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Unlocking the Antioxidant and Antimicrobial Potential of Flavone and Amide-rich Fractions from Conchocarpus macrocarpus (Rutaceae) Leaves

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Abstract

Conchocarpus macrocarpus (Rutaceae) is an endemic species in Brazil, whose chemical and biological properties are poorly understood. This study aimed to evaluate the antioxidant and antimicrobial potential of the butanolic partition phase extracted from *C. macrocarpus* leaves collected in Espírito Santo, Brazil. *In vitro* antioxidant assays, including DPPH radical scavenging and Folin-Ciocalteu assay, and cytotoxicity and antioxidant activity in RAW cells using the MTT method were performed. Additionally, antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* was investigated. Results showed that the flavone-rich fraction exhibited the strongest antioxidant activity, as observed for the PF-BuOH G2.1 fraction that showed values of 237.2 ± 1.4 mg TE/g of extract and 118.2 ± 1.6 mg GAE/g of extract. However, the samples did not show protective effects against hydrogen peroxide. Furthermore, the flavones and amides present in the PF-BuOH G2.1 fraction displayed antimicrobial activity against *C. albicans*, showing a minimum inhibitory concentration of 1.25 mg/mL at the highest microbial load ($1.5x10^5$ CFU/mL). Our findings provide insights into the chemical composition and biological potential of *C. macrocarpus* and highlight the promising applications of the BuOH partition phase and its fractions.

1 Introduction

Biodiversity remains a critical source of natural products used in medicines. About two-thirds of the global population may rely on drugs derived from plant sources (Tlili et al. 2019). Despite Brazil has the largest number of angiosperm species, only a small portion of the native flora, approximately 5%, has undergone phytochemical investigations for potential biological activities (Dutra et al. 2016), highlighting the vast untapped potential of Brazil's natural resources in terms of novel drug discovery and development.

Besides the large biodiversity, Brazil is home to many plant genera with a high degree of endemism. *Conchocarpus* J.C. Mikan, a genus that comprises 47 species predominantly found in neotropical regions (Groppo et al. 2021), has many species that are endemic or native to Brazil. Despite its rich diversity, only six species were evaluated for their biological potential.

Antiacetylcholinesterase activity was described for the crude extract and isolated compounds from the stem of *Conchocarpus fontanesianus* (A.St.-Hil.) Kallunki & Pirani (Cabral et al. 2012), where alkaloids, such as skimiamine, were correlated to the biological potential. Trypanocidal activity for leaf hexanic extracts of *Conchocarpus. gaudichaudianus* (A.St.-Hil.) Kallunki & Pirani [currently = *Dryades gaudichaudiana* (A.St.-Hil.) Groppo, Kallunki & Pirani] (Ranieri Cortez et al. 2009), *Conchocarpus marginatus* (Rizzini) Kallunki & Pirani (Bellete et al. 2012) and *Conchocarpus longifolius* (A.St.-Hil.) Kallunki & Pirani (Mafezoli et al. 2000), leaf and stem methanolic extract of *C. heterophyllus* (A.St.-Hil.) Kallunki & Pirani (Ambrozin et al. 2008), and leaf methanolic extract of *C. inopinatus* Pirani (Bellete et al. 2012) were already described. In all trypanocidal activity assays, quinoline and acridine alkaloids such as haplotusine were associated to the activity. In addition, antioxidant, cytotoxic, antimicrobial, and antifungal activities were verified for the volatile oil of *C. fontanesianus* (Cabral et al. 2016).

Usually, the main compounds found in species of the genus are alkaloids, mainly derivatives of anthranilic acid, such as quinolinic described for *C. fontanesianus* (Cabral et al. 2012), C. *gaudichaudianus* (Ranieri

Cortez et al. 2009), C. *heterophyllus* (Ambrozin et al. 2008)d *longifolius* (Mafezoli et al. 2000). In addition, coumarins have been described for *C. fontanesianus* (Cabral et al. 2012), flavonoids for *C. heterophyllus* (Ambrozin et al. 2008), while steroids, triterpenes and various apolar components of cuticular waxes have been described for *C. macrocarpus* (Engl.) Kallunki & Pirani, *C. inopinatus*, *C. macrophyllus* J.C.Mikan, *C. mastigophorus* Kallunki, *C. obovatus* (Nees & Mart.) Kallunki & Pirani, *C. ruber* (A.St.-Hil.) Bruniera & Groppo (Silveira et al. 2022) (See the review in Table S1).

Conchocarpus macrocarpus is an intriguing species endemic to Brazil, found primarily in the states of Rio de Janeiro and Espírito Santo. The species stands out in the genus due to distinctive features such as sessile flowers, robust and erect inflorescences, large and rough fruits, and coriaceous leaves with reticulate-venous unifoliolate. Despite its unique morphology, there is still little knowledge regarding the chemical composition and potential biological activities of this species. To date, the only chemical study conducted on *C. macrocarpus* pertains to the composition of its cuticular wax (Silveira et al. 2022), leaving much to be explored in terms of its extracts and partition phases for potential therapeutic applications.

The vast diversity of plant species offers a river of possibilities for the discovery of novel compounds with therapeutic potential. In fact, plants have been a major source of drugs for centuries and continue to play a critical role in the development of new treatments for a variety of diseases (Dutra et al. 2016). In accordance with this approach, the primary objective of the study was to investigate the potential antioxidant and antimicrobial activities of the butanolic partition phase of *C. macrocarpus*, and to identify the major compounds that may contribute to these activities. Through our research, we aim to shed light on the biological potential of this understudied plant species and provide valuable insights seeking for future drug-discovery efforts.

2 Materials and methods

2.1 Plant material, leaf extract (LE) and partition phases (PF) production

Leaves of *Conchocarpus macrocarpus* were collected from multiple individuals in Cachoeiro do Itapemirim, Espírito Santo (voucher specimen J.R.Pirani et al. 3530). After drying in an air-circulating oven at 60°C, the leaves were ground into a fine powder using a knife mill. The resulting 800 g of powder underwent cold maceration with 70% ethanol at a ratio of 1 g:100 mL, under constant agitation for 10 days with three changes of solvent in days 2, 5, and 8. The resulting extracts were filtered, concentrated on a rotary evaporator, and lyophilized until completely dry (yielding 100.5 g of leaf extract, LE = 12.6%). Subsequently, the LE was dissolved in 20% ethanol and partitioned with solvents of increasing polarity (hexane – Hx, dichloromethane – DCM, ethyl acetate – AcOEt, and butanol – BuOH), using 150 mL of each solvent three times. The butanolic partition phase (PF-BuOH = 11.6 g) was then subjected to further fractionation (Fig. 1).

2.2 Sephadex column chromatography

PF-BuOH was subjected to Sephadex LH20 column chromatography. The mobile phase was determined as a series of eluents prepared at an increasing polarity grade, such as hexane:dichloromethane (7:3, 1:1, 3:7, 0:10), dichloromethane:ethyl acetate (7:3, 1:1, 3:7, 0:10), ethyl acetate:methanol (7:3, 1:1, 3:7, 0:10), and pure methanol, which was used until complete cleaning of the column. The fractions obtained were concentrated, monitored by thin layer chromatography (TLC), and grouped according to the chemical profile similarity of each sample, which resulted in seven groups (PF-BuOH G1 (12.7 mg), PF-BuOH G2 (10.5 g), PF-BuOH G3 (700.6 mg), PF-BuOH G4 (8.3 mg), PF-BuOH G5 (31.3 mg), and PF-BuOH F65 (79.0 mg). The PF-BuOH G2 group was again partitioned with methanol and chloroform, generating two new partition phases: PF-BuOH G2.1 (7.5 g) and PF-BuOH G2.2 (3.0 g), the first being the methanol-soluble part, and the second the chloroform-soluble part (Fig. 1).

2.3 High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD)

HPLC-DAD analyses were performed using the Agilent 1260 system. Chromatograms were obtained with detection at 280 nm and 352 nm, using a Zorbax C18 column (150 mm × 4.6 mm x 3.5 µm) at 45 ^{o}C. As the mobile phase, a gradient of 0.1% acetic acid (AcOH) and acetonitrile (CH_3CN) was used, starting with 10% CH_3CN (0–6 min), increasing to 15% (6–7 min), maintaining isocratic for 15 min, followed by an increase to 50% (22–32 min) and 100% (32–42 min), remaining isocratic for eight minutes. The solvent flow was 1.0 mL/min. The sample volume injected for analysis was 3 µL.

2.4 High Performance Liquid Chromatography coupled with high resolution mass spectrometry (HPLC-MS/MS)

The analysis of the PF-BuOH was performed on a chromatograph (Shimadzu - model) coupled to a mass spectrometer (Maxis - model) equipped with an electron-spray ionization (ESI) source. The chromatographic conditions were the same of HPLC-DAD analysis. The conditions of the mass spectrometer were ionization in positive mode, with a capillary potential of 4000 V, sample-drying temperature of 200 ^{o}C, and nebulizer gas (Nitrogen) flow of 5 $L.min^{-1}$ with a pressure of 35 psi. Mass spectra were acquired in full scan mode in the range of m/z = 50 to 1200. A slope of 20 to 75 eV of collision energy was used in the analyses.

2.5 Obtaining molecular networks

The data obtained through HPLC-MS/MS were used for the construction of molecular networks using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data were filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were

then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks.

2.6 Quantification of amides and flavones

Apigenin and ferulic acid were dissolved in MeOH (1 mg/mL) to prepare stock solutions. These solutions were then serially diluted to the desired concentrations, ranging from 1.56 to 200 µg/mL for ferulic acid and 1 to 150 µg/mL for apigenin. All solutions were submitted to HPLC-DAD analyses as described above. The calibration curves were constructed by plotting the peak area against the concentration of each compound. The final values were expressed in mg/g of extract.

2.7 Biological activities

2.7.1 *In vitro* antioxidant activity assay

Samples were evaluated by DPPH radical scavenging assays (Pires et al. 2017) and Folin-Ciocalteu (Torres et al. 2018). For this, the samples were solubilized in methanol at a concentration of 2 mg/mL. The protocols were performed in a 96-well microplate and the absorbance readings were performed in a microplate reader (SynergyTM H1). Assays were performed in procedural triplicates.

For the DPPH assay, an 80 µM solution of DPPH in methanol was prepared at the time of testing. In each well of the microplate, 200 µL of the DPPH solution and 20 µL of each sample were added. After incubating the microplate for 30 min at room temperature, the absorbance was checked at 517 nm. Values were calculated in Trolox equivalent (TE) per gram of extract (mg TE/g). In the assay with the Folin-Ciocalteu reagent, an aliquot of 20 µL of the samples was added in each well of the microplate to 200 µL of ultrapure water and 20 µL of the Folin-Ciocalteu reagent (Dinâmica®). Between 5–8 min, 60 µL of a saturated sodium carbonate solution (250 mg/mL used after the formation of a precipitate) was added to this mixture. After 30 min, absorbances were read at 760nm. Using a gallic acid calibration curve (0-120 µg/mL), values were calculated in gallic acid equivalent (GAE) per gram of extract (mg GAE/g).

2.7.2 Evaluation of cytotoxicity and antioxidant activity of samples in RAW cells lines

MTT reduction Assay

RAW 264.7 macrophages (ATCC® TIB-71™,) cells were grown in a culture flask using Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS (10% v/v) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and maintained in a 5% CO_2 humidified at 37°C. The medium was changed every three days. To analyze the citotoxicity effect of the samples on cell viability it was done by MTT reduction. The cells were grown into 96-well plates until they reached a density of 1×10^4 cells/well. The medium was changed and it was not added FBS, cell lines were kept under starvation for 24 h. The next day, the medium was replaced with FBS and with samples at a concentration of 0.5 mg/mL. The negative control was cell culture without sample. After 24 h it was dome the MTT assay to evaluated the citotoxicity. The medium containg samples was removed and it was added a new medium having 1 mg/mL of MTT and it was

incubated for 4 h at 37°C. After this period, the supernadant was removed and the purple formazan crystal was solubilized in ethanol (Mosdam 1983). The absorbance was measured at 570 nm with a Biotek Epoch microplate spectrophotometer (Instruments Inc., Winooski, VT, USA). The negative control (without samples) were normalized as 100% MTT reduction and the values were correlated to negative control. The experiment was performed in triplicate.

Hydrogen peroxide Assay

To assess the extent of injury, a modified method from Ouyang et al. (2011) was utilized. RAW 264.7 cell line was seeded in 96-well plates at a density of 4 x 10^3 cells/well and incubated in DMEM medium supplemented with 10% FBS for 24 hours at 37°C with 5% CO_2. The medium was then replaced with serum-free medium containing hydrogen peroxide at different concentrations ranging from 0.1 to 5 mM, and incubated for 1 hour at 37°C. After that, the medium was replaced with DMEM medium containing 10% FBS, followed by incubation for another 24 hours at 37°C. Cells viability was determined using the MTT assay as described above. The concentration of hydrogen peroxide that promoted a 50% MTT reduction was considered as the optimal injury condition.

The antioxidant activity of the *C. macrocarpus* samples was evaluated by treating RAW 264.7 cell line concomitantly with 3 mM hydrogen peroxide and different samples concentratios (0.1 and 0.25 mg/mL). RAW 264.7 cell line was seeded at a density of 4 x 10^3 cells/well in 96-well plates and incubated in DMEM medium supplemented with 10% FBS for 24 hours at 37°C with 5% CO2. The DMEM medium was then replaced with a medium having samples plus 3 mM hydrogen peroxide, and it was incubated for 2 hours at 37°C. After this period, the medium was replaced with DMEM supplemented with 10% FBS, followed by incubation for 24 hours at 37°C. The antioxidant capacity of the samples was determined using the MTT assay as described above. A positive control was prepared using DMEM medium containing 10% FBS and 3 mM hydrogen peroxide, while a negative control was prepared using DMEM medium supplemented with 10% FBS without hydrogen peroxide.

2.7.3 Antimicrobial assay

Initially, the samples were evaluated at a concentration of 5 mg/mL against *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli*. Each microorganism was tested at four concentrations, 1.5x10^5 CFU/mL, 1.5x10^4 CFU/mL, 1.5x10^3 CFU/mL and 1.5x10^2 CFU/mL. Then, the samples with a significant inhibition result at this concentration were submitted to further analysis with different dilutions.

For the assay, the microdilution broth assay was used, following the methodology described by Martins et al. (2019a) and Martins et al. (2019b). Inoculums were prepared from colonies grown on Mueller Hinton agar (MH) (Oxoid) for *Staphylococcus aureus* and *Escherichia coli* and Sabouraud dextrose (SD) (Oxoid) for *Candida albicans*. The colonies were suspended in sterile saline solution, homogenized, and adjusted to the used concentrations.

A 1:2 dilution was used in MH broth (for bacteria) and SD (for yeast) medium, to which 10 µL of samples were added in 190 µL of the medium, in 96-well microplate wells (Costar), obtaining final concentrations per

well of 5.00 mg/mL, 2.50 mg/mL, 1.25 mg/mL, $6.25x10^{-1}$ mg/mL, $3.13x10^{-1}$ mg/mL, $1.56x10^{-1}$ mg/mL. Two wells were left as standards, one negative (only culture medium, no microorganism) and one positive (culture medium plus inoculum), in concentrations of 0.15 and 0.18 mg/mL. All tests were performed in procedural duplicates. After being filled, the microplates were incubated at 36°C for 24 h. Subsequently, 2 µL of each well were transferred to Petri dishes containing MH and SD agar media and incubated at 36°C for 24h to read the minimum microbial concentration.

2.7.4 Statistical analyses

The statistical analysis in this study involved a one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. A significance level of $p < 0.005$ was used. The one-way ANOVA was conducted to verify significant differences among the means of the measured variables across different groups. The Tukey post hoc test was then employed to perform pairwise comparisons between groups and identify specific groups with statistically significant differences.

3 Results

3.1 Identification and quantification of major compounds

Seven major compounds were annotated in PF-BuOH through HPLC-DAD, HPLC MS/MS, and comparison to GNPS platform, which provides a suggested identification based on database searching and HPLC-MS/MS fragmentation. However, it should be noted that the identification process using these methods may have some limitations, and there may be some uncertainty in the identification. For instance, determining the sugar position in glycosylated flavonoids may not be possible with absolute certainty. Table 1 presents the maximum absorption values in UV-visible spectra and the main m/z values from the mass spectrum. The molecular network analysis revealed two different groups of compounds based on MS/MS fragmentation patterns (Fig. 2).

Peaks 1, 2, and 3 showed similar UV-visible spectra, with maximum absorption (λ max) bands between 220 and 316 nm, characteristic of compounds derived from cinnamic acid (Caldas et al. 2019), in addition to similar MS/MS fragmentation patterns evidenced by the analysis of the molecular network (Table 1, Fig. 2A).

Peak 1 was annotated as *p*-coumaroyl agmatinebased on the mass spectral data obtained in positive ion mode. Upon protonation, *p*-coumaroyl agmatine typically forms $[M+H]^+$, which was observed in this study. Fragmentation of the $[M+H]^+$ ion led to the formation of several characteristic fragment ions. One prominent fragmentation pathway involves the loss of the *p*-coumaroyl group from the parent molecule, leaving behind the agmatine moiety. This loss of the *p*-coumaroyl group can be observed in the fragment ion at m/z 147, while the agmatine moiety can be observed in the fragment ion at m/z 248. This fragmentation pattern has already been reported by Pihlava (2014).

Peak 2 was annotated as feruloyl putrescine based on its characteristic fragments observed in MS^2. The compound had a molecular ion at m/z 265.1538, and yielded ions at m/z 177.0575 $[M+H-88]^+$, which was due to the loss of putrescine. In addition, the fragment ions at m/z 177.0545, 145.0285 and 117.0332 were

characteristic of this compound. The fragmentation pathway involved the cleavage of the C-N bond between the putrescine and ferulic acid moieties, resulting in the formation of a fragment ion at m/z 177 and m/z 145, corresponding to the ferulic acid moiety., while the ion at m/z 117 was likely formed from further fragmentation of the ferulic acid moiety. This fragmentation pattern is consistent with previous reports of Feruloyl Putrescine analyzed by M^S and MS^2, as described by Pihlava (2014).

Peak 3 was annotated as feruloyl agmatine based on its characteristic fragmentation pattern observed in MS^2. In positive ion mode, feruloyl agmatine undergoes protonation to form $[M+H]^+$, which then undergoes fragmentation. One observed fragmentation is the cleavage of the C-N bond between the agmatine and ferulic acid moieties, resulting in the formation of a fragment ion at m/z 177 and 145, corresponding to the ferulic acid moiety, which is also observed in peak 2. However, feruloyl agmatine can be distinguished from feruloyl putrescine (peak 2) by the cleavage of the ester bond between the ferulic acid and agmatine moieties, resulting in the formation of a unique fragment ion at m/z 248, corresponding to the agmatine moiety.

Chromatographic peaks 4, 5, 6, and 7 exhibited comparable UV-Vis absorption spectra, featuring maximum absorption (λ max) at around 270 nm and 334 nm, consistent with values reported for various flavone-type flavonoids derived from apigenin (Lachowicz et al. 2020) (Table 1). Moreover, these compounds displayed analogous fragmentation patterns in MS^2, and the consistency in the fragmentation pattern of these compounds supports their correlation in the GNPS analyses (Fig. 2B).

The fragments obtained were grouped and matched against the GNPS library, leading to the proposal of four glycosylated flavones: Isovitexin 2"-O-arabinoside (4), Isovitexin-2"-O-rhamnoside (5), Vitexin (6) and Saporanin (7). The fragmentation patterns were compared with those reported for flavones identified by Abrankó et al. (2011). In positive ion mode, all of these compounds gets protonated to form $[M+H]^+$, which then undergoes fragmentation.

Peak 4 was annotated as Isovitexin 2"-O-arabinoside by analyzing its fragmentation pattern in MS^2. The main fragmentation observed was the breaking of the glycosidic bond between the flavonoid (Isovitexin) and the arabinose moiety, resulting in the formation of a fragment ion at m/z 433, which corresponds to the Isovitexin aglycone moiety. Other fragments, such as the aglycone moiety at m/z 285, corresponding to the loss of the arabinose moiety, were also observed.

Peak 5 was annotated as Isovitexin-2”-O-rhamnoside based on its characteristic fragmentation pattern observed in MS^2. One of the main fragmentations observed was the cleavage of the glycosidic bond between the flavonoid (Isovitexin) and the rhamnose moiety, resulting in the formation of a fragment ion at m/z 433, corresponding to the Isovitexin aglycone moiety. Another fragment ion observed at m/z 313 corresponds to the rhamnose moiety.

Peak 6 was annotated as Vitexin based on its characteristic fragmentation pattern observed in MS^2. The main fragmentation observed was the cleavage of the glycosidic bond between the flavonoid (apigenin) and the glucose moiety, resulting in the formation of a fragment ion at m/z 269, corresponding to the apigenin aglycone moiety. Another major fragment was observed at m/z 283, corresponding to the apigenin aglycone

moiety with a loss of one carbon dioxide (CO_2) molecule. The loss of the C-ring fragment at m/z 165 is due to the breakage of the C-C bond between the C2 and C3 atoms of the flavone ring in apigenin, which leads to the formation of a carbocation on the C3 atom. This carbocation is unstable and can undergo a series of rearrangements and subsequent fragmentation to form the m/z 165 ion. This fragmentation pathway is a common occurrence in flavones and is known as the retro-Diels-Alder (RDA) fragmentation (He et al. 2012).

The peak 7, Saponarin, also known as apigenin-6-C-glucoside, was annotated based on its characteristic fragmentation pattern observed in MS^2. Saponarin is a flavonoid glycoside that can undergo fragmentation in mass spectrometry. A prominent fragment observed was at m/z 183, corresponding to the loss of the glucose moiety. This fragment is a result of the elimination of a water molecule from the aglycone ion. The fragment ion at m/z 165, corresponding to the loss of the C-ring, was also observed, which is a common occurrence in flavones and is known as the retro-Diels-Alder (RDA) fragmentation. Other fragments that were observed include a fragment ion at m/z 313, corresponding to the intact glucose moiety, and fragment ions at m/z 415, 397, and 379, which are likely to be related to the rearrangements and subsequent fragmentation of the apigenin aglycone moiety.

The quantification results of amides and flavones in the analyzed samples (mg/g of extract) have revealed intriguing findings, as summarized in Table 2. The PF-BuOH sample exhibited the presence of *p*-coumaroyl agmatine(0.9 mg/g), Feruloyl putrescine (0.9 mg/g), and Feruloyl agmatine (1.2 mg/g). Interestingly, the PF-BuOH G2.1 sample displayed enriched levels of these amides, with concentrations per gram of extract as follows: *p*-coumaroyl agmatine(1.7 mg/g), Feruloyl putrescine (1.7 mg/g), and Feruloyl agmatine (12.8 mg/g). Additionally, the PF-BuOH G4 sample contained Feruloyl agmatine (0.9 mg/g).

Regarding flavones, the PF-BuOH sample contained four types: Saponarin (0.6 mg/g), Vitexin 2"-O-arabinoside (1.3 mg/g), Isovitexin-2"-O-rhamnoside (1.3 mg/g), and Vitexin (3.3 mg/g). The PF-BuOH G1 sample contained Vitexin 2"-O-arabinoside (0.1 mg/g of extract) and Vitexin (1 mg/g of extract). Remarkably, the PF-BuOH G2.1 sample exhibited the highest levels of flavones, with Saponarin (9.3 mg/g), Vitexin 2"-O-arabinoside (1.2 mg/g), Isovitexin-2"-O-rhamnoside (15.9 mg/g), and Vitexin (19.7 mg/g). PF-BuOH F65 presented Isovitexin-2"-O-rhamnoside (1.9 mg/g) and Vitexin (0.4 mg/g). Notably, PF-BuOH G2.2, PF-BuOH G3, PF-BuOH G4, and PF-BuOH G5 samples did not show detectable levels of flavones. These findings underscore the variations in both the quantity and types of flavones and amides across the different samples.

3.2.1 Antioxidant activity

The antioxidant potential of the butanolic partition phase and the fractions and subfractions obtained were evaluated *in vitro* using the DPPH and Folin-Ciocalteu assays, as summarized in Table 3. Remarkably, the PF-BuOH G2.1 sample exhibited the highest antioxidant activity, with values of 237.2 ± 1.4 mg TE/g of extract in the DPPH assay and 118.2 ± 1.6 mg GAE/g of extract in the Folin-Ciocalteu assay (highlighted in bold in Table 3). Moderate activity was observed for PF-BuOH, with values of 131.8 ± 3.4 mg TE/g of extract in the DPPH assay and 102.3 ± 4.2 mg GAE/g of extract in the Folin-Ciocalteu assay. In contrast, weak activity was observed for PF-BuOH G2.2, with values of 103.8 ± 4.6 mg TE/g of extract in the DPPH assay and 83.8 ± 1.6

mg GAE/g of extract in the Folin-Ciocalteu assay, as well as for PF-BuOH F65, with values of 124.4 ± 75.9 mg TE/g of extract in the DPPH assay and 34.6 ± 8.3 mg GAE/g of extract in the Folin-Ciocalteu assay.

3.3 Evaluation of toxicity and antioxidant activity of samples in RAW cells

RAW 264.7 cell line (murine macrophages) was cultured with the addition of samples (PF-BuOH, PF-BuOH G4, PF-BuOH F65, PF-BuOH G2.1 and PF-BuOH G2.2) at concentration of 0.5 mg/mL. All samples showed signs of cytotoxicity, with PF-BuOH G2.2 leading to reduction of cell viability with MTT reduction values below 15%. Therefore, samples with a moderate cytotoxicity with MTT reduction values between 24% – 52%, were evaluated at lower concentrations (0.1 mg/mL and 0.25 mg/mL). At a concentration of 0.1 mg/mL, all samples, except PF-BuOH G2.1, were considered not cytotoxic (Fig. 3A).

The antioxidant activity of non-cytotoxic samples at 0.1 mg/mL was evaluated in RAW 264.7 cell line exposed to hydrogen peroxide. No protective action was observed in cells subjected to oxidative stress due to the presence of samples (Fig. 3B), that is, no difference was observed between the percentage of MTT reduction in the positive control (RAW 264.7 cells + hydrogen peroxide) and in the samples (RAW 264.7 cells + hydrogen peroxide + samples).

3.4 Antimicrobial activity

In the initial screening, all samples (PF-BuOH G2.1, PF-BuOH G2.2, PF-BuOH G4) except PF-BuOH F65, showed growth inhibition of *Candida albicans* and *Staphylococcus aureus*, but not influenced *Escherichia coli* (Table S2). The minimum inhibitory concentration of the samples that showed inhibition in the previous assay was determined using only *Candida albicans* and *Staphylococcus aureus* (Table 4). In general, greater inhibition of *C. albicans* growth was observed, even at higher microorganism loads, with lower MIC values associated with lower concentrations of microorganisms. The PF-BuOH G2.1 sample was the most efficient in inhibiting *C. albicans* with MIC = 1.25 mg/mL at the highest microbial load ($1.5x10^5$ CFU/mL). The same sample was the only one that showed activity against *Staphylococcus aureus*.

4 Discussion

4.1 Chemical characterization

The identification of three compounds derived from hydroxycinnamic acids, which showed similar fragmentation patterns and were grouped together in the same cluster of the molecular network, can be attributed to their biosynthesis pathways. Amides derived from hydroxycinnamic acids, like those identified in the butanolic partition phase, are known antifungal compounds, and are often classified as phytoalexins. These compounds are synthesized by plants in response to infections caused by pathogens and are widely known as strong antimicrobial and antibacterial agents (Darzuli et al. 2021). Many amides from plant sources have been reported, particularly from the Piperaceae and Rutaceae, which are known to produce a variety of secondary metabolites associated with plant defense processes (Kumar et al. 2018).

For instance, Dala-Paula et al. (2019) reported that *Citrus* species infected by pathogens tend to produce many amides, such as feruloyl putrescine, resulting in a more bitter taste to the juice. Amides have also been

previously reported for *Conchocarpus* species, including N-trans-feruloyltyramine (moupinamide) in *C. gaudichaudianus* (Ranieri Cortez et al. 2009)d *fontanesianus* (Cabral et al. 2016). However, to the best of our knowledge, this is the first report of *p*-coumaroyl agmatine, feruloyl putrescine, and feruloyl agmatine in this genus.

Flavonoids, on the other hand, are widely distributed in plants and are particularly common in species of Rutaceae, Zingiberaceae, Scrophulariaceae, Leguminosae, Ranunculaceae, and Myrtaceae (Iwashina 2000; Wang et al. 2019).

The flavones reported in this study have been previously reported in other species of the Rutaceae. Vitexin, for example, has been described by Noori et al. (2019) in *Ruta graveolens* L., in *Erythrochiton brasiliensis* Nees & Mart (Baj et al. 2017), and in *Euodia daniellii* (Benn.) Hemsl. (Yoo et al. 2002). Isovitexin-2”-O-arabinoside and isovitexin-2”-O-rhamnoside were found in the fruit peel of *Fortunella margarita* (Lour.) Swingle (synonym of *Citrus japonica* Thunb.) (Sadek et al. 2009). Saponarin was found in the ethanolic extract of *Feronia limonea* Scheff. along with Vitexin and other flavonoids (Intekhab and Aslam 2009; Kulkarni et al. 2021). This flavonoid has also been reported in the leaf extract of *Limonia acidissima* Groff (Dhakar et al. 2019) and *Citrus hystrix* DC (Umran et al. 2020), among other species in the family. As far as we can tell, these flavonoids have not been previously described in other *Conchocarpus* species. However, other C-glycosylflavones such as isoswertisin, 6,8-di-C-arabinosylpigenin, and 6-C-glucosyl-8-C-arabinosilgenkwanin have been reported in *Conchocarpus guyanensis* (Pulle) Kallunki & Pirani (Wirasutisna et al. 1986).

4.2 Assessing the Antioxidant Potential through *In vitro* and *In vivo* Methods

The antioxidant activity of the butanolic partition phase of *C. macrocarpus* leaf extract and its fractions was evaluated using the DPPH assay, yielding promising results. Significantly, the fractionation of PF-BuOH led to a remarkable improvement in antioxidant activity, with the PF-BuOH G2.1 fraction demonstrating a substantial increase from 131.8 ± 3.4 mg TE/g of extract (for PF-BuOH) to an impressive 237.2 ± 1.4 mg TE/g. These findings surpassed the reported antioxidant capacities of some Rutaceae species, highlighting the potency of the *C. macrocarpus* extract.

Li et al. (2018) defined an antioxidant capacity of 216 ± 0.066 mg TE/g of extract as strong for *Zanthoxylum avicennae*. Notably, the antioxidant capacity of PF-BuOH G2.1, with a value of 237.2 ± 1.4 mg TE/g, exceeded even this significant threshold.

In comparison to other Rutaceae plants, the antioxidant activity of the *C. macrocarpus* extract stood out prominently. For instance, the water extract of *Toddalia asiatica* (L.) exhibited a DPPH value of 122 ± 3.03 mg TE/g, while the ethyl acetate extract demonstrated a significantly lower value of 2.14 ± 0.002 mg TE/g. This disparity in antioxidant activity can be attributed to the higher phenolic content in the water extract, measuring 50.9 ± 0.52 mg GAE/g of extract, compared to 33.7 ± 0.39 mg GAE/g for the ethyl acetate extract (Lobine et al. 2021). These findings further underscore the notable antioxidant potential of the *C. macrocarpus* extract within the Rutaceae family.

In line with our findings, Fejzić and Ćavar (2014) observed similar results when assessing the antioxidant capacity of *Citrus* species. The orange peel extract exhibited the highest total phenol content (232.5 mg GAE/g) and one of the lowest IC_{50} values (19.15 ± 0.24 mg/mL). Conversely, lemon juice displayed the highest IC_{50} value (78.11 ± 6.70 mg/mL) and the lowest total phenol content (102.2 mg GAE/g).

The Folin-Ciocalteu method is widely used as an indicator of the antioxidant capacity of compounds (Aryal et al. 2019). Notably, the presence of these phenolic compounds is closely associated with the measured antioxidant activity (Fejzić and Ćavar 2014). Consistent with prior research, our study revealed that samples exhibiting higher levels of total phenols, as determined by the Folin-Ciocalteu method, also demonstrated greater antioxidant capacity in the DPPH assay.

Phenolic compounds, particularly flavonoids, are well-known natural products recognized for their potent antioxidant properties, which have been extensively investigated in previous studies (Ciumărnean et al. 2020; Heim et al. 2002; Moure et al. 2001). Therefore, the observed high antioxidant activity in the PF-BuOH G2.1 sample can be attributed to the presence of these powerful flavones. These findings emphasize the potential of natural products, specifically flavones, as valuable sources of potent antioxidants that can be explored for the development of novel therapeutics and dietary supplements.

Among all the fractions obtained, the PF-BuOH G2.1 sample exhibited the highest antioxidant activity. This can be attributed to the presence of various flavones, including Vitexin (19.3 mg/g of extract), Isovitexin-2"-O-rhamnoside (15.9 mg/g of extract), Vitexin 2"-O-arabinoside (1.2 mg/g of extract), and Saponarin (9.3 mg/g of extract). Flavones are the primary flavonoids identified in Rutaceae species, and they were found to be the major constituents of the butanolic phase, as previously reported (Yoro et al. 2020).

On the other hand, while the butanolic partition phase samples showed promising *in vitro* antioxidant activity, they did not provide significant protection against oxidative stress induced by hydrogen peroxide in RAW cells (murine macrophages). Previous studies have reported cellular protection against hydrogen peroxide for Rutaceae species, such as *Clausena excavata* Burm. f., where the presence of quercetin and myricetin in extracts was associated with strong protection against oxidative stress (Albaayit et al. 2015). Similarly, Alam et al. (2020) found that the foliar extract of *Zanthoxylum armatum* DC, which had high phenolic compound content, including quercetin, exhibited good *in vitro* antioxidant activity (DPPH) as well as strong cellular protection against hydrogen peroxide. However, in *Citrus paradisi* Macf., despite its antioxidant potential against the DPPH assay, no significant protection against hydrogen peroxide was observed in evaluated cells (Lado et al. 2016), a finding consistent with the present study. Zhang et al. (2008) identified three essential structural requirements for the effectiveness of cellular protection by flavonols such as rutin, including the 3-hydroxyl group on the C ring, the 3',4'-orthodihydroxy group (catechol structure) on the B ring, and the 2,3-double bond conjugated to the 4-oxo function in the C ring. Flavones derived from apigenin, such as those identified in *Conchocarpus macrocarpus*, do not possess two of these requirements and therefore have little or no cell protective activity, as reported by Surveswaran et al. (2007).

This study emphasizes the importance of conducting multiple assays to evaluate the potential health benefits of natural products. While the fractions obtained from *Conchocarpus macrocarpus* showed promising antioxidant activity *in vitro*, the lack of protection against hydrogen peroxide-induced oxidative

stress in RAW 264.7 cell lines suggests that further investigation is necessary to understand the potential health benefits of these fractions. Moreover, the structural requirements for effective cell protection against hydrogen peroxide should be taken into consideration when studying the potential health benefits of flavonoids and other phenolic compounds.

4.3 Antimicrobial assay

The results of this study demonstrate that the extracts tested exhibited greater antimicrobial activity against the Gram-positive bacteria *S. aureus* and the fungus *C. albicans*, while no activity was observed against the Gram-negative bacteria *E. coli*. The observed variation in the pattern of growth inhibition between Gram-positive and Gram-negative bacteria can be attributed to the fundamental differences in the composition of their cell walls. Gram-negative bacteria possess a more complex cell wall structure, which includes an outer membrane containing lipopolysaccharides, that can limit the permeability of drugs and antimicrobial compounds. This characteristic has been linked to the lower susceptibility of Gram-negative bacteria to certain classes of antibiotics and underscores the importance of identifying novel compounds with activity against these microorganisms (Lee et al. 2008).

Antimicrobial potential of Rutaceae species has been widely studied, and several studies have reported significant activity against a variety of microorganisms. For instance, the ethanolic extract of *Coleonema album* (Thunb.) Bartl. & H.L. Wendl. showed promising activity with a minimum inhibitory concentration (MIC) ranging from 0.195 mg/mL to 0.234 mg/mL for *C. albican*s and 0.729 mg/mL for *S. aureus*. The acetone extract also demonstrated antimicrobial activity, with the MIC ranging from 0.299 mg/mL to 0.338 mg/mL and 1.666 mg/mL to 2.083 mg/mL for *C. albicans* and *S. aureus*, respectively. The coumarin aglycones were found to be responsible for the observed activity (Esterhuizen et al. 2006).

Similarly, the methanolic extract and furanoquinoline alkaloids isolated from *Teclea afzelii* Engl. (Rutaceae) exhibited significant antimicrobial activity against various microorganisms, including *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, and *C. albicans* (Kuete et al. 2008). The dichloromethane extract of *Conchocarpus fontanesianus* also showed antifungal activity against *C. albicans*, with the alkaloids flindersine and methoxyflindersine being responsible for this activity (Cabral et al. 2016).

Regarding the present study, the results demonstrated weak activity of sample PF-BuOH G2.1 against *S. aureus* at the highest extract concentration (5 mg/mL) and the lowest microbial load evaluated ($1.5x10^2$ CFU/mL). However, this same sample were found to be active against *C. albicans*, with an MIC of 1.25 mg/mL. The same flavones present in this sample, as previously discussed for antioxidant activity or amides such as feruloyl putrescine (1.7 mg/g), feruloyl agmatine (12.8 mg/g), and *p*-coumaroyl agmatine 1.7 mg/g) could be responsible for the observed activity against *C. albicans*. In addition, a study by Ivanov et al. (2020) reported that flavones have antifungal potential against *C. albicans*, which could further explain the higher activity of PF-BuOH G2.1 among the evaluated samples. Furthermore, the PF-BuOH G2.1 sample had the highest flavone and amides content, which strengthens the hypothesis of the involvement of these compounds in the antimicrobial activity observed.

Overall, these findings suggest that *Conchocarpus* species could be an important source of antimicrobial compounds and could potentially lead to the discovery of novel drugs for the treatment of infectious

diseases caused by various microorganisms.

To summarize, this study sheds light on the promising antioxidant and antimicrobial properties of the butanolic partition phase and its fraction groups, particularly PF-BuOH G2.1, which exhibited antioxidant activity with values of 237.2 ± 1.4 mg TE/g of extract and 118.2 ± 1.6 mg GAE/g of extract in the DPPH and Folin-Ciocalteu assays, respectively. Additionally, the high flavone and amides content in the PF-BuOH G2.1 sample was associated with the inhibition of *Candida albicans*, with a MIC of 1.25 mg/mL at the highest microbial load ($1.5x10^5$ CFU/mL). Of note, this study presents the first report of four glycosylated flavones (Saporanin, Isovitexin 2"-O-arabinoside, Isovitexin-2"-O-rhamnoside, and Vitexin) and three amides derived from hydroxycinnamic acid (*p*-Coumaroyl agmatine, Feruloyl putrescine, and Feruloyl agmatine) in the genus *Conchocarpus*, highlighting its potential as a rich source of unexplored bioactive compounds. These findings could pave the way for the development of novel natural antioxidant and antimicrobial agents from *Conchocarpus*. Future studies are warranted to elucidate the mechanisms of action of these compounds and their potential for therapeutic applications. Overall, this study contributes to our understanding of the chemical composition and biological activity of *Conchocarpus* species and underscores their potential for the discovery of new bioactive compounds with diverse applications.

Declarations

Authors' contributions:

ERS and DYACS conceptualized and designed the study, critically revised the manuscript, and actively contributed to data discussions. PBT and ERS conducted the *in vitro* antioxidant activity, and actively participated in data discussions. ERS prepared the extracts, performed statistical analyses, conducted the experiments, and identified the compounds. KCS and HAOR performed the *in vivo* antioxidant experiments. IBS and JSS assessed the antimicrobial activity. All authors critically reviewed and approved the final version of the manuscript.

Data availability

All data are included in this article (and its supplementary information files).

Compliance with ethical standards

Conflict of interest:

The authors declare that they have no conflicts of interest.

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Tables

Table 1 Identified compounds detected in butanol partition phase obtained from leaf extract of *Conchocarpus macrocarpus*

Peak	t.r. (min)	Compounds	Mass $[M+H]^+$	Mass diference	Error (PPM)	Positive ionization Ms/Ms	UV max (nm)
1	3.0	*p*-Coumaroyl agmatine	277.1643	1.004	3609	277 ➡ 218; 248; 147; 119	220; 316
2	3.5	Feruloyl Putrescine	265.1521	0.002	5	265 ➡ 177; 145; 117	222; 316
3	5.9	Feruloyl agmatine	307.1752	,0.001	3	307 ➡ 248; 177; 145	226; 316
4	10.6	Saponarin	595.1611	0.003	5	595 ➡ 415; 397; 379; 367; 337; 313; 283; 183; 165; 121	230; 270; 334
5	13.9	Isovitexin 2”-*O*-arabinoside	565.1519	0.003	5	565 ➡ 433; 397; 287	236; 270; 332
6	14.6	Isovitexin-2”-*O*-rhamnoside	579.1691	0.003	4	579 ➡ 433; 367; 313	228; 268; 338
7	15.0	Vitexin	433.1095	0.004	9	433 ➡ 313; 269; 283; 165	228; 270; 338

Table 2 Quantification (mg/g of extract) of amides and flavones in butanol partition phase and its groups/fractions obtained from leaf extract of *Conchocarpus macrocarpus*

Samples[#]	Amides			Flavones			
	p-Coumaroyl agmatine	Feruloyl putrescine	Feruloyl agmatine	Saponarin	Vitexin 2"-O-arabinoside	Isovitexin-2"-O-rhamnoside	Vitexin
PF-BuOH	0.9	0.9	1.2	0.6	1.3	1.3	3.3
PF-BuOH G1	-	-	-	-	0.1	-	1
PF-BuOH G2.1	1.7	1.7	12.8	9.3	1.2	15.9	19.7
PF-BuOH G2.2	-	-	-	-	-	-	-
PF-BuOH G3	-	-	-	-	-	-	-
PF-BuOH G4	-	-	0.9	-	-	-	-
PF-BuOH G5	-	-	-	-	-	-	-
PF-BuOH F65	-	-	-	-	-	1.9	0.4

[#] For samples preparation see Fig. 1. Target compounds were quantified using ferulic acid and apigenin calibration curves. (-) indicates non-detectable levels of the compounds

Table 3 *In vitro* antioxidant activities of butanol partition phase and its groups/fractions obtained from leaf extract of *C. macrocarpus* using DPPH and Folin-Ciocalteu assays

Sample[#]	DPPH mg TE/g of extract	Folin-Ciocalteu mg GAE/g of extract
PF-BuOH	131.8 ± 3.4	102.3 ± 4.2
PF-BuOH G1	-	-
PF-BuOH G2.1	**237.2 ± 1.4**	**118.2 ± 1.6**
PF-BuOH G2.2	103.8 ± 4.6	83.8 **±** 1.6
PF-BuOH G3	-	-
PF-BuOH G4	-	-
PF-BuOH G5	-	-
PF-BuOH F65	124,4 ± 75,9	34.6 ± 8.3

[#] For samples preparation see Figure 1. The data are expressed as mg of Gallic Acid Equivalents (GAE) and mg Trolox Equivalents (TE) per g of the extract.

Table 4 Minimum inhibitory concentration (MIC) of butanol partition phase and its groups/fractions obtained from leaf extract of *C. macrocarpus* against *Candida albicans* and *Staphylococcus aureus*. The values in the table correspond to the concentration of the samples in mg/mL. (-) sample not tested at this microbial load. (x) sample tested without inhibitory activity. (*) The values correspond to all concentrations evaluated

Samples#	*Candida albicans*				*Staphylococcus aureus*			
	Concentration of microorganisms (CFU/mL)							
	$1,5x10^5$	$1,5x10^4$	$1,5x10^3$	$1,5x10^2$	$1,5x10^5$	$1,5x10^4$	$1,5x10^3$	$1,5x10^2$
PF-BuOH	-	X	-	-	-	-	-	-
PF-BuOH G2.1	1,25	X	-	-	-	-	X	5
PF-BuOH G2.2	2,5	X	-	-	-	-	-	-
PF-BuOH G4	X	X	-	-	-	-	-	-
Clorexidina					<0,18*			
Anfoterecina B	<0,15*							

For samples preparation see Figure 1. Chlorhexidine and Amphotericin B were the positive controls.

Figures

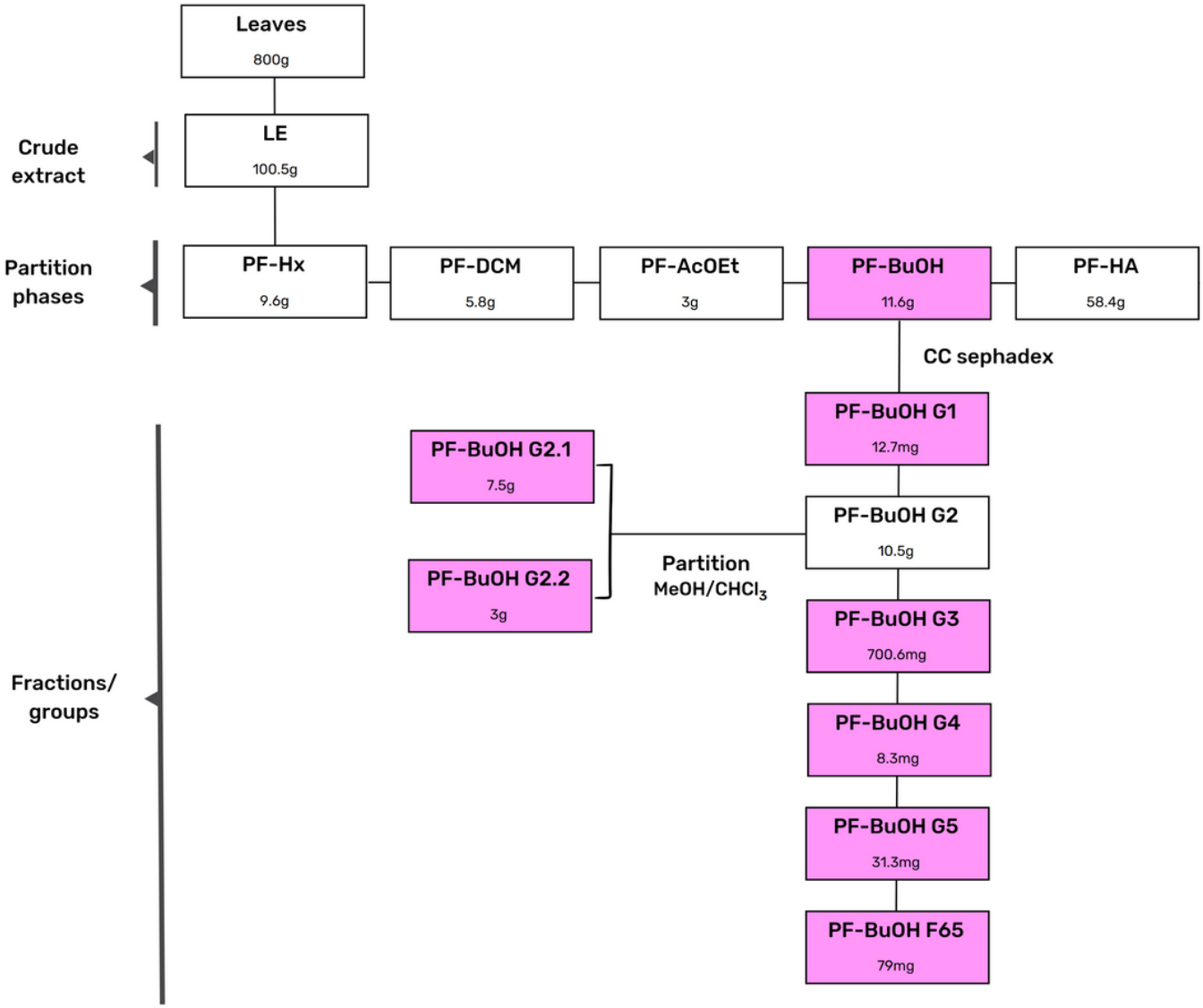


Figure 1

Samples production from dried leaves of *Conchocarpus macrocarpus.* Partition Phases (PF) using increasing polarity solvents (Hexane – Hx, Dichloromethane – DCM, Ethyl Acetate – AcOEt, Butanol – BuOH, and Hydroalcoholic – HA) were obtained from crude hydroethanolic Leaf Extract (LE). The PF-BuOH phase was subjected to additional fractionation using an open-top column packed with Sephadex LH20. The fractions were grouped into elution groups (G) based on TLC similarities. Further partition phases were generated using methanol (G2.1) and chloroform (G2.2) from PF-BuOH G2. Fraction F65 represents a single fraction obtained from the open-top column. Pink highlight corresponds to the samples used in the biological assays

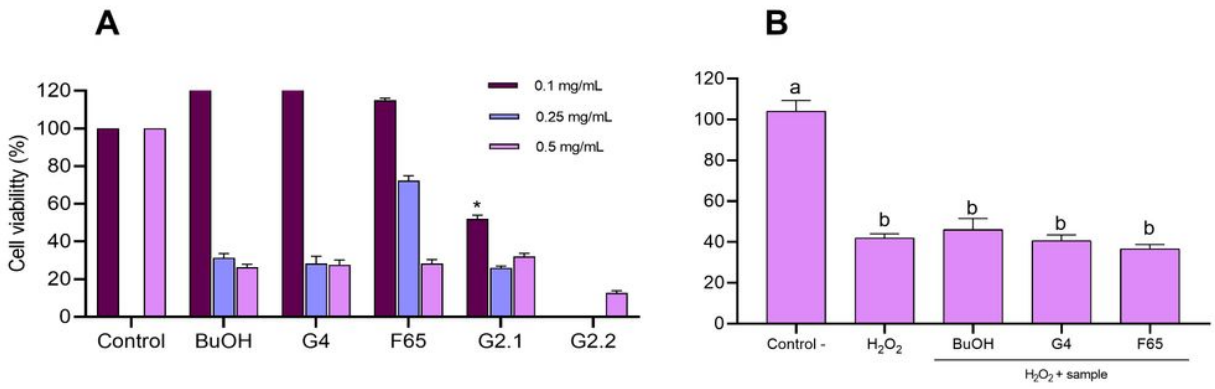


Figure 2

Molecular network of compounds from the butanolic partition phase obtained from the crude leaf extract of *Conchocarpus macrocarpus*. Smaller circles represent minor compounds; In green (amides) and pink (flavones) are the major compounds and the red lines highlight the link between these metabolites.

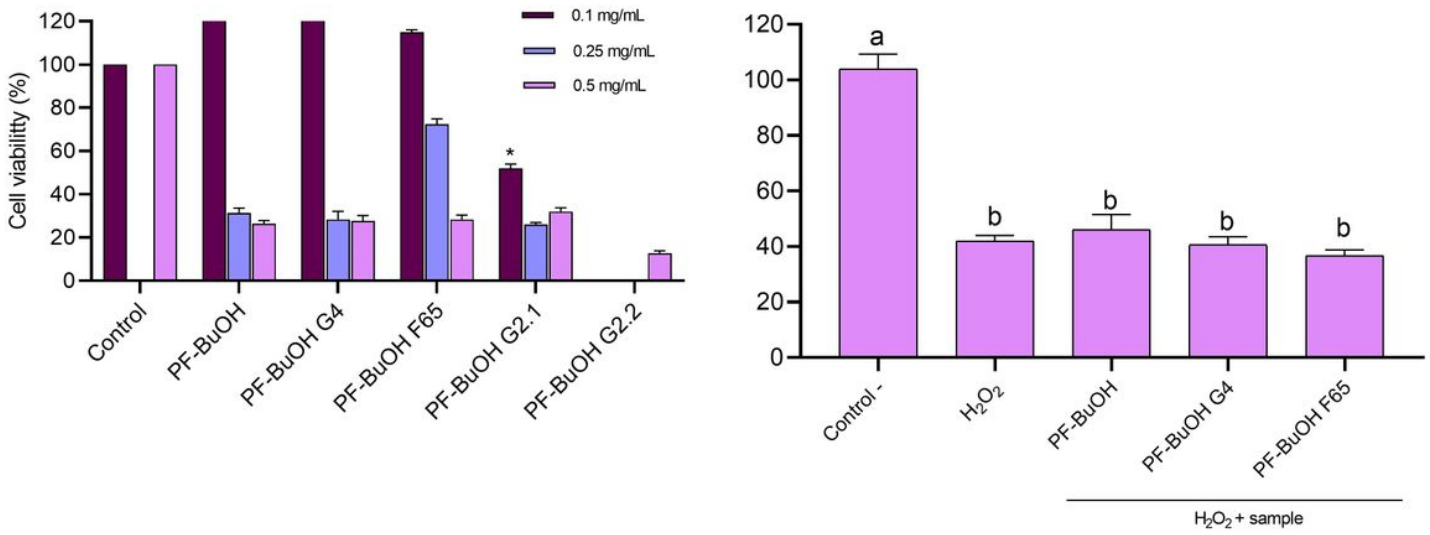


Figure 3

Toxicity and antioxidant activity of butanol partition phase and its groups/fractions obtained from leaf extract of *Conchocarpus macrocarpus* in RAW cells. For samples preparation see Figure 1. (**A**) Cell viability of RAW cells treated with samples at concentrations of 0.1 mg/mL, 0.25 mg/mL and 0.5 mg/mL, using the MTT method. The samples were dissolved in DMEM medium containing serum. (*) indicates cytotoxic samples at the lower evaluated concentration. (**B**) Cell viability of RAW cells exposed to hydrogen peroxide added of samples at 0.1 mg/mL. The samples were dissolved in DMEM medium containing FBS. Different letters indicate significant differences (one-way ANOVA followed by Tukey, $p < 0.005$)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Stable1.docx
- STable2.docx