GREEN CHEMISTRY: AZEOTROPIC SOLUTION EXTRACTION AND PURIFICATION OF THE BIOACTIVE COMPOUND SPILANTHOL FROM JAMBU (*Acmella oleracea (L.) R. K. Jansen).*

Aila T. Castelo Branco[1*], Fernanda L. A. Silva[2], Maria A. M. Castelo Branco[3], Maria J. B. Silva[4], José de A. R. do Rêgo[5], Davi do S. B. Brasil[6], Nathiel S. Moraes[7].

[1] *Chemical Engineering, UFPA/ITEC, Belém/Brazil.*
[2] *Chemical Engineering, UFPA/ITEC, Belém/Brazil.*
[3] *Biotechnology, UFPA/ICB, Belém/Brazil.*
[4] *PPGCMA, UFPA/ICEN, Belém/Brazil.*
[5] *UFPA/ICEN, Belém/Brazil.*
[6] *UFPA/ITEC, Belém/Brazil.*
[7] *PPGCMA, UFPA/ICEN, Belém/Brazil.*
* *ailacastelobranco@gmail.com*

ABSTRACT

Acmella oleracea is a species native to the Amazon. In the study of the chemical activities of jambu, spilanthol is its main bioactive, and is of great relevance in the pharmaceutical industry, being mostly extracted via supercritical fluid or hydroalcoholic solutions. In this work, the extraction is performed by an acidic azeotropic solution with the aim of enhancing its antioxidant activity. The raw material was liquefied with an azeotropic solution of citric acid:distilled water:ethanol, divided into two processes, the first (J1) filtered immediately, the second (J2) sonicated for one hour after 24 hours in BOD at 6°C and filtered. Column chromatography was carried out on both samples and the fractions obtained containing spilanthol and chlorophyll (S1, S2 and CL2) were subjected to UV-vis scanning, ATR-FTIR, ABTS, DPPH and FRAP analyses. The graphical analyses showed that the extractions and column purifications of the bioactives were successful. The antioxidant analyses showed excellent results, expressed in μM TE/g, for spilanthol (DPPH 927-917, ABTS 2077-1673 and FRAP 1357-1218) and chlorophyll (DPPH 149, ABTS 460, FRAP 744). It can be concluded that the extraction, under the conditions used, was successful, opening up possibilities for acid extractions of bioactives from jambu.

Keywords: Jambu. Green extraction. Spilanthol. Antioxidant.

1 INTRODUCTION

Acmella oleracea (L.) R.K. Jansen belongs to the Asteraceae family and is native to the Amazon[2]. In Brazil, it is widely cultivated in the northern region, more precisely in Pará, where it is known as jambu. In the last years it has been the subject of much research and study in other countries such as Mexico, India, Colombia and the United States, as it has important chemical and biological properties[3]. Other scientific names are also given to this species such as: *Spilanthes oleracea* L., *Spilanthes acmella* var. *oleracea* (L.), *Spilanthes fusca*, *Spilanthes oleracea fusca* (Lam.) D. C.[2]. Therefore, all these names refer to the same species.

Figure 1 *Acmella oleracea* (L.) R. K. Jansen.

In the study of the chemical activities of jambu, they refer to spilanthol as its main bioactive compound. Spilanthol is an alkylamine (alkaloid), present in the genus Acmella and found in all parts of the plant, in higher concentration in its inflorescences. Biosynthetically, spilanthol is composed of α-linolenic acid and the amino acid valine, conferring both hydrophilic and lipophilic properties due to the presence of the relatively polar amide and the less polar fatty acid. Several species containing alkylamides have been used in traditional medicines, including those from Asteraceae, Solanaceae and Piperaceae families. Numerous biological activities have already been attributed to spilanthol, including[1,2,3,4]: analgesic, antinociceptive, anxiolytic, anti-inflammatory, antimutagenic, antifungal, bacteriostatic, insecticidal, antiparasitic, and antioxidant activities.
In short, the extractions developed to obtain the active ingredient of the genus Acmella are aqueous, hydroalcoholic, methanolic or with supercritical fluid. The aim of this study is to extract and separate spilanthol with a hydroalcoholic acid azeotropic solution in order to enhance the antioxidant activity of this bioactive.

2 MATERIAL & METHODS

The raw material was obtained from the "ver-o-peso" street market, sanitized under running water, removing parts that were not interesting to this research, such as very fibrous stems and all the roots, thus using flowers, leaves and thin stems. The obtained mass of jambu was liquefied in a 2:1 (m/v) ratio of solute and extracting solution. The extracting solution, called citric acid azeotropic solution (AZC sol.), was obtained from a mixture of citric acid P.A., distilled water and ethanol P.A. in a 3:8:1 (m/v/v) ratio.

Half of the volume of this mixture (100 mL) was called sample J1 and filtered through qualitative paper immediately, while the other half, called sample J2, was subjected to cooling for 24 hours in a Biochemical Oxygen Demand (BOD) at 6°C, then taken to an ultrasound bath heated to 47°C for 1 hour and filtered through qualitative paper, called sample J2. Following this process, the two mixtures obtained were subjected to chromatography on a silica gel 60 column (70-230 mesh) using ethyl acetate, ethanol and water in a 1:3:1 (v/v/v) ratio as the eluent. The ethanol fractions of the two samples containing spilanthol and the water fraction of the J2 sample containing chlorophyll, a by-product of the extraction of the bioactive from the plant, were subjected to analysis.

The analyses carried out were scannings on the Shimadzu UV-1800 Ultraviolet-visible (UV-vis) spectrophotometer and the Bruker's Vertex 70v Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectrometer and antioxidant assays.

The antioxidant assays carried out were:

The first, using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) method[8], carried out in triplicate using 30 µL of sample in 3 mL of previously prepared ABTS radical, which was left to react for 6 minutes, then read in a UV spectrum, zeroed in ethanol, at the 734 nm range. The result was expressed in µM Trolox Equivalent (TE)/g of sample from the Trolox standard curve (100 µM-2000 µM);

The second, by the 1,1-diphenyl-2-picrylhydrazyl (DPPH)[10] method, performed in triplicate, beginning with the reaction of 5.085 mL of ethanolic DPPH solution and 150 µL of sample for 30 minutes, read, afterwards, at the 515 band of the UV spectrum, zeroed in ethanol. The percentage of inhibition was obtained in µM TE/g from the Trolox standard curve (50 µM-1000 µM);

The last, using the ferric reducing antioxidant power (FRAP)[9] method, which consisted of transferring an aliquot of 90 µL of the sample, while adding 270 µL of distilled water, as well as 2.7 mL of the FRAP reagent. The mixture was homogenized in a vortex and kept in a water bath at 37 °C for 30 minutes, then read at 595 nm. The FRAP reagent was used as a blank to calibrate the spectrophotometer, the antioxidant activity was calculated based on a Trolox standard curve (160 µM-1600 µM) and the final concentration was expressed in µM TE/g.

3 RESULTS & DISCUSSION

The samples were purified in a chromatography column to separate the chlorophyll found, discard the ethyl acetate fractions and preserve the best ethanol fractions, whose golden yellow hue contained spilanthol. For the fresh sample called J1, after purification, the sample S1, containing spilanthol, was obtained from the ethanol eluent after purification. For the sonicated sample J2, the ethanol fraction also contained spilanthol and was called S2. In addition, the distilled water fraction contained chlorophyll and ws called CL2. The 5 samples and the extracting solution without sample were subjected to UV-vis and ATR-FTIR, as shown in figure 2, while the numerical results of the antioxidant analysis can be seen in table 1.

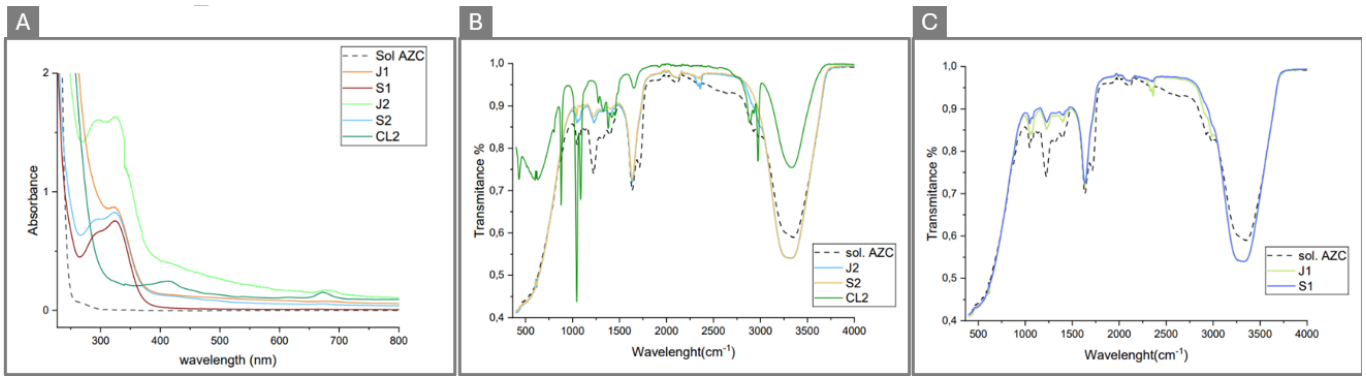

Figure 2 UV-vis and ATR-FTIR scanning graphs of jambu comparing the fresh and sonicated extractions and the extracting solution, with image A comparing the absorbance capacity with wavelength, in nanometers, while images B and C compare the percentage transmittance with wavelength, in centimeters^{-1}. **A:** UV-vis scanning of *Acmella oleracea*. **B:** ATR-FTIR scanning of the fresh *Acmella oleracea* sample. **C:** ATR-FTIR scanning of the sonicated *Acmella oleracea* sample. **Source:** Authors (2024).

Image A shows a significant difference in the behavior of the fresh and sonicated extractions, J1 and J2. For J1, there was a peak only in the 200-400 range; following the fractionation, in the same range appeared a perfect and clearly drawn spilanthol curve. Still using the same image A, sample J2 obtained a more complex graph: in addition to the 200-400 curve representing spilanthol, characteristic chlorophyll curves were observed in the ranges of 400-450 and 650-700, separated after fractionation in a column, generating the S2 graph for spilanthol and the Cl2 graph for chlorophyll, proven by comparison[4,6,7]. Still in the same range, J2 obtained a more interesting graph: in the ranges of 400-450 and 600-700, there was chlorophyll extraction only in S2, well demonstrated after column fractionation, generating the graphs S2 for spilanthol and Cl2 for chlorophyll, proven by

comparison[5,7,8]. Regarding the FTIR, shown in images B and C, J1, J2, S1 and S2, there was indicated slight differences; the samples before fractionation expressed peaks in the ranges of 3200-2800 and 1500-900, which were smoothed out in the S1 and S2 samples, indicating a decrease in C-H bonds and single bonds; another important decrease was within 2250-2500, indicating a decrease in the triple bonds between carbons or carbon-nitrogen, opening up the possibility that the citric acid used in the extraction was removed when the spilanthol was purified, given that the important undulations for demonstrating spilanthol continued to be expressed in the 4 samples. Graph 3 also shows CL2, and the double bonds of aromatic rings, esters and ketones in the range of 1800-1400 were weak compared to the single bonds expressed within 900-1400 of the same compounds, in line with the UV graph of chlorophyll, so it was identified as chlorophyll B[7].

Table 1: Antioxidant activity of spilanthol and chlorophyll samples, demonstrated by the results of ABTS, DPPH inhibition and FRAP assays.

Sample	ABTS (µM TE/g of the sample)	DPPH (%ini / µM TE/g)	FRAP (µM TE/g)
S1	86^{e} / 2077^{a}	70^{d} / 927^{b}	1357
S2	68^{e} / 1673^{a}	69^{d} / 917^{b}	1218
CL2	17^{e} / 460^{a}	9^{d} / 149^{b}	744
sol. AZC	5^{e} / 170^{a}	8^{d} / 125^{b}	392

Captions: a, b, c: µM TE/g sample; d, e: % inhibition of the radical by the sample. **Source:** Authors (2024).

The results of the antioxidant activity of the spilanthol and chlorophyll samples are shown in table 1. The extracting solution (sol.AZC) showed low results, such as 5% inhibition for ABTS and 8% inhibition for DPPH, but the extractions carried out using this solution obtained surprising results. While the chlorophyll obtained good results for FRAP and low DPPH and ABTS inhibition, the spilanthol samples obtained very high results. Compared to each other, S1 and S2 obtained slightly different results, with a significant difference only in ABTS, in which S1 inhibited 86% of the radical and S2 inhibited 68% of the radical. in the literature, the DPPH[6] results of spilanthol for different heat treatments of aqueous extracts came out within 12-41% inhibition; ABTS in ethanolic extracts[11] in the different parts of jambu varied between 4-16 µM TE/g of sample; and FRAP for sonicated methanolic extraction[4] of fresh and sonicated samples, the results were 42-103 µM TE/g per sample; which means that each literature presented a type of solvent subjected to variables that directly influenced their results. The heat treatment undergone in this work did not directly interfere with the high antioxidant results, as seen in table 1, but it did have an impact on the graphical demonstrations and the identification of *Acmella oleracea* chlorophyll.

4 CONCLUSION

This study achieved its objective to isolate, identify and significantly increase the antioxidant activity of the bioactive of interest, spilanthol. Furthermore, one of the extraction methods succeeded in isolating and identifying the chlorophyll extracted from *Acmella oleracea*. Therefore, two different methods of subjecting the material to the same solvent were explored, opening up possibilities for acid extractions of the bioactives present in jambu. In view of the importance of these compounds in the pharmaceutical field, it has become feasible to further develop this new methodology in subsequent studies, so that the antioxidant capacity of spilanthol can be more well utilized and recurrent.

REFERENCES

[1] DÉCIGA-CAMPOS, M. et al. Pharmacological and Toxicological Profile of Extract from Heliopsis l ongipes and Affinin. Drug Development Research, [s. l.], v. 73, n. 3, p. 130-137, 2012.

[2] LORENZI, H.; MATOS, F. J. A. Plantas medicinais no Brasil: nativas e exóticas. São Paulo: Instituto Plantarum de Estudos da Flora, 2002.

[3] NAKATANI, N.; NAGASHIMA, M. Pungent alkamides from Spilanthes acmella L. var. oleracea Clarke. Bioscience, Biotechnology, and Biochemistry, [s. l.], v. 56, n. 5, p. 759- 762, 1992.

[4] NEVES, D. A. CHEMICAL CHARACTERIZATION, DETERMINATION OF THE PRESENCE OF POLYCYCLIC AROMATI HYDROCARBON, AND EVALUATION OF THE EFFECT OF THE BOILING PROCESS IN CHEMICAL COMPOSITION, ANTIOXIDANT CAPACITY AND ANTIMICROBIAL ACTIVITY OF JAMBU (Acmella oleracea (L.) R.K. Jansen). Thesis (Ph.D.) Faculty of the Food Engineering of the University of Campinas. Campinas. 2018.

[5] NUNES, K. D. N. M. Thermal processing and phenological stage in the antioxidant activity of jambu. Thesis (Doctorate) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Ciências Agronômicas. Botucatu, p. 67. 2016.

[6] PETTERSON, A. R.; Lima, V. A.; Anaissi, F. J. CHLOROPHYLL EXTRACTED FROM INDUSTRIAL WASTE FROM MATE-HERB (Ilex paraguaiensis) A POSSIBILITY OF CIRCULAR ECONOMY. Chem. Nova 45 (07) - 2022. https://doi.org/10.21577/0100-4042.20170877.

[7] PINHEIRO, M. da S. P. ANALYSIS OF THE SPILANTHOL CONTENT IN THE SPECIES Acmella oleracea (L.) R. K. JANSEN (Jambu. Final course work (full degree in chemistry) - Faculty of Chemistry, Federal University of South and Southeast Pará. Marabá-PA. 2015.

[8] RE, R; Pellegrini, N et al. Trolox ASSAY. Int. Antioxid. Res. Center, Guy's, King's St Thomas' Sch. Biomed. Sci. Kings Coll. Campus London SE1 9RT UK 26. Free Radic. Biol. Med. 1999, 26, 1231-1237, doi:10.1016/s0891-5849(98)00315-3.

[9] RUFINO, M. S. M.; Alves, R. E et al. Metodologia científica: determinação da atividade antioxidante total em frutas pelo método de redução do ferro (FRAP). Fortaleza: Embrapa Agroindústria Tropical, 2006. 4 p. (Technical Communication).

[10] SILVEIRA, A. C; Kassuia, Y. S.; Domahovski, R. C.; Lazzarotto M (2018) Adapted DPPH method: a tool to analyze antioxidant activity of yerba mate fruit pulp in a quick and reproducible way Adapted DPPH method: a tool to analyze antioxidant activity of yerba mate fruit pulp in a quick and reproducible way. Technical Communication 421 - Embrapa, 2018.

[11] WEINTRAUB, L. et al. Antioxidant Activity and Antiproliferative Effects of Acmella alba, Acmella oleracea, and Acmella calirrhiza. The FASEB Journal, [s. l.], v. 34, n. S1, p. 1-1, 2020.

ACKNOWLEDGEMENTS

I am greatly thankful to the laboratories LEVAP, LABNANO, LABA, LEPRON, and of the UV spectroscopy, all laboratories from UFPA, and their coordinators for all the support in conducting this work, especially to my supervisor Nathiel Sarges Moraes, Dr. Lindalva Ferreira and laboratory technicians Rafaela Pinheiro, Graziela Telles and Samara Marques for their teachings, collaboration, patience, and dedication to the advancement of natural product science.