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Comparison of different extraction methods of Brazilian "pacová" (*Renealmia petasites* Gagnep.) oilseeds for the determination of lipid and terpene composition, antioxidant capacity, and inhibitory effect on neurodegenerative enzymes

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ABSTRACT

Pacová (*Renealmia petasites* Gagnep.) is a Brazilian native plant, usually cultivated in south regions of the country. Pacová was previously reported concerning their possible health benefits, mostly from folk medicine. However, only few works relates the health benefits with the composition of the fruit parts. In this context, this work aimed to bring, for the first time in literature, the chemical characterization in respect to lipid and terpene composition of *R. petasites* oilseed, performed by three different extraction methods (supercritical fluid extraction (SFE) with CO_2, Soxhlet with petroleum ether (SOX), and maceration with hexane (MAC)). SFE was most selective for MUFAs, PUFAs, sesqui- and diterpenes. The main terpene identified in all extracts was 2-carene. The extracts presented poor AChE inhibition, and SOX presented potential inhibitory effect against lipoxygenase activity. Overall, *R. petasites* oilseed is a natural source of terpenes and their potential health benefits are highly encouraged to be investigated.

1. Introduction

Renealmia petasites belongs to the Zingiberaceae family. Some more popular species from the same family include *Zingiber officinale* Roscoe (ginger) and *Elettaria cardamomum* (L.) Maton (cardamom), both well recognized for their use in food as spices and herbs. The *Renealmia petasites* Gagnep. (vernacular name: pacová or picova) fruit is a native plant from Brazil, found in the south and southwest regions (Yazbek et al., 2019). A local community in Ubatuba - São Paulo, Brazil suggests heart benefits when consuming the oil extracted from their seeds (Yazbek, dos Santos, & Braga, 2018) . However, the chemical characterization of *R. petasites* seeds is still unknown, making it difficult to establish a correlation of its composition with potential health benefits. The genus *Renealmia* is commonly found in countries with tropical rainforests, e.g., Brazil, Colombia, Mexico, etc. Among the species that most caught researchers' attention are *R. alpinia* and *exaltata*, which have been used to diseases treatment in indigenous communities of South America as a natural medicine practice (Gómez-Betancur & Benjumea, 2014). Recently, Soares et al. (2021) identified eight compounds (mostly phenolics) in the same plant species of this work, although they have only investigated rhizomes, stems and leaves. The fruit, including pulp, seeds, and peel, also found application due to high antioxidant capacity (Arroy, Ruiz-Espinosa, Luna-Guevara, Luna-Guevara, Hernández-Carranza, Ávila-Sosa, & Ochoa-Velasco, 2017), but the lack of studies using the

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seeds suggests that the investigation of its bioactivity deserves attention. The chemical characterization for *Renealmia alpinia* extracts shows an extensive list of terpenes reported elsewhere (Lognay et al., 1991; Maia, Andrade, Carreira, & da Silva, 2007). High contents of β-caryophyllene, β-pinene and spathulenol are found in leaf extracts (Maia et al., 2007), while α-phellandrene, limonene and β-pinene are the main constituents in the seed extracts (Lognay et al., 1991).

Lately, studies concerning human diseases' prevention and their relation with natural bio compounds have gained great relevance, with special attention to neurodegenerative diseases. Pathologies such as Alzheimer's, Parkinson's, multiple sclerosis, and other neurological disorders harm society and are estimated to overcome cancer as the second most common cause of death among the elderly by the 2040s (Ansari, Siraj, & Inamdar, 2010). Their leading cause is aging because of the progressive degeneration of the nervous system's structure and functionality. Unfortunately, current treatments can only alleviate the symptoms, motivating studies to put their efforts into progress prevention. Terpenes and terpenoid compounds have been associated with neuroprotective effects (Sánchez-Martínez et al., 2021), in which their antioxidant and anti-inflammatory properties could help understanding the mechanisms of action for such biological activity (do Nascimento et al., 2018). These remarks raise some positive prospects for the recovery of these compounds from natural sources.

At present, studies aiming to obtain extracts from *Renealmia* involve hazardous solvents utilization as methanol, hexane, and dichloromethane (Lognay et al., 1991; Sekiguchi, Shigemori, Ohsaki, & Kobayashi, 2001). These solvents offer numerous disadvantages for further application such as the traces of solvent remaining in the extracts, making them unfeasible for use in food or pharmaceuticals, which involves human consumption; or even the utilization of an environmentally unfriendly process, going against the current awareness of achieving natural products through sustainable technologies. An alternative process, which is gaining visibility in the last two decades, is the supercritical fluid extraction (SFE) to obtain natural products from vegetable matrices. The most common solvent used to obtain lipid extracts is CO_2, once it is safe to handle, it can be recycled through the process (environmentally friendly), and it is easily separated from the final product. Additionally, it is a tunable density solvent, i.e., it offers high selectivity power to obtain a specific compound. CO_2 has moderate critical properties (Tc ≈ 31 °C, and *Pc* ≈ 7.4 MPa), which allow the preservation of the thermolabile bioactive compounds during the extraction process (Brunner, 2005).

To the best of our knowledge, no work has evaluated extracts' biological activities from *R. petasites* seeds. In this context, this work aimed to apply SFE with CO_2 to obtain lipid extracts from *R. petasites* seeds, comparing their lipid and terpene profile with the extracts obtained by conventional methods (Soxhlet (SOX) with petroleum ether, and maceration (MAC) with hexane). The extracts were also compared concerning neuroprotective effects, assessing the inhibition activity of acethylcholinesterase and lipoxygenase, two enzymes often associated with Alzheimer's disease.

2. Materials and methods

2.1. Sample pretreatment

Sun-dried pacová fruits were gently donated by the company Virga Gim do Brasil, located at Fazenda Guadalupe in Pirassununga, SP, Brazil (22°02′15.1″S 47°21′55.3″W), which uses the fruit as a spice to enhance flavor in their drink production. First, seeds were manually separated from the rinds and dried pulp. As a pretreatment, the reduction of the seeds' particle size was achieved by grinding them in a domestic blender for approximately 2 min. After, the seeds were stored in the freezer at −18 °C until being subjected to the respective extraction process.

2.2. Conventional extraction

2.2.1. Dynamic Maceration

Maceration was performed according to the methodology described in Bouallegue, Allaf, Besombes, Younes, and Allaf (2019) with some modification. Approximately 1.5 g of dried and milled seeds were mixed with 15 mL of hexane (Honeywell, Charlotte, NC, United States) in a porcelain glass at room temperature. Next, the mixture was constantly subjected to maceration for 5 min. This procedure was repeated three times. The final solution (hexane and extract solution) was filtered under vacuum, and the solvent was evaporated using a constant high purity (99.99%) nitrogen (White Martins, Rio de Janeiro, Brazil) flow. The procedure was performed in duplicate and the extracts were kept under freezing at −18 °C until the analysis of biological activities.

2.2.2. Soxhlet

The solvent reflux in an intermittent process was applied to pacová seeds in a Soxhlet apparatus. Petroleum ether (Neon, Suzano, Brazil) was used to extract the non-polar compounds from the seeds. Approximately 10 g of ground seeds were inserted in cotton thimbles, and 160 mL of solvent was refluxed at 80 ± 5 °C with an average of 10–15 solvent refluxes an hour, until no solvent was observed in the solvent flask. In order to calculate the exact yield, two aliquots of 5 mL of each extract were dried in an air circulation oven at 90 °C for 24 h to obtain the actual yield of the extracts, assuring that all solvent was evaporated. The solvent in the remaining extract was evaporated under vacuum, at 35 °C in a rotary evaporator, until no solvent could be condensated. The procedure was performed in duplicate, and the extracts were kept under freezing at −18 °C until of the analysis of biological activities.

2.3. Supercritical fluid extraction (SFE)

SFE was performed in pacová seeds, following the procedure described in Viganó et al. (2016) with some modification. Approximately 35 g of dried and milled seeds were packed into a stainless-steel vessel with 50 mL inner volume, where the void was filled with glass beads. The extraction condition was defined based on a previous study with seeds, which also used supercritical CO_2 as solvent (Viganó et al., 2016), and it was fixed at 35 ± 1 MPa and 40 ± 5 °C with a flow rate of 1.06×10^{-4} kg CO_2/s. The static time was 50 min whereas extraction time was 40 min, as no significant amount of extract was observed after that. Solvent to feed ratio (S/F) was calculated based on the mass of solvent used, resulting in 7.5 g CO_2/g feed. CO_2 was used with 99.9% purity (White Martins, Rio de Janeiro, RJ, Brazil), passing through a serpentine submerged in a cooling bath at −10 °C and pumped into the system using a booster Maximator M−111 CO_2 (Nordhausen, Germany). The process temperature was controlled by a heating bath Marconi MA-184 (Piracicaba, SP, Brazil). The same bath circulated water in a heating jacket around the extraction cell to maintain the process' constant temperature. The non-polar extract was collected in amber glass flasks after complete depressurization of the system, and the global yield was calculated according to Equation (1). The process was performed in duplicate, and the extracts were kept under −18 °C until characterization and biological activity assays.

$$X_0(\%, w/w) = 100 \times \left(\frac{E}{F}\right) \quad (1)$$

where,X_0 is the global yield in percentage;E is the extracted mass collected at the end of SFE;F is the dried pacová seed mass used in SFE.

2.4. Fatty acids and terpenes profiling by GC-qTOF-MS analysis

The identification of the major fatty acids and terpenes in the extracts was performed using gas chromatography (GC) coupled to quadrupole time-of-flight mass spectrometry (qTOF-MS). Derivatization

followed the protocol described by Fiehn (2016) with slight modification. First, 10 μL of extract solution (10 mg/mL ethanol) were dried under constant nitrogen flow for 10 min. 10 μL of methoxyamine hydrochloride [MeOX] (40 mg/mL pyridine) solution was added into the dried extract, shaken at 750 rpm, 60 °C, for 15 min. Next, 90 μL of MSTFA (*N*-methyl-N-(trimethylsilyl) trifluoroacetamide) with 1% TMCS (tri-methylchlorosilane) and 2 μL of myristic acid (d27) were added and the mixture was shaken in the thermomixer (750 rpm, 37 °C, 30 min). The derivatized samples were transferred to vials and closed immediately, waiting 2 h before the analysis. Samples (diluted and derivatized) were analyzed in a 7890B Agilent system (Agilent Technologies, Santa Clara, CA, United States) coupled to a quadrupole time-of-flight mass spectrometry detector (qTOF-MS) 7200 from the same brand that uses electron impact ionization (EI) source. Separation of molecules was first achieved using a capillary column Agilent Zorbax DB5-MS + 10 m Duragard (30 m × 250 μm × 0.25 μm). Injection volume was 1 μL, at a split ratio 10:1 at a total flow rate of 9.05 mL/min. Helium was the carrier gas (0.8 mL/min). The temperature was set to start at 60 °C, and it was elevated to 325 °C at a temperature rate of 10 °C/min, staying at 325 °C for 10 min. The mass spectrometry parameters are described as follows. EI at 70 eV, filament source temperature 250 °C, *m/z* scan range 50–600 amu at 5 spectra/s. The software Agilent Mass Hunter Unknown Analysis tool was used to deconvolute the chromatograms. Identifying the unknown compounds was performed with the help of NIST and FIEHN updated mass spectrum data libraries.

2.5. Antioxidant capacity

Terpenes are the compounds most frequently associated with the antioxidant capacity in lipid extracts. They work against oxygen reactions preserving food, and giving functionality to some products (anti-aging, supplements, etc.). Soxhlet, maceration and SFE extracts of pacová seeds were evaluated according to their antioxidant capacity values obtained by DPPH and ORAC-lipophilic assays as described in sections 2.5.1 and 2.5.2, as follows.

2.5.1. DPPH

The radical-scavenging assay DPPH is based on the electron-transfer reaction. DPPH of the pacová seed extracts followed the methodology previously described in Brand-Williams, Cuvelier, and Berset (1995). Extracts solution (50 mg/mL methanol) was mixed with DPPH reagent (1.8×10^{-4} M) and dissolved in methanol, in the concentration range 3–20 mg/mL. The mixture was incubated for 30 min and the absorbance was measured at 516 nm in a microplate reader SynergyHTX (BioTek Instruments, Winooski, VT, United States). The EC_{50} value was calculated as the extract's concentration required to achieve 50% of the maximum absorption value (blank DPPH reagent), in mg extract/mL.

2.5.2. Lipophilic oxygen radical absorbance capacity (ORAC-L)

The lipophilic antioxidants presented in pacová seed extracts also had their antioxidant capacity determined using ORAC-L as described in Huang, Ou, Hampsch-Woodill, Flanagan, and Deemer (2002) with some modifications. Unlike in DPPH, the principle of ORAC-L assay is based on hydrogen-atom transfer reaction. First, a calibration curve was built (10–150 μM) from a Trolox solution (1500 μM) in acetone:water (1:1, v/v) prepared with 7% (w/v) randomly methylated cyclodextrin (RMCD) (TCI Chemicals, Tokyo, Japan). Samples were prepared in the same solution at concentrations of 0.026–2.6 mg/mL. Briefly, 25 μL of blank, Trolox (Sigma-Aldrich, Madrid, Spain) solution or sample extract, 150 μL of fluorescein (0.012 μM) and 25 μL of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) (0.0128 M) (TCI Chemicals, Tokyo, Japan) solutions diluted in phosphate buffer (pH = 7.4), were added in that order in quadruplicate in a black microplate in the absence of light. The microplate reader Cytation 5 Multi-mode reader (BioTek Instruments, Winooski, VT, United States) was configured to read fluorescence kinetics for 1 h every 5 min, at temperature 37 °C and wavelengths of 485 ± 20 nm (excitation), and 530 ± 20 nm (emission). The results were calculated based on net areas under the kinetic curve (AUC sample – AUC blank) and expressed as mg Trolox equivalent (TE)/g of extract.

2.6. Biological activity assays

2.6.1. Acetylcholinesterase (AChE) inhibition

The inhibitory activity of AChE was measured in the pacová extracts following the methodology described in Ellman, Courtney, Andres, and Featherstone (1961) with the adaptations proposed in Sánchez-Martínez et al. (2021). Extract samples (2 mg/mL) or positive control galantamine (0.0125 mg/mL) (Sigma-Aldrich, Madrid, Spain) were dissolved in ethanol:water (1:1, v/v) solution, in that order. A brief description of the reagents and their respective solvents is given: substrate acetylthiocholine iodide (ACth) (Sigma-Aldrich, Madrid, Spain) was diluted in water milli-Q. The reagent 4-fluoro-7-sulfanoylbenzofurazan (ABD-F) (TCI Chemicals, Tokyo, Japan) was diluted in buffer Tris-HCl 150 mM (pH = 8); stock enzyme solution (AChE) type VI-S from Electrophorus electricus (Sigma-Aldrich, Madrid, Spain) was prepared in Tris-HCl 150 mM (pH = 8) with 0.1% of BSA (bovine serum albumin) (Sigma-Aldrich, Madrid, Spain).

First, ACth concentrated at 3.2 mM was used to determine the enzymatic velocity constant (or K_m). ACth concentrations ranged from 0.1067 to 1.0667 mM/μL. Consecutively, 50 μL of ethanol:water (1:1, v/v), 25 μL of ABD-F at 0.125 mM, 100 μL of buffer (pH = 8), and 25 μL of the enzyme AChE at 0.8 U/mL were added in the black microplate in triplicate. The microplate was read in a microplate reader SynergyHTX (BioTek Instuments, Winooski, VT, United States) every 10 s for maximum 15 min. The fluorescent parameters are given: temperature = 37 °C; wavelength excitation = 389 ± 20 nm and emission = 513 ± 20 nm. The K_m constant was determined by the substrate concentration needed to achieve half of the maximum enzyme reaction velocity. The AChE inhibition assays were assembly using the same reaction principle, respecting the following order: I) Extracts concentrated in a range 50–500 μg/mL; II) 100 μL of buffer (pH = 8); III) 25 μL of AChE solution at 0.8 U/mL; IV) 25 μL of ABD-F solution, and V) 50 μL of ACth solution at the calculated K_m.

An incubation period of 10 min in the absence of light was done after step III. The microplate reading parameters were the same described previously to determine K_m. In order to compare inhibition values with a positive control, the same procedure was done substituting the extract for galantamine (0.4–4 μg/mL). Values were obtained in triplicate and the calibration curves were built to obtain AChE inhibition percentage, which was calculated according to Equation (2). Results were expressed as IC_{50} (%) (i.e. extract concentration required to inhibits the activity of AChE in 50%).

$$\%Inhibition = \frac{\overline{V}_{blank} - \overline{V}_i}{\overline{V}_{blank}} \times 100 \quad (2)$$

where:$\overline{V}i$Indicates the calculated mean of the enzymatic velocity at *i* extract concentration and,$\overline{V}blank$ refers to the mean velocity of the enzyme when no extract was added.

2.6.2. Lipoxygenase (LOX) inhibition

The LOX assay followed the similar procedure presented for AChE assay, with some modification. The buffer used was Tris-HCl 150 mM with pH = 9, enzyme lipoxygenase (TCI Chemicals, Tokyo, Japan) was solubilized in buffer (0.208 U/mL), the substrate was linoleic acid (Sigma-Aldrich, Madrid, Spain) stock solution 7 mM in ethanol:water (1:4, v/v) (before Km calculation) and the reagent was fluorescein 1 μM. Extracts were dissolved in ethanol:water (1:4, v/v) until 0.25–1.5 mg/mL. After adding the extract solution in the specified concentrations (7.14–428.57 μg/mL, depending on the sample), 75 μL of fluorescein, 100 μL of the substrate, and 75 μL of the enzyme were added in a black

96-well-microplate and read in a microplate reader SynergyHTX (Bio-Tek Instruments, Winooski, VT, United States) every 10 s for 15 min. The fluorescent parameters are given: temperature = 27 °C, wavelength excitation = 485 ± 20 nm and emission = 530 ± 20 nm. To compare inhibition values with positive control, the same procedure was done substituting the extract for quercetin (Sigma-Aldrich, Madrid, Spain) at 0.85 mg/mL (25–250 μg/mL). Values were obtained in triplicate. The calibration curves were built to obtain LOX inhibition percentage, which was calculated according to Equation (2). Results were expressed as IC_{50} (%).

3. Results and discussion

3.1. Extraction yield

The pacová fruit parts were separated manually. Different extraction processes aiming to recover the non-polar fraction of pacová seeds were performed in this work. Seeds presented a firm aspect, and after triturated, a blank wax was observed. The global yields (%, w/w) for SFE, Soxhlet, and maceration are presented in Table 1.

All extracts presented a dark-orange color and intense aroma. Maceration presented the higher yield recovery of lipid extracts. This extraction's advantage is its physical principle, in which no heat is applied, resulting in a higher content in volatiles, perhaps justifying its higher yield. Therefore, it is reasonable to infer that a significant content of thermolabile compounds might be present in pacová seeds. On the other hand, the SFE, which applies lower temperature than Soxhlet method, offers the advantage of using a green solvent and the recovery of a solvent-free-product (Brunner, 2005). In this work, there were no significant differences observed in the yield of pacová seed extracts obtained by SFE and SOX. In their studies, Singh, Kiran, Marimuthu, Isidorov, and Vinogorova (2008) tested different solvents in Soxhlet extractor to obtain oleoresin from *Elettaria cardammon* seeds, finding 3.9–4.6% yield in a dry basis. The results found in this work are in agreement with the seeds extract yield (except for maceration where no temperature is applied), despite no work reporting extraction yield of *R. petasites* seeds was found in literature for a more reasonable comparison.

3.2. Characterization of the lipid extracts

The pacová seed extracts obtained by different extraction methods were characterized according to their fatty acids profile and terpenes content, as described in sections 3.2.1 and 3.2.2, respectively. Antioxidant capacity results are presented in section 3.2.3.

3.2.1. Fatty acids profile

An unknowns analysis strategy, based on peak detection, deconvolution and MS database search (i.e., NIST and Fiehn databases) was performed for the characterization of the lipid fraction of pacová seed extracts. Derivatization was carried out to improve the detectability of saturated fatty acids (SFA), and unsaturated fatty acids (such as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)), which were identified as trimethylsilyl (TMS) derivatives. The results revealed the presence of 13 major fatty acids in the target extracts, as summarized in Table 2. According to the results, SFE contained almost 46% of oleic acid, the most abundant fatty acid in SFE extracts, followed by palmitic and stearic acids. Similarlly, oleic and palmitic acids were also the most abundant fatty accids in SOX extracts, totalizing almost 30 and 20%, respectively. Likewise, MAC extracts were classified as richer in oleic and palmitic acids, in concentrations very similar to SOX extracts. Among the beneficial properties attributed to oleic acid, antimicrobial activity is enhanced due to the long unsaturated chain (Zheng et al., 2005). Even though there are not reported studies about the oilseed composition of *R. petasites*, Parry et al. (2006) characterized cardamom (from the same family of pacová, Zingiberaceae) oilseed extracted by cold pressing. They found a composition very similar to those obtained in this work: oleic and palmitic acids, in concentrations of 49.2 and 26.4%, respectively.

SFE extracts had considerably lower content of saturated fatty acids (SFA), which is accounted in SOX and MAC mostly by palmitic, behenic, lignoceric, and hexacosanoic acids. Moreover, arachidic acid was less concentrated in SFE than in SOX and MAC extracts, contributing to the smallest SFA level in SFE extracts. On the other hand, MUFAs were more concentrated in SFE, followed by MAC and SOX extracts. As noticed in Table 2, despite the higher content in palmitic acid in the SFE extract, supercritical CO_2 was more selective to extract MUFAs and PUFAs from pacová seeds than the other organic solvents. MUFAs characteristics include a double bond and a liquid aspect at room temperature, and they are found in a wide variety of oils, especially those from nuts and seeds (Gillingham, Harris-Janz, & Jones, 2011). The considerably higher content in MUFAs (here represented by the oleic acid) is of great importance when considering these molecules are less susceptible to oxidation than PUFAs (Owen et al., 2004), which is a positive quality attribute of the lipid extract. Besides, some essential health benefits associated with a high intake of MUFAs were elsewhere reported (Jakobsen, O'Reilly, Heitmann, Pereira, & Bälter, 2009). For instance, dietary MUFA seemed to decrease the risk of cardiovascular health diseases, but the lipidemic effects of individual SFA (usually present in high content MUFA oils) should be considered in assessing cardiovascular risks (Gillingham et al., 2011).

In general, SFE extract was less concentrated in SFA, known by their adverse effects on coronary heart disease risk (Siri-Tarino, Sun, Hu, & Krauss, 2010). However, the overall nutritional aspects of the pacová extracts should be deeply investigated (depending on their final application) to establish an adequate intake that does not harm consumer's health. For instance, virgin olive oils demonstrated their healthy benefits due its high antioxidant properties mainly because of the presence of phenolics and squalene (Owen et al., 2004). The oleic acid in olive oils accounts with around 70% of the total fatty acids (Matthäus, 2011). Similar to olive oils, pacová seed extract also demonstrated to be rich in oleic acid, making these oils less susceptible to lipid oxidation. In contrast, another oil widely used in food industry is palm oil, which contains approximately 44% of palmitic acid and 40% of oleic acid of the total fatty acid content (Mba, Dumont, & Ngadi, 2015). In this work, similar composition in fatty acids was observed for pacová seed extracts. Despite the controversial benefits of oils rich in pamitic acid, there is no clear evidence palm oil has a negative role in health (Fattore & Fanelli, 2013). Despite the remaining gaps in the context of dietary fats and health bennefits, studies are encouraged to deeply investigate the presence of oleic acid and other antioxidants such as carotenoids, terpenes and tocols, and their potential as bioactive compounds (Mba et al., 2015).

Table 1
Global yield of pacová seed extracts obtained by different extraction methods.

Extraction method	Solvent	Yield (%, w/w)
Soxhlet (SOX)	Petroleum ether	3.13 ± 0.11^{a}
Supercritical fluid extraction (SFE)	Supercritical CO_2	4.15 ± 0.78^{a}
Maceration (MAC)	Hexane	8.27 ± 1.43^{b}

Equal letters indicate no statistical difference between values at a 95% of confidence level from Tukey's test.

3.2.2. Terpenes profile

The GC-qTOF-MS unknowns analysis of pacová seed extracts also allowed the identification of the main terpenes of these samples, as summarized in Table 3. SFE extract showed the higher number of identified terpenes (considering a minimum relative area of 0.5%), accounting for a total of 20 compounds. In this extract, 2-carene, labdadiene, and spathulenol were the most abundant terpenes. All extracts contained mono-, sesqui-, and diterpenes. The intense aroma of the

Table 2
Fatty acids identified as trimethylsilyl (TMS) derivatives of pacová seed extracts obtained by different extraction methods.

Fatty acid	Molecular formula	Main fragments (*m/z*)	RT (min)	Match factor	Relative area percent (%)		
					SFE	SOX	MAC
Myristic acid	$C_{14}H_{28}O_2$	75.0263; 117.0365; 285.2259	16.52	82	0.17 ± 0.02	0.26 ± 0.01	0.24 ± 0.01
Pentadecanoic acid	$C_{15}H_{30}O_2$	73.0461; 117.0365; 299.0723	17.51	76	0.19 ± 0.01	0.21 ± 0.01	n.d.
trans-Palmitoleic acid	$C_{16}H_{30}O_2$	75.0268; 129.0367; 311.2404	18.28	86	0.93 ± 0.01	n.d.	0.23 ± 0.03
Palmitic acid	$C_{16}H_{32}O_2$	73.0469 ;117.0370 ;313.2575	18.47	88	40.02 ± 0.01	19.02 ± 1.19	23.93 ± 1.28
Eicosapentaenoic acid	$C_{20}H_{30}O_2$	95.0812; 183.1129; 319.2105	19.34	70	0.77 ± 0.06	n.d.	n.d.
Heptadecanoic acid	$C_{17}H_{34}O_2$	55.0533; 129.0367; 327.0240	19.39	70	0.10 ± 0.00	n.d.	n.d.
Linoleic acid	$C_{18}H_{32}O_2$	67.0536; 81.0689; 337.2554	19.99	91	4.04 ± 0.31	3.60 ± 0.49	1.52 ± 0.24
Oleic acid	$C_{18}H_{34}O_2$	55.0533; 117.0370; 339.2722	20.05	93	45.66 ± 0.38	29.70 ± 1.43	36.15 ± 0.94
Stearic acid	$C_{18}H_{36}O_2$	73.0469; 117.0370; 341.0239	20.27	88	7.72 ± 0.58	8.71 ± 1.21	7.73 ± 0.14
Arachidic acid	$C_{20}H_{40}O_2$	73.0469; 117.0370; 369.1156	21.93	97	0.31 ± 0.01	12.26 ± 0.54	8.95 ± 1.07
Behenic acid	$C_{22}H_{44}O_2$	73.0468; 117.0366; 397.3476	23.47	86	n.d.	16.16 ± 0.74	13.76 ± 0.43
Lignoceric acid	$C_{24}H_{48}O_2$	73.0468; 117.0366; 425.3782	24.83	77	n.d.	6.96 ± 0.30	5.41 ± 0.36
Hexacosanoic acid	$C_{26}H_{52}O_2$	73.0468; 117.0366; 453.4090	26.17	82	n.d.	3.02 ± 0.01	2.09 ± 0.29
Saturated (SFA)					48.60 ± 0.64[b]	66.61 ± 1.44[a]	62.10 ± 1.03[a]
Monounsaturated (MUFA)					46.59 ± 0.39[a]	29.70 ± 2.03[b]	36.38 ± 1.37[c]
Polyunsaturated (PUFA)					4.81 ± 0.25[a]	3.60 ± 0.69[a]	1.52 ± 0.34[b]

n.d. = not detected. Equal letters in the same row indicate no statistical difference between values at a 95% of confidence level from Tukey's test.

Table 3
Terpenes composition in pacová seed extracts.

Terpene name*	Molecular formula	Main fragments (*m/z*)	RT (min)	Match factor (%)	Relative area percent (%)		
					SFE	SOX	MAC
3-Thujene	$C_{10}H_{16}$	77.0382; 91.0538; 93.0700	6.34	89	n.d.	3.72 ± 0.42	0.84 ± 0.03
γ-Terpinene	$C_{10}H_{16}$	65.0355; 93.0660; 121.0969	6.44	87	5.04 ± 0.02	n.d.	n.d.
Terpinen-4-ol	$C_{10}H_{18}O$	43.0517; 71.0816; 93.0660	8.51	91	1.17 ± 0.04	n.d.	n.d.
α-Terpineol	$C_{10}H_{18}O$	59.0462; 93.0660; 121.0969	8.71	93	2.30 ± 0.33	0.49 ± 0.05	n.d.
Sabinol	$C_{10}H_{16}O$	81.0659; 91.0505; 92.0561	8.84	90	1.11 ± 0.01	n.d.	n.d.
3-Carene	$C_{10}H_{16}$	77.0348; 93.0660; 121.0969	10.30	86	0.59 ± 0.06	n.d.	n.d.
2-Carene	$C_{10}H_{16}$	93.0700; 121.1014; 136.1232	10.78	81	24.16 ± 4.88	71.56 ± 0.87	78.02 ± 0.97
α-Cubebene	$C_{15}H_{24}$	105.0697; 119.0853; 161.1321	11.07	90	n.d.	0.84 ± 0.07	0.86 ± 0.01
Caryophyllene	$C_{15}H_{24}$	79.0505; 93.0660; 105.0656	11.34	91	1.49 ± 0.29	n.d.	n.d.
α-Santalene	$C_{15}H_{24}$	41.0390; 79.0537; 94.0733;	11.59	93	n.d.	2.93 ± 0.13	1.73 ± 0.00
cis-α-Bergamotene	$C_{15}H_{24}$	41.0391; 93.0705; 119.0855	11.88	88	5.56 ± 0.69	2.46 ± 0.21	1.54 ± 0.02
Aristolene	$C_{15}H_{24}$	79.0541; 91.0542; 105.0696	12.18	92	n.d.	1.07 ± 0.16	1.31 ± 0.01
Alloaromandendrene	$C_{15}H_{24}$	91.0505; 119.0811; 204.1853	12.32	95	4.60 ± 0.54	n.d.	n.d.
Germacrene D	$C_{15}H_{24}$	91.0541; 105.0692; 161.1329	12.44	78	n.d.	1.73 ± 0.04	2.81 ± 0.46
Ylangene	$C_{15}H_{24}$	105.0656; 133.0107; 161.1285	12.57	89	8.17 ± 1.12	n.d.	n.d.
β-Bisabolene	$C_{15}H_{24}$	41.0388; 67.0538; 93.0705;	12.69	88	n.d.	0.86 ± 0.08	0.59 ± 0.00
Spathulenol	$C_{15}H_{24}O$	43.0545; 93.0660; 119.0811	13.76	95	13.65 ± 0.88	8.69 ± 0.38	7.23 ± 0.13
Globulol	$C_{15}H_{26}O$	67.0509; 93.0660; 107.0810	14.11	93	1.90 ± 0.17	n.d.	n.d.
Isospathulenol	$C_{15}H_{24}O$	43.0517; 91.0505; 119.0811	14.40	93	1.74 ± 0.01	n.d.	n.d.
τ-Cadinol	$C_{15}H_{26}O$	43.0517; 109.0963; 161.1285	14.66	81	0.97 ± 0.11	n.d.	n.d.
Spathulenol (2)	$C_{15}H_{24}O$	43.0545; 93.0660; 119.0811	15.44	95	2.29 ± 0.04	n.d.	n.d.
Isospathulenol (2)	$C_{15}H_{24}O$	43.0517; 91.0505; 119.0811	15.65	76	1.67 ± 0.20	n.d.	n.d.
Isospathulenol (3)	$C_{15}H_{24}O$	43.0517; 91.0505; 119.0811	16.56	88	1.29 ± 0.11	n.d.	n.d.
Alloaromadendrene (2)	$C_{15}H_{24}$	91.0505; 119.0811; 204.1853	18.83	83	3.01 ± 0.07	n.d.	n.d.
Labdadiene	$C_{20}H_{34}$	41.0390; 95.0853; 137.1323	21.57	89	16.83 ± 0.16	5.66 ± 0.35	5.07 ± 0.40
Labdadiene (2)	$C_{20}H_{34}$	79.0505; 91.0505; 95.0813	23.97	84	2.47 ± 0.06	n.d.	n.d.

n.d. = not detected. *Numbers in brackets represent a compound isomer. Classification of terpenes according to number of carbon atoms: C_{10} = monoterpenes; C_{15} = sesquiterpenes; C_{20} = diterpenes.

target extracts suggests a high content of highly volatile compounds. CO_2 was more selective for sesqui- and diterpenes, achieving 46.34 and 19.3% in its composition, respectively. On the other hand, SOX and MAC showed a higher concentration in monoterpenes, accounting for almost 80% of their composition (Table 3). The monoterpene 2-carene was the major compound in all extraction methods, and the solvents used in SOX and MAC extraction were more selective in its concentration than CO_2 used in SFE. Thus, the other three common terpenes (labdadiene, spathulenol, and *cis*-α-bergamotene) seemed to appear largely in SFE extracts. In their work, Bagheri, Manap, and Solati (2014) demonstrated that SFE (30 MPa and 40 °C) using CO_2 was more selective to terpenes in *Piper nigrum* essential oil than hydrodistillation, corroborating the results of this work. Fig. 1 shows the relative area % of the prevailing terpenes found in each extraction method applied, while a piece of complete information regarding composition can be seen in Table 3.

Renealmia petasites Gagnep. oilseed presented a unique terpene chemical profile in comparison with the most studied species so far, *Renealmia alpinia* (Lognay et al., 1991; Maia et al., 2007; Sekiguchi et al., 2001). For instance, Lognay et al. (1991) reported the characterization of *Renealma alpinia* Rott. seed oleoresin, and α-phellandrene, β-pinene, and limonene were the main terpenes. The authors performed steam distillation to concentrate the volatile fraction, finding a smaller number of terpenes (16) than the SFE extract in the present work (20), possible due to thermal degradation of some compounds.

2-Carene was previously reported by Salim et al. (2016) in *Zingiber anamalayanum* specie, also from the Zingiberaceae family. The authors reported the chemical profile of the rhizome oil, obtained by GC–MS. In such extracts, δ-2-Carene was predominant among terpenes, representing 52.83%, followed by camphene (9.83%), and *endo*-Fenchol (9.42%). Their findings suggested that the oil has a potential cytotoxic activity

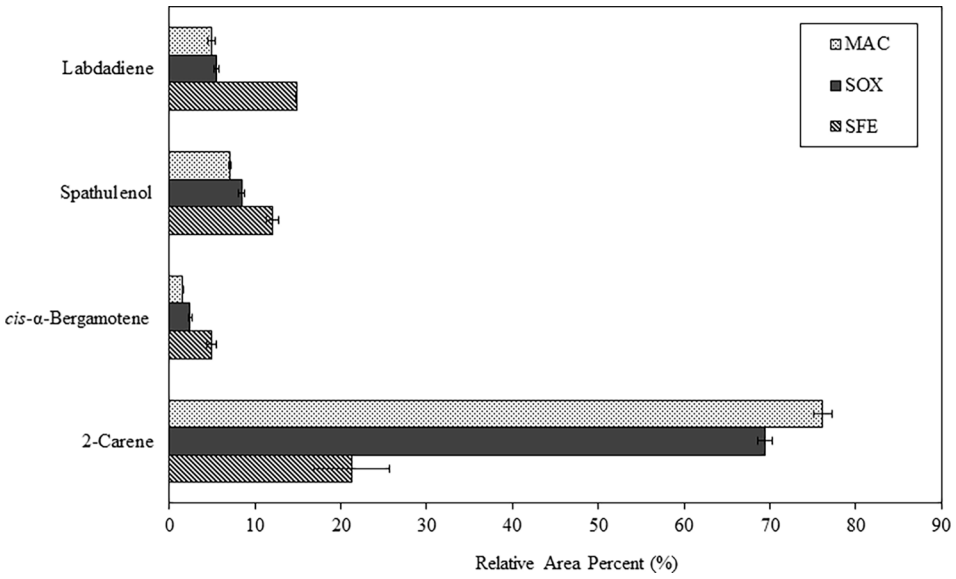

Fig. 1. Major terpenes in pacová seed extracts.

against DLA cells, where 50 μg of extract/mL was able to inhibit 89% of the cells. The biotransformation of the monoterpenes 2-carene and 3-carene originates oxygenated terpenes that find application in antifungal and antibacterial mechanisms, playing an essential role in plant defense (Dvorakova, Valterova, Saman, & Vanek, 2011). In fact, oxygenated mono- and sesquiterpenes were found to be the main constituents in different species of Zingiberaceae family (Mesomo et al., 2013).

The main sesquiterpene alcohol identified in this work was spathulenol, a volatile compound derived from alloaromadendrene (do Nascimento et al., 2018), also identified in the SFE extract of this work (Table 3). do Nascimento et al. (2018) evaluated antioxidant capacity, antiproliferative effects, and antimycobacterial activity of essential oil of *P. guineense* leaves. The authors observed potential therapeutical effects of the essential oil, attributing it to synergy associated with some sesquiterpene alcohols as spathulenol and α-bisabolol. Labdadiene was isolated and quantified in *Alpinia zerumbet*, in different parts of the plant, by Chompoo, Upadhyay, and Fukuta (2012). The authors found the highest concentration in the seeds (1 mg/g of ethanolic extract), followed by rhizomes (0.81 mg/g of aqueous extract) and pericarp (0.75 mg/g of aqueous extract). In the present work, we have identified labdadiene and one of its isomers, this last exclusively in the SFE extract. The last common terpene is *cis*-α-bergamotene, also identified in the heartwood of *Cedrus brevifolia* (Douros, Christopoulou, Kikionis, Nikolaou, & Skaltsa, 2019) and essential oil of *Aeollanthus suaveolens* (de Oliveira Ferraz et al., 2020).

The best terpene selectivity of SFE with CO_2 allowed the identification of some compounds not found in MAC or SOX extracts, namely γ-terpinene, caryophyllene and alloaromadendrene.

3.3. Antioxidant capacity

The antioxidant capacity of the extracts was determined by DPPH and ORAC-Lipophilic methods. The results are presented in Table 4.

As observed in Table 4, the pacová seed extracts presented good antioxidant capacities. SOX presented higher DPPH scavenging capacities, followed by SFE and MAC, respectively. On the other hand, ORAC-L, an important free-radical scavenging method, did not present any significant difference between SFE and SOX's antioxidant capacity. Parry et al. (2006) compared antioxidant capacity by DPPH of different seed oils, finding cardamom (from the same family of pacová) seed oil 4.34 and 2.56 times more efficient than parsley and onion oilseed,

Table 4
Antioxidant capacity for pacová seed extracts.

	SFE	SOX	MAC
DPPH EC_{50} (mg of extract/ mL)	2.15 ± 0.09^{a}	1.59 ± 0.06^{b}	2.35 ± 0.08^{c}
ORAC-L EC_{50} (μg of extract/ mL)	14.5 ± 0.5^{a}	14.85 ± 0.79^{a}	27.91 ± 1.9^{b}
ORAC-L value (mg TE/ g of extract)	111.15 ± 14.26^{a}	93.67 ± 4.37^{a}	43 ± 3^{b}

Equal letters in the same row indicate no statistical difference between values at a 95% of confidence level from Tukey's test.

respectively. The same authors also performed ORAC, obtaining 235.65 mg TE/g extract for cardamom oilseed, higher than the values obtained to the pacová seed extracts in the present study, suggesting that more antioxidant compounds were present in cardamom seeds. A recent work from Soares et al. (2021) found good DPPH IC_{50} results (<0.1 mg of extract/mL) for hydroethanolic extracts of stems, leaves and rhizomes of *R. petasites*. Likewise, Chompoo et al. (2012) evaluated antioxidant capacity from different *Alpinia zerumbet* parts (from the same family as pacová), including seeds. The authors found the best DPPH IC_{50} result for the aqueous seed extract ($IC_{50} = 0.01$ mg/mL), where no significant differences were observed compared with BHT, a commercial antioxidant. According to the same authors, the ethanolic seed extracts presented an IC_{50} more than ten times higher than aqueous extract, which is possibly related to the less phenolic content in ethanolic seed extract. The results indicated that extracts rich in polar compounds surpass the antioxidant capacity of terpene-rich extracts from the seeds, as evidenced in this work. However, this information does not limit the discovery of other essential health benefits associated with these compounds.

3.4. Biological activities (AChE and LOX assays)

AChE and LOX can play key roles in neurological disorders due to their regulation in cholinergic system and neuroinflammatory response, respectively (Sánchez-Martínez et al., 2021). Fig. 2 present the IC_{50} values for pacová seed extracts for AChE and LOX assays, respectively.

The AChE inhibition improves the cholinergic function due to the increase of the acetylcholine neurotransmitter in Alzheimer's disease patients (Mushtaq, Greig, Khan, & Kamal, 2014). The AChE inhibition for pacová seed extracts is in accordance with previous studies that also

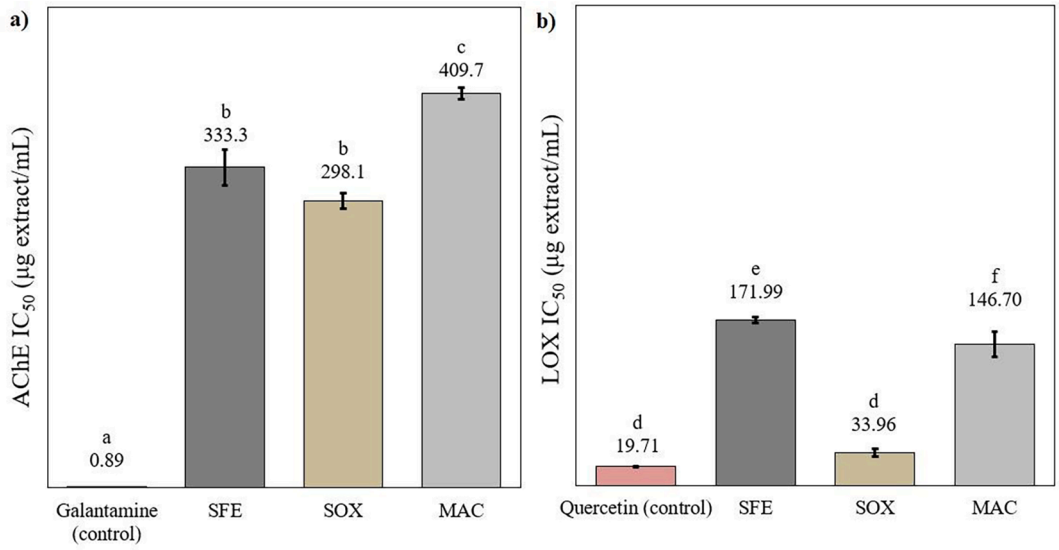

Fig. 2. a) AChE and b) LOX Inhibitory extract concentration (IC_{50}). Same letters indicate no statistic difference between values at a 95% of confidence level from Tukey's test.

tested oilseeds in AChE inhibition, resulting as poor inhibitors (IC_{50} > 200 µg/mL). However, Czernicka, Ludwiczuk, Rój, Marzec, and Jarzab (2020) used thin layer chromatography (TLC) to report the terpenes found in *Zingiber officinale* diethyl ether roots extract as potential AChE inhibitors, obtaining satisfactory inhibitory results for α-curcumene, α-sesquiphellandrene and α-zingiberene. Although many terpenes were identified in *R. petasites* in the present study, two possibilities may have led to the poor AChE inhibition: first, the identified compounds may not present bioactivity on the enzyme inhibition or, second, the synergy of the high number of compounds present in the seeds extract did not present a satisfactory effect on enzyme inhibition. According to a review done by Santos et al. (2018), the plant extracts that better inhibit AChE are obtained from roots and leaves, whereas seed extracts reported in literature always presented an IC_{50} > 233 µg/mL. The same authors analyzed over than 50 plant species in their compilation, but none is representative of the Zingiberaceae family. However, Khatri and Juvekar (2016) used different extract concentrations of root extracts from curcumin (*Curcuma longa,* from Zingiberaceae family) in Parkinson's induced mouse, observing, after 21 days of treatment, a significant decrease of almost 50% in AChE activity at 100 mg of curcumin extract/kg dosage.

As for LOX activity, the concentration of extract needed to inhibit this enzyme was significantly lower for SOX extracts than SFE and MAC. Considering the similarity in terpene profile and ORAC values for both SOX and MAC, we believe the inhibition might be associated with other non-identified compounds presented in the SOX extract. Neuroinflammation can play an important role in the well-functioning of neural reactions, but when oxidative stress in brain cells is over control, inflammation would worsen a neurodegenerative disorder, so inhibition of LOX might be helpful (Giannopoulos et al., 2014). In scientific literature, anti-inflammatory activity from *R. petasites* was only reported in respect of joint inflammation. Soares et al. (2021) evaluated the *R. petasites* extracts from leaves, stem and rhizomes in an anti-inflammatory model *in vivo*. The authors noted a decrease in leukocyte influx, representing the potential therapeutical strategy in such an inflammatory process. Chompoo et al. (2012) evaluated the *Alpinia Zerumbet* polar extracts from different plant parts in the inhibitory efficiency of some skin diseases-related enzymes (collagenase, elastase, hyaluronidase, and tyrosinase). The authors found potential inhibitory effects for aqueous rhizome extract for all the enzymes. Moreover, regarding the same work, it is important to note that no correlation was observed between antioxidant capacity and enzyme inhibition since the best antioxidant (aqueous seed extract) was considered a poor inhibitor for the selected enzymes. The mentioned results indicate that natural plant extracts play an important role in biological activities and should be widely investigated.

4. Conclusion

Terpene and lipid profiles of *R. petasites* Gagnep. oilseed were reported for the first time in literature. 2-Carene, spathulenol, labdadiene and *cis*-α-bergamotene were the prevailing compounds for all extraction methods. The lipid extracts demonstrated good antioxidant capacities, although extracts richer in polar compounds seemed to present improved antioxidant potential, according to previous studies regarding fruit seeds from the Zingiberaceae family. SFE recovered more sesqui- and diterpenes than SOX and MAC. However, the possibility of using SFE extracts as food ingredients should be better investigated regarding its nutritional concerns because of the high content in palmitic acid. In addition, the present work suggests that supercritical CO_2 could be further applied to investigate fractionation processes of terpenes and/or lipid content. AChE IC_{50} showed poor efficiency, similarly to other plant seed extracts already reported in the literature. Nevertheless, LOX IC_{50} results, demonstrated promising inhibitory concentration for SOX extracts compared to the other tested extraction methods, even though more detailed studies are needed to establish a precise relationship between anti-inflammatory activities and pacová seed extracts.

CRediT authorship contribution statement

Luana Cristina dos Santos: Conceptualization, Data curation, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gerardo Álvarez-Rivera:** Formal analysis, Writing – review & editing. **José David Sánchez-Martínez:** Writing – review & editing. **Julio César Flores Johner:** Investigation, Methodology. **Francisco Manuel Barrales:** Investigation. **Alessandra Lopes de Oliveira:** Conceptualization, Writing – review & editing. **Alejandro Cifuentes:** Funding acquisition, Resources, Writing – review & editing. **Elena Ibáñez:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Julian Martínez:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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