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Phytochemical analysis and biological activity from *Clidemia capitellata* leaves (melastomataceae)

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Palavras-chave

Clidemia Capitellata
Metabólitos Secundários
Antioxidante
Antimicrobiano

Keywords

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Resumo: Esta pesquisa determinou o perfil fitoquímico e avaliou as atividades fotoprotetora, antimicrobiana e antioxidante em extratos de folhas de *Clidemia capitellata*, obtidos com hexano, acetato de etila e metanol. Cromatografia em camada delgada foi usada para identificar os metabólitos secundários; os taninos, flavonoides e fenólicos totais foram dosados; a atividade antimicrobiana foi determinada por Concentração Inibitória Mínima e Concentração Microbicida Mínima nas bactérias *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, e na levedura *Candida albicans*; e a atividade antioxidante foi avaliada pelos métodos de Poder Redutor, sequestro de DPPH e fosfomolibdênio. As folhas de *C. capitellata* apresentam flavonoides, taninos, triterpenos, esteroides, antraquinonas e óleos essenciais. Os taninos, flavonóides e fenólicos totais foram extraídos em maiores quantidades com metanol, solvente cujo extrato também apresentou melhor atividade antioxidante, sendo mais eficaz em relação ao poder redutor. O extrato com acetato de etila apresentou melhor atividade contra *P. aeruginosa* and *S. marcescens*, mas nenhum apresentou atividade fotoprotetora dentro dos valores estabelecidos pela legislação brasileira.

Abstract: This research determined the phytochemical profile and evaluated the photoprotective, antimicrobial and antioxidant activities in extracts of *Clidemia capitellata* leaves, obtained with hexane, ethyl acetate and methanol. The phytochemical profile was determined through thin layer chromatography; tannins, flavonoids and total phenolics were measured; the antimicrobial activity was determined by Minimum Inhibitory Concentration and Minimum Microbicide Concentration in bacteria *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis* and *Enterococcus faecalis*; *Escherichia coli*, *Serratia marcescens* and *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, and the yeast *Candida albicans*. Antioxidant activity was evaluated through the methods of Reducing Power, DPPH sequestration and phosphomolybdenum. The *C. capitellata* leaves show flavonoids, tannins, triterpenes, steroids, anthraquinones and essential oils; tannins, flavonoids and total phenolics were extracted in greater amounts with methanol, solvent whose extract also showed better antioxidant activity, being more effective in relation to reducing power. The ethyl acetate extract showed better activity against *P. aeruginosa* and *S. marcescens*, but none showed photoprotective activity within the values established by Brazilian legislation.

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Introduction

The use of plants for the treatment, cure and prevention of diseases is one of the oldest medicinal practices and can be considered the origin of pharmacy and modern medicine (SALMERÓN-MANZANO *et al.*, 2020). Until the mid-nineteenth century, plants were the main therapeutic agents used by people (DEKEBO, 2019) and their role in medicine is still relevant today.

The therapeutic properties of plants have also given rise to many drugs, and have gained popularity because of their natural origin, availability in local communities and ease of administration, plus a useful alternative treatment option in case of side effects and drug resistance (SHUANG; WNG, 2020).

Thus, plants are extracted and processed for direct consumption as herbal medicines or prepared for experimental purposes in the search for therapeutic compounds. Preparation for experimental purposes involves proper and timely collection, expert authentication, proper drying and milling, followed by extraction, determination of quantity and quality of bioactive compounds, added to fractionation and, when applicable, isolation of the bioactive compound (ABUBAKAR; HAQUE, 2020).

The Brazilian flora is rich in different types of plants, but only a fraction of this potential is known and properly used compared to what still needs to be explored. In this scenario is Melastomataceae, the sixth largest family of Angiosperms in Brazil, with 68 genera and more than 1,500 species, being present in the most diverse plant formations (GOLDENBERG *et al.*, 2012).

Among the Melastomataceae genera is *Clidemia*, with 175 species distributed in the Neotropics, and in Brazilian territory, 50 of them are found from the Amazon to Santa Catarina. They are erect shrubs and sub-shrubs, rarely lianas or epiphytes (MATSUMOTO; MARTINS, 2009).

Plants of *Clidemia* genus, despite having medicinal properties for the treatment of various diseases, have few records in the scientific literature, as is the case of *Clidemia capitellata*, a species native from southern Mexico to Colombia and the West Indies, but which has become naturalized in several areas of Brazil (CHEN *et al.*, 2018). The plant is a shrub, widely distributed in environments such as forest edges, clearings and riverbanks and, in addition, has abundant glandular trichomes on the stem, leaves, flowers and fruits (ACERO-MURCIA, 2016).

Despite the well-described morphology, studies that examine phytochemistry and biological activity are scarce. Considering the importance of this type of investigation, whether for characterization and better knowledge of the species, or for its application in society, this article analyzed the phytochemical characteristics and evaluated the antimicrobial, photoprotective and antioxidant activities in extracts of *C. capitellata* leaves.

Materials and methods

Plant material

About of 2g of *C. capitellata* aerial parts were collected, identified by the botanist Dr. Rita de Cássia Pereira, curator of the herbarium of the Agronomic Institute of Pernambuco (IPA), and deposited in the IPA herbarium under number 93041.

Obtaining extracts

C. capitellata leaves were placed in an oven with controlled temperature (40 °C) and constant air renewal for 4 days, to dry. Subsequently, the leaves were ground in a knife mill and extracted by exhaustive maceration, with hexane (Hex), ethyl acetate (AcOEt) and methanol (MeOH), for 7 days at room temperature and protected from light.

Phytochemical analysis

Phytochemical profile

The phytochemical profile analysis was performed by Thin Layer Chromatography (TLC) according to Wagner; Bladt (2001). The extracts were tested at 5 mg/mL. As stationary phase, plates with silica gel F254 were used, and as mobile phase hexane, ethyl acetate and methanol in the proportions: hexane extract (Hex:AcOEt – 7:3), ethyl acetate extract (Hex:AcOEt – 6:4), and methanol extract (Hex:AcOEt:-MeOH – 2:6:2).

The extracts were applied to the base of the silica plate and placed to elute in the chromatographic vat with the eluents. The plates with certain developers were observed at 254 nm and 365 nm, and the visible aspects were drawn. Other developers required heating to 100°C until the appearance of colored bands that were observed in the visible and ultraviolet ranges.

Dosing of total phenolics

Dosing of total phenolics was performed according to Pieroni *et al.* (2011), with modifications. In a test tube, 0.2 ml of extract (500 mg/ml), 0.5 ml 10% Folin-Ciocalteu (v/v) and 1 ml 7.5% sodium carbonate solution (Na_2CO_3) were added. After shaking, the systems remained for 30 minutes in the dark, at room temperature. Posteriorly, 3 ml of distilled water was added and the reading was taken at 760 nm. A standard curve with gallic acid was drawn, and the phenol content was expressed in milligrams of gallic acid equivalent per gram of extract (mg EAG/g). All analyzes were performed in triplicate.

Dosing of total tannins

This analysis was performed according to Shad *et al.* (2012). In a test tube,500 µL of extract (500 mg/mL) and 2.5 mL10 % Folin-Ciocalteu were added. After stirring for 3 minutes, 2 ml 20% sodium carbonate (Na_2CO_3) were added and system stayed in rest for 2 h in the dark before a spectrophotometer reading be performed at 725 nm. A standard tannic acid curve was drawn up. The tannin content was expressed in milligrams equivalent of tannic acid per gram of sample (mg EAT/g). All analyzes were performed in triplicate.

Dosing of total flavonoids

The analysis was performed following Lima *et al.* (2020), where 1 mL extract (500 mg/mL), 4 mL distilled water and 300 µl 25% sodium nitrite ($NaNO_2$) were added to a test tube. After 5 minutes, 300 µL 10% aluminum chloride ($AlCl_3$), 2 mL 1 mol/L sodium hydroxide (NaOH) and 2.4 mL distilled water were added, and then the reading at 510 nm was made. A standard curve was drawn using rutin, and the flavonoid content was expressed as milligrams equivalent of rutin per gram of sample (mg ER/g). All analyzes were performed in triplicate.

Biological activity

Antimicrobial activity

Micro-organisms

Bacteria and yeast fungi strains used were obtained from the Collection of Microorganisms of the Department of Antibiotics of the Federal University of Pernambuco (UFPEDA). For this assay, four gram-positive bacteria were used: *Staphylococcus aureus* (UFPEDA 01), *Micrococcus luteus* (UFPEDA 06), *Bacillus subtilis* (UFPEDA 16) and *Enterococcus faecalis* (UFPEDA 138); three gram-negative bacteria: *Escherichia coli* (UFPEDA 224), *Serratia marcescens* (UFPEDA 398) and *Pseudomonas aeruginosa* (UFPEDA 39); an alcohol-acid-resistant: *Mycobacterium smegmatis* (UFPEDA 71); and the yeast *Candida albicans* (UFPEDA 1007).

Minimum Inhibitory Concentration (MIC) and Minimum Microbicide Concentration (MMC)

The determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Microbicide Concentration (MMC) was carried out using the broth microdilution methodology according to NCCLS (2002; 2003), in sterile 96-well plates, suitable for microdilution.

Initially, 90 µL of the Muller Hinton Broth (MHB) medium were added to each well, and from the third column (A3), 90 µL of the extract at a concentration of 16 mg/mL. This aliquot was homogenized and transferred to the fourth column (A4) and so on, until the twelfth column (A12), which received the extract at a concentration of 0.03 mg/mL. This last aliquot, after being homogenized, was discarded. Finally, 10 µl of the microorganism suspension were added to each well. Therefore, each well had a final volume of 100 µL (90 µL of medium and extract and 10 µL of the microorganism).

The plates with the medium, extract and microorganism were incubated for 24 hours (37 ºC) for bacteria, and 48 h (28 ºC) for yeast. Subsequently, 30 µL of rezasurin were added for qualitative analysis of the microbial growth in the test wells and determination of the relative antimicrobial activity of each sample dilution. To determine the Minimum Microbicide Concentration (MMC), an aliquot of 5 µL of the concentrations that showed activity in the MIC plate was replicated in Petri dishes containing Mueller Hinton Agar (MHA). These plates were incubated at 37 ºC for 24 h for bacteria, and 28 ºC for 48 h for yeast. The MMC was considered the lowest concentration of the extract where there was no cell growth on the MHA surface.

Antioxidant Activity

Reducing Power

The reducing power was evaluated according to Waterman; Mole (1994), where 100 µL of samples were used (concentration of 500 ppm) and 8.5 mL of distilled water, 1 mL 0.1 mol/L $FeCl_3$ solution were added. After 3 min, 1 mL 0.08 mol/L potassium ferricyanide solution was added, and homogenized. The reading at 720 nm was performed after 15 min. A standard curve was constructed using ascorbic acid, and the results obtained were expressed in milligrams equivalent to ascorbic acid per gram of extract (mg EAA/g). All analyze were performed in triplicate.

Free radical scavenging activity (DPPH)

The DPPH (2,2-diphenyl-1-picrylhydrazi) free radical scavenging activity was performed using the descriptive method by Cavin *et al.* (1998) with modifications. In test, 0.1 ml of the extract was placed in 3.9 ml 0.004% DPPH. After resting for 30 min, in the dark, the reading was taken at 517nm. All analyze were performed in triplicate.

Phosphomolybdenum Complex Reduction Assay

For the phosphomolybdenum complex reduction assay (PRIETO *et al.*, 1999), the extract was prepared at a concentration of 200 µg/mL, as well as the standard used, ascorbic acid. In test tubes, 0.3 mL aliquots of each sample were placed and 3 mL phosphomolybdenum complex reagent solution was added. The tubes were closed and incubated at 95 °C for 90 minutes. After cooling, absorbance readings were performed at 695 nm. The blank consisted of 0.3 mL methanol and 3 mL of reactive. The entire trial was performed in triplicate.

Photoprotective activity

The evaluation of the photoprotective activity *in vitro* was carried out according to Mansur *et al.* (1986). This test assesses whether the extracts provide protection against UVA and UVB radiation. The extract

was diluted in ethanol to obtain 100 µg/mL. Absorbance readings were performed at 290 nm and 320 nm (UVB and 320 radiation), and 400 nm (UVA radiation). The analysis was performed in triplicate, and the sun protection factor (SPF) was calculated using the equation:

$$SPF = FC \cdot \sum \frac{320}{290} \cdot EE(\lambda) \cdot I(\lambda) \cdot Abs(\lambda)$$

where *FC* = 10(constant); *EE* = erythemogenic effect; *I* = intensity of the sun; and *Abs* = absorbance of the sample. The constants *EE* and *I* were pre-defined by Mansur (1984).

Statistical analysis

The results were compared to means, using the Sisvar statistical program (FERREIRA, 2011). The values differed from each other by the Scott-Knott test at the 5% level.

Results and discussion

Extraction and Phytochemical profile

From 150g of the leaves, 9.1g (6%) of hexane extract, 9.2g (6%) of ethyl acetate extract and 13.8g (9%) of methanol extract were obtained. Based on the results, it can be stated that the methanolic extract achieved a higher yield.

Checking the yield of the extraction process is an important step in the phytochemical processing itinerary for the identification of bioactive constituents. The selection of an extraction technique is also important for standardizing products for removal of desirable soluble constituents and for scaling-up purposes at the pilot plant level (DHAMANI *et al.*, 2016).

C. capitellata leaves present six classes of nine metabolics studied, namely: flavonoids, tannins, triterpenes, steroids, anthraquinones and essential oils. The extract that presented the greatest variety of classes was ethyl acetate. In Table 1 is showed the classes of compounds detected in the hexane, ethyl acetate and methanol extracts of *C. capitellata* leaves by the thin layer chromatography method using solvents of different polarities.

Table 1 - Phytochemical profile of *C. capitellata* leaves. (+): detected; (–): Not detected; Hex: hexane extract; AcOEt: ethyl acetate extract; MeOH: methanol extract.

Metabolic Classes	Extraction Solvent		
	Hex	AcOEt	MeOH
Alkaloids	–	–	–
Coumarins	–	–	–
Steroids	+	+	–
Flavonoids	–	+	+
Essencial oils	–	+	–
Anthraquinones	+	–	–
Saponins	–	–	–
Tannins	–	+	+
Triterpenes	+	–	–

Source: Prepared by the authors.

In some phytochemical analyzes carried out by Silva (2012), in the plant species *Miconia cabucu*, of the same family than *C. capitellata*, the metabolic classes tannins, flavonoids and coumarins were identified, not detecting the presence of anthraquinones, saponins, cardiotonic heterosides and alkaloids. The absence of alkaloids in the extracts reinforces a chemotaxonomic characteristic of the Melastomataceae (BOMFIM *et al.*, 2020).

An analysis performed with *Clidemia hirta* L. cultivated in three basal media (Murashige and Skoog, modified Quoirin and Lepoivre, and Woody Plant Medium) showed that the samples exhibited different contents in saponins, phenolics and flavonoids (LOPEZ *et al.*, 2016).

Thus, the relative accumulation of metabolites may be linked to the extraction conditions, but also to the growth medium or the tissue considered (LABARRERE *et al.*, 2019). The situation can even occur in specimens of the same plant species growing under different environmental conditions. (SAMPAIO *et al.*, 2016), making it possible to find culture conditions that favor specific classes of compounds.

Dosing of total phenolics, tannins and flavonoids

The Table 2 presents the results obtained in the determinations of tannins, flavonoids and total phenolics of *C. capitellata* leave extracts, where means followed by vertically distinct letters differ from each other at 5% probability by the Scott-Knott Test. The hexane extract was dosed, but its concentrations were very low compared to the standard curve, not obtaining any valid result.

Table 2 - Determination of total tannins, flavonoids and phenolics in *C. capitellata* leaves. AcOEt: ethyl acetate extract; MeOH: methanol extract; mg EAG, EAT, ER/g: milligram equivalent to gallic acid, tannic acid and rutin.

Extract	Phenolics (mg EAG/g ext)	Flavonoids (mg ER/g ext)	Tannins (mg EAT/g ext)
AcOEt	325,5 ± 0,003[b]	181,3 ± 0,003[b]	188,93 ± 0,15[a]
MeOH	628,4 ± 0,021[a]	270,1 ± 0,003[a]	301,6 ± 0,004[b]

Source: Prepared by the authors.

Metabolite concentrations varied with the solvent used, so as different metabolite extraction methods result in different metabolite recoveries (SER *et al.*, 2015). In this work, in the determination of tannins, flavonoids and total phenolics, the methanol extract obtained higher values than the ethyl acetate extract, being phenolic compounds in greater quantity when extracted with both solvents, although a statistical difference is not necessary when compared to the other compounds.

These values for phenolic compounds can be explained by the fact they are components of electron transport chains in mitochondria and chloroplasts, in addition to other functions such as involvement in oxidative reduction processes, likely regulation of plant growth and development, as they affect the biosynthesis of indolyl-3-acetic acid (BABENKO *et al.*, 2019), and the highest content of secondary metabolites obtained when methanol was used as an extractant can due to the interactive abilities that influence their greater solubility in methanol, which is a polar protic solvent (FELHI *et al.*, 2017).

The differences between this study and previous studies in the content of phenols and flavonoids may reflect factors such as extraction conditions, seasonality and collection location. For example, Bomfim *et al.* (2021) verified *C. capitellata* leaves were extracted with ethyl acetate under slightly different conditions from the work presented here and their content was much lower, with a content of 205.95 mg EAG/g and 143.99 mg EQ/g for total phenols and total flavonoids, respectively.

In a study carried out by Santos; Couto (2018), the dry extract of the plant species *Miconia albicans*, belonging to the same family, presented 367.193 ± 10.52 mg equivalent of catechin/g in the methanolic extract at a concentration of 1,000 µg/mL, which indicates that the species studied shows a higher concentration of these compounds since the extract was dosed at a concentration of 500 µg/mL.

Oliveira (2017) related the EtOAc and MeOH extracts of *Miconia prasina*, which also showed that the methanolic extract excelled in phenolic content compared to other extracts, obtaining 139.3 ± 8.69 milligrams equivalent of tannic acid, however again, the species presented higher content of these compounds.

Antimicrobial activity

Table 3 shows the results of the antimicrobial activity tests that were carried out through microdilution in broth.

Table 3 - Minimum Inhibitory Concentration (MIC) and Minimum Microbicide Concentration (MMC) in mg/mL of *C. capitellata* extracts. *S. Aureus* **(01)**, *M. Luteus* **(06)**, *B. Subtilis* **(16)** and *E. Faecalis* **(138)**, *E. Coli* **(224)**, *S. Marcescens* **(398)**, *P. Aeruginosa* **(39).**

Extracts	Microorganisms						
	Gram-positives				Gram-negatives		
	01	06	16	138	39	224	398
HEX	1/4	1/4	2/4	2/4	1/2	4/4	2/4
AcOEt	0.5/2	0.5/0.5	1/4	1/2	0.12/1	0.5/4	0.06/0.5
MeOH	0.5/2	0.5/1	2/2	0.5/0.5	0.5/1	2/2	1/8

Source: Prepared by the authors.

Prior to the analysis of microdilution in broth there was the disk diffusion test or in this test the extracts did not show activity against the alcohol-acid-resistant microorganism: *M. smegmatis* and the yeast *C. albicans*, therefore these microorganisms have not been tested in broth microdilution to determine their MIC/CMM.

When comparing the extracts of the studied plant species, it is possible to infer that the ethyl acetate extract was the most promising of the extracts, as MIC and MMC obtained against the gram-negative bacteria *P. aeruginosa* (0.12/1 mg/mL) and *S. marcescens* (0.06/0.5 mg/mL) were relevant, as this concentration is considered promising when working with extracts. *P. aeruginosa* is one of the bacterium evaluated as having the highest risk due to its rapid development of antibiotic resistance, a serious public health problem.

Barroso (2015) when studied *Miconia ferruginata* crude extract, a species belonging to the same family as *C. capitellata*, observed no antimicrobial activity by the method MIC, so when comparing the two species it is possible to state that *C. capitellata* showed better result.

The mode of action of secondary metabolites depends on their chemical structure and properties, and can affect the microbial cell in several ways: disruption of plasma membrane function and structure (including the efflux system), interaction with membrane proteins, disruption of DNA / RNA synthesis and function, destabilization of proton driving force with ion leakage, inhibition of enzymatic synthesis. Alkaloids, for example, can intersperse with DNA and interrupt transcription and replication, and inhibit cell division (GORLENKO *et al.*, 2020).

Antioxidant Activity

In the results obtained in the antioxidant analysis by the phosphomolybdenum reduction method, DPPH scavenging and reducing power, the methanol extract has a greater antioxidant activity compared to the ethyl acetate extract. Table 4 shows the results obtained in the analyzes to obtain the antioxidant activity of *C. capitellata* leave extracts through three tests.

Table 4 - Antioxidant activity of *C. capitellata.* AcOEt: Ethyl acetate extract; MeOH: Methanol extract; mg EAA/g: milligram ascorbic acid equivalent per gram of sample; DPPH: 1,1 – Definil-2-Picril-Hidrazila.

Extract	Reducing power (mg EAA/g)	DPPH (%)	Phosphomolybdenum (%)
AcOEt	274,8 ± 0,02[a]	42,02 ± 0,03[a]	25,47 ± 0,06[a]
MeOH	378,0 ± 0,00[b]	67,99 ± 0,04[b]	40,03 ± 0,19[b]

Source: Prepared by the authors.

The greatest antioxidant activity in methanolic one can be because a correlation between the total phenolic content and the antioxidant capacity that is attributed to its chemical structure added to the high concentrations found. According to Sousa *et al.* (2007), the phenols have an ability to scavenge free radicals due to the presence of phenols, promoters of its metal chelating potential and its redox properties, which causes presenting an efficient antioxidant activity.

Normally, higher levels of phenolics and flavonoids lead to better DPPH sequestering activity (KADRI *et al.*, 2011; FELHI *et al.*, 2016), however, the *C. capitellata* leave extracts evaluated here had better power activity reducer. According to KOBUS-CISOWSKA *et al.* (2020), there are divergences in the discussion of issues related to the antioxidant activity of extracts regarding the content of phenolic compounds, because in certain cases can have a correlation between individual polyphenol content and antioxidant activity, but others have reported a reduction or lack of it.

Usubillaga *et al.* (1997) proved that the species of the genus *Solanum* that have a higher concentration of secondary metabolics also have a higher antioxidant potential. In another study. Santos; Couto (2018) verified the plant species *M. albicans* showed inferiority when compared to the methanolic fraction of *C. capitellata*, since the inhibition presented was 49.45 ± 0.005% of the methanol extract of *M. albicans* against 67.99 ± 0.04 % of the methanol extract of *C. capitellata*.

Commonly, the *in vitro* antioxidant property of plant extracts is determined by different traditional techniques (POURMORTAZAVI *et al.*, 2017) such as those used in this work. It is widely accepted that, to characterize the properties of antioxidant agents, different validated reference methods are needed, as the antioxidant activity measured in an individual assay only reflects the chemical reactivity under the specific conditions applied in that assay (OUERGHEMMI *et al.*, 2016).

Photoprotective Activity

The SPF values found for the hexane, ethyl acetate and methane extracts were less than 6 at the concentration tested (data not shown). According to BRASIL (2012), for any product to be suitable for application in cosmetics with the objective of photoprotection, it must present an SPF equal to or greater than 6, which guarantees low protection and allows its indication for skin that is not very sensitive to sunburn. Thus, no extract is indicated according to the standards required by legislation for use as sunscreen, however, the MeOH extract is close to that established by law, with FPS = 4,23 ± 5,56.

Conclusion

C. capitellata leaves have a great diversity of metabolic classes, being phenolic compounds, flavonoids and tannins were extracted in greater quantities with methanol. The ethyl acetate extract showed antimicrobial activity against *P. aeruginosa* and *S. marcescens*, and methanolic had greater antioxidant activity, being more effective in relation to reducing power.

Phytochemical studies are important in the biochemical characterization of plants, as well as evaluating the biological properties, in addition to recording substances from plant drugs that may be another option for application in society.

The results showed that this plant has medicinal potential to use as antimicrobial or antioxidant. Based on the little literature on this genus and plant species, it is worth noting that, in addition to promising results in all analyzes performed, further research is needed for better use. The present study will help researchers as basic data for future research in exploring the potential of this species as an enhancement of antioxidants and antimicrobials.

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