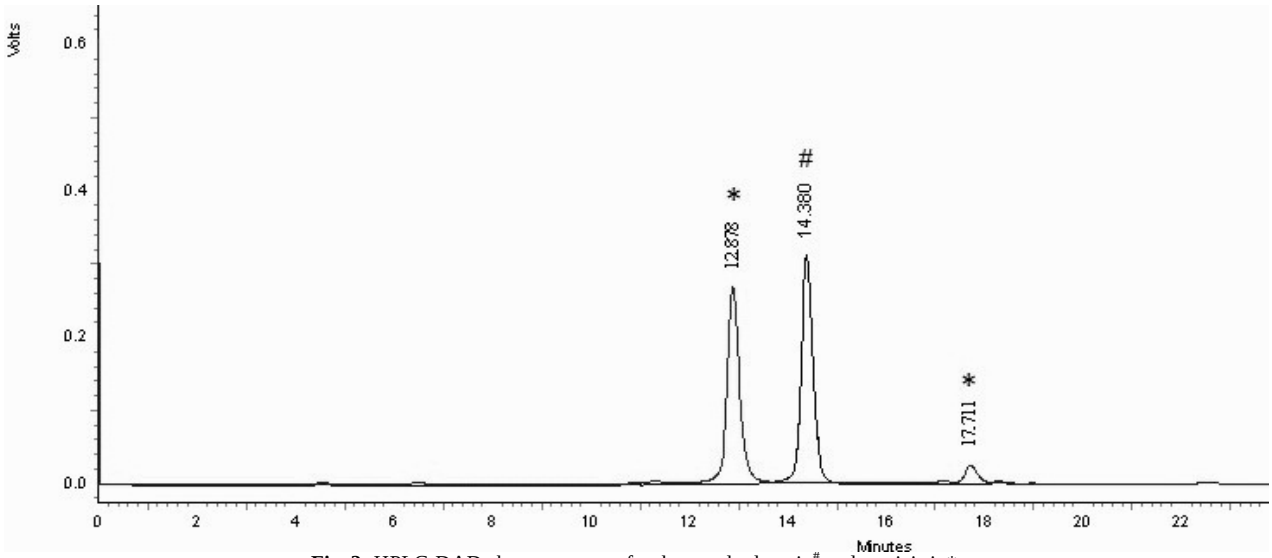

Fig. 2: HPLC-DAD chromatograms for the standards rutin[#] and myricitrin*

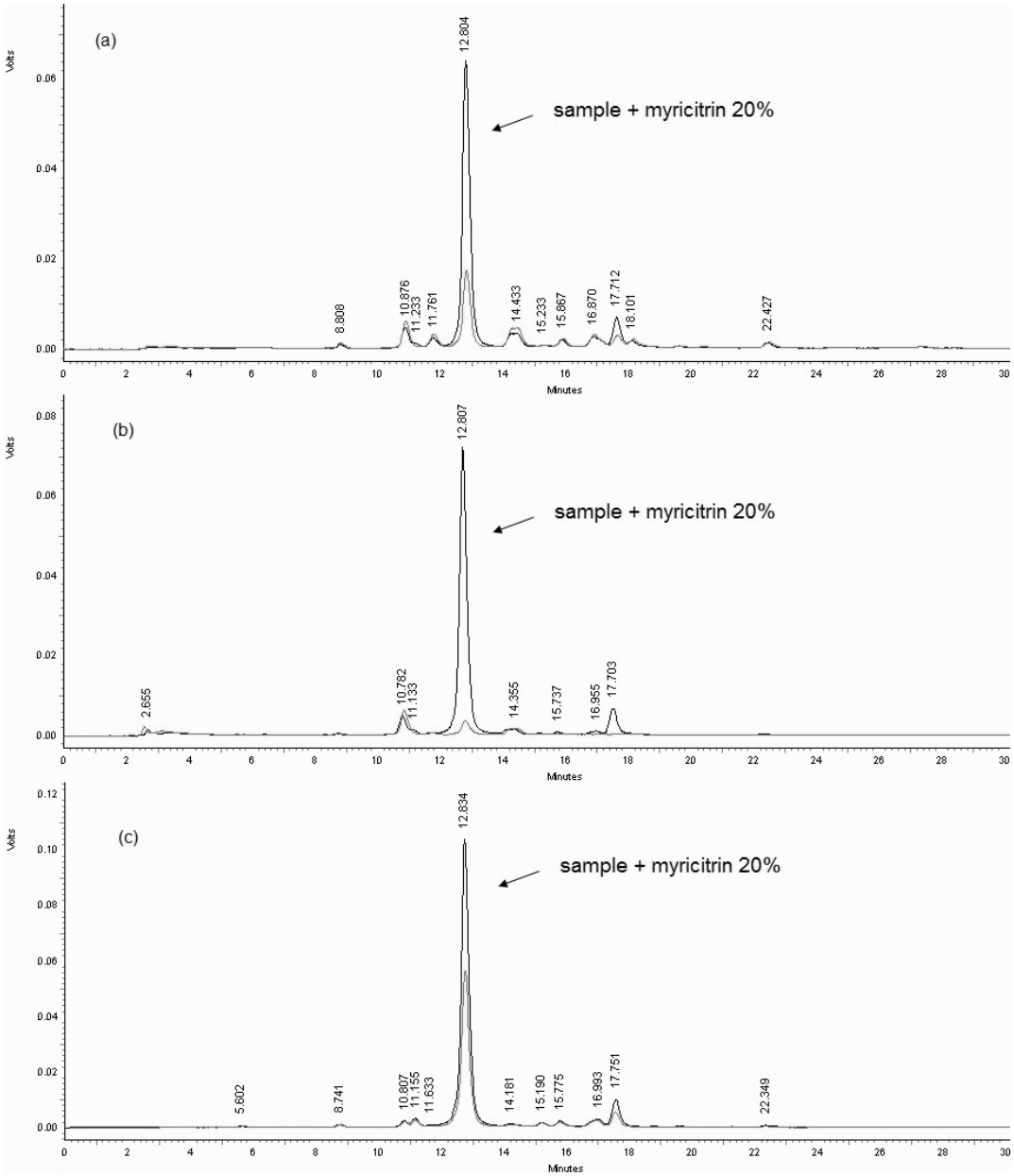

Fig. 3: continued….

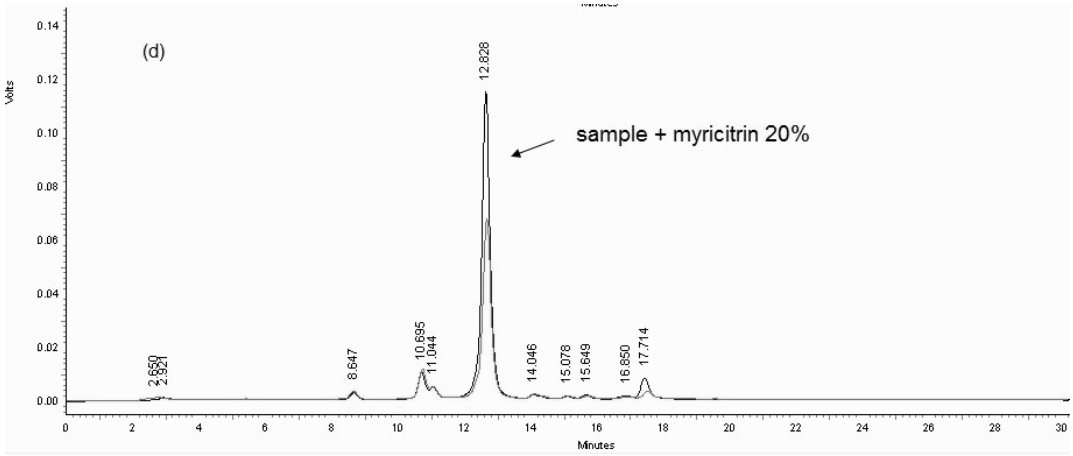

Fig. 3: HPLC-DAD chromatograms of fractions of *M. splendens* and *M. palustris* recorded at 354 nm using co-injection of myricitrin standard (20%): (a) ethyl acetate fraction of *M. splendens* , (b) butanol fraction of *M. splendens* , (c) ethyl acetate fraction of *M. palustris*, (d) butanol fraction of *M. palustris*.

The major compound in myricitrin showed the same retention time as that of the fractions (12.8 min), as seen in Fig. 2. The peak at 14.3 min relates to rutin and a small signal at 17.7 min is an impurity, since the myricitrin standard was an isolated compound and not a commercial standard.

The myricitrin standard was therefore used for the co-injection analysis in order to confirm the presence of this flavonoid in the samples. The chromatograms with and without co-injection were superimposed to check for similarity (Fig. 3). A substantial increase in the peak at 12.8 min for the flavonoid myricitrin was observed in the co-injection chromatograms.

According to the chromatograms, it can be inferred that the flavonoid myricitrin is present in all fractions with the exception of the butanol fraction of *Myrcia splendens* (Fig. 3). The chromatographic profiles were almost the same for the other three fractions with an increase in the area of the compound at 12.8 min. The profile of the co-injection chromatogram for the butanol fraction of *M. splendens* (Fig. 3-b) did not indicate the presence of the flavonoid myricitrin and in Fig. 1-b it can be seen that the most intense peak is at a retention time of 11.1 min and not at 12.8 min as in the case of other fractions.

The liquid-liquid partitioning of a crude hydroalcoholic extract of species leaves resulted in the isolation of myricitrin (**1**) from the ethyl acetate fraction of *M. splendens* and *M. palustris*. Physical data, spectroscopic data (^{1}H and ^{13}C NMR, IR), and comparison with the data from the literature were utilized to identify the compound **1** (Agrawal, 1989).

Myricitrin has been isolated from other species of the *Myrcia* genera and shown to exert multiple pharmacological activities. The aqueous partition of the methanolic extract of *M. uniflora* and its isolated myricitrin were able to inhibit thyroid peroxidase activity in vitro (Ferreira *et al*., 2006). The methanolic extract and ethyl acetate-soluble portion obtained from leaves of *Myrcia multiflora* demonstrated the inhibition of aldose reductase and α-glucosidase. One of the components isolated was myricitrin which also showed potent inhibitory activity (Matsuda *et al*., 2002b).

CONCLUSIONS

This study demonstrated the antioxidant activity of the more polar fractions of the two *Myrcia* species obtained in the liquid-liquid partitioning. The antioxidant activity may be related to the flavonoid composition of the extracts, most fractions being mainly composed of myricitrin.

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