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Phytochemical and in Vitro Biological Activity from Epicuticular Wax of the *Copernicia prunifera* (Mill.) H.E. Moore

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HIGHLIGHTS

- Phytochemical characterization of *Copernicia prunifera* wax extracts.
- Antioxidant activity higher in mature leaf extracts in *Copernicia prunifera*.
- Significant cytotoxic activity against glioblastoma cells.

Abstract: The study aimed to characterize the phytochemical, antioxidant and hemolytic activity, and cytotoxicity of aqueous extracts of epicuticular wax from young (AEYL) and mature (AEML) leaves of *Copernicia prunifera*, a palm native to Brazil that adds value to both the local and the country's economy. Qualitative phytochemical analysis and physical-chemical tests (Fourier transform infrared spectroscopy (FTIR-ATR) and differential pulse voltammetry) were performed. Quantitative analyses were carried out for total phenols, antioxidant activity by 2.2-azinobis-3-ethylbenzothiazoline-6-sulfonic (ABTS + •) and in vitro cytotoxic and hemolytic activity in human tumor cell lines and erythrocytes, respectively. Tannins, saponins and reducing sugars in all extracts were detected and total phenolic content was 56.48 ± 21.91 mg and 158.52 ± 39.27 in AEYL and AEML, respectively. Antioxidant activity was 34.9 ± 4.8 µg in AEYL and 100.3 ± 15.2 µg in AEML. The AEML extract presented higher total phenol content and greater cytotoxicity against the SF-295 glioblastoma cell line (IC_{50} = 27.58 µg/mL) when compared to AEYL (IC_{50} = 77.59 µg/mL), reinforcing its potential as a source of bioactive compounds.”. Hemolytic activity of AEYL was lower than in AEML, and both presented concentrations capable of causing hemolysis above those effective against tumor cell lines. The results obtained showed to have potential as a source of bioactive compounds and that add value to its production chain.

Keywords: Arecaceae; carnauba; ceriferous powder; cytotoxic activity; hemolytic activity.

INTRODUCTION

Copernicia prunifera (Mill.) H.E. Moore), known colloquially as carnauba, carandaúba, carnaíva, carnaíba, and carnaúva, is native to the Caatinga vegetation of the semi-arid Northeast region of Brazil, with additional occurrences in the North and Midwest regions. This palm species is also found in Equatorial Africa, Colombia, Ceylon, Ecuador, and Thailand [1]. One of the remarkable features of the carnauba palm is its leaf surface, which is adorned with thick, irregularly arranged layers of epicuticular wax, commonly referred to as ceriferous powder. This wax is particularly abundant in younger leaves and serves various ecological and industrial functions. It can be manually or mechanically extracted [3].

Epicuticular wax, a complex mixture of chemical components, is hydrophobic and is subdivided into intracuticular (incorporated into the cutin) and epicuticular (outermost layer) wax [4]. Its disposition and composition vary from species to species, being influenced by climatic factors and plant growth stage [5, 6]. This complex wax can be understood as an adaptation of plants to adverse environments, acting as a physical and chemical barrier to protect the plants in various ways, from controlling incident radiation to defense against attacks by pathogenic microorganisms and insects. It can be found mainly covering the surface of leaves and together with stomata it forms an integrated system responsible for preserving the structural and functional equilibrium of the leaf [4, 7, 8, 9]. This is the main raw material used in the manufacture of carnauba wax, a product used in various industrial sectors due to its physical-chemical properties, and which has enormous economic importance in domestic and foreign markets, on the use in conservation and food processing [10] and widely used in controlled drug delivery systems, providing an increase in the effectiveness and reduction of the toxicity of bioactive agents (antimicrobials, antioxidants and antineoplastics). Brazil's Northeast Region is the world's main producer and exporter of the product [11, 12, 13, 14].

Carnauba wax powder can be classified into two types: Type A or eye powder, which is taken from young, still closed leaves and processed into light-yellow type I wax, which has greater economic value and more refined applications. Type B or straw powder is extracted from mature open leaves and is used to make the darker type III and IV waxes. The waxes can also vary, depending on the degree of impurity and shape of the wax bodies (bars or scales). The wax powder of young leaves is less pigmented because of its lower chlorophyll concentration [15, 16, 17, 18, 19, 20].

The inorganic components present in the epicuticular wax obtained from carnauba are aluminum, calcium, copper, iron, magnesium, manganese, sodium, and zinc [20], while the organic components are principally aliphatic esters, alpha-hydroxy and kinematic aliphatic esters, free acids and alcohols, hydrocarbons, and resins [9, 18, 19, 21, 22, 23] and in addition gallic acid, chlorogenic acid and catechin [18, 19].

Studies of the biological activities of carnauba wax powder have been mostly concerned with isolated chemical components, such as: β-1.3-glucanase and chitinase proteins, isolated from Type B wax [24], that have fungicidal activity against pathogenic fungi [25], triterpenoids with antiprotozoal activities against intracellular amastigotes and trypomastigote forms of *Leishmania infantum* and *Trypanosoma cruzi*, respectively [21], p-methoxy cinnamic acid with hypoglycemic, antioxidant and hypolipemic properties [8, 26, 27], antioxidant and antifungal activities against dermatophyte fungi *Microsporum canis* and *Trichophyton rubrum* [20].

Although the search for natural products in carnauba wax as a source of bioactive components for disease treatment has gathered pace in recent years and the economic importance and diversity of the chemical compounds discovered have increased, studies of the biological activity and phytochemistry of the wax are few and under-exploited and most have concerned chemical compounds isolated from the industrialized wax. In contrast, the current study focused on characterizing the phytochemistry and evaluating the biological activity of aqueous extracts made from the raw, unprocessed wax powder of the leaves of *Copernicia prunifera*.

MATERIAL AND METHODS

Plant Material and Extraction

Samples of wax powder from young and mature leaves of Copernicia prunifera were obtained in July 2017 at the Piauí state (S3º22’4.53” W41º46’25.9572”), located in the municipality of Caxingó, Brazil. A voucher specimen of the sampled plant (HDELTA registration number 4596) was deposited at the Delta do Parnaiba Herbarium (HDELTA). Access to genetic patrimony for this project has been officially registered on the database of the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (Ministério do Meio Ambiente) under the registration code A04D452.

Aqueous extracts of wax powder from young leaves (EAFJ) and mature leaves (EAFM) were made according to the methodology of [28], with some modifications. Aqueous extracts were prepared to simulate the traditional mode of use of *C. prunifera* and due to their lower toxicity and greater applicability in future pharmacological formulations.

Chemicals and reagents

All reagents used were of analytical grade. Acetic acid, acetic anhydride, aluminium trichloride and calcium carbonate were purchased from Vetec Química. Acetone, ethanol, methanol, sodium carbonate, sulphuric acid and hydrochloric acid were purchased from Neon Comercial Ltds. Reagentes Analíticos. Gallic acid, Folin & Ciocalteu's phenol reagent and DPPH (2,2-diphenyl-1-picrylhydrazyl) and quercetin from Sigma-Aldrich.

Qualitative Phytochemical Analysis

The methodology used to carry out the qualitative phytochemicals was the one recommended by [29] and [30]. Tests were performed for phenols, tannins, general flavonoids, saponins, alkaloids, organic acids, polysaccharides and reducing sugars. All analyses were performed in triplicate.

Fourier Transform Infrared Spectroscopy (FTIR-ATR)

To identify the functional organic groups, analysis of Fourier Transform Infrared Spectroscopy (FTIR-ATR) was performed on a Shimadzu IRAffinity-1S spectrometer, in the spectral range of 4000-700 cm-1 of resolution, by the ATR (Attenuated Total Reflectance) technique. In all samples, 45 scans were made in a zinc selenide crystal (Se-Zn). To detect possible modifications caused by the extraction, the composition of the extracts and that of the natural epicuticular wax powder were compared.

Differential Pulse Voltammetry (DPV)

The redox capacity of the extracts was assessed by the Differential Pulse Voltammetry (DPV) technique using the Potentiostat / Galvanostat Autolab PGSTAT128N (EcoChemie, Utrecht, Netherlands) with the software NOVA 1.6, and three printed electrodes (Dropsens): carbon working electrode, Ag / AgCl (silver chloride) reference electrode, and auxiliary carbon electrode, in a potential window from -0.2 V to + 1 V for 180 s. Gallic acid (1mg / mL) and quercetin (1mg / mL) solutions were used as an analysis reference standard. To determine the voltammetry, an aliquot of 5 µL of solution for each extract (1mg / mL) and 45 µL of 0.1 M HCl solution (pH 4.3) was added to the surface of the working electrode.

Total Phenols

The quantification of total phenolics (TPC) was carried out using the methodology described by [31] with modifications and determined by the Folin-Ciocalteu method. Samples were obtained using 1 mg of lyophilized and solubilized extract in 1 mL of ultrapure water to produce a stock solution. To each 25 µL of extract, 25 µL of Folin-Ciocalteu reagent and 75 µL of ultrapure water were added. The samples were incubated for 6 minutes in the absence of light, and then 100 µL of 7.5% sodium carbonate solution (Na_2CO_3) was added. For the blanks and the standard, the extracts were substituted with 25 µL of ultrapure water and 25 µL of gallic acid, respectively. The absorbances of the samples were read on a spectrophotometer at 593 nm. The concentration of total phenolics was obtained by using a standard curve of gallic acid (10-200 µg of AG / mL of water) expressed in equivalents to gallic acid (GAE) per gram of sample (GAE / g sample). The reading of the absorbances was carried out in an ELISA microplate.

Determination of total phenolic and total flavonoid content

The concentration of total phenolics was measured using the method by [31], with some modifications. An aliquot (1 mL) of appropriately diluted extracts or standard solutions of gallic acid (30, 50, 80, 100, 200 mg/L) or extract (1 mg/mL) was added to a 25 mL volumetric flask containing 9 mL of deionized water. After preparing a reagent blank using deionized water, 1 mL of Folin and Ciocalteu's phenol reagent was added, and the mixture was shaken. After 5 min, 10 mL of 7% Na_2CO_3 solution was added with mixing. The solution was then immediately diluted to volume (25 mL) with deionized water and mixed thoroughly. After incubation for 90 min, the absorbance versus the prepared blank was read at 750 nm using a Shimadzu UV-1800 JP spectrophotometer. Total phenolic content of the samples was determined using a 10-calibration curve made with a gallic acid standard and the results expressed as mg of gallic acid equivalent (GAE) per g of dried

extract. The total flavonoid content was determined using a method previously described by [31]. An aliquot of 1 mL of plant extract in ethanol (200 µg/mL) was mixed with 1 mL of aluminum trichloride in ethanol (20 mg/mL) and a drop of acetic acid, and then diluted with ethanol to 25 mL. The absorbance at 415 nm was read after 40 min using a Shimadzu UV-1800 JP spectrophotometer. Blank samples were prepared from 1 mL of plant extract and a drop of acetic acid, and then diluted to 25 mL with ethanol. The total flavonoid content was determined using a calibration curve of standard quercetin and expressed as mg of quercetin equivalent (QE/g of dried extract). All samples were analyzed in duplicate.

Antioxidant Activity

The determination of antioxidant capacity was performed using the ABTS•+ method, as described by Giao and coauthors [32], following the methodological parameters detailed in the aforementioned study, which involves the generation of ABTS radical chromophore by the oxidation of ABTS with potassium persulfate. The radical was produced by reaction between 0.007 mol/L ABTS and 0.14 mol/L potassium persulfate solutions. For the assay, ABTS•+ radical solution was diluted in 0.005 mol/L pH 7.4 PBS (phosphate-buffered saline) to an absorbance of 0.7 (± 0.02) at 734 nm. The reading of the absorbances was performed 6 minutes in a 6405 / UV-Vis-Spectrophotometer, at 734 nm, and the results were expressed in VEAC (antioxidant capacity equivalent to ascorbic acid).

Cytotoxicity Activity

The cytotoxicity test of the extracts was performed in the National Laboratory of Experimental Oncology (LOE) of the Federal University of Ceará (UFC). The tumor cell lines used were HCT-116 (Human-colon), SF295 (Glioblastoma), PC3 (Prostate) and HL60 (Leukemic), provided by the National Cancer Institute (USA). The cultivation was carried out in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% antibiotics, maintained in an oven at 37 °C in an atmosphere containing 5% CO_2.

The cytotoxicity determination was made by the colorimetric method based on the conversion of salt (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) into formazan blue, by mitochondrial enzymes found only in metabolically active cells [33]. The tests were carried out at a concentration of 300 µg/mL, for the initial cytotoxic screening based on previous studies with crude extracts, as it allows the identification of samples with relevant inhibitory activity while avoiding excessively high concentrations that may mask differential effects among cell lines. Tests were carried out in the four tumor cell lines, and doxorubicin was used as a positive control. The cells were plated at concentrations of 0.7×10^6 cells/ml for the HCT-116 strain, 0.3×10^6 cells/ml for HL60 and 0.1×10^6 cells/ml for the SF-295 and PC3 strains. After 24 hours, the cells were processed and incubated with the extracts for 72h in an oven at 5% CO_2 at 3°C. After incubation and centrifugation, the supernatant was removed and added to the 150 µL samples of MTT (10%) and RPMI 1640, and incubated for 3h. The absorbance was read after dissolving the formazan precipitate in 150 µL of pure DMSO (dimethyl sulfoxide) in a plate spectrophotometer (Multimode Detector, DTX 880, Beckman Coulter) at 595 nm.

An intensity scale was used for the initial screening to evaluate the samples according to cytotoxic potential: little activity (inhibition of cell growth ranging from 1% to 50%), moderate activity (50% to 75%), and a lot of activity (75% to 100%).

Hemolytic Activity

The hemolytic activity was assessed following the methodology of Marani and coauthors [34]. Blood samples were voluntarily provided (self-donated) by one of the authors with informed consent, and the procedure followed institutional biosafety guidelines. , who is O Rh negative. EDTA was employed as an anticoagulant and added to the blood samples, which were then centrifuged at 3600 rpm for 10 minutes to separate the plasma from the erythrocytes.These were washed three times and resuspended using 0.85% saline in a 1:10 (v / v) ratio to obtain a 2.5% (v / v) suspension of erythrocytes. An aliquot of 400 µL was taken from this suspension and diluted in 3600 µL saline to 0.85% in the proportion 1:4 (v/v).

Samples at concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.6 µg/mL and 7.8 µg/mL were obtained by using 1 mg of each crude extract and solubilized in 1mL of saline solution (0.085%) with 1% of DMSO. In the tests, 150 µL of extract at each concentration and 150 µL of the erythrocyte suspension were used. The samples were homogenized, incubated for 30 minutes at 37°C, and then centrifuged for one minute at 8000 rpm. After this, 100 µL of supernatant was taken for reading in an ELISA reader at 492 nm. Triton X (0.01%) was used as the positive control, and 0.85% saline as the negative control. All tests were performed in triplicate. The percentage hemolytic activity of the extracts was expressed by the

equation: % Hemolysis = (AbsE - AbsS) / (AbsT - AbsS) x 100, where % Hemolysis = percentage of hemolysis, AbsE = absorbance of extracts, AbsS = absorbance of saline, AbsT = absorbance of Triton X.

Statistical analysis

The tests of cytotoxicity were performed in triplicate in two independent experiments. The results were analyzed using the mean ± standard deviation (SD) of the percentage inhibition of cell growth using the GraphPad Prism 5 program. All the obtained results (phenols, antioxidant, hemolytic and activities) were expressed as mean ± SD standard deviation.

RESULTS

Qualitative Phytochemical Analysis

I n both extracts, the phytochemical tests showed the presence of condensed tannins, saponins and reducing sugar. Alkaloids were detected only in AEYL and flavanols in AEML (Table 1).

Table 1. Secondary metabolites in aqueous extracts of epicuticular wax *Copernicia prunifera* (Mill.) H.E. Moore. AEYL - Aqueous extract of epicuticular wax from young leaves; AEML - Aqueous extract of epicuticular wax from mature leaves.

Secondary Metabolites	Samples	
	EAFJ	EAFM
Phenols	-	-
Condensed or catechic tannins	+	+
Hydrolyzable or pyrogallic tannins	-	-
Tannin using copper acetate	+	+
Anthocyanins and anthocyanidins	-	-
Flavones, flavonols, and xanthones	-	+
Chalcones and aurones	-	-
Flavanones	-	-
Saponins	+	+
Alkaloids	+	-
Organic acids	-	-
Polysaccharides	-	-
Reducing sugar	+	+

Fourier Transform Infrared Spectroscopy (FTIR-ATR)

No significant difference was observed in the Fourier transform infrared spectroscopy (FTIR-ATR) analyses between the absorption spectra of extracts, probably because of overlapping bands (Figure 1). However, when the extracts were compared to the spectra of waxes it was possible to verify that bands of 2911, 2846, 2844, and 1731 cm-1 are characteristic of natural waxes, although little evidenced in the extracts.

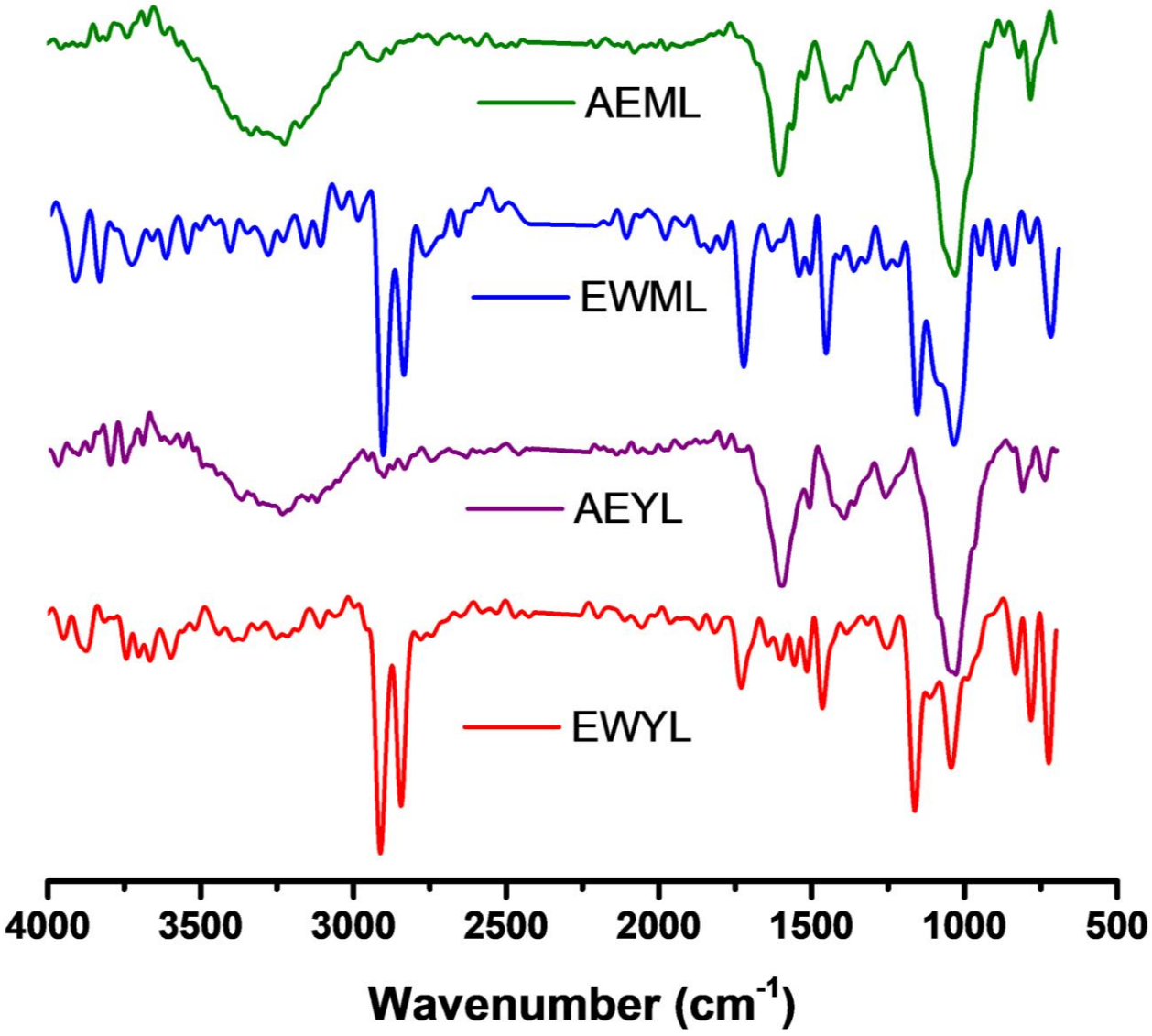

Figure 1. Infrared absorption spectrum (FTIR-ATR) of epicuticular wax (EWYL and EWML) and aqueous extracts of young (AEYL) and mature (AEML) leaves from *Copernicia prunifera*. (Mill.) H.E. *(Green) AEML - Aqueous extract of epicuticular wax from mature leaves, (Blue) EWYL - Epicuticular wax from mature leaves, (Purple) AEYL - Aqueous extract of epicuticular wax from young leaves, (Red) EPYL - Epicuticular wax from young leaves.

In both extracts, the recorded absorption bands of 3234 cm-1 and those between 1200 - 1000 cm-1 indicate a strong presence of alcohols and phenols, characterized by axial deformation vibrations of O-H and C-O [23, 35]. Also, the bands between 1200 - 1000 cm-1 present in all the spectra indicate the presence of ethers (C=O) and aromatic esters (C-O).

Differential Pulse Voltammetry (VPD)

When plant extracts are electroactive, their profiles can be directly determined by electrochemical methods. In the differential pulse voltammogram obtained, the blue curve (-) represents the oxidation of the electrolyte support (HCl 0.1 M pH 3), while the red and purple curves (-,-) show the oxidation of the extracts in 0.1 M HCl (pH 3) solution, and the orange and green curves (-,-) show the oxidation of the quercetin and gallic acid standards, respectively (Figure 2).

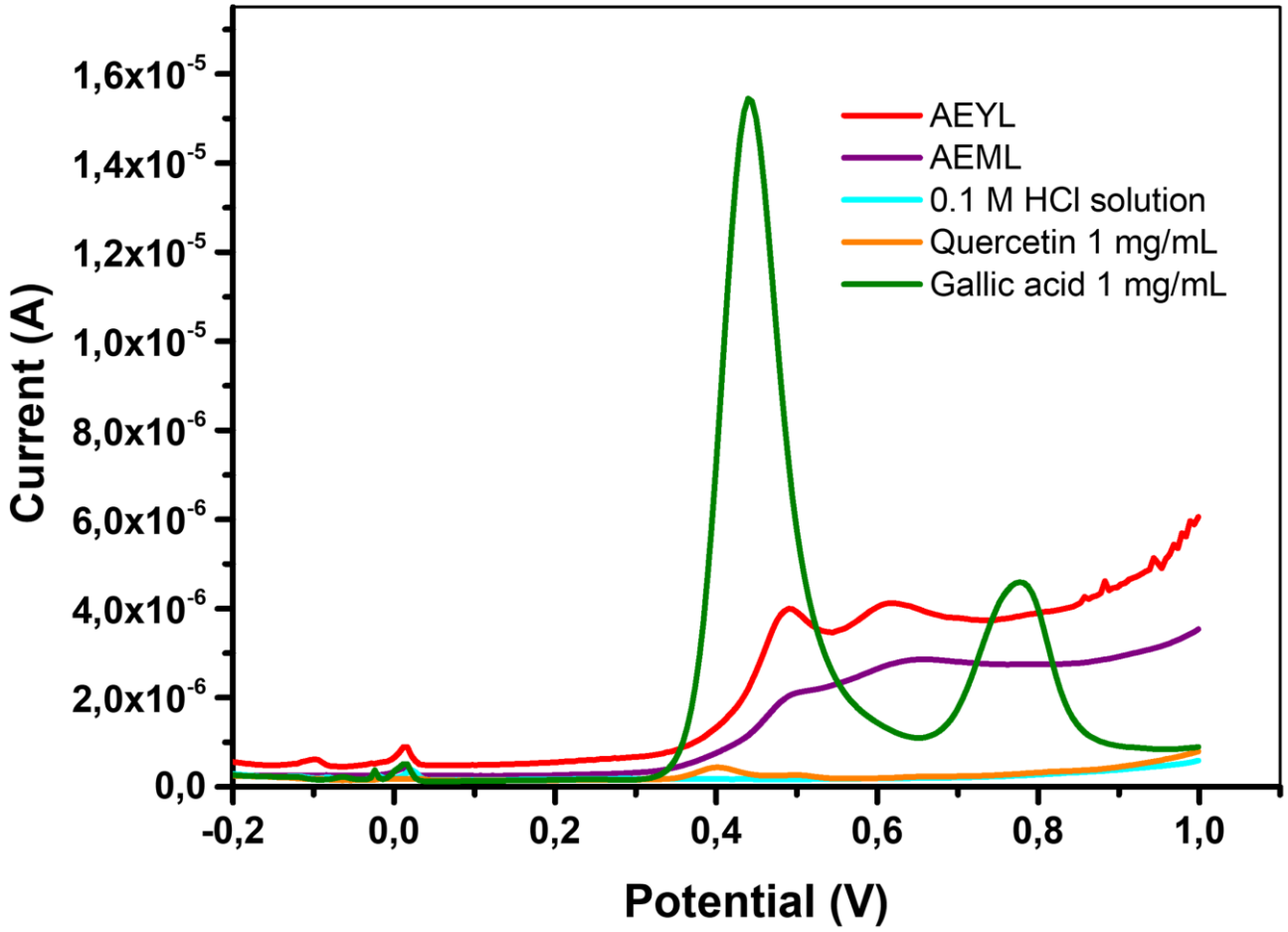

Figure 2. Differential pulse voltammograms obtained from young leaf epicuticular wax extracts (AEYL) and mature leaf epicuticular wax extracts (AEML) from *Copernicia prunifera* and phenolic acid standards. *Extracts and phenolic acid standards: (-) 1 mg/mL AEYL, (-) 1mg/mL AEML, (-) 0.1 M HCl, (-) 1mg/mL quercetin, (-) 1mg/mL gallic acid. Initial potential (Ei) = -0.2 V, final potential (Ef) = 1.0 V, pulse amplitude = 50 mV, scan rate = 50 mV/s, working electrode: printed carbon, reference electrode: Ag/AgCl (KCl3M) and against electrode: platinum.

Total Phenols and Antioxidant Activity

The results obtained in the quantification of total phenolics and antioxidant activity by the ABTS+• method show differences, with lower values for AEYL than for AEML (Table 2).

Table 2. Total phenol content (FT) and antioxidant activity by the ABTS+• method in aqueous extracts of young leaves (AEYL) and mature (AEML) leaves of *Copernicia prunifera* (Mill.) H.E. Moore. AEYL - Aqueous extract of epicuticular wax from young leaves, AEML - Aqueous extract of epicuticular wax from mature leaves.

	Total phenols (mg GAE/g) mean ± SD	**ABTS**
		(µg VEAC/mg extract) mean ± SD
AEYL	56.48 ± 21.91	34.9 ± 4.8
AEML	158.52 ± 39.27	100.3 ± 15.2

Cytotoxic Activity

The cytotoxic screening of the extracts showed activity ranging from moderate (PC3, HL60, and HCT116) to high (SF-295), expressed as percentage inhibition of cell growth. In evaluating the minimum inhibition concentration (IC50), the efficiency of aqueous extract activity against the tumor strains was demonstrated, since they showed cytotoxic potential against the glioblastoma lineage (SF-295) with IC50 of 27.58 and 77.59 µg/mL for AEML and AEYL, respectively. For the other strains, the IC50 varied from 97.20 to 154.3 µg/mL for the AEML, and 178.2 to 244.2 µg/mL for the AEYL, which showed that mature leaves had greater cytotoxic activity than young ones (Table 3).

Table 3. Cytotoxic activity in vitro of epicuticular wax's aqueous extracts of young (AEYL) and mature (AEML) leaves from *Copernicia prunifera*. SF-295 (glioblastoma), PC3 (prostate), HL60 (leukemic), HCT116 (colon). (IC50) minimum inhibitory concentration. Doxorubicin was used as a positive control.

Cell line	AEYL		AEML		Doxorubicin
	Growth inhibition (%)	IC_{50} ($\mu g.mL^{-1}$)	Growth inhibition (%)	IC_{50} ($\mu g.mL^{-1}$)	IC_{50} ($\mu g.mL^{-1}$)
SF-295	92.31±4.5	77.59 (65.43-92.01)	84.6 ± 3.17	27.58 (21.16-35.94)	0.25 (0.22-0.28)
C3	63.53 ± 9.2	244.2 (207.8-286.9)	85.95±8.39	112.1 (91.69-137.1)	0.44 (0.34-0.54)
HL60	74.19±5.65	178.2 (146.9-216.1)	64.93±9.74	154.3 (127.1-187.4)	0.01 (0.005-0.01)
HCT-116	70.60±6.0	222.4 (187.1-264.3)	88.40±6.0	97.20 (77.15-122.5)	0.11 (0.08-0.14)

Hemolytic Activity

The results showed that AEYL had a hemolytic activity of 60% at the highest concentration (500 µg/mL), while the AEML had more than 60% hemolytic activity at 250 µg/mL, and 100% in the highest concentration (Figure 3).

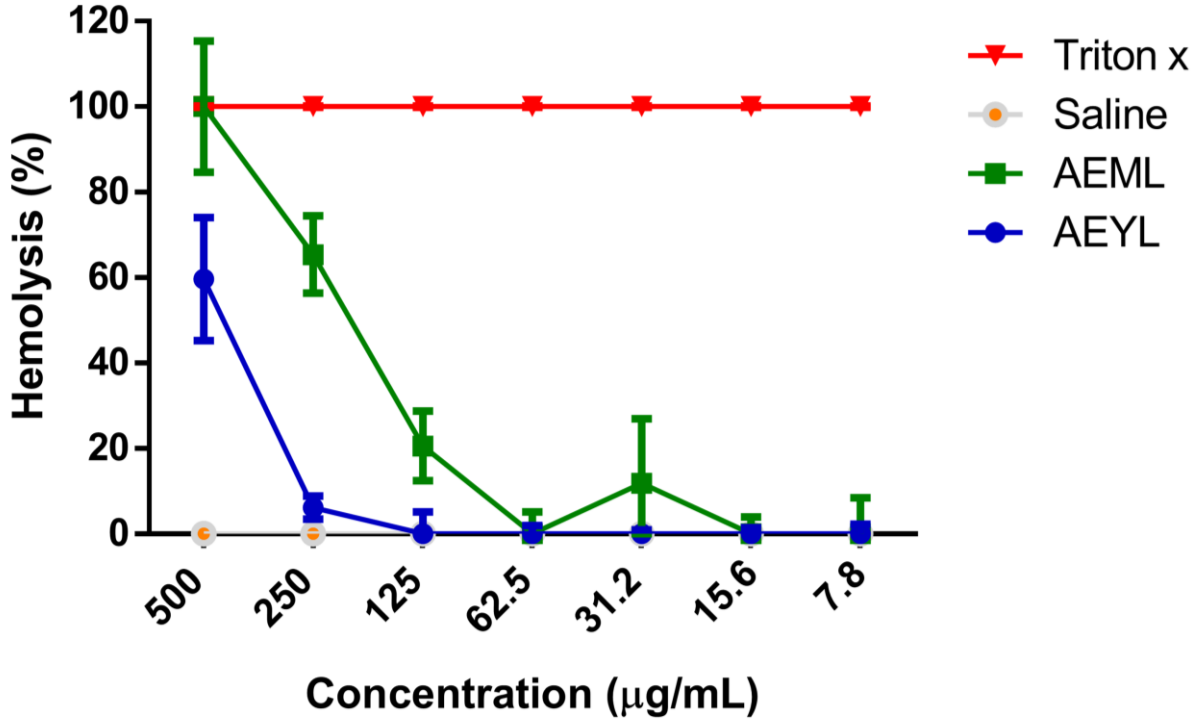

Figure 3. Hemolytic activity of aqueous extracts of young (AEYL) and mature (AEML) leaves from Copernicia prunifera. *(AEYL) - Aqueous extract of epicuticular wax from young leaves, (AEML) - Aqueous extract of epicuticular wax from mature leaves.

DISCUSSION

The phytochemicals found corroborate the results obtained in other studies for the same species [20,36, 37]. According to the literature, the composition of plant cuticular waxes is influenced by climatic factors and growth stages, and may include triterpenes, flavonoids, and tannins [6,38,39,40].

There are several classes of phenols, such as: flavonoids, phenolic acids (derived from benzoic and cinnamic acids), simple phenols, coumarins, tannins, lignans and tocopherols [41,42]. They can occur in simple form or with a high degree of polymerization associated with sugars and proteins [43].

Condensed tannins (procyanidins) are primarily found in the form of esters or heterosides. While soluble in water, they have a propensity to form insoluble (precipitated) complexes when in contact with water containing alkaloids, gelatins, or proteins. Additionally, they exhibit high reactivity when exposed to heavy metals like copper (Cu) and iron (Fe), or specific enzymes, leading to color changes [44]. These observations align with the test results obtained in the current study.

Saponins can be found occasionally in species of Arecaceae and they are more evident in aqueous solutions, where they cause persistent foams. Flavonoids are widely distributed in the angiosperms [44, 45], as are alkaloids, pharmacologically active nitrogenous compounds [46] and phenolic acids, which can be derived from benzoic or cinnamic acid.

Compounds derived from secondary plant metabolism are widely distributed in plants and structurally variable, which explains their multifunctionality, as well as providing numerous benefits to the plant, these compounds are also responsible for different kinds of biological activity [41,47]. Phytochemical screening is the first stage in finding which bioactive molecules are present in plant extracts. The cytotoxic and hemolytic effects observed in our study may be attributed to the presence of saponins, tannins, and flavonoids, which are known for their membrane-interacting and antiproliferative properties. Saponins, while associated with hemolysis, have also been reported to induce apoptosis in tumor cells, suggesting a dual role in both efficacy and toxicity. Condensed tannins and flavonoids, on the other hand, are strongly correlated with antioxidant activity and may contribute to cytotoxicity through oxidative stress modulation. This integrated interpretation suggests that the chemical complexity of *C. prunifera* extracts underlies their multifaceted biological effects.

Fourier Transform Infrared Spectroscopy (FTIR-ATR)

The observation that there was no significant difference in the absorption spectra between the extracts using Fourier transform infrared spectroscopy (FTIR-ATR) may initially seem puzzling. However, this can likely be attributed to overlapping bands within the spectra, which could mask any discernible distinctions between the extracts. What becomes intriguing is the comparative analysis of the extracts against the spectra of waxes. This comparison reveals characteristic bands at 2911, 2846, 2844, and 1731 cm-1, which are indicative of natural waxes. Although these bands were less evident in the extracts, their presence suggests the retention of certain wax components.

These bands are associated with various compounds inherent to natural waxes, including alkanes, aldehydes, acids, carboxylates, alcohols, steroids, and hydrocarbons [4,48]. Furthermore, they indicate the presence of additional compounds such as terpenes, phenols, and saponins, distinguished by their characteristic functional groups (C-H, O-H, and C = O, respectively) [23,49]. The absorption bands observed in all spectra between 1650 – 1515 cm-1 indicate angular deformation of C-H and N-H of amides, indicating the presence of proteins [35, 50]. Bands of 833 and 782 cm-1 correspond to the symmetrical angular deformation outside the plane of N-H of primary and secondary amides, further suggesting the presence of proteins.

The bands of 1600 cm-1, 1515 cm-1 and 830 cm-1 present only in the waxes corroborate the results of Vandenburg and Wilder [51] and indicate the presence of C=C and C=C bonds in aromatic rings. The 833 cm-1 absorption band observed in the EAFJ and the young leaf wax powder refers to silicon (Si) compounds, which suggests angular deformation of silicon-hydrogen (Si-H) or axial deformation of silicon-oxygen (Si-O). This is corroborated by X-ray fluorescence analysis (XRF), which demonstrated that silicon is one of the chemical elements present in the carnauba leaf and that this band can also be part of the angular deformation outside the C-H plane of phenols (naphthalene) [48]. Although FTIR provided insights into functional groups, it does not allow precise compound identification. Future studies should employ complementary techniques such as LC-MS/MS or HPLC-DAD to fully characterize the chemical constituents of the extracts.

Differential Pulse Voltammetry (DPV)

In the present study, anode peaks were observed between 0.40 and 0.48 V, which can be attributed to the oxidation of different polyphenols with typical anode signals at 0.44 V, 0.40 V, 0.48 V, and 0.49 V for oxidation of gallic acid, quercetin, AEYL, and AEML, respectively. These results corroborated those of Magarelli and coauthors [52], which showed phenolic acids with an anode peak at approximately 0.4 V.

This value can be attributed to the oxidation of hydroxyl groups in the aromatic ring, which leads to the conversion of semiquinone to O-quinone. Based on this anodic peak and the voltammetry determination of phenolic acids present in the extracts, gallic acid showed a better maximum oxidation current result than that obtained for the quercetin standard, and therefore is considered the best standard for the determination of total phenolic acid content in the extracts.

Phenolic acids can be divided into two groups: derivatives of benzoic acid, which include gallic acid, and derivatives of cinnamic acid, more commonly found in plants. Phenolic acids can link together or combine with other compounds, they are easily oxidized in alkaline media and their extraction is based on their polarity. They act by sequestering radicals and sometimes as metal chelators that initiate and propagate the oxidative process [43,53].

Total Phenols and Antioxidant Activity

The total phenol levels obtained were lower than those reported by [54], made using ethanol leaf extracts (1454.54 ± 12.56 mgEAG/g), and [20] (116.89 ± 3.69 for young leaves, 191.75 ± 2.55 for mature leaves). Total phenols have also been reported for other plant parts, such as the roots by [42] (250 ± 8.2 mgEAG/g) and the fruit by Silva and coauthors [41] who found 44.60 mgGAE/g and 33.2 mgGAE/g in ethanol (EE) and hexane (EH) extracts, respectively.

In addition to the extraction time and methodology adopted, other factors may interfere or influence the detection and quantification of the phenolics, such as: sample size, nature of the compound, storage conditions, the standards used, and the presence of interferents like waxes, chlorophylls, fats, and terpenes [20,43,55,56,57].

Phenolic compounds from plant extracts have received much attention from researchers, mainly about their pharmacological and biological properties, especially antioxidant activity, because of their redox properties that enable them to act as donors of hydrogen atoms, reducing agents, and oxygen suppressors [43,42,55,58]. Thus, the higher the total phenol content of the extract, the greater the antioxidant activity, depending on the methods used.

Erenler and coauthors [59] isolated bioactive compounds with antiproliferative and antioxidant activities from *Origanum rotundifolium*, highlighting the relevance of phenolic compounds and flavonoids as primary antioxidant agents. Likewise, our results indicate that phenolic compounds in infusions contribute significantly to the overall antioxidant activity, corroborating the findings of this study. A correlation between total phenolic concentration and antioxidant capacity was also observed in our study, aligning with [60], who demonstrated the antioxidant efficacy of *Origanum majorana* extracts, revealing a strong association between phenolic content and the observed antioxidant activity.

Variations in chemical composition between species, such as *Echinacea purpurea* and *Echinacea pallida*, result in different antioxidant capacities [61]. Our results showed that different medicinal plant species, even within the same genus, can exhibit significant variations in their antioxidant activity due to differences in their phytochemical profiles. Flavonoids isolated from *Allium vineale* demonstrated substantial antioxidant capacity, highlighting their central role in radical neutralization [62]. Likewise, our findings confirm that flavonoids in the *Copernicia prunifera* are primarily responsible for the total antioxidant activity, reaffirming the essential role of these bioactive compounds.

The analysis of *Althaea officinalis* highlights the influence of extraction methods and the nature of phenolic compounds on antioxidant activity [63]. This study highlighted the effectiveness of moisture scavenging, similar to the method used in our study, resulting in efficient protection of antioxidant compounds. Therefore, the extracts investigated in our work demonstrate an antioxidant capacity consistent with previous observations, emphasizing the importance of phenolic compounds and flavonoids as the main antioxidant agents.

The ABTS+• method is based on the reduction or loss of absorbance of the cation ABTS•+, which results in the discoloration of the solution [64]. The Folin-Ciocalteu method, used in the determination of total phenols, is also based on their redox properties and thus provides a significant adjunct to ABTS+• analysis. Assays of phenols by the Folin-Ciocalteu method help to determine the contribution of these compounds to the observed antioxidant activity.

Cytotoxic Activity

Antitumor activity has been associated with a variety of secondary metabolites produced by plants, among which phenolic and flavonoid compounds stand out as the most important, since they have been associated with the inhibition of several types of cancer, such as those of the colon, esophagus, liver, breast, skin, and lung [47]. Man and coauthors [65] highlighted the significant anti-cancer properties of saponins.

In an investigation of the activity of total flavonoids of *Cotinus coggygria* Scop. Wang and coauthors [66] reported that the in vitro and in vivo inhibition of cancerous cells of glioblastoma by these compounds. Likewise, Cho and coauthors [67] identified phenols and flavonoids in hexane extracts of the roots of *Pluchea indica* (L.) Less. and verified dose-dependent suppression of glioblastoma cell growth and induction of cell death by autophagy. It was inferred that the extract activity may have been related to the presence of these compounds.

Studies conducted on palms (Arecaceae) have shown that some species have cytotoxicity capability. [35] used green synthesis of zinc oxide nanoparticles (ZnONPs) obtained from fruit extracts of *Borassus flabellifer* L. against two tumor strains, HT29 (colon), and MCF7 (breast). Ramazani and coauthors [68] showed cytotoxic activity of n-hexane extracts obtained from aqueous fruit extracts of the date palm *Phoenix dactylifera* L. against two tumor cell strains, A2780 (ovary) and A172 (glioblastoma). Bello and coauthors [69]

used aqueous extracts of *Hyphaene thebaica* L. fruits for biosynthesis of silver nanoparticles (AgNPs), which in turn were used to evaluate antiproliferative activity of three human tumor strains: PC3 (prostate cancer), MCF7 (breast), and HepG2 (liver). Bello and coauthors [69] used caffeic acid from methanol extracts of *Phoenix dactylifera* against a HepG2 (lung carcinoma) tumor strain. Omran and coauthors [49] investigated the protective effects of *Phoenix dactylifera* polyphenols against MCF7 (breast cancer).

Many of the agents employed in cancer treatment are derived from natural sources and were discovered from cytotoxicity tests that measured inhibition of proliferation of cancer cells in vitro or ex vitro [70]. According Cragg and Newman [71], the impact of cancer on the world in 2020 was 19.3 million new cases of cancer in the world (18.1 million, if cases of non-melanoma skin cancer are excluded) and for Brazil, the estimate for the triennium from 2023 to 2025 points out that there will be 704 thousand new cases of cancer, 483,000 if non-melanoma skin cancer cases are excluded. As a result, the search for natural products as new sources of bioactive compounds for the treatment of neoplasia has been increasing and is showing promising results [33,72,73]. Glioblastoma remains one of the most aggressive and treatment-resistant cancers, with a median survival of less than two years despite surgery, radiotherapy, and chemotherapy. The strong cytotoxic effect of AEML against SF-295 cells is therefore highly relevant, as it indicates the potential of *C. prunifera* extracts as a natural source for novel adjuvant or primary therapies in glioblastoma management. Considering the scarcity of effective treatments, the identification of bioactive compounds in this species may contribute to the ongoing search for alternative strategies against this devastating malignancy. One limitation of this study was the absence of assays using non-tumor cell lines, which prevents a conclusive assessment of selectivity. Future investigations should include such analyses to better evaluate the therapeutic window and safety profile of the extracts.

Hemolytic Activity

The hemolytic activity of all extracts was dependent on the concentration in our results. In vitro tests of hemolysis have been used to screen for toxicological evaluation of different plant species by various authors [22,74,75].

One possible cause of hemolytic activity of the extracts is the presence of saponins in some palm species. Among the various pharmacological properties of saponins, hemolytic activity is a negative feature due to their ability to lyse erythrocytes. Studies have reported hemolytic activity of saponins [76, 77]. The presence of tannins must have led to the low hemolytic activity of the EAFJ extract since these compounds have been shown to precipitate hemoglobin [44].

We note here that the extract concentrations capable of causing hemolysis are greater than those that showed cytotoxic activity against the tumor cell lines we assessed. Thus, AEML and AEYL may be a promising source of bioactive compounds in the search for new treatments for the neoplasia concerned. It is noteworthy that the cytotoxic concentrations effective against tumor cells were lower than those inducing significant hemolysis, suggesting a preliminary indication of selectivity. This observation, while limited in the absence of non-tumor cell assays, reinforces the potential therapeutic window of the extracts and warrants further investigation in normal cell models and in vivo systems to confirm safety and tolerability.

Future studies should focus on bioguided fractionation and compound isolation, complemented by advanced analytical techniques such as LC-MS/MS and NMR, to precisely identify the molecules responsible for the observed bioactivities. Additionally, in vivo studies and mechanistic assays (apoptosis induction, cell cycle arrest, ROS generation) would provide valuable insights into the mode of action and therapeutic relevance of these extracts.

CONCLUSION

The qualitative phytochemical tests in aqueous extracts of epicuticular wax from young and mature leaves of *Copernicia prunifera* indicated the presence of condensed tannins, saponins, and reducing sugars in both extracts, with alkaloids detected only in AEYL and flavanols exclusively in AEML. FTIR analysis suggested that the main organic constituents of the extracts are compounds containing aldehyde groups, esters, ethers, and aromatic rings. The phenolic content and antioxidant activity assessed by the ABTS+• method demonstrated a direct relationship with the biological activities observed, particularly due to the contribution of phenolic compounds and flavonoids. The cytotoxic screening against tumor cell lines revealed activity ranging from moderate (PC3, HL60, and HCT116) to high (SF-295), with the AEML extract exhibiting remarkable potency against glioblastoma cells (IC_{50} = 27.58 µg/mL), a finding of particular relevance given the scarcity of effective therapies for this aggressive cancer. Although hemolytic activity was observed, the effective cytotoxic concentrations were lower than those inducing significant hemolysis, suggesting a preliminary therapeutic window and supporting the safety potential of these extracts. Altogether, these results

highlight the great biological potential of *C. prunifera* as a promising source of bioactive compounds for the development of new drugs. Future studies should include bio-guided fractionation, compound isolation, and advanced analytical techniques such as LC-MS/MS and NMR to identify active molecules, as well as mechanistic and in vivo assays to confirm efficacy and safety, thereby advancing these extracts from preliminary screening to potential pharmacological applications.

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