Detection of flavonoids in *Alpinia purpurata* (Vieill.) K. Schum. leaves using high-performance liquid chromatography

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ABSTRACT: The species *Alpinia purpurata* is scarcely cited as to ethnopharmacology and phytochemistry. This study aimed to analyze bioactive compounds through high-performance liquid chromatography (HPLC). Hydroalcoholic crude extract was obtained from *A. purpurata* dried leaves. Folin-Ciocalteau method was used to quantify total phenols, using gallic acid as standard. The obtained result was 15.6 mg GAE g^{-1}. The crude extract was partitioned with the solvents ethyl acetate and butanol, followed by thin-layer chromatography (TLC) and HPLC. The flavonoids kaempferol-3-*O*-glucuronide and rutin were detected at a higher concentration in ethyl acetate and butanolic extracts. The butanolic extract contains the highest flavonoid percentage (94.3%). *A. purpurata* presents important flavonoids of therapeutic use, already verified for *A. zerumbet*. This is the first study verifying the presence of flavonoids in *A. purpurata* extracts.

Key words: TLC, polyphenols, medicinal plants, rutin, Zingiberaceae

RESUMO: Detecção de flavonóides em folhas de *Alpinia purpurata* (Vieill.) K. Schum. por cromatografia líquida de alta eficiência. A espécie *Alpinia purpurata* apresenta poucas citações referentes a etnofarmacologia e fitoquímica. Este estudo propõe a análise de substâncias bioativas através da técnica de cromatografia líquida de alta eficiência (CLAE). O extrato bruto hidroalcóolico foi obtido a partir de folhas secas de *A. purpurata*. A quantificação de fenóis totais foi realizada pelo método de Folin-Ciocalteau, usando ácido gálico como padrão. Como resultado, foi verificado 15,6 mg EAG g^{-1}. O extrato bruto foi particionado com os solventes acetato de etila e butanol e depois analisado por cromatografia em camada delgada e CLAE. Nos extratos acetato de etila e butanólico foi detectada a presença dos flavonóides kaempferol-3-*O*-glicuronídeo e rutina, em maior concentração. O extrato butanólico contém a maior porcentagem de flavonóides (94,3%). Esta espécie possui flavonóides importantes no uso terapêutico, já antes verificados para a espécie *A. zerumbet.* Este é o primeiro trabalho que verifica a presença de flavonóides em extratos de *A. purpurata*.

Palavras-chave: CCF, polifenóis, plantas medicinais, rutina, Zingiberaceae

INTRODUCTION

The genus *Alpinia* (Zingiberaceae family, Alpinioideae subfamily, Alpinieae tribe) is native to tropical and subtropical Asia (Kress et al., 2002). Nowadays, it is cultivated in several places around the world due to the attractive beauty of its inflorescences and its therapeutic potential (Soares de Moura et al., 2005; Victório, 2008). In addition, these plants are important sources of raw material for many useful products: foods, spices, medicines, perfumes, dyes and fiber paper (Tomlinson, 1969).

A. purpurata (Vieill.) K. Schum (red inflorescence) is an herbaceous perennial plant, internationally known in the ornamental plant market as potted plant, landscape accent and cut flower (Morón, 1987; Kress et al., 2002). An ethnobotanical study developed in a community from Trujillo State, Venezuela, investigated the use of its flowers as decoction for cough (Bermúdez & Velásquez, 2002).

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Zoghbi et al. (1999) analyzed *A. purpurata* essential oil composition, which showed notable antibacterial activity. However, studies about *A. purpurata* phytotherapeutic potential are scarce; most scientific works with this species are directed to the improvement of its production as an ornamental plant, including the evaluation of postharvest treatment as an alternative to chemical insecticides, indications of harvest and postharvest procedures, tolerance to fumigation, biological control of pests, *in vitro* storage of multiple shoots and micropropagation (Morón, 1987; Dekkers et al., 1991; Illg & Faria, 1995; Hara et al., 1997; Chen & Paull, 1998; Anderson & Gardner, 1999; Gonzalez & Mogollon, 2001; Sangwanangkul et al., 2008).

The presence of *A. purpurata* in several parts of Brazil allows easy access to it. Thus, this species is an available resource to phytotherapeutic treatment. Several species of the Zingiberaceae family present antioxidant property mainly due to the considerable presence of flavonoids such as rutin, quercetin, alpinetin and different types of kaempferol in the genus *Alpinia* (Table 1) (Williams & Harborne, 1977; Mpalantinos et al., 1998; Vankar et al., 2006). Pugialli et al. (1993) studied Zingiberaceae chemotaxonomy and considered that flavonoids and their structural variety are taxonomic markers. Zingiberaceae family is at a higher level within the superorder Zingiberiflorae due to the use of protection mechanisms of phenolic hydroxyl groups: glycosylation and methylation. Investigations of phytochemical compounds have been important tools to study plant classification and evolution (Kaplan & Gottlieb, 1982). This study proposes a phytochemical approach to *A. purpurata* based on the chemotaxonomy of the genus *Alpinia*, which presents high therapeutic potential (Bleier & Chirikdjian, 1972; Mendonça et al., 1998; Mpalantinos, 2001; Kim et al., 2006).

The aim of the present study was to evaluate the phytochemistry of hydroalcoholic extracts from *A. purpurata* leaves for the presence of the flavonoids rutin, kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucuronide, reported in the scientific literature for their therapeutic action.

MATERIAL AND METHOD

Plant material

A. purpurata leaf samples were collected from plants growing in the city of Rio de Janeiro in the Federal University of Rio de Janeiro (Rio de Janeiro State, Brazil). The voucher specimen was identified and deposited at the Herbarium of Rio de Janeiro Botanical Garden under the accession number RB 433484.

Preparation of extracts and Fractions

A. purpurata leaves were collected from adult plants in the morning; then, the plant material was dried and ground in 70% ethanol for a week. After the first extraction, leaves were kept in ethanol (100%) until exhaustive extraction. Crude extracts were filtered and dried through evaporation at 60ºC in a rotary evaporator and through freeze-drying. From 1009 g dried leaves, 112.5 g dried crude extract was obtained. The yield was calculated as percentage, according to the formula: (crude extract weight/plant material weight) x 100. A 59.4g crude extract fraction was resuspended in methanol:water (9:1, v/v) and partitioned in different solvents of increasing polarity range: hexane, dichloromethane, ethyl acetate and *n*-butanol. Hexane partition was separated and evaporated to dryness (4.1 g). The residue was dried

TABLE 1. Flavonoids reported for the genus *Alpinia*.

Flavonoids	Source	Reference
rutin	*A. zerumbet* (leaves)	Mpalantinos et al., 1998
quercetin-3-*O*-glucoside	*A. zerumbet* (leaves)	Mpalantinos et al., 2001
kaempferol	*A. tonkinensis* (rhizome)	Zhang et al., 2003
	A. officinarum (rhizome)	Bleier & Chirikdjian, 1972 Kim et al., 2006
kaempferol-3-*O*-glucuronide	*A. zerumbet* (leaves)	Mpalantinos et al., 1998
kaempferol-3-*O*-rutinoside	*A. zerumbet* (leaves)	Mpalantinos et al., 1998
catechin and epicatechin	*A. zerumbet* (leaves)	Mendonça et al., 1998; Mpalantinos et al., 1998
alpinetin	*A. zerumbet* (leaves)	Mendonça et al., 1998
	A. zerumbet (seeds)	Krishna & Chaganty, 1973
	A. katsumadai (seeds)	Rao & Lin, 1998
	A. henryi	Wang et al., 2001
galangin and 3-*O*-methyl galangin	*A. officinarum* (rhizome)	Tao et al., 2006
**	*A. officinarum* (roots)	Tunmann & Tkotz, 1972

**The abstract did not cite.

using a rotary evaporator until methanol elimination; then, the aqueous layer was further partitioned using solvents and evaporated to dryness in order to obtain dichloromethane (0.29 g), ethyl acetate (0.31 g) and *n*-butanol (4.8 g) partitions. Each solvent extractor (50 to 60 mL) was used five times.

Flavonoid standards

Flavonoids of the kaempferol class were isolated from *Alpinia zerumbet* Roxb. and identified using Nuclear Magnetic Resonance (NMR) (Mpalantinos et al., 1998). Rutin was purchased from Merck. Kaempferol-3-*O*-glucuronide had 82% purity, kaempferol-3-*O*-rutinoside, 91%, and rutin, 98%. Purity was verified using three replicate injections of standards into HPLC.

Evaluation of phenolic compound content

Total phenolic compounds were determined using the Folin-Ciocalteau method. Hydroalcoholic extracts were dissolved in ethanol (70%) at 1mg mL^{-1}. A 0.5 mL aliquot of diluted extract and 2 mL Folin-Ciocalteau reagent (10%) were added after 3 min, together with 2 mL of 7.5% sodium carbonate, and mixed. The mixture was homogenized and incubated at 50°C for 30 min. Absorbance was measured at 740 nm in a spectrophotometer using gallic acid as standard. Two controls were used: (1) Folin-Ciocalteau + sodium carbonate and (2) crude extract solution. Phenolic compounds in crude extracts were quantified through the regression equation of calibration curves: $y = 0.0229x + 0.0968$ ($R^2 = 0.9993$), and expressed as mg gallic acid equivalents (GAE) per 1 g dried leaves. All measurements were done in triplicate. The analyses included *A. purpurata* and *A. zerumbet* samples collected in June (2006) and February (2007).

Thin-layer chromatography (TLC)

Aliquots of standards, and crude, ethyl acetate and butanolic extracts were spotted on TLC plate (silica gel 60 $F_{254\ nm}$, Merck) and developed in ethyl acetate, formic acid and distilled water (65/20/15, v/v/v) mobile phase. TLC was observed under UV spectrum at 254 and 360 nm before and after spraying with NP/PEG reagent. The flavonoid standards of rutin (Rf = 0.69), kaempferol-3-*O*-rutinoside (Rf = 0.76) and kaempferol-3-*O*-glucuronide (Rf = 0.83) were verified in the extracts after concomitant running with standards (Victório et al., 2007).

High-performance liquid chromatography (HPLC)

Crude, ethyl acetate and butanolic extracts were dissolved in methanol (70%) at 20 mg mL^{-1} and filtered under vacuum; HPLC-UV analyses were performed in a Shimadzu apparatus equipped with SPD-M10A diode array detector, LC-10AD pump and CBM-10 interface. Data were obtained and processed in a reversed phase column (Lichrosorb RP-18, 25 cm x 5 mm), at room temperature. Separation was done in the following mobile phase: MilliQ water + 0.1% phosphoric acid (A) and methanol (B): 1-10 min (30% B); 20 min (40% B); 60 min (100% B). The prepared mobile phase was degassed using ultrasonic agitation. After 61 min, the gradient was recycled to the initial conditions and held for 10 min before a new injection. The flow rate was kept constant at 1 mL min^{-1} and peaks were detected at 254 nm and 360 nm. All chemicals used in the analysis, such as methanol and phosphoric acid, were of HPLC grades and were purchased from Merck. MilliQ water was used in HPLC mobile phase and sample preparation. Standards were dissolved in 70% methanol at 1 mg.mL^{-1} and analyzed in the same elution. Injections were done in triplicate. Linearity was observed in the concentration range 0.0078 - 0.0625 mg mL^{-1} rutin and 0.01325 - 0.25 mg mL^{-1} kaempferol-3-*O*-glucuronide. Flavonoids in the extracts were quantified against calibration curves of standards, where *y* is the peak area and *x* the concentration in mg mL^{-1} (Figure 1).

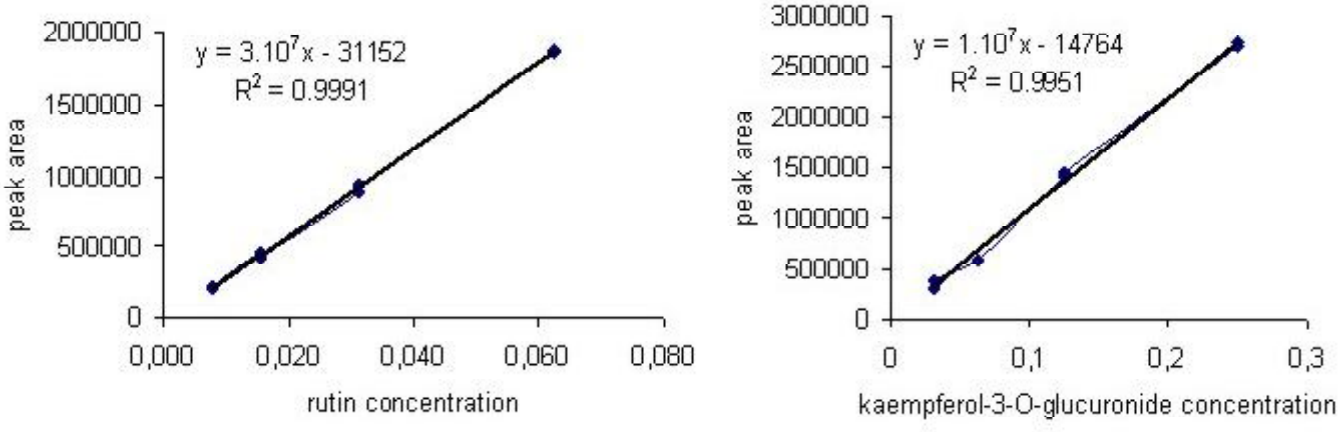

FIGURE 1. Calibration curves of rutin and kaempferol-3-*O*-glucuronide standards.

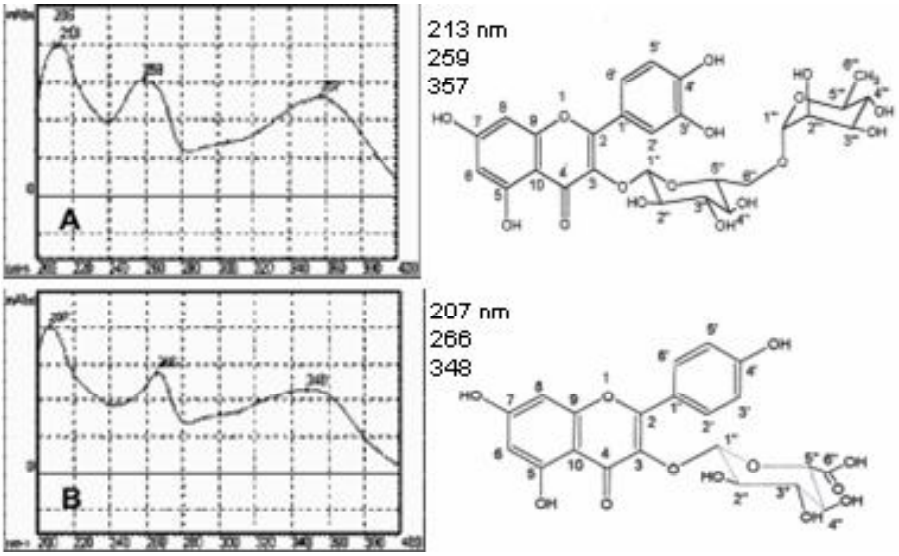

FIGURE 2. UV spectra of (A) rutin (RT= 32.5 min) and (B) kaempferol-3-*O*-glucuronide (RT= 35.2 min).

Flavonoid detection

Flavonoids were detected through retention times (RT), ultraviolet spectrum compared with flavonoid standard spectrum (Figure 2) and coinjection with authentic samples analyzed under the same conditions. For coinjection, a mixture (1:1, v/v) of extracts at 20 mg mL^{-1} and standard at 1 mg mL^{-1} was prepared. The purity of each flavonoid peak in Figures 4 and 5 was assessed by comparing the UV spectra at upslope and downslope inflexion points for both wavelengths (254 and 360 nm).

RESULT AND DISCUSSION

Phenolic compounds present antioxidant activity; thus, they are considered important therapeutic agents. As already known, antioxidants reduce the effects of excessive free radical production in critically ill patients affected by diseases like cancer, cardiovascular disturbances, and brain dysfunction (Atoui et al., 2005). Folin-Ciocalteau method can quantify the presence of flavonoids and other phenolic compounds in plant material. There were significant differences in total phenolic content ($p<0.03$) between *A. purpurata* and *A. zerumbet* in both evaluated months (Figure 3). *A. zerumbet* was used for comparison since it is greatly employed in folk medicine and has been extensively reported concerning phytochemistry and phytotherapy.

The chromatographic profile of *A. purpurata* shows rutin and kaempferol-3-*O*-glucuronide peaks, similarly to those for *A. zerumbet* (Figure 4) in opposite proportions. These flavonoids were verified in ethyl

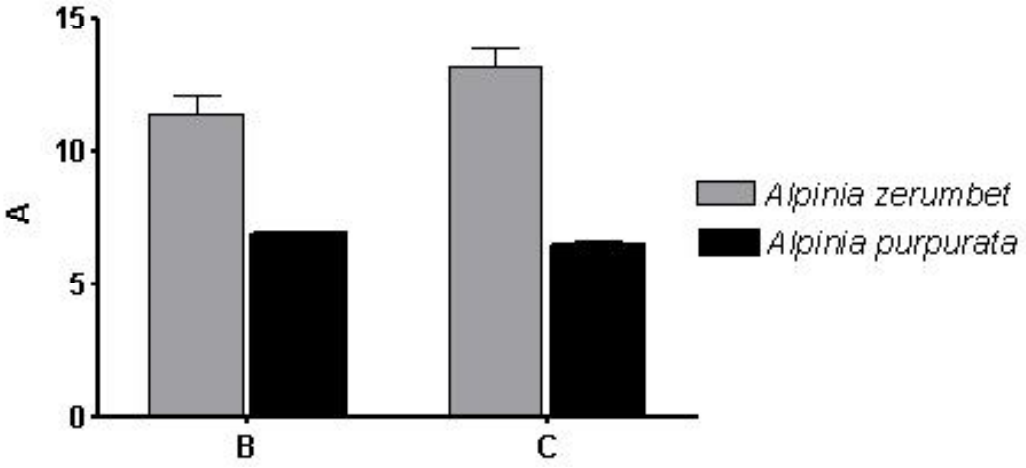

FIGURE 3. Comparison of total phenolic content extracted from *Alpinia zerumbet* and *A. purpurata* collected in two different periods of the year. A (total phenols in mg GAE g^{-1} dried leaves), B (June, 2006) and C (February, 2007).

acetate and butanolic extracts between 20 and 40 min (Figure 5). The main difference was detected between 40 and 60 min, when *A. zerumbet* crude extract showed more compounds in the chromatographic profile than *A. purpurata* (Figure 4). The flavonoid kaempferol-3-*O*-rutinoside was not detected through TLC or HPLC in the extracts. HPLC and TLC methods were reproducible. UV spectrum of peaks 1 and 3 of the chromatogram (Figure 5) are characteristic of flavonoids; however, for an accurate identification, they should be isolated in SEPHADEX column using polar solvents from ethyl acetate and butanolic fractions, followed by structural elucidation using the spectroscopy technique. Rutin and kaempferol-3-*O*-glucuronide were isolated from *A. zerumbet* and also detected in *A. purpurata* at the following concentrations, respectively: 17.8 and 8.7 mg g^{-1} dried leaves (ethyl acetate) and 356 and 85.5

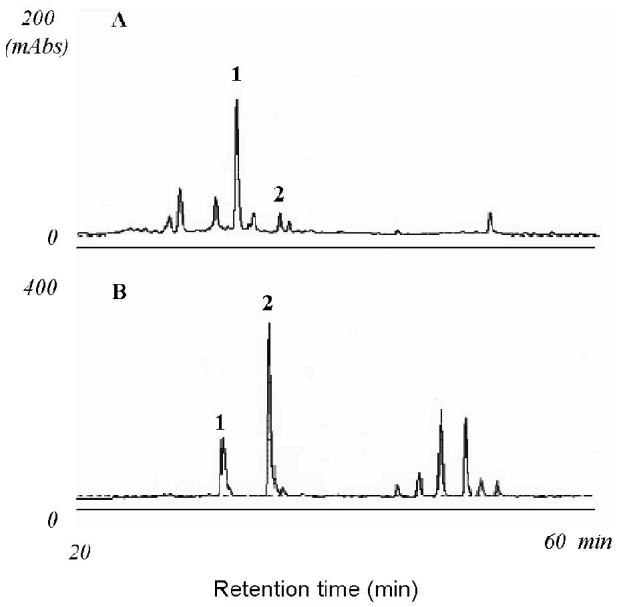

FIGURE 4. Comparison between chromatographic profiles of crude extracts from *Alpinia purpurata* (A) and *A. zerumbet* (B): rutin (1) and kaempferol-3-*O*-glucuronide (2). Values obtained at 254 nm.

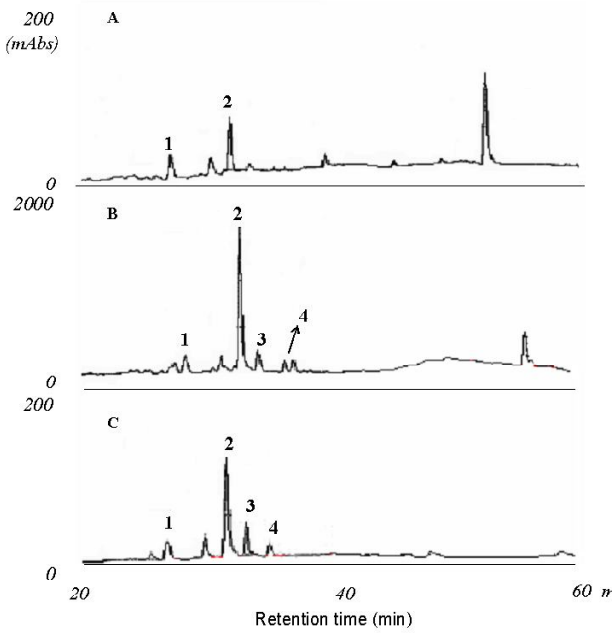

FIGURE 5. Chromatographic profile of *Alpinia purpurata*: crude extract (A), butanolic extract (B) and ethyl acetate extract (C). Rutin (2) and kaempferol-3-*O*-glucuronide (4). Values obtained at 254 nm.

TABLE 2. Quantitative analysis of flavonoids rutin and kaempferol-3-*O*-glucuronide in extracts of dried leaves of *Alpinia purpurata*. Values obtained at 254 nm.

		Flavonoids					
		Rutin			kaempferol-3-*O*-glucuronide		
Extracts	Yielding %	RT	Area	Concentration (mg g^{-1} dried leaves)	RT	Area	Concentration (mg g^{-1} dried leaves)
Crude	11.1						
Acetate	0.06	31.35	1845848	17.8	34.70	278400	8.7
Butanolic	0.9	30.63	16414602	356	34.65	1895737	85.5

Data represent mean of triplicates.

mg g^{-1} dried leaves (butanol) (Table 2). Rutin had the highest concentration in both extracts. This flavonoid is widely distributed in the Plant Kingdom and, despite its hydrophilic character, it presents several therapeutic applications such as antioxidant activity, besides reducing arteriosclerosis risks and increasing vein tone, which improves the blood flow (Wojcicki, 1995; Kreft et al., 2006). Recent studies have reported the application of flavonoids of the kaempferol class to treat Alzheimer's disease (De Melo & Costa, 2005). Mpalantinos et al. (1998) suggested that rutin, kaempferol-3-*O*-glucuronide and kaempferol-3-*O*-rutinoside are responsible for the effects of lower blood pressure. In an analysis of *A. purpurata* crude extract other compounds were obtained between 40 and 60 min RT but were not identified (Figure 5). No previous research has been conducted into *A. purpurata* phytochemistry. This study is the first to report such flavonoids in this species, also confirming the significant presence of flavonoids in different *Alpinia* species. In addition, these results indicate the close phytochemical relationship between *A. zerumbet* and *A. purpurata*, which represent important phytoterapeutic resources.

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