

**Extraction of bioactive metabolites from sucupira seeds (*Pterodon emarginatus*) using
cachaça**

Extração de metabólitos bioativos de sementes de sucupira (*Pterodon emarginatus*) com cachaça

**Extracción de metabolitos bioactivos de semillas de sucupira (*Pterodon emarginatus*)
utilizando cachaça**

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Abstract

This work aimed to evaluate the presence of the bioactive metabolites from sucupira seeds in prepared herbal medicines obtained by the immersion of the seeds in cachaça were evaluated by varying the physical-chemical characteristics of the cachaça used; the phytotherapeutic preparation method (extraction time of plant material) and storage conditions (with and without seeds immersed). The chemical profile of the extracts was determined GC-MS. The presence of some bioactive metabolites was observed, which were extracted with the cachaça of lower alcohol concentration (44.5%) after two months of contact with the seeds. These

metabolites were not identified in samples stored for four months. The cachaça with an alcohol concentration of 46.5%, after 2 months of maceration, promoted the extraction of β -elemene, only and the others with contents of 48.5%, 49.5% and 50.4%, respectively, extracted the bioactive metabolites: spathulenol, D-germacrene, δ -cadinene, caryophyllene and β -elemene in the maceration periods of 15 days and 2 months. In the extracts that were stored without seeds, the metabolites spathulenol and δ -cadinene were the only ones that remained present in all samples after two and four months of storage. The extraction of all metabolites was possible with the crushed and macerated seeds remaining for 2 months in cachaça with an alcohol content greater than 45%.

Keywords: Sucupira seed; GC-MS; Herbal medicines; Sesquiterpenes.

Resumo

Este trabalho teve como objetivo avaliar a presença de metabólitos bioativos de sementes de sucupira em fitoterápicos preparados pela maceração de sementes de sucupira em cachaça, variando-se as características físico-químicas da cachaça utilizada; o método de preparo do fitoterápico (tempo de extração do material vegetal) e as condições de armazenamento (com e sem sementes imersas). O perfil químico dos extratos foi determinado por CG-EM. Observou-se a presença de alguns metabólitos bioativos, que foram extraídos com a cachaça de menor teor alcoólico (44,5%) após dois meses de contato com as sementes. Esses metabólitos não foram identificados em amostras armazenadas por quatro meses. A cachaça com concentração de álcool de 46,5%, após 2 meses de maceração, promoveu a extração do β -elemeno, apenas e as demais com teores de 48,5%, 49,5% e 50,4%, respectivamente, extraíram os metabólitos bioativos: espatulenol, D -germacreno, δ -cadieno, cariofileno e β -elemeno nos períodos de maceração de 15 dias e 2 meses. Nos extratos armazenados sem sementes, os metabólitos espatulenol e δ -cadieno foram os únicos que permaneceram em todas as amostras após dois e quatro meses de armazenamento. A extração de todos os metabólitos foi possível com as sementes trituradas e maceradas permanecendo 2 meses em cachaça com teor de álcool superior a 45%.

Palavras-chave: Semente de sucupira; CG-EM; Fitoterápicos; Sesquiterpenos.

Resumen

Este trabajo tuvo como objetivo evaluar la presencia de metabolitos bioactivos de semillas de sucupira en hierbas medicinales preparadas pela maceração de sementes de sucupira em cachaça, variando como características físico-químicas de la cachaza utilizada; el método de

preparación fitoterápico (tiempo de extracción del material vegetal) y como condiciones de almacenamiento (con y sin semillas sumergidas). El perfil químico de los extractos se determinó mediante CG-EM. Se observó la presencia de algunos metabolitos bioactivos, los cuales fueron extraídos con menor contenido alcohólico (44,5%) luego de dos meses de contacto con las semillas. Estos metabolitos no se identificarán en muestras almacenadas durante cuatro meses. La cachaça con una concentración de alcohol de 46.5%, luego de 2 meses de maceración, promovió la extracción de elemento β , solo y las demás con contenidos de 48.5%, 49.5% y 50.4%, respectivamente, extrajeron los metabolitos bioactivos: espatulenol, D-germacreno, δ -cadieno, cariofileno y β -elemento en los períodos de maceración de 15 días y 2 meses. En los extractos almacenados sin semillas, los metabolitos espatulenol y δ -cadieno fueron los únicos que permanecieron en todos ellos como los dos después y los cuatro meses de almacenamiento. La extracción de todos los metabolitos fue posible con semillas trituradas y maceradas permaneciendo 2 meses en cachaça con un contenido de alcohol superior al 45%.

Palabras clave: Semilla de sucupira; CG-EM; Hierbas medicinales; Sesquiterpenos.

1. Introduction

Since the dawn of humanity, the use of plants for the prevention and treatment of disease has been a common practice. According to the World Health Organization (WHO), 65-80% of the world population currently depends on these plants as a form of basic health care (Veiga & Pinto, 2005), mainly in developing countries. The therapeutic actions of medicinal plants can be achieved through their direct use or through the preparation of pharmaceutical products, called phytotherapies (ANVISA, 2018). The safe and effective use of a medicinal plant and a herbal medicine depends on the absence of toxic substances from the plant species itself or from contaminants (such as heavy metals), as well as on the presence of bioactive secondary plant metabolites (Simões et al., 2017). The chemical profile of a medicinal plant can be affected by factors involved in planting, cultivating and collecting the species. The chemical composition of a herbal medicine will be affected by factors that involve the storage of plant material after harvest, extraction processes and storage of the product (Brasil, 2006).

Among the homemade herbal preparations, the extract obtained by macerating the parts of the vegetable in brandy, cachaça or white wine, is one of the most common. To prevent and treat inflammatory conditions, one of the frequently used extracts is that of

sucupira seeds. White sucupira (*Bowditchia nitida*, *Pterodrom emarginatus*, *Pterodrom abruptus*, *Pterodrom appariciori*, *Pterodrom polygalaeiflorus*) is a medicinal plant whose seeds are rich in bioactive terpenes, such as: germacrene-D, β -elemene, spathulenol, caryophyllene, humulene, geranylgeraniol, δ -cadinene and copaene (Hoscheid & Cardoso, 2015).

The present work was performed to evaluate the manner in which the conditions for preparation and storage of the sucupira seed extract affect the chemical profile of the product. Since that the biological proprieties of the prepared medicine depends on the chemical composition. In this study, the duration of maceration, characteristics of the cachaça used and storage conditions of the product were verified. The metabolites present in the extracts were identified by gas chromatography coupled with mass spectrometry (GC-MS).

2. Materials and Methods

2.1 Methodology

Crushed sucupira seeds (*Pterodrom emarginatus*) were macerated in cachaças of different alcoholic contents, with storage time variation to evaluate the variation of these parameters in the extraction of bioactive metabolites (Bustamante et al., 2010; Passos et al., 2018).

2.2 Seed acquisition

White sucupira seeds were purchased in the central market of Belo Horizonte, MG, Brazil in February 2016.

2.3 Characterization of the cachaças

2.3.1 Determination of alcohol concentration

The alcohol concentration was determined according to EEC Regulation No. 2676/90 (adapted for cachaça) using an alcoholmeter (IAL, 2008).

2.3.2 Determination of soluble solids

Based on the method used by the Centro de Tecnologia Canavieira (CTC), approximately 40.00 g of sucrose was weighed in a beaker and sufficient distilled water was

added for the complete dissolution of the sugar. The solution was transferred to a 200.00 mL volumetric flask, the volume was completed with distilled water and the solution was homogenized to furnish 20% m v⁻¹ solution (CTC, 2011).

Subsequently, 2.5, 5.0, 10.0 and 15.0% m v⁻¹ solutions were prepared in 100.00 mL volumetric flasks from a 20% m v⁻¹ stock solution. With the aid of a bench refractometer, an analytical curve was constructed, and the graph of concentration versus refractive index was constructed. The refractive indices of all the prepared solutions were determined. Finally, the refraction indices of the cachaças were measured, and the concentration of soluble solids was determined.

2.3.3 Determination of total acidity

The total acidity was determined by volumetric titration with a standardized solution of 0.01 N NaOH using phenolphthalein as the indicator (Brasil, 2005).

2.4 Seed maceration

The sucupira seeds were ground manually until fragments of about one cm diameter were obtained. To prepare each system, 20 g of seeds was measured in duplicate and transferred to an amber flask, to which 100 mL of cachaça was added. Five distinct brands of cachaça were used. The vials were allowed to stand at room temperature, protected from heat and light, for a period of 15 days, after which 50 mL of the liquid portion was removed from each vial to leave the residue in the initial system. Chromatographic analysis of each liquid portion taken from the extracts was performed.

2.5 Evaluation of the influence of storage conditions

Systems with and without seeds were stored away from light and heat for periods of 60 and 120 days, after which time, aliquots were removed to perform the chromatographic analyses.

2.6 Chromatographic analysis

A 1000 µL aliquot of each system was diluted with 500 µL of the corresponding cachaça in properly cleaned and dried 2-ml vials. Samples of each cachaça were also

subjected to chromatographic analysis. The analyses were performed in a gas chromatograph coupled to a mass spectrometer (Agilent Technologies Model: 7890A and Agilent 5975C inert MSD Triple-Axis Detector and quadrupole analyser). A capillary column of fused silica (HP-5) was used, with a helium flow of 1.3 mL min⁻¹. The column was heated at a programmed temperature from 120 °C for 2 minutes to 240 °C at 3 °C min⁻¹ and then hold for 2 min.

The identification of the substances present in the extracts was performed through the joint analysis of the chromatograms and mass spectra corresponding to each peak present in the chromatograms. To confirm the identity of each substance, the retention indices were calculated by the Kovats method, which consisted of the injection of a certified standard mixture of C9-C22 hydrocarbons, from which an analytical curve was constructed that served as a basis for performing the calculations of the values obtained from the Kovats method for each sample compound (Adams, 2007).

3. Results and Discussion

3.1 Characterization of the cachaças

Table 1 - Alcohol content described on the label and measured, soluble solids and volatile acidity of the cachaças used in the preparation of the extracts.

<i>Cachaça</i>	<i>Alcohol conc. (% v/v)</i>		<i>Total soluble solids (°Brix)</i>	<i>Volatile acidity (mg/100 mL anhydrous alcohol)</i>
	<i>Label</i>	<i>Experimental</i>		
C1	38.0	44.5	14.48	41.70
C2	nd	46.5	15.22	25.80
C3	46.0	48.5	15.96	64.20
C4	43.0	49.5	14.85	62.10
C5	50.8	50.4	16.03	32.70

nd – Concentration is not declared on the label. Source: Own authorship.

The physical-chemical parameters determined for each cachaça used to extract the metabolites from sucupira seeds were shown in Table 1.

A small difference between the labeled and the experimental alcohol content was observed in all the samples. The determination coefficient (R^2) for the equation of the line used to calculate the sugar concentration in cachaças was equal to 0.9967, indicating the

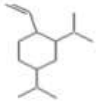

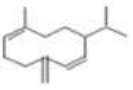

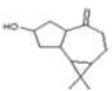
adjustment of the data. Using the straight-line equation ($y = 736.98 - 982.65$), the refractive index of the cachaça was determined, and then the soluble sugar content in the cachaças was determined (Table 1). The concentrations of soluble solids ranged from 14.48 to 16.03 °Brix, whereas the variation of the acidity was 25 to 62 mg/100 mL of anhydrous alcohol. All of these parameters are in accordance with the normative instruction number 13 of 2005 for cachaça and cane spirits (Brasil, 2005).

3.2 Substances identified

The retention times and Kovats indices obtained for the secondary metabolites that generated the most intense peaks in the chromatograms of the samples are shown in Table 2. These compounds were the sesquiterpenes spathulenol, D-germacrene, caryophyllene, β -elemene and δ -cadinene. The pharmacological activities described in the literature for these substances are the following: spathulenol - antioxidant, anti-inflammatory and antimicrobial activity against *Mycobacterium tuberculosis* (Nascimento et al., 2018); caryophyllene - anti-inflammatory, antioxidant, sedative, anxiolytics, antidepressant, anticonvulsant, antitumor (colon, skin and pancreas), antimicrobial action against *Streptococcus mutans* (Francomano et al., 2019); β -elemene - anti-inflammatory (mainly neurological action), antineoplastic activity against cancer cells resistant to platinum drugs, such as ovarian tumor (Shamsizadeh et al., 2017; Lee et al., 2012); δ -cadinene - anti-inflammatory, antimicrobial activity against *Streptococcus pneumoniae* and larvicide, acts on the malaria vector mosquito *Anopheles stephensi* (López et al., 2011; Russo & Marcu, 2017); germacrene-D - potent antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* bacteria and cytotoxicity in cancer cells such as skin melanoma, colon and liver carcinoma, breast and cervical adenocarcinoma and lung fibroblasts (Montanari et al., 2011; Oliveira et al., 2015).

Table 2 shows the bioactive metabolites detected at work, together with their structure, retention time and theoretical and calculated kovats.

Table 2 - Sucupira substances identified in the extracts and their chromatographic parameters.

<i>Metabolite</i>		<i>Tr (min)</i>	<i>Theoretical Kovats</i>	<i>Calculated Kovats</i>
β -Elemene		7.6	1398	1402
Caryophyllene		8.3	1428	1433
Germacrene-D		9.9	1499	1498
δ -Cadinene		10.9	1537	1535
Spathulenol		12.7	1577	1588

Source: Own authorship.

3.3 Chemical profile of the samples

3.3.1 Influence of variation of the maceration period on the chemical profile of the systems

Table 3 - Bioactive metabolites present in the samples macerated for 15, 60 and 120 days.

<i>Metabolites</i>	<i>15 dias</i>					<i>60 dias</i>					<i>120 dias</i>				
	<i>C1</i>	<i>C2</i>	<i>C3</i>	<i>C4</i>	<i>C5</i>	<i>C1</i>	<i>C2</i>	<i>C3</i>	<i>C4</i>	<i>C5</i>	<i>C1</i>	<i>C2</i>	<i>C3</i>	<i>C4</i>	<i>C5</i>
β -Elemene	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+
Caryophyllene	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Germacrene D	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
δ -Cadinene	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Spathulenol	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+

(+) Presence and (-) Absence. Source: Own authorship.

The results presented in Table 3 refer to the bioactive metabolites identified in the extracts and the duration of maceration.

Bioactive metabolites were only observed in the extracts macerated for 60 days in systems prepared with cachaça (C1) of low alcohol content. In systems macerated for 15 days, these metabolites might not have been extracted because the lower alcohol content implies a higher polarity of the system and the bioactive substances in question are sesquiterpenes. Because they are apolar, they will be more extensively extracted in less polar systems. The absence of these metabolites in systems macerated for 120 days might have occurred because of evaporation or degradation in the system. Such observations show that the extracts of sucupira seeds prepared by maceration for a period of less than 60 days or more than 120 days in cachaças whose alcohol content is 44% will be ineffective for the treatment of inflammation symptoms.

In cachaças with higher alcohol concentrations, which correspond to a lower polarity of the extractive system, there is still the presence of metabolites with the greater contact time of the seeds with the cachaça. This observation indicates that there are no degradation reactions of the metabolites when the period of the maceration is extended, which demonstrates the stability of these metabolites in this system. Such preparations, because they contain the metabolites with anti-inflammatory action, will be effective in the treatment of this symptom if they reach the therapeutic doses.

A peculiar behavior was observed with the metabolite β -elemene, which had a shorter retention time (7.6 min) because of its weaker interaction with the stationary phase, which is of medium polarity (HP-5ms). This fact demonstrates the greater volatility and lower polarity of the β -elemene in relation to the other compounds. Thus, its absence in hydroalcoholic solutions (cachaça) of lower alcohol content (C1) and greater polarity is explained because it is difficult to extract this compound in a short period of time. On the other hand, the 60-day period favored the extraction of this metabolite, but the 120-day time favored its volatilization or even degradation.

Spathulenol, an oxygenated metabolite, was extracted in greater quantity in cachaça (C2) under the three storage times. This is a very unique behavior because the medium did not contain less alcohol nor greater acidity, a combination that contributes to the greater polarity of the extractor system and, consequently, greater possibility of extraction in view of the greater polarity of this metabolite in relation to the others. This fact suggests that there are other factors that influence extraction, in addition to synergism or antagonism between them.

In general, samples from C3, C4 and C5 cachaça showed a similar behavior in the extraction of bioactive compounds when macerated at 15, 60 and 120 days (table 3). The extraction power of the three samples is probably due to their physical properties, since they had a higher alcohol content, a higher content of soluble solids and greater acidity, except for sample 5, whose acidity was 32.70 mg / 100 mL of anhydrous alcohol. The synergism between these properties favored the extraction of the active ingredients from sucupira seeds.

3.3.2 Stability of extracts during storage

Table 4 - Bioactive metabolites in seedless samples immersed during 15, 60 and 120 days.

Metabolites	15 dias					60 dias					120 dias				
	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5
β -Elemene	-	-	+	+	+	-	-	+	-	+	-	-	-	-	-
Caryophyllene	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-
Germacrene D	-	+	+	+	+	-	-	+	-	+	-	-	-	-	-
δ -Cadinene	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
Spathulenol	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+

(+) Presence and (-) Absence. Source: Own authorship.

The metabolites identified in the sucupira extracts after removal of the seeds and storage for 60 and 120 days were shown in Table 4. In general, a loss of bioactive metabolites was observed with the increase in the storage time of the extracts.

The removal of the seeds from the extracts after 15 days, but storage for another 45 days without the seeds (60 days) again showed a different behavior in the extraction power and stability of the metabolites of C3, C4 and C5 cachaças, with better results for the C3 and C5 where the 5 bioactive metabolites were identified up to 60 days. The similarity found in the two cachaças was the presence of a higher content of soluble solids, which led us to assume that the greater amount of sugars in the cachaça favored the permanence or stability of the active compounds in up to 60 days.

Storage for 60 days in cachaça C2 led to the disappearance of germancreen-D, whereas in cachaça C4, no germacrene-D or β -elemene was found. This is probably due to degradation or evaporation. Therefore, these extracts have lower anti-inflammatory, antitumor, antimicrobial and insecticidal activities because of the absence of those

compounds. In cachaças with higher alcohol levels, the metabolites can still be found. Thus, these extracts still retain the initial pharmacological characteristics.

After 120 days of storage, only spathulenol and δ -cadinene remain present. The other metabolites were not identified, probably because they were volatilized or degraded. They were identified in extracts whose seeds remained for 120 days, as is shown in Table 3.

4. Conclusion

The ideal time of extraction of the bioactive metabolites of sucupira seeds in cachaça during the maceration process and the storage period of the herbal medicine obtained is directly influenced by the alcohol content of the beverage. Bioactive metabolites were only found in cachaças containing 44% alcohol if the seeds were macerated for 60 days and, after filtration, the product was stored for less than 120 days. On the other hand, maceration of the extracts prepared with cachaças of higher alcohol contents resulted in the extraction of bioactive metabolites within 15 days of contact between the plant material and the extraction system. Filtration to remove seeds from these systems is not advisable because, after 120 days of storage, the extracts that were stored with the seeds contained all the bioactive metabolites, as opposed to those that were stored for the same period without the seeds, in which only δ -cadinene and spathulenol were found.

Such observations highlight the importance of carrying out future studies that make it possible to assess the manner in which the variation in the conditions of preparation and storage of herbal products can affect their chemical profiles by affecting the presence of bioactive metabolites and, consequently, the safety and efficacy in the use of such products.

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