

Contribution to the Study of Phytosterols and Phenolics from the Barks and Leaves of the *Trichilia catigua* A. Juss., Meliaceae

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ABSTRACT

Trichilia catigua is a Brazilian native species known as “catuaba”. This study investigated the chromatographic features hydroalcoholic extracts that were obtained by different techniques, from barks and leaves. The chromatographic analysis has demonstrated the presence of chlorogenic acid, epicatechin and catechin, as well as the presence of β -sitosterol and stigmasterol in both analyzed plant parts. The best results were those when it was used turbo-extraction, extraction using Soxhlet apparatus and ethanol 50%.

Keywords: *Trichilia catigua*; Catuaba; Chromatography

1. Introduction

The *Trichilia catigua* A. Juss., Meliaceae barks are very requested as industrial and export raw material. Pre-clinical studies, with the ethanolic crude extract obtained from the barks of the *T. catigua*, have showed vasorelaxing [1], antinociceptive [2], anti-inflammatory [3] and anti-depressive [4] activities. A mixture of two flavolignanes that were isolated from ethyl acetate portion of *T. catigua* barks has showed antimicrobial activity [5]. NMR and GC-MS of chloroformic portion from *T. catigua* barks has showed the presence of alkaloids, β -sitosterol, stigmasterol and campesterol [6]. Cinchonain was isolated from crude hydroalcoholic extract of *T. catigua* barks and a HPLC-UV method was validated for its quantification in commercialized herbal medicines and barks of “catuaba” in Brazil [7]. Botanical studies which in order to accomplish that anatomical characterization of the *T. catigua* bark and leaves were carried out for pharmacognostical purposes [8].

The aim of this study was to compare the performance of five extraction methods of the compounds from *T. catigua* barks and leaves using chromatographic techniques.

2. Experimental

2.1. Plant Material

The botanical material was collected in São Miguel do Oeste, with altitude between 522 to 536 meters, latitude 26°46'950" to 26°47'006" and longitude 53°30'612" to 53°30'910". The samples were dried in a greenhouse at 50°C for 24 hours and then grinded in a hammers mill with a 3 mm sieve. The botanical material was identified by the botanist Dr. Gerdt Hatchbach and a voucher was deposited in Curitiba's botanical garden over the number 306253.

2.2. Extraction Procedures

Both of extractions, barks and leaves, were performed with ethanol 96% (v/v) and ethanol 50% (v/v). It was used 5 g of dried botanical material in all methods. The final volume was concentrated to 50 mL. The tested were tested: maceration (with 50 mL of solvent for 24 hours); sonication (with 50 mL of solvent in ultrasound bath Bransom model 2210 for one hour); turbo extraction (five extractions with 10 mL of solvent with the Ultra Turrax IKA model T25 basic apparatus at 24,000 rpm for 5 minutes each, after each extraction the solution was centrifuged and the supernatant filtered to a 50 mL volumetric flask), reflux (with 50 mL of solvent under reflux

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on a hot plate for one hour) and Soxhlet (with 200 mL of solvent under reflux hot plate for five hours and then concentrated with a rotary evaporator at 50°C to 50 mL).

2.3. GC Analysis

Analyses were carried out on a Shimadzu 14-B gas chromatograph equipped with a flame ionization detector, with programmable heating ramps and interface CBM-101. The data acquisition program used was Class GC. It was used a J & W Scientific DB-17 fused silica capillary column (30 m × 0.53 mm, 1 µm of film thickness) for the explorative method. Hydrogen was the carrier gas at 30 kPa. The temperature gradient was: initial temperature 100°C with 1 min hold, heating ramp at 7°C/min up to 260°C with 66 min hold. The injector temperature was 260°C and the detector temperature was 280°C. The samples were injected with a split ratio of 1:10 and the injection volume was 4 µL. It was used a Phenomenex ZB-1 fused silica capillary column (30 m × 0.53 mm, 0.5 µm of film thickness) for the quantitative method. Hydrogen was the carrier gas at 30 kPa. The temperature gradient was: initial temperature 100°C with 1 min hold, heating ramp at 8°C/min up to 270°C with 35 min hold and heating ramp at 40°C/min up to 300°C with 5 min hold. The injector temperature was 280°C and the detector temperature was 300°C. The samples were injected in split mode at 1:10 and the injection volume was 4 µL. The reference standards stigmasterol, β -sitosterol, lupeol, α -amyrine and β -amyrine and internal standard tocopherol acetate were diluted in ethanol. The samples were prepared adding 25 mL of the ethanolic crude extract in 5 mL of distilled water and extracted five times with 20 mL of petroleum ether with agitation of the solution in vortex. After each extraction the solution was centrifuged and the ethereal portions were gathered and evaporated to dryness at 50°C. The residue was resuspended with ethanol, 500 µL of internal standard solution at 1 mg/mL was added, and the extract was transferred in a 5 mL volumetric flask and brought to volume with ethanol. The solution was centrifuged prior to the injection. The presence of the identified substances was confirmed by the addition of 0.1 mg·mL⁻¹ of the reference standards in the samples of the extracted crude extracts.

2.4. HPLC Analysis

Both qualitative and quantitative HPLC analyses were developed in a Merck-Hitachi LaChrom Elite high pressure liquid chromatograph equipped an L-2450 DAD detector, L-2300 column oven, L-2130 quaternary pump and L-2200 autosampler. The data acquisition program used was EZChrom Elite. Separations were performed in a Waters X-Terra RP-18 column (250 × 4.6 mm, 5 µm) associated to an equivalent pre-column. The column oven

operated at 25°C. The mobile phase was phosphoric acid at 0.2% in sulfuric acid 0.01 M (A) and acetonitrile 90% in purified water (B). The DAD detector operates from 200 to 400 nm and chromatograms were acquired at 280 nm for the explorative method and at 326 nm on the quantitative method. The qualitative method used was: 90% to 88% of (A) in 25 min, 63% of (A) in 75 min, 10% of (A) in 77 min, hold 6 min and down to initial conditions in 7 min at a flow rate of 0.7 mL/min. The quantitative method used was: 80% to 77% of (A) in 9 min with a flow rate of, 10% of (A) in 10 min, hold 4 min and return to initial conditions in 6 min at a flow rate of 0.7 mL·min⁻¹. The standards stock solutions of gallic acid, epicatechin, chlorogenic acid, caffeic acid, catechin, coumarin, *p*-coumaric acid, ferulic acid and rutin were prepared in methanol and then diluted with the acidic mobile phase. The barks and leaves crude extracts were diluted four times in the acidic mobile phase for the qualitative method and 7 times for the quantitative method. The presence of the phenolic compounds was confirmed by the addition of 0.1 mg·mL⁻¹ of standard in the samples in the samples of crude extracts diluted in acidic mobile phase.

2.5. Statistical Analysis

All the quantifications were performed in triplicate. Significant differences were statistically evaluated using analysis of variance (one-way ANOVA) and the Tukey test on untransformed data. For all tests, statistical significance was set at $p < 0.05$. STATISTICA® software was used for statistical analysis of the data.

3. Results and Discussion

A CG method for phytosterols and terpenoids detection was developed with high efficiency in the separation of stigmasterol, β -sitosterol, lupeol, α -amyrine and β -amyrine. This explorative method was used to obtain the bark and leaves extract crude fingerprint to identify the presence of these substances ethanolic by co-injection with standards.

The chromatograms have demonstrated the coincidence of peaks, in the same relative retention time for the stigmasterol with RRT of 1.163 in the barks and 1.165 in the leaves. Also the presence of β -sitosterol was verified by the presence of a peak on 1.262 in the barks and 1.268 in the leaves. Between the samples and the standards confirmation was done with addition of genuine standards to the extracts and it was observed an increase of the corresponding peaks areas without showing new peaks or any kind of peak shape distortion in the chromatogram.

It was observed the presence of β -sitosterol in a high concentration therefore this compound was the chosen one for quantitative analysis. It was used a more polar chromatographic column (ZB-1) in order to reduce the

analysis time and it still has efficiently separated β -sitosterol (RRT = 1.159) from the other extract's substances, in 60 minutes, reducing the time of the analysis and the overall operational costs. The chromatographic extracts profile obtained with the column ZB-1 was very similar to the preview obtained with the DB-17 column. This methodology was validated to be used in the quantification of β -sitosterol on extractive efficiency tests. The data regarding the validation are showed in **Table 1**.

The Soxhlet extract with ethanol 50% has presented the highest quantity of β -sitosterol for the extraction of both barks and leaves, in a yield of 396 $\mu\text{g/g}$ and 777

$\mu\text{g/g}$ respectively. On the other hand, the maceration method was the least efficient in the β -sitosterol extraction with ethanol 50%, where the yield was 37, 38 $\mu\text{g/g}$ and 28, 29 $\mu\text{g/g}$ on the barks and leaves respectively. All the other extractive methods tested have presented the higher extraction yields with ethanol 96% as a solvent. It was observed that the second best extractive method was the turbo extraction with ethanol 96%, therefore the solvent renewal in the extractive process helps the extraction of β -sitosterol, as both Soxhlet and turbo extraction methodologies used solvent renewal in the extractive process (**Table 2**).

Table 1. Results of GC method validation.

	Analyzed Parameter	Specification	Obtained Results	
Specificity	RRT correlation with standard peak	Minimum 95%	B-sitosterol 97.81	
	Resolution versus other peaks	Minimum 1.0	Complies	
Linearity	Correlation coefficient	$r^2 \geq 0.99$	$r^2 = 0.99832$	
Precision	Method	Analysis RSD $\leq 5\%$	1.0%	
	Intermediate	Analysts RSD $\leq 5\%$	Analyst 01	0.72%
			Analyst 02	0.74%
			Analyst 03	0.38%
Accuracy	Addition of standards	Recovery 95 to 105%	Concentration 1 50 $\mu\text{g/mL}$	99.67%
			Concentration 2 100 $\mu\text{g/mL}$	100.23%
			Concentration 3 150 $\mu\text{g/mL}$	100.10%
Robustness	Carrier gas velocity alteration	RSD $\leq 5\%$ respect to standard conditions	0.29%	
	Column batch change	RSD $\leq 5\%$ respect to standard conditions	0.22%	
	Column oven temperature alteration	RSD $\leq 5\%$ respect to standard conditions	0.25%	

*RSD% = relative standard deviation.

Table 2. Statistical analysis of the β -sitosterol and chlorogenic acid content based on different extractive methods.

Extraction method	Material and Solvent	Average content of β -sitosterol ($\mu\text{g/g}$)	Standard deviation	Variance	Average content of chlorogenic acid ($\mu\text{g/g}$)	Standard deviation	Variance
Soxhlet	Barks EtOH 50%	396.31 ^a	3.803	23.066	1932.91 ^a	1.970	3.882
	Leaves EtOH 50%	777.14 ^b	1.786	3.191	1565.77 ^b	1.782	3.175
	Barks EtOH 96%	252.33 ^c	3.940	15.526	951.24 ^c	2.061	4.248
	Leaves EtOH 96%	581.65 ^d	1.320	1.743	927.25 ^d	0.655	0.429
Reflux	Barks EtOH 50%	57.44 ^e	2.194	4.814	1823.40 ^e	3.593	12.912
	Leaves EtOH 50%	64.17 ^f	0.345	0.119	1560.92 ^b	2.203	4.851
	Barks EtOH 96%	230.49 ^g	1.980	3.920	551.20 ^f	1.754	3.078
	Leaves EtOH 96%	399.193 ^a	2.217	4.913	534.70 ^g	2.324	5.399
Turbo extraction	Barks EtOH 50%	190.62 ^h	1.263	1.595	2046.41 ^h	1.357	1.842
	Leaves EtOH 50%	294.70 ⁱ	3.081	16.654	1702.37 ⁱ	1.800	3.238
	Barks EtOH 96%	313.33 ^j	2.229	4.967	475.87 ^j	0.514	0.264
	Leaves EtOH 96%	534.03 ^l	3.846	14.788	278.07 ^l	0.250	0.063
Maceration	Barks EtOH 50%	37.38 ^m	1.596	2.548	1592.02 ^m	1.674	2.803
	Leaves EtOH 50%	28.29 ⁿ	1.955	3.821	1199.14 ⁿ	1.881	3.539
	Barks EtOH 96%	221.58 ^o	0.621	0.385	424.07 ^o	1.344	1.807
	Leaves EtOH 96%	366.11 ^p	3.699	22.084	195.40 ^p	0.406	0.164
Sonication	Barks EtOH 50%	51.92 ^e	0.542	0.294	1645.22 ^q	2.561	6.558
	Leaves EtOH 50%	53.32 ^e	3.419	11.692	1436.60 ^r	0.878	0.771
	Barks EtOH 96%	170.34 ^s	0.613	0.376	426.83 ^o	1.647	2.714
	Leaves EtOH 96%	288.74 ⁱ	2.451	6.005	274.16 ^l	0.774	0.600

*Different letters mean different contents at 95% of significance.

The temperature influences positively the β -sitosterol extraction, because the Soxhlet method was more efficient than turbo extraction. The β -sitosterol is more soluble in ethanol than in water and it justify a better yield with ethanol 96% when it was compared to ethanol 50%. However, the extraction in Soxhlet with ethanol 50% has presented a larger yield of β -sitosterol than the extraction with ethanol 96%. This fact can be explained due the presence of water in the solvent, allied to the heat soften the vegetable material, that facilitate the ethanol penetration into the tissues. These results are in agreement with the literature [9] which demonstrated the superior efficiency of the terpenoid and steroids ethanol's extraction with Soxhlet in tobacco's leaves.

According to statistical analyses, most of the extraction methods and solvent concentrations were different at 5% of significance, except for the extraction of the barks and leaves with ethanol 50% reflux and sonication extractions. In the species *T. catigua* the leaves presented a larger content of β -sitosterol that the barks in all extractive methods tested (**Table 2**).

A HPLC-DAD method was developed in order to investigate the presence of phenolic compounds in *T. catigua* bark and leaves. An explorative method was used to obtain the chromatographic fingerprint and it was identified the presence of phenolic compounds on extracts. This method was able to efficiently separate the gallic acid, epicatechin, catechin, chlorogenic acid, caffeic acid, cumarine, p-cumaric acid, ferulic acid and rutin standards.

The crude extracts were injected without the needing of sample preparation. The analysis of the chromatograms has demonstrated the coincidence of the retention time of catechin, chlorogenic acid and epicatechin peaks in both plant parts. The identity of these compounds was con-

firmed by the addition of genuine standards in the extracts, and the analysis of the peak purity was accomplished by DAD detector software, which was satisfactory on excluding the co-elution of different substances. The presence of chlorogenic acid was not mentioned in this species yet. The chlorogenic acid was identified as the phenolic compound with the highest concentration on plant parts and a new method for its quantification was developed to reduce the analysis time from 90 down to 20 minutes using the same chromatographic conditions. This methodology was validated to be used in the chlorogenic acid quantification in the extractive efficiency tests. Data regarding the method validation are showed in the **Table 3**.

According to **Table 2**, the turbo extraction method with ethanol 50% presented the highest content of chlorogenic acid on the barks and leaves extract with yields of 2046 $\mu\text{g/g}$ and 1702 $\mu\text{g/g}$ respectively. In agreement with the phytosterols study, the maceration method was the least efficient for the extraction of chlorogenic acid with a content of 1592 $\mu\text{g/g}$ and 1199 $\mu\text{g/g}$ from barks and leaves respectively, approximately 25% less than the turbo extraction method. The second best extractive method was Soxhlet with ethanol 50%, confirming that solvent renewal in the extractive process is important to the highest yield achievement. The superior efficiency of the turbo extraction can be due to the pulverization of the drug material particles during the extractive process that exposes the internal content of the cells, facilitating the action of the extracting solvent. The presence of water in the solvent again caused the softening of the plant tissue facilitating the ethanol extraction of the components. According to the statistical analyses, most of extraction methods and solvent concentrations tested were different at 95% of significance level except for: extraction of the leaves with ethanol 50% on reflux and Soxhlet for chlorogenic

Table 3. Results of HPLC method validation.

	Analyzed Parameter	Specification	Obtained Results
Specificity	Peak Purity	Minimum 95%	Chrologenic acid 99.72
	Resolution versus other peaks	Minimum 1.0	Complies
Linearity	Correlation coefficient	$r^2 \geq 0.99$	$r^2 = 0.999442$
	Method	Analysis RSD $\leq 5\%$	1.0%
Precision	Intermediate	RSD $\leq 5\%$	Analyst 01 1.21%
			Analyst 02 0.97%
			Analyst 03 1.25%
Accuracy	Standards addition	Recovery 95 to 105%	Concentration 1 6.70 $\mu\text{g/mL}$ 100.75%
			Concentration 2 13.30 $\mu\text{g/mL}$ 100.18%
			Concentration 3 20.00 $\mu\text{g/mL}$ 99.92%
Robustness	Mobile phase pH alteration	RSD $\leq 5\%$	0.33%
	Column batch change	RSD $\leq 5\%$	1.24%
	Column oven temperature alteration	RSD $\leq 5\%$	1.08%

*RSD% = relative standard deviation.

acid; extraction of the barks with ethanol 96% on maceration and sonication for chlorogenic acid; extraction of the leaves with ethanol 50% on turbo extraction and ethanol 96% on sonication for β -sitosterol. The barks presented a higher content of chlorogenic acid than the leaves in all of extractive methods and the leaves presented a higher amount of β -sitosterol than the barks in all the extractive methods.

4. Conclusions

The analytical methodologies developed in this study demonstrated efficiency, safety and reproducibility, being characterized as viable proposals for quality control routines of the species *T. catigua* barks and leaves. The chromatographic analysis demonstrated the presence of chlorogenic acid for the first time in the barks and leaves of the species and confirmed the presence of β -sitosterol and stigmasterol [6]. All the obtained results of the accomplished analyses are in agreement with the method's validation parameters required by the ANVISA [10].

In the studied conditions it was demonstrated that the best technique for chlorogenic acid extraction is the turbo extraction with ethanol 50% and for extraction of β -sitosterol is Soxhlet with ethanol 50%. These results can be useful to future extraction tests for the process in industrial scale. The presented data in this study demonstrated that the *T. catigua* barks and leaves have a similar chemical composition, which basically differs in the percentage concentrations of the secondary metabolites. Parallel chemical and pharmacological studies could be done to develop a leaves extract that possesses similar pharmacological properties. This fact could reduce the chance of extinction of the species because it could substitute the use of the barks, which actually are collected extensively from native trees. The trees cultivation could be proposed for the production of leaves, facilitating the process of the plant harvest and turning the process ecological [11].

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