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Flavonoid extraction from Alpinia zerumbet (Pers.) Burtt et Smith leaves using different techniques and solvents

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Abstract: The current study aims to verify the best method for a rapid and efficient extraction of flavonoids from *Alpinia zerumbet*. Dried leaves were extracted using distillated water and ethanol 70% by extraction methods of shaking maceration, ultrasonic, microwave and stirring. By the application of TLC and reversed-phase HPLC techniques the rutin and kaempferol-3-*O*-glucuronide were detected. Ethanol 70% was more efficient for flavonoids extraction than water. No significant yielding variation was verified for ultrasonic, microwave and stirring methods using ethanol 70% (11 to 14%). The relative concentration of rutin and kaempferol-3-*O*-glucuronide, respectively, was higher by ultrasonic (1.5 and 5.62 mg g^{-1} dried leaves, respectively) and by microwave (1.0 and 6.64 mg g^{-1} dried leaves) methods using ethanol. Rapid and simplified extraction proceeding optimize phytochemical work and acquisition of secondary metabolites.

Keywords: high performance liquid chromatography; maceration; microwave; ultrasonic; Zingiberaceae.

Introduction

Alpinia zerumbet (Pers.) Burtt *et* Smith (Zingiberaceae) is an herbaceous perennial plant with wide use in Brazil. This plant is among the most cited in folk medicine. It is indicated to treat arterial hypertension [1]. The flavonoids of this species present remarkable medicinal properties and are related to its main biological activity [2-4]. Mpalantinos *et al*. [3] isolated and identified the flavonoids rutin and kaempferol-3-*O*-glucuronide by NMR, which have a high medicinal value in therapeutic uses.

Parameters as time, solvent, temperature and extraction technique influence secondary metabolites extraction. For this reason different procedures have been used for acquisition of biologically active compounds from crude plant extracts. Recent studies mentioned the ultrasonic and microwave methods as efficient in secondary metabolites extraction [5-7]. Solvent type and method of extraction are fundamental factors to consider for optimizing yield extraction [8]. The purpose of this work was to develop and evaluate efficient and simple procedures for extraction of flavonoids from *A. zerumbet* leaves in short time.

Material and methods

Materials

Samples of *Alpinia zerumbet* (Pers.) Burtt *et* Smith leaves were collected in Rio de Janeiro (Rio de Janeiro state, Brazil), at "Núcleo de Pesquisas em Produtos Naturais" in the Universidade Federal do

Rio de Janeiro. Voucher specimen was identified and deposited in the Herbarium of Rio de Janeiro Botanical Garden, accession number RB 433485.

All chemicals used in analysis as methanol and phosphoric acid were of HPLC grades and were purchased from Merck. MilliQ water was utilized to HPLC mobile phase and sample preparation. Kaempferol-3-*O*-glucuronide was isolated from *Alpinia zerumbet* and identified by Nuclear Magnetic Resonance (NMR) [3]. Rutin was purchased from Merck.

Preparation of extracts

Leaves of *A. zerumbet* were collected from adult plants, in the morning, then plant material was dried for 3 days in stove (60ºC) and macerated in 70% ethanol or distillated water, in the same proportion of 1 g dried leaves/20 mL solvent (10% w/v).

It was evaluated four extraction methods from dried leaves: maceration in shaker at 100 rpm, ultrasonic bath (40 kHz, Thornton Unique, model 1400 USC), microwave (PANASONIC - Auto Sensor Diet, full power) and stirring (Table 1). In the microwave extraction, the suspensions were irradiated under microwaves in pre-setting procedures (3 s power on, 60 s off) for three times to the desired temperature about 60 and 70ºC. The temperature was measured after turn off the microwave using a thermometer into extracts in Becker. For the ultrasonic extraction, the 30 mL flask containing 1 g of dried leaves plus 20 mL of one of extracting solvents was partially immersed into the ultrasonic bath and temperature was controlled.

Preliminary results obtained by different extraction time 15 and 30 min for ultrasonic and 1 and 2 days for maceration techniques did not indicate significant differences in relation to extraction time presented in this study.

Crude extracts were filtered in vacuum using Whattman® filter (110 mm Ø, 1). Aqueous extracts were frozen, then liophylizated and the hydroalcoholic extracts were evaporated to dry under reduced pressure at 60ºC. The dried weight was measured. The yielding was defined as follows: (crude extract weight/plant material weight) x 100. The crude extract obtained by each extraction technique was analyzed directly by TLC and HPLC.

TLC analysis

Aliquots of standards and crude extracts were spotted on TLC plate (silica gel 60 $F_{254\ nm}$, Merck) and developed in different mobile phases below. The elution 5 was more appropriated for rutin and kaempferol-3-*O*-glucuronide detection. Components were visualized under ultraviolet light (λ 254 and 366 nm, Model UVGL-58 Upland/EUA) and detected by spraying the TLC plates with reagent NP (2-aminoethyl-phenyl-borate, 1 $mg.L^{-1}$ in ethanol, Spectrum) and PEG (5% poliethyleneglycol-4000, Fluka). The flavonoids standards rutin (Rf = 0.37) and kaempferol-3-*O*-glucuronide (Rf = 0.64) was verified in extracts after concomitant running with standards and they were visible as yellow and orange fluorescent spots.

Table 1. Specifications of extraction methods.

	Maceration	Ultrasonic	Microwave	Stirring
Extracting solvents		distillated water and 70% ethanol		
Temperature[a]	25ºC	40 and 60ºC	60 and 70ºC	50 and 60ºC
Extraction time	3 d	45 min	3x (3 s)	60 min

[a] Data are related to water and ethanol 70% temperatures, respectively.

1	ethyl acetate/ethanol/acetic acid/water	(16:1.5:1.0:1.0, v/v/v/v)
2	ethyl acetate /ethanol/ acetic acid/water	(7.5:2.0:0.5:0.5, v/v/v/v)
3	ethyl acetate /ethanol/ acetic acid/water	(27:1:0.5:0.5, v/v/v/v)
4	ethyl acetate / acetic acid /formic acid/water	(100:11:11:27, v/v/v/v)
5	ethyl acetate /formic acid/water	(65:20:15, v/v/v)
6	chloroformic/methanol	(70:30, v/v)

HPLC analysis

Crude extracts were dissolved in methanol (70%) at 10 mg.mL^{-1}, filtered in vacuum and HPLC analyses were performed on an apparatus Shimadzu equipped with SPD-M10A diode array detector, LC-10AD pump and CBM-10 interface, UV-vis detector. Data were acquired and processed by a reversed-phase column (Lichrosorb RP-18, 25 cm x 5 mm), ambient temperature. The solvent system used was a gradient of MilliQ water + 0.1% phosphoric acid (A) and methanol (B). The gradient was as follows: 1-10 min (30% B); 20 min (40% B); 60 min (100% B). The flow rate was 1 mL.min^{-1}. The prepared mobile phase was degassed using ultrasonic agitation. The elution was monitored at 254 nm and 360 nm. Flavonoids were identified by comparison of HPLC retention times, UV spectra and co-elution with authentic samples analyzed in the same conditions. Standards were dissolved in methanol 70% at 1 mg.mL^{-1} and analyzed in the same elution. For co-elution, it was prepared a mixture (1:1, v/v) of extracts at 10 mg.mL^{-1} and standard at 1 mg.mL^{-1}. The injections were repeated three times. Determination of the content of the flavonoids was performed by the external standard method, using authentic standards.

Linearity was observed in concentration range of 0.0078 – 0.0625 mg.mL^{-1} of rutin (y= 3.10^{-7} x – 31152, R= 0.9991, n= 9) and 0.01325 – 0.25 mg.mL^{-1} of kaempferol-3-*O*-glucuronide (y= 1.10^{-7} x – 51000, R= 0.9951, n= 9). Each determination was carried out in triplicate. Quantification of flavonoids in the extracts was obtained against these calibration curves of standards, where *y* is peak area and *x* concentration in mg.mL^{-1}.

Results and Discussion

Table 2 shows the extraction yielding obtained for each extraction technique. Regarding the extractive techniques, the most yielding was obtained after ethanol extraction in comparison with distillated water. The lowest yielding was found by maceration technique for both solvents.

Hydroalcoholic crude extracts analysis using HPLC revealed six main compounds, among them the peak corresponding to rutin (TR: 31.42 min) and kaempferol-3-*O*-glucuronide (TR: 34.49 min) can be observed right after 30 min, without interference of other components (Figure 1). These flavonoids were also verified through TLC.

Chromatographic profiles of crude extracts obtained through different extraction methods and solvents were similar. The greater variation was achieved in relation to relative content of flavonoids (Figure 2). The visualization of chromatographic profiles for each extraction technique and solvent used permit to evaluate the qualitative and quantitative variations in secondary metabolites content. In addition, these data present compound profiles related to the biological effects and medicinal use.

The highest flavonoids extraction occurred at 70% ethanol, by all techniques extraction (Figure 2). Aqueous, alcoholic and hydroalcoholic extracts are commonly used in researches with plant crude extracts [9]. The results in Figure 2 also indicate that these 4 extraction techniques reach significant differences in content of flavonoids in the same material content. The use of ultrasonic and microwave

Table 2. Yielding from dried leaves extraction of *Alpinia zerumbet*.

Extracting solvents	Yielding extraction (%, w/w)			
	Maceration (3 days)	Ultrasonic (45 min)	Microwave 3x (3 s)	Stirring (60 min, 50ºC)
Aqueous	3.7	6.7	11	-
Hydroalcaholic	8.2	13	13.5	14

Values indicate the averages of the three replicates.

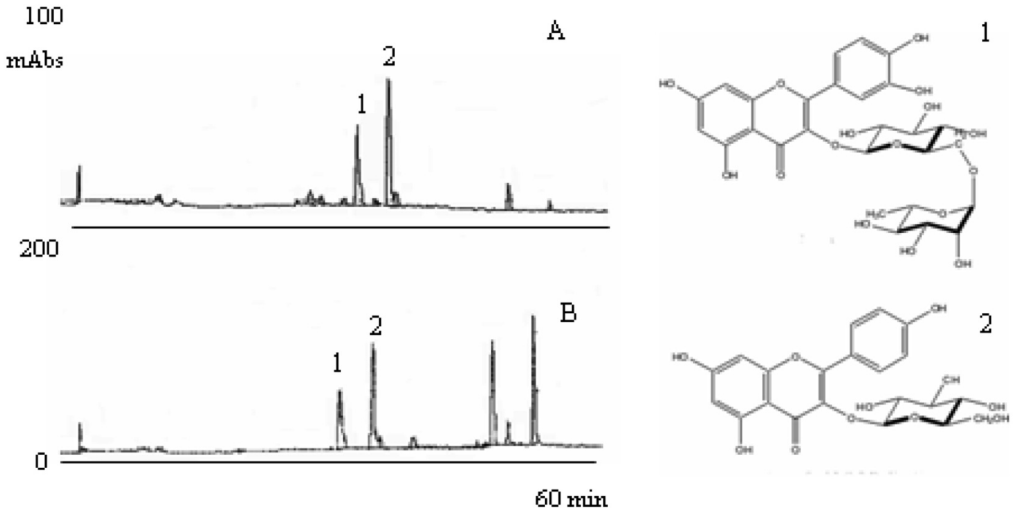

Figure 1. Chromatographic profiles (HPLC) of aqueous (A) and hydroalcoholic (B) extracts of *Alpinia zerumbet* obtained by ultrasonic extraction: rutin (**1**, RT 31.42 min) and kaempferol-3-*O*-glucuronide (**2**, RT 34.49 min) at 360 nm.

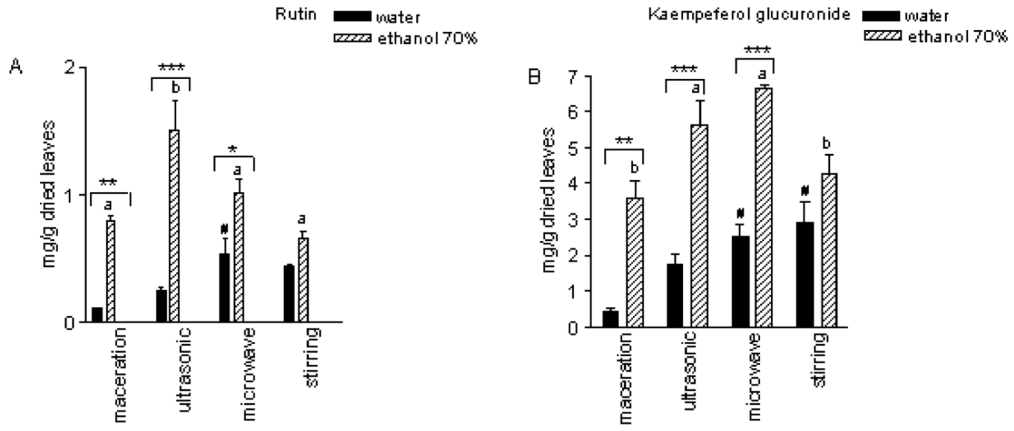

Figure 2. Flavonoid contents in aqueous and hydroalcoholic extracts of *Alpinia zerumbet* obtained by HPLC, comparing different extraction techniques. Each value consisted of average ± SD. Equal letters indicate no statistical differences among extraction techniques considering 70% ethanol as solvent, $p<0.05$. Comparing the extractor solvents, *$p<0.05$, **$p<0.01$ and ***$p<0.001$ indicate statistical differences for each isolated technique; #$p< 0.05$ considering aqueous extract in relation to maceration technique (Bonferroni, n= 3).

techniques provide a high flavonoid extraction, followed by stirring (Figure 2). By comparison with the conventional techniques, ultrasonic and microwave techniques resulted in high levels of flavonoid extracted with the advantage of saving time and solvent. These methods have been reported by the efficiency in extracting secondary metabolites [5,6,10]. Yang and Zhang [11] veri-

fied the efficiency and short extraction time of flavonoids using ultrasonic technique. The use of microwave for extracting biologically compounds is recent. Some reports have shown its positives results for extracting phenolic compounds, essential oil, flavonoids and alkaloids, more effective than conventional extraction methods [6,12,13].

Extraction methods, involving heating, raised the efficiency of solvents. Microwave, ultrasonic and stirring may improve the flavonoid extraction using as water as 70% ethanol. These techniques present in common, besides higher temperature, a reduced extraction time in comparison with maceration in shaker.

TLC and HPLC chromatographic techniques are wide used and favorable for flavonoid detection [14]. Reversed-phase HPLC is one of the most employed techniques for the analysis of flavonoids [15]. Some results using HPLC were reported for *Alpinia officinarum* and *A. purpurata* species [16,17]. The described HPLC procedures could be useful for the qualitative and quantitative analysis of flavonoids in crude extracts, especially from Zingiberaceae family that has a pronounced presence of flavonoids in its species [18].

Conclusion

From the results obtained in the current study, the relative proportion of these flavonoids was reduced by maceration conventional technique, while microwave and ultrasonic techniques in combination with 70% ethanol solvent were the most efficient. It may suggest that microwave and ultrasonic methods using 70% ethanol are suitable for fast extraction of flavonoids in a simple way, also considering extraction yield and extraction time. These methods also permit the acquisition of flavonoids from little raw plant material.

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Cristiane P. Victorio, Celso Luiz S. Lage, Ricardo M. Kuster. Extração de flavonóides de Alpinia zerumbet (Pers.) Burtt et Smith utilizando diferentes técnicas e solventes.

Resumo: O presente estudo teve como objetivo verificar a melhor metodologia de extração para rápida e eficiente obtenção de flavonóides a partir de *Alpinia zerumbet*. Folhas secas foram extraídas com água destilada e etanol 70%, utilizando as metodologias de extração: maceração sob agitação, ultrassom, microondas e agitador. Para verificação dos flavonóides rutina e kaempferol-3-*O*-glicuronídeo foram utilizadas as técnicas de CCD e CLAE em fase reversa. O solvente etanol 70% foi mais eficiente como extrator. Para as metodologias ultrassom, microondas e agitador, não houve variação significativa para o rendimento utilizando etanol 70% (11 a 14%). A concentração relativa de rutina e kaempferol-3-*O*-glicuronídeo respectivamente foi maior pelos métodos de extração ultrassom (1,5 e 5,62 mg g^{-1} folha seca) e microondas (1,0 e 6,64 mg g^{-1} folha seca) utilizando etanol 70%. Procedimentos rápidos e simplificados de extração otimizam o trabalho fitoquímico e a obtenção de metabólitos secundários.

Palavras-chave: cromatografia líquida de alta eficiência; maceração; microondas; ultrassom; Zingiberaceae.

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