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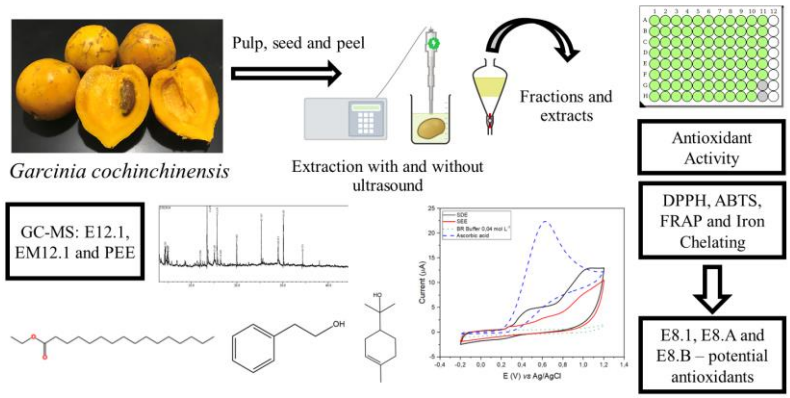
Evaluation of Antioxidant Activity of Pulp and Seed Extracts from *Garcinia cochinchinensis* and Chemical Characterization of its Essential Oil

Elton Kazmierczak* [a], André Victor Bassani [a], Maycon Hudson Contador [a], Sandra Grzygorczyk [b], Christiana Andrade Pessôa [a], Cássia Gonçalves Magalhães [a], and Romaiana Picada Pereira [a]

Garcinia cochinchinensis is native from Vietnam, used to cure allergy, itches and skin diseases. Considering the lack of studies about the chemical profile as well as the biological activities of *G. cochinchinensis,* this research aimed to evaluate antioxidant properties from its pulp and seeds extracts/fractions. Pulp was submitted to two different methods of extraction (liquid-liquid extraction with and without ultrasound extraction). Seed extracts were obtained with dichloromethane and ethyl acetate. Among these, the essential oil from the peel fruits of this species was analyzed by GC-MS. The antioxidant activity of extracts from pulp and seed was evaluated by the DPPH•, $ABTS^{\bullet+}$, FRAP, Iron (II) chelating methods, cyclic voltammetry and differential pulse voltammetry. Ethyl acetate in pH 8 and 4 fractions exhibited high values of phenolics and flavonoids content and showed effective antioxidant activity by the tested mechanisms. The essential oil from the peel is constituted by fatty esters and terpenes. Seed ethyl acetate extract exhibited expressive antioxidant activity in pH 6 via differential pulse voltammetry method. These promising fractions will be explored to isolate active phytochemicals and further the continuation of studies related to the evaluation of the antioxidant potential *in vivo* models.

Graphical abstract



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1. Introduction

Aerobic organisms can suffer damage by excess of reactive oxygen species (ROS), which may be free radical and non-free radical [1]. The oxidative stress is the condition of imbalance between the concentrations of reactive oxygen

[a] Department of Chemistry, State University of Ponta Grossa (UEPG). Av. General Carlos Cavalcanti - Uvaranas, ZIP CODE 84030-900, Ponta Grossa, Paraná, Brazil. [b] Department of Pharmacy, State University of Ponta Grossa (UEPG). Av. General Carlos Cavalcanti - Uvaranas, ZIP CODE 84030-900, Ponta Grossa, Paraná, Brazil. *Corresponding author. E-mail: **kazmierczak.elton@gmail.com**

species (ROS) and reactive nitrogen species (RNS) and antioxidant species, with the predominance of oxidant species. High production and concentration of ROS and low concentration of endogenous defense antioxidants lead to an accumulation of free radicals of oxygen, which induces cellular damage, including lipid peroxidation, damage in DNA and inducing other health problems [2, 3]. In normal conditions, there is a balance between prooxidant and antioxidant levels. However, in oxidative stress conditions, ROS can contribute to the development of some diseases and metabolic disorders, such as cancer, Alzheimer diseases, Parkinson, diabetes and obesity [3-6]. One way to treat oxidative stress and associated diseases is to use dietary rich with antioxidants from natural and artificial sources [3].

Natural or synthetic antioxidants, when present at low concentration in comparison with an oxidizable substrate, inhibits or prevents oxidation of this substrate. [5] Several natural plant antioxidants have no danger for dietary humans, in contrast with the synthetic antioxidants used in the food industry, which can be responsible for liver damage and carcinogenesis [3]. For this reason, research about the potential of natural products as antioxidants is important.

Among natural plants with antioxidant activity, species from the *Garcinia* genus (syn. *Rheedia*, Clusiaceae) is a relevant source. This genus is composed of more than 550 species, which are distributed in Asia, Africa, Australia, Polynesia and South America. Species from *Garcinia* have a variety of secondary metabolites which present diversified biological activities with therapeutics and medicinal uses, including anti-inflammatory, analgesic, antioxidant, cytotoxic against cancer cells and potential role as antimetabolic syndrome and disorders, such as diabetes and obesity [7-11].

Garcinia genus is rich of secondary metabolites, such as phenolic acids, flavonoids, xanthones and benzophenones and its polyprenylated forms. *G. cochinchinensis* is native from Viet Nam. In Vietnamese folk medicine, bark of this plant is used to treat allergy, diseases and threatened abortion [11, 12]. Popularly, *G. cochinchinensis* is known as false mangosteen or yellow mangosteen because of its yellow color in the young fruit stage, which is confused with *G. mangostana* [9, 13]. Farinazzi-machado et al. [14] studied the effect of leaves and pulp juices of *G. cochinchinensis* in the food intake, behavior and obesity in animal models. It was observed that the pulp juices intake reduces the weight and visceral fat after 40 days, when compared with the control group. Among the isolated metabolites, polyisoprenylated benzophenones isolated from pericarp from *G. cochinchinensis* showed strong activity against MCF-7 (breast adenocarcinoma), HeLa (human cervical cancer) and NCI-H460 (human lung cancer) human cancer cell lines [11].

The choice of extraction method is an important part in research of chemistry of natural products, since the profile chemical profile of plant extracts is influenced by that. Traditional techniques such as percolation, maceration, and Soxhlet extraction are used to prepare *Garcinia* extracts [15]. However, maceration followed by liquid-liquid extraction with variation of pH is a good method for conduct the isolation of phenolic compounds, which can change to organic phase or aqueous phase depending of pH. [16-17]. This method was effective for extracting phenolic compound by its different polarity accordingly to used solvent and the pH. In pH 8, for example, flavonoids and phenolics acids can be extracted with medium polarity using ethyl acetate. This occurs because the present base attacks phenolic acid hydroxyl, resulting in the respective salt in aqueous phase and medium polarity flavonoids in organic phase. Farinazzi-machado et al. [18] used only percolation extraction with different solvents to obtain extracts from pulp and leaves of *G. cochinchinensis*, different of this research, which seek obtain rich extracts with phenolic compounds using liquid-liquid partition that is based in [16].

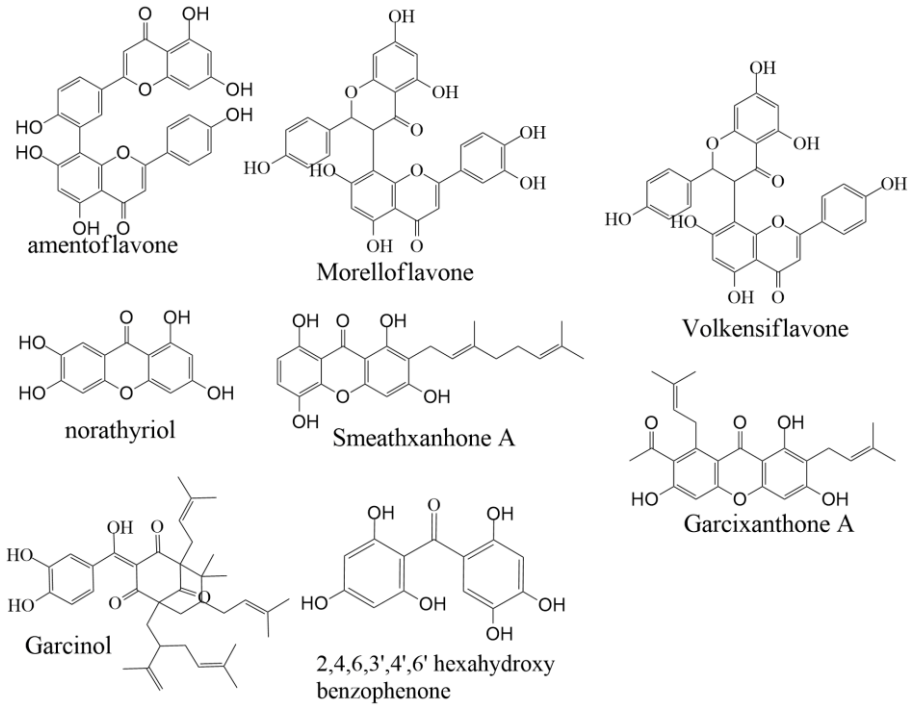

Fig. 1. Examples of secondary metabolites present in genus *Garcinia*.

Ultrasound-assisted extraction is an eco-friendly technique that offers advantages such as reducing solvent

use, extraction time, and energy consumption. This method was applied to extract biflavonoids from *Garcinia madruno* [15] and polysaccharides from *G. mangostana* [19]. Ultrasound is an emerging technique with a wide range of applications in research, especially in synthesis reactions and extractions. The extraction mechanism involves the formation of microbubbles through the cavitation phenomenon, where pressure variations caused by ultrasonic waves lead to the formation, growth, and collapse of these microbubbles. This sequence generates microjets that disrupt plant membranes, releasing secondary metabolites [20, 21]

Chemical profile of *Garcinia* genus can be known by several approaches. Li et al. [22] explored the metabolomic profile of *G. oblongifolia* through UPLC-QTOF-MS. Benzophenones, flavonoids and xanthones were verified in this species, mainly in leaves and branch extracts. Metabolites from *Garcinia* genus with antioxidant activity include amentoflavone, morelloflavone, volkensiflavone, xanthones norathyriol, smeathxanthone, garcinexanthone A and benzophenones 2,4,6,3',4',6'-hexahydroxybenzophenone and garcinol [8, 9, 22], illustrated in Fig. 1.

The consumption of fruits is increasingly encouraged due to this potential as natural antioxidants sources, which show fewer side effects [23]. In this context, research that prove the effectiveness of this activity in fruits that are few studied is relevant. In the best of our knowledge, there is not study related to *G. cochinchinensis* fruits grown in Brazil. Considering the lack of studies about the chemical profile as well as the biological activities of *G. cochinchinensis*, the aim of this study was to evaluate the antioxidant activity *in vitro* assays and electrochemical assays of pulp and seed extracts from this species, as well as to characterize using GC-MS essential oil from peel and pulp of *G. cochinchinensis*.

2. Material and Methods

2.1 Chemicals and equipment

All chemicals and solvents used were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid), potassium peroxydisulfate ($K_2S_2O_6$), iron sulphate ($FeSO_4$), 1,10-phenanthroline, iron chloride III ($FeCl_3$), anhydrous sodium sulfate (Na_2SO_4), ascorbic acid (AA), Folin-Ciocalteu reagent, aluminum chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), gallic acid and quercetin were obtained from suppliers Sigma Aldrich®, Merck®, Dinâmica® and Reatec®. The solutions were prepared with ultrapure water or methanol solvent.

The absorbances of the samples were read and registered in Biotek μQuant® (Synergy H1 Hybrid Reader) spectrophotometer of plate. For electrochemical assays, an Autolab® Multi Autolab/M204 potentiostat/galvanostat was used. The analyses in GC-MS were carried out using a GCMS2010 Plus (Shimadzu, Japan) equipment coupled to a triple quadrupole mass spectrometer TQ8040 and auto sampler AC 5000.

2.2 Plant Material

The fruits of *G. cochinchinensis* were collected in March 2020 at the campus from State University of Londrina (UEL, -23.3146 S, -51.2144 W), in Londrina, Paraná. The exsiccate of this vegetable is deposited in the UEL herbarium, with registration FUEL 54604. The collected fruits were selected based on their healthy appearance. The fruits were washed, peeled and their seeds were removed manually using a knife.

2.3 Preparation of Extracts of *G. cochinchinensis*

The fruits pulp (1.5 kg) was previously washed and cut. This sample was subjected to hydrodistillation for approximately 2.5 h. A liquid-liquid extraction of the aqueous phase was performed with chloroform (3 x 60 mL) to obtain the chloroform extract (CE). After, the aqueous solution was alkalized to pH 12, and extracted with ethyl acetate (3 x 70 mL), thus obtaining ethyl acetate extract pH 12 (EA12). After obtaining (EA12), it was extracted once again with ethyl acetate and methanol (70:30) to obtain the ethyl acetate/methanol extract pH 12 (EM12). The same procedure was continued, adjusting the pH to 8 and then acidifying to 4, obtaining the ethyl acetate pH 8 extract (EA8) and ethyl acetate pH 4 extract (EA4).

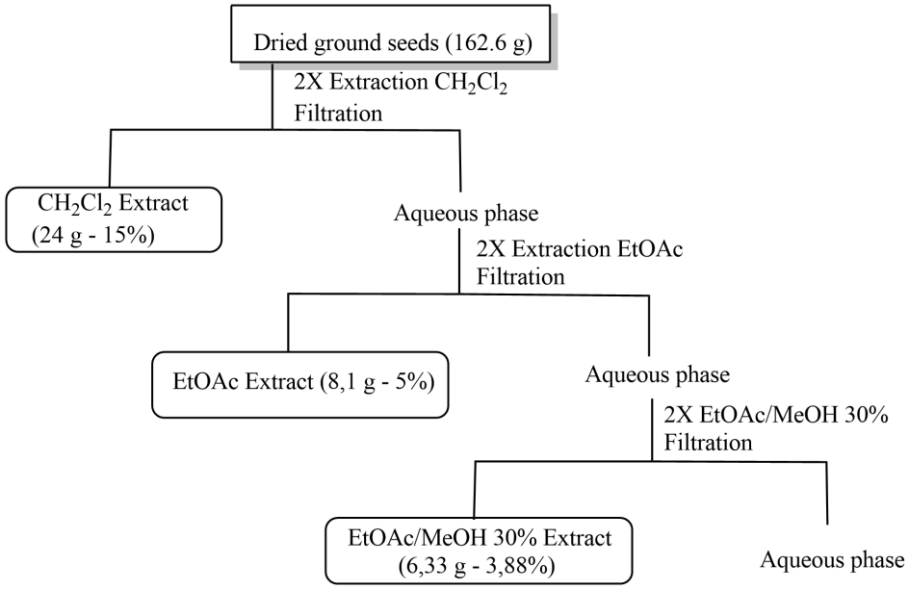

Fig. 2. Flowchart of extraction of *G. cochinchinensis* seeds.

The seeds were dried at 45 ºC for 72 hours. Seed shells (19.0 g) were separated from seeds, (162g), which were

ground and submitted to the extraction process with a sequence of solvent with increasing polarity. The extraction was made in series with three organic solvents in duplicate (250 mL) or mixture of solvent, under heating at 60 ºC for 30 minutes. The following extracts were obtained: seed dichloromethane extract (SDE), seed ethyl acetate extract (SEE) and seed ethyl acetate/methanol 30% extract (SEME). The flowchart of extraction is present in Figure 2.

The extracts were dried using anhydrous sodium sulfate, Na_2SO_4, filtered and then concentrated in a rotary evaporator at approximately 50 ºC.

2.3.1 Ultrasound Assisted Extraction of *G. cochinchinensis* pulp

The protocol used for extraction of fruits and its antioxidants was based in [20]. The sliced vegetable sample (700 g) was subjected to ultrasound-assisted extraction in an ultrasound device (sonifier from Sonics & Materials Inc model Vibra-Cell VC 505, 500 W, 20 kHz) with a probe coupled to a conical microtip of 12 mm diameter titanium.

The conditions of extractions were: 200 mL ultrapure water and ethanol (H_2O:EtOH, 1:1), 25 kHz frequency sound wave and extraction the time was 1 h. The extract obtained (EUA) was concentrated using a rotary evaporator under reduced pressure. EUA was again solubilized with 200 mL ultrapure water and ethanol (H_2O:EtOH, 1:1). From this aqueous phase, liquid-liquid extraction with chloroform (3 x 60 mL) was performed to obtain chloroform extract A (CA). The pH of the aqueous phase was adjusted to 12 and the extraction was carried out with ethyl acetate (3 X 100 mL) (E12A). This procedure was repeated with the ethyl acetate, but adjusting the pH to 8 and 4, obtaining the (E8A and E4A). The flowchart containing the extraction scheme is shown in Figure 3.

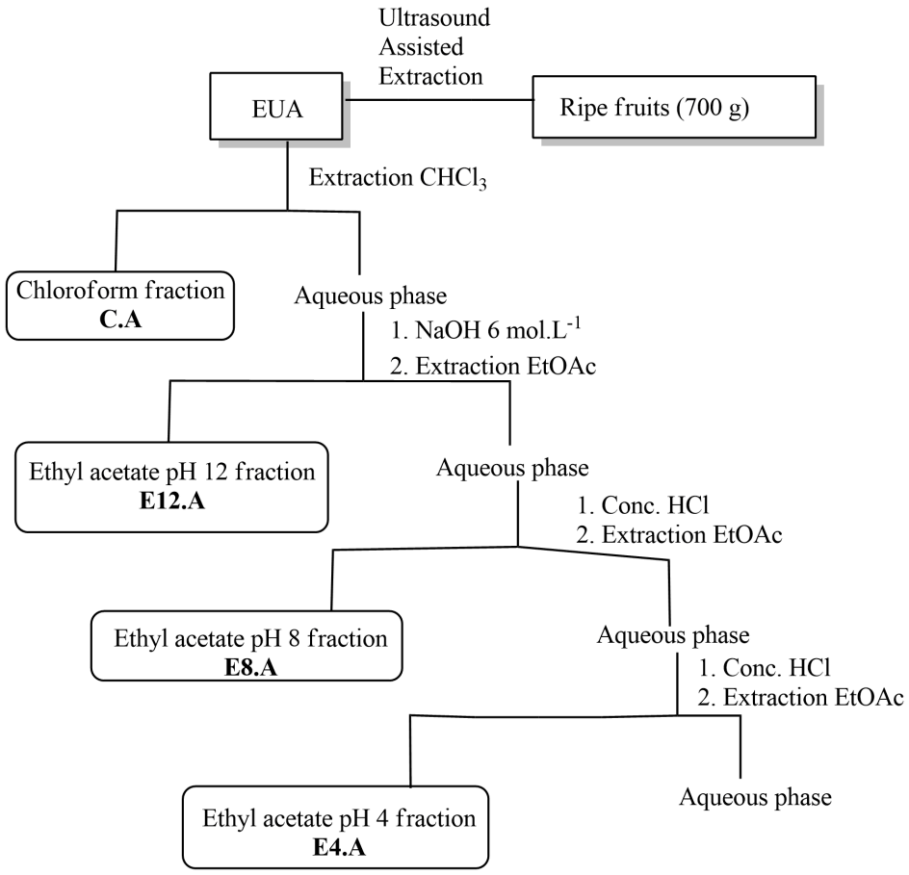

Fig. 3. Flowchart of liquid-liquid extraction of extracts obtained by ultrasound of *G. cochinchinensis* pulp.

At the end of all extractions, the organic phases were dried using anhydrous sodium sulfate, Na_2SO_4, filtered and concentrated in a rotary evaporator at approximately 50 ºC.

2.3.2 Ultrasound Assisted Extraction of peel fruits

The peel of the fruits (27.6 g) was removed and separated for extraction assisted by ultrasound with ethanol (400 mL) for 30 minutes. The conditions of extraction were: 25 kHz frequency of sound wave and amplitude of 25%. The procedure was carried out in triplicate. After that, the sample was filtered and a liquid-liquid extraction with hexane was performed. The hexane phase was concentrated using a rotary evaporator under reduced pressure at 50 ºC and it was denominated as peel essential oil (PEO).

2.4 Determination of Total Phenolic Content (TPC)

The determination of total phenolic content (TPC) was carried out according to the protocol from [24] with modifications. A calibration curve with gallic acid as standard (0.25 mg/mL) was prepared. The samples were diluted in ultrapure water until 500 µg/mL. In a 96-well ELISA plate, 100 µL of samples, 125 µL of Folin-Ciocalteu reagent (10% v/v) and 100 µL of sodium carbonate, Na_2CO_3, 7.5% (w/v) were added. After adding all reagents in the order described, the plate was incubated in the dark for 30 minutes. The absorbance was measured at 765 nm. The results were

expressed in terms of mg gallic acid equivalents (GAE) per g sample.

2.5 Determination of Total Flavonoids Content (TFC)

The determination of total flavonoids content (TFC) was carried out according to the protocol elaborated by [25] and with minor adaptations from [17]. The evaluation of pulp and seeds extracts was measured spectrophotometrically with minor modifications. For this assay, 100 µL of extracts (500 µg/mL) was added in 96 well microplates, after mixing 100 µL of $AlCl_3$ (10% w/v) solution in methanol. This mixture was incubated for 30 minutes, and subjected to spectroscopic analysis at 415 nm. A standard curve was carried out with quercetin and it was prepared at a concentration range of 0 to 100 µg/mL. The results were expressed as mg QE (quercetin equivalent)/g of extract. The analyses were realized in triplicate.

2.6 DPPH• radical scavenging assay

The determination of scavenging of radical DPPH• was performed as described in [26]. The test samples were initially prepared as a solution in MeOH (1000 µg/ml) and diluted at 100 µg/ml. In order to determine the inhibitory concentration 50% (IC_{50}) the samples at the concentration of 100, 50, 25, 12,5, 6,25, 3,125 µg/ml and ascorbic acid as positive control were used. In a 96-well microplate, 20 µL of sample solution, 80 µL ethanol and 100 µL DPPH• solution (120 µmol/L) were added in each well. For the blank, 180 µL of ethanol and 20 µL of the sample solution were added. For the control, 100 µL of ethanol and 100 µL of DPPH• solution were added. The reaction mixture was incubated for 30 min in the dark and the absorbance at 510 nm was measured with a microplate reader. The inhibition was calculated as follows:

$$I = \frac{Abscontrol - Abssample}{Abscontrol - Absblank} \quad \text{Eq. (1)}$$

where $Abs_{control}$ is the absorbance of the mean of the triplicate of control, Abs_{sample} is the absorbance of the samples. The experiment was carried out in triplicate (n = 3) and each time consisted of three repetitions.

2.7 ABTS•+ radical scavenging assay

The antioxidant activity was also evaluated by scavenging of radical ABTS•+ (2,2''-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) using the protocol from [27] with minor modifications. For this assay, 5 mL of 7 mM of ABTS stock solution was mixed with 88 µL of 140 mM potassium peroxydisulfate ($K_2S_2O_6$) and after stocked in the dark room for 16 hours before using it. The ABTS•+ solution was diluted with phosphate buffer pH 7.4 (10 mM) with dimethyl sulfoxide (DMSO) until obtain absorbance of 0.7 at 734 nm. For the evaluation, 20 µL of the sample in different concentrations (3,125 – 100 µg/mL) was added to 96 wells microplate. After this, 80 µL of phosphate buffer pH 7,4 (10 mM) and 100 µL of ABTS•+ working solution were added. Then, the plate was incubated for 30 min. The blank was prepared as 100 µL of sample and 100 µL of phosphate buffer. The positive control was prepared using ascorbic acid in the place of the samples. The reading of the plate was carried out in a spectrophotometer at 734 nm. The results were expressed as IC_{50} (µg/mL) as Eq. 1.

2.8 Reducing Power Assay

The ferric-reducing antioxidant power (FRAP) was carried out according to a method reported by [28] with minor modifications. It was prepared the following solutions for this assay: 0.160 g of $NH_4Fe(SO_4)_2{\cdot}12\ H_2O$ in 2 mL of HCl (1 M) and *o*-phenanthroline (0.01 M). These two solutions were mixed and diluted with distilled water until 100 mL. In 96 well microplates, 150 µL of the samples (500 µg/mL) and 100 µL of Fe^{+3}-phenantroline complex were added and incubated for 30 min in a dark room. A standard curve was prepared with ascorbic acid in the place of the samples, but using different concentrations for the curve. The increase of red-orange color indicated the presence of the ferrous-phenanthroline complex (Fe^{2+}-*o*-phen), evaluated by the measure of the absorbance at 510 nm. The results were expressed as mg AA (acid ascorbic equivalent)/g of the sample.

2.9 Fe^{2+} chelation assay

The capacity of chelation of ferrous ion was adapted by the method described by [29] with minor modifications. 150 µL of the samples (500 µg/mL) were placed in 96 well microplate and mixed with 100 µL of ferrous sulfate, $FeSO_4$ (500µM). The microplate was incubated for 30 minutes. Then, 45 µL of *o*-phenanthroline (0.25% w/v) was added. For the blank, the samples and *o*-phenanthroline solutions were mixed. The standard curve was prepared using 45 µL *o*-phenanthroline solution and 100 µL $FeSO_4$ (500 µM to 50 µM). The absorbance was measured at 510 nm in a spectrophotometer. The Fe (II) chelating capacity was calculated with respect to the reference of the standard curve, which was calculated by the equation of the straight line. The results were expressed in terms of percentage of Fe (II) ions chelated.

2.10 Electrochemical assay

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed using an Autolab® Multi Autolab/M204 potentiostat/galvanostat at room temperature. The NOVA 2.1.7 software was used in a three-electrode system consisting of a glassy carbon working electrode (GCE), a saturated Ag/AgCl reference electrode, and a Pt wire as counter electrode. All measurements were carried out in a 0.04 M Britton-Robinson (BR) buffer solution with pH varying from 2 to 10. The GCE surface was polished with 1.0 µm and 0.5 µm alumina/water slurries on a flat pad, followed by sonication in ultrapure water for 5 minutes [30]. CV experiments were carried out in a potential window of -0.2 V to +1.2 V (vs. Ag/AgCl) at a scan rate of 100 mV s^{-1}. DPV measurements were conducted in the potential window of -0.2 V to 0.8 V (vs. Ag/AgCl) with a step potential of 5 mV, a modulation amplitude of 50 mV, a modulation time of 0.05 s, and an interval time of 0.5 s. The concentrations of extract and ascorbic acid added in the electrochemical cell were 2000 mg/L.

2.11 GC-MS analysis

The samples EA12, EM12 and PEE were analyzed by Gas Chromatography coupled with Mass Spectrometry (GC-MS). A GCMS2010 Plus (Shimadzu, Japan) equipped with a triple quadrupole mass spectrometer TQ8040 and an auto sampler AC 5000, with an SH-Rtx-5MS column (Shimadzu), was used. The low polaritiy column used was SH-Rtx-5MS (30m x 0,25mm x 0,25 µm). GC-MS spectra were obtained using the following conditions: the carrier gas was helium, the flow rate

for this column was 1.00 mL/min, splitless model injection, injection temperature was set at 180 ºC, oven temperature program was held at 60 ºC for 2 min and then increased by rate of 4 ºC/min to 240 ºC and held to 250 ºC for 6 min.

The identification of the components was based on the comparison with their respective mass spectra matching against commercial libraries (Wiley, MassFinder 2.1 Library and NIST 98). RI values were calculated with the help of a series of linear alkanes C8–C20 on the low polarity column SH-Rtx-5MS.The compounds were identified and mentioned with its retention time (min) and area (%). Each sample was analyzed twice.

2.12 Statistical Analysis

All assays of antioxidant activity were carried out in triplicates. The experimental results were expressed as mean ± standard deviation. The data were analyzed using one-way analysis variance (ANOVA) using software Origin version 2018.

3. Results and Discussion

3.1 Total phenolic compounds (TPC) and flavonoids (TFC)

Total phenolic content (TPC) and total flavonoids content (TFC) of the fraction from pulp and extracts from seed of *G. cochinchinensis* are shown in Table 1. The acoustic phenomenon of ultrasound contributed to the best extraction of phenolic compounds and flavonoids [30, 31], as can be observed between EA4 (4.36 ± 0.8 mg GA g^{-1}) and E4.A (20.23 ± 0.08 mg GA g^{-1}) for TPC and 2.81 ± 0.74 mg QE g^{-1} and 12.58 ± 0.84 mg QE g^{-1} for TFC. Another argument that highlights the good performance of extraction assisted by ultrasound is the weight of pulp used in the extraction. 700 g was used in extraction assisted by ultrasound and 1.5 kg was used in the only maceration followed by sequential liquid liquid extraction. It can demonstrate that the ultrasound was able to extract the same amount or more metabolites for a minor amount of sample. The highest values of TPC and TFC are found in fractions of ethyl acetate in pH 8 and 4 of these three extractions of pulp, such as EA8 (25.3 ± 0.23), E8.A (24.6 ± 0.23) for TPC. About total flavonoids content (TFC), similar way occurs with the fractions of the ethyl acetate in pH 8 and 4, with exceptions, such as E12.1 (14.18 ± 0.45 mg QE g^{-1}) have the highest content of flavonoid among fractions of same extraction. Extracts from seed showed high content of phenolics and flavonoids compounds, among two extracts ethyl acetate solvent was more efficient in extraction of phenolic and flavonoids compounds because of medium polarity of this solvent, which shown values of TPC and TFC 115.25 ± 0.092 mg GA g^{-1} and 49.45 ± 0.79 mg QE g^{-1}, respectively. This phenomenon is in line with [32], in which the authors studied antioxidant activity of seeds, epicarps and leaves of *G. madruno* of sequential liquid liquid extraction obtaining hexane, dichloromethane, ethyl acetate and methanol extracts. The extract obtained with ethyl acetate showed highest values of total phenolic compound.

The chloroform extract exhibited a lower proportion of phenolics and flavonoids (Table 1) if it is compared to those obtained with ethyl acetate, in the same extraction. Very polar solvents, such as water and others with low polarity extract low amounts of phenolic compounds and flavonoids. [33] discuss the uses of different solvents in extraction of peel of *G. mangostana* to obtain antioxidant compounds. They indicated that medium polarity solvents, such as ethyl acetate and acetone or methanol and ethanol, can extract more easily and to obtain more amount reducing agents compared to the non-polar solvents [33]. The pH variation was also taken into account in this work regarding fractionations for better extraction of phenolic compounds and flavonoids. Therefore, it is possible to obtain more expressive values of antioxidant activity due to the higher concentrations of phenolic compounds. The content of total phenolic compounds and flavonoids increased in the extracts obtained with ethyl acetate at pH 8, in the three fractionations. The adjustment from pH 12 to 8 allowed the solution to be slightly basified, causing the formation of phenolate ions from the phenolic compounds that they have a pKa of around 4 to 4.5, arising from the resonance of phenols through the reaction with hydroxide ions from the basic environment, and flavonoids with higher acidity hydrogens remain intact in ethyl acetate. Above pH 9.5, ionization of more acidic protons may occur, which makes the extraction difficult, it can become more difficult for the phenolate ions to be solubilized in the aqueous phase. This justifies why phenolic compounds do not remain at a high level in the extracts obtained at pH 12 compared to those obtained at pH 8. As the pKa of the organic phase solution is adjusted, in the way that the ionic strength of the phenolate ions migrate easily to the aqueous phase that will be extracted with ethyl acetate at pH 8 [16].

Table 1. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of extracts obtained with different polarity from liquid liquid extraction and extraction assisted by ultrasound of pulp and seed extracts from *G. cochinchinensis*.

Source	Samples	TPC (mg GA g^{-1}.) *	TFC (mg QE g^{-1}) *
Pulp	CE	10.42 ± 0.48^{d}	5.65 ± 1.47^{de}
	EA12	4.93 ± 0.11^{f}	14.18 ± 0.45^{a}
	EA8	25.3 ± 0.23^{b}	10.11 ± 0.16^{bc}
	EA4	4.36 ± 0,8^{f}	2.81 ± 0.74^{ef}
	C.A.	4.43 ± 0.4^{f}	2.85 ± 0.78^{ef}
	E12.A	4.79 ± 0.05^{f}	2.86 ± 0.48^{ef}
	E8.A	24.6 ± 0.23^{b}	11.45 ± 0.19^{abc}
	E4.A	20.23 ± 0.08^{bc}	12.58 ± 0.84^{ab}
Seed	SDE	64.90 ± 0.015^{g}	14.77±0.043^{ab}
	SEE	115.25 ± 0.092^{h}	49.45±0.79^{g}

Note: *Values presented as mean ± standard deviation (n=3) Means followed by different letters (a-h) differ statistically in the same column (Newman-Keuls at p > 0.05). Tukey's test was applied at a 5% probability level.

Farinazzi-machado et al. [18] evaluated non-sequential liquid-liquid extraction of the fruits of *G. cochinchinensis* and used the extract in acetone to determine the TPC and TFC. The values obtained by [18] were 469.6 ± 114.9 mg gallic acid 100 g^{-1} of the fruit for total phenolic compounds, 89.6 ± 14.7 mg of rutin 100 g^{-1} of the fruit and 14.9 ± 2.43 mg quercetin g^{-1} for flavonoids. The difference in this case can be due to the technique used in the extraction, as well as the nature of the solvent used [34]. Studied the antioxidant activity, and the determination of phenolics compounds, minerals and vitamins of *G. cochinchinensis* and other species from Cambodian. The total phenolic content of methanol extract of *G. cochinchinensis* reported was 28.29 ± 2.9 µg GA mg^{-1}. In present work, it was observed that the ethyl acetate in pH 8 and 4 fractions showed the highest content of phenolics compounds and flavonoids when compared with ethyl acetate pH 12 and chloroform fraction in both extraction methods. The portion of content of phenolic compounds in chloroform fractions can be explained by presence of hydroxylated

xanthones with non-polar carbonic chain or hydroxylated poly isoprenylated benzophenones, which are soluble in low polarity solvent.

3.2 Antioxidant activity

There are several ways to represent the antioxidant activity of a compound or extract. A curve can be created and represented by the equivalent of a known standard, such as mg AA (ascorbic acid) equivalent/g sample. For the antioxidant activities in question, the representation of 50% inhibitory concentration (IC_{50}) was used, which is the concentration necessary to inhibit 50% of the radicals in the experiment. In other words, the lower the IC_{50} value, the greater the antioxidant activity of the compound or extract analyzed as well as the quantity or concentration necessary to inhibit the radical is smaller. In Table 2 the IC_{50} values of all *G. cochinchinensis* extracts from the pulp are reported.

Table 2. Antioxidant activity of extracts from *G. cochinchinensis* pulp.

Samples	DPPH• IC_{50} (µg/mL) *	$ABTS^{\cdot+}$ IC_{50} (µg/mL) *	FRAP (mg AA g^{-1}) *	Chelation of Fe^{2+} (%) *
CE	86.70 ± 11,04^{c}	43.14 ± 14^{c}	2.9 ± 0.02^{b}	21.3 ± 4^{d}
EA12	364.7 ± 91.3^{a}	1843.7 ± 268^{d}	3.5 ± 0.3^{b}	81.7 ± 5^{ab}
EA8	34.0 ± 5.1^{c}	40.08 ± 8.6^{b}	47.26 ± 0.2^{a}	25.7 ± 8^{d}
EA4	174.9 ± 44.7^{ab}	37.16 ± 6.27^{c}	3.73 ± 0.3^{b}	65.7 ± 3^{abc}
C.A.	366.9 ± 130.6^{ab}	65,08 ± 0,93^{c}	8.52 ± 0.3^{b}	42.7 ± 4^{cd}
E12.A	774.3 ± 285.5^{ab}	14,44 ± 0,85^{a}	7.7 ± 0.3^{b}	81.3 ± 2^{ab}
E8.A	30.01 ± 0.9^{c}	15,14 ± 1,25^{a}	46.2 ± 0.1^{b}	89 ± 8^{a}
E4.A	24.8 ± 1.4^{c}	33,02 ± 17,81^{b}	5.93 ± 0.3^{b}	83 ± 12^{ab}
AA	12.46 ± 1.7^{d}	9.96 ± 1.4^{a}	-	-

Note: *Values presented as mean ± standard deviation (n=3) Means followed by different letters (a-h) differ statistically in the same column (Newman-Keuls at p > 0.05). Tukey's test was applied at a 5% probability level. AA = ascorbic acid.

Nguyen et al. [35] reported the antioxidant and antimicrobial activities of six species of *Garcinia*. The extracts were prepared with n-hexane, ethyl acetate and methanol starting with Soxhlet methanol extract of these species plant material. It was observed that great values of antioxidant activity were exhibited by methanol and ethyl acetate extracts, when compared with n-hexane extract. This is in accordance to the present work, which ethyl acetate in pH 8 and 4 is higher free radical scavenging compared to chloroform fractions in DPPH• and $ABTS^{\cdot+}$ assays. The antioxidant assay of the extracts and fractions from *G. cochinchinensis* of scavenging of DPPH• and $ABTS^{\cdot+}$ is based on a single electron transfer (SET) and hydrogen atom transfer (HAT) reaction, because hydroxyl groups in phenolics compounds or unsaturated compounds are able to donate electrons or hydrogen atoms to stabilizing radicals [36].

The fractions obtained with ethyl acetate in pH 8 and 4 showed the highest antioxidant activity in the DPPH• and $ABTS^{\cdot+}$ assays. The extracts EA8 (34,0 ± 5,1 µg/mL), E8.A (30,01 ± 0,9 µg/mL) and E4A (24,8 ± 1,4 µg/mL) presented the best inhibition values for the DPPH• scavenging assay. The $ABTS^{\cdot+}$ radical is prepared from non-radical ABTS by reacting with a strong oxidizing agent, such as persulfate ion. $ABTS^{\cdot+}$ is a blue-green radical cation with maximum absorption at 734 nm, which gradually loses its color intensity when it reacts with antioxidant species [37]. The EA8, E8.A and E4A fractions showed near values of IC_{50} for $ABTS^{\cdot+}$ scavenging assay. Some fractions, such as E8.A (15,14 ± 1,25 µg/mL) were near, statistically, to ascorbic acid IC_{50} (9.96 ± 1.4µg/mL). IC_{50} values higher than 50 µg/mL were observed for the extracts CE, EA12, EA4, C.A and E12.A, which is related to the low antioxidant activity (Table 2). This may have occurred due to the low content of phenolic compounds and flavonoids in those extracts, as well as the difficulty in donating hydrogen atoms or transferring electrons from the compounds present in those extracts. The DPPH• radical capture assay is among the most commonly used assays to evaluate antioxidant activity. This is a method based on electron transfer (SET), with the transfer of hydrogen atoms being a reaction mechanism that is only a marginal route in the test. DPPH• is a stable radical with a purple color and soluble in ethanol. After reacting with antioxidants, electrons or hydrogen atoms, it loses its purple color, becoming colorless or slightly yellowish. The colorless compound formed is also called 2,2-diphenyl-picrylhydrazyl or reduced DPPH [37]. However, it is important to observe because are not only phenolic compounds responsible for scavenging of DPPH• and $ABTS^{\cdot+}$ radicals, but also other xanthones and benzophenones, with unsaturated bonds or hydroxylated aromatic rings. [38]. These data are in agreement with the study of [39], where the antioxidant activity of the leaves, roots and fruits of *G. xanthochymus* was evaluated. The authors performed fractional extraction with petroleum ether, ethyl acetate, n-butanol, in a similar way to the present work. The extracts obtained with ethyl acetate and n-butanol of the fruits, leaves and root exhibited lowest IC_{50} for DPPH• and $ABTS^{\cdot+}$ scavenging assays. This indicates that intermediary polarity solvent can extract good content of phenolic compounds with great antioxidant activity.

The FRAP evaluation indicates the capacity of isolated compounds, extracts or fractions in the reaction that occurs in the one-electron redox reaction, generally redox reactions that reduce Fe^{3+} to Fe^{2+}. Therefore, the mechanism presents in FRAP is SET, because it measures the capacity of potential antioxidant donor one electron to reduce some compounds, mainly transition metals and other pro-oxidant agents. [38,40] The need to investigate the ability to reduce iron ions is in their involvement in the oxidative process, increasing the concentration of ROS. Consequently, worsening OS. Iron participates in the Fenton reaction, present in the body, with the Fenton reaction being an advanced oxidative process (AOP), [41, 42]. Some ROS are moderately reactive, such as superoxide, $O_2^{\cdot-}$, like most biological molecules. However, through the Haber-Weiss reaction catalyzed by the Fenton reaction, other extremely reactive ROS are generated [42]. Fe^{3+} reacts with superoxide to form Fe^{2+} (Reaction 1), this cation reacts quickly with hydrogen peroxide, H_2O_2, producing iron III ions, radical hydroxyl and hydroxyl ions (Reaction 2), ROS that are extremely harmful to the body [42]. The combination of reactions 1 and 2 results in the Haber-Weiss reaction (Reaction 3) catalyzed by iron, which ends up being possible to occur *in vivo* [39, 40, 42]. According with results present in

Table 2, the ethyl acetate in pH 8 fraction in three extractions showed higher FRAP values being E8.1 (47.26 ± 0.2 mg AA g^{-1}) and E8.A (46.2 ± 0.1 mg AA g^{-1}), These fractions have significant values of antioxidant potential in the methods above mentioned, supported by the presence of phenolic and flavonoids compounds.

$Fe^{3+} + O2^{\bullet-} \rightarrow Fe^{2+} + O_2$ Reaction 1

$Fe^{2+} H_2O_2 \rightarrow Fe^{3+} + OH^- + HO\bullet$ Reaction 2 - Fenton Reaction

$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO\bullet + OH^-$ Reaction 3 – Haber-Weiss Reaction

The iron chelating capacity in terms of percentage of chelated iron was more significant for the extracts E8.A (89%), E4.A (83%), E12.A (81%) and E12.1 (81%, Table 2). The presence of phenolic and flavonoid compounds in these extracts confirms their chelating capacity, as these antioxidant species can chelate the transition metal and inhibit or delay its participation in lipid oxidation via the Fenton reaction [40]. The evaluation of antioxidant activity is made when a complex is formed between the antioxidant and the metallic ion Fe^{2+}. In the present work, however, the chelating agent 1,10-phenanthroline was used instead of EDTA to chelate excess iron that was not chelated by the compounds present in the samples. Thus, the maximum absorbance was measured at 510 nm, which is read for the 1,10-phenanthroline-Fe^{2+} complex (Fig. 4), since the loss of absorbance means that, after reacting with the samples, a metal-antioxidant complex was formed and the metal's chelation capacity could be quantified spectrophotometrically. Therefore, what is read is the 1,10-phenanthroline-Fe^{2+} complex after the chelation reaction. Consequently, the percentage of iron ions, Fe^{2+}, chelated by the samples was determined [43].

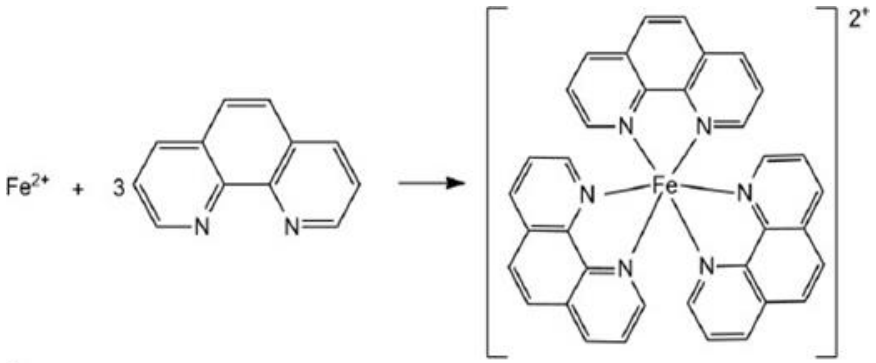

Fig. 4. Reaction of chelating of Fe^{2+} with 1,10-phenanthroline.

Chelation of transition metals prevents their participation in reactions that generate oxidizing species, such as Fenton and Haber-Weiss reactions [45, 46]. Considering the presence of flavonoids in the extracts, the interaction between the flavonoid and the metal can lead to the formation of a chelate, which occurs when the metal is linked to the flavonoid structure in three possible sites: (a) between group 5 -hydroxy and 4-carbonyl group; (b) between the 3-hydroxyl and the 4-carbonyl group; and (c) between the 3',4'-dihydroxy groups located in ring B. [49] The antioxidant activity of ethanolic extracts of the leaves, pericarp and pulp from *G. mangostana*, *G. atroviridis* and *G. hombroniana* were evaluated, as well as their ability to chelate iron [47]. The results demonstrated that the leaves of *G. hombroniana* (approximately 60% chelated Fe II) had a greater iron chelating capacity than *G. mangostana* (33%) and *G. atroviridis* (29%). Among the pericarps evaluated, *G. mangostana* (78%) showed greater chelation than the other two (*G. hombroniana*, 45% and *G. atroviridis*, 11%, respectively). As for the pulps, that of *G. hombroniana* had a greater chelating capacity (30%) than that of the species *G. mangostana* (19%) and *G. atroviridis* (17%). In the present work, however, it was evaluated fractionated extracts and rich of phenolics and phenolics compounds, which justifies the difference between the values obtained and the values published by [49] who worked only with ethanolic extract in extraction by maceration.

As mentioned previously, the uses of medium polarity solvent, ethyl acetate for example, favor obtaining rich extract in phenolic compounds and flavonoids as observed in Table 1. These results reflect the antioxidant activity values obtained in the SDE and SEE. Although SEE showed better antioxidant activity than SDE in DPPH, ABTS and FRAP methods, SDE can chelate 22% of Fe^{2+}, while compared SEE chelated 11%. Low polarity compounds present in SDE, such as polyprenylated benzophenones and hydroxylated xanthones or others, if having bidentate ligand parts can bind with metals, chelating them. The iron chelating ability is not just caused by binding with flavonoids compounds, but can be related to the presence of certain groups depending of bi- or tridentate structure. For example, derivatives of hydroxamic acids are good chelators and catecholate chelators show weaker iron chelating binding [51]. For DPPH• and $ABTS^{\bullet+}$, IC_{50} of SEE is lower than SDE. This is in agreement with TPC and TFC values. For FRAP, the SEE (28.4±1.2 mg AA g^{-1}) exhibited higher reducing power than SDE (19.94±0.7mg AA g^{-1}).

Table 3. Antioxidant activity of extracts from *Garcinia cochinchinensis* seed.

Samples	DPPH• IC_{50} (µg/mL) *	$ABTS^{\bullet+}$ IC_{50} (µg/mL) *	FRAP (mg AA g^{-1}) *	Chelation of Fe^{2+} (%) *
SDE	195.52 ± 0.92^a	127.9 ± 10.15^a	19.94±0.7^a	22 ± 3^a
SEE	37.53 ± 0.4^b	46.24 ± 5.45^b	28.4±1.2^b	11 ± 2^b
AA	10.32 ± 0.1^c	10.26 ± 0.5^c	-	-

Note: *Values presented as mean ± standard deviation (n=3) Means followed by different letters (a-h) differ statistically in the same column (Newman-Keuls at p > 0.05). Tukey's test was applied at a 5% probability level. AA = ascorbic acid.

Phenolic compounds are secondary metabolites containing one phenol group in the structure. As phytochemicals, phenolic compounds have a role to protect from oxidative stress by neutralizing the free radical by transfer of electrons, reducing the pro-oxidant species, donating hydrogen or chelating metal. These metabolites present in *Garcinia* genus and other plants can break chain reactions of ROS by formation of a phenoxy-radical intermediate [35]. The antioxidant potential of flavonoids, benzophenones and xanthones is influenced by the amount of hydroxyl substituents in the aromatic ring and the position of other substituents. The HAT reaction mechanism in xanthones, for example, can scavenge free radicals by transferring labile hydrogen atoms to radicals. For example, xanthones with one, two or three hydroxyl groups are able to scavenge radicals when one electron from hydroxyl's oxygen neutralizes the radical resulting in a SET mechanism. After that, the hydroxyl's hydrogen transfers its electron to oxygen and is transferred to radical, its HAT mechanism. Xanthone loses its hydrogen from hydroxyl group and is stabilized by resonance. Generally, the increasing number of phenolic hydroxyl groups increase the antioxidant potential, because the greater number of phenolic hydroxyls in xanthones can provide conducive conditions to scavenge free radicals faster than respective xanthones with a minor number of phenolic hydroxyl groups [7, 35, 38, 48]. Nguyen et al. [11] isolated benzophenones and four xanthones from *G. cochinchinensis* pericarp, including dulxanthone A that is trihydroxylated and others three xanthones also are hydroxylated can be potential antioxidant present in some fraction of this study.

3.3 Electrochemical antioxidant assay

Electrochemical techniques can provide valuable information about electron transfer reactions without the need for prior analyte treatment, offering rapid and highly sensitive responses [49]. Using the GCE, only in the presence of BR buffer (pH 6.0), before the addition of the extract to the electrochemical cell, no peaks were observed. After the addition of the extract in the electrochemical cell, only anodic peaks were observed, demonstrating the redox activity of the extract when solubilized in ethyl acetate and dichloromethane (Fig. 5). The potential values ranged from 0.27 V, 0.62 V and 0.97 V for the extract in ethyl acetate (SEE) and from 0.44 V and 1.02 V for the extract in dichloromethane (SDE). Both extracts exhibit lower potentials compared to ascorbic acid (E = 0.63 V), but their current responses are significantly lower than those of the reference compound. Samples with lower oxidation potentials have greater electron donating capabilities and, as a result, greater antioxidant capacity [50]. According to Hacke et al. [17], the observed electrochemical responses are related to the oxidation of phenolic compounds at the electrode surface, providing evidence of the antioxidant activity of the extract. The oxidation of these phenolic compounds, regardless of pH, produces polymeric compounds in irreversible reactions, without presenting cathodic peaks [51].

DPV is an electroanalytical technique due to its high sensitivity and ability to distinguish specific analytes from background currents [52]. The technique was also performed at pH 6, where the results previously obtained using CV were verified with greater sensitivity. The potential values obtained for SEE were 0.22 V and 0.32 V, and for SDE they were 0.34 V, while for ascorbic acid it was 0.35 V (Figure 5). The current values for the extract in ethyl acetate were higher, demonstrating that the solubilization of the extract in that solvent is greater, providing a higher concentration of electroactive compounds [53]. These results indicate that SEE has higher antioxidant activity than SDE and ascorbic acid.

To investigate the pH dependence of the SEE's antioxidant activity, DPV was performed at pH 2, 4, 7, and 10 (Figure 6a). It was observed that as the pH increased, the potential decreased (Table 4), leading to higher antioxidant activities. However, above pH 7, the current decreased due to the unavailability of antioxidant compounds caused by their deprotonation. At pH 6, the presence of two peaks at close potentials indicates the presence of other compounds that may be acting as antioxidant agents, which are exclusively favored by this pH. A linear relationship was observed between the potential and the pH, with an increase in reducing power at higher pH (Figure 6b).

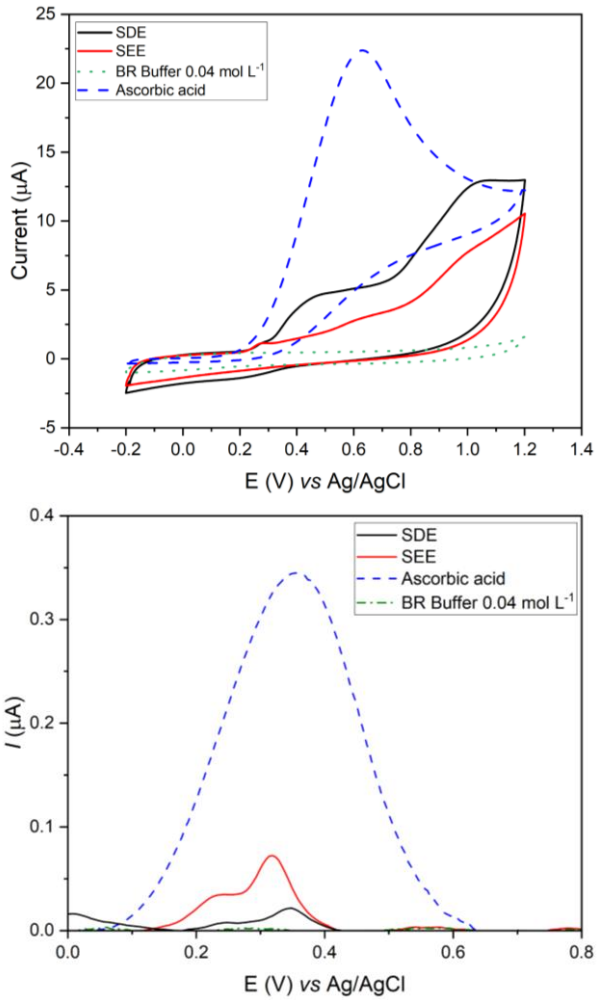

Fig. 5. Cyclic voltammogram of *G. cochinchinensis* seed in ethyl acetate extract (SEE) and seed dichloromethane extract (SDE) compared to BR buffer 0.04 mol L^{-1} and ascorbic acid.

Table 4. pH Values and potentials for SEE in Differential pulse voltammetry.

pH	Potential (V)
2	0.5 ± 0.025
4	0.43 ± 0.0215
6	0.32 (higher peak) ± 0.016
7	0.26 ± 0.013
10	0.12 ± 0.016

The relationship between peak potential and pH provides us with valuable information about the amount of electrons transferred in the extract oxidation process based on the

Nernst equation (Eq. 2). The experimental equation presented a slope of 0.050 V/pH, a value close to the theoretical value of 0.059 V/pH, indicating the transfer of a single electron (*n*) and proton. This process is known as a proton-coupled electron transfer (PCET), and occurs in the oxidation of phenolic compounds [54].

$$E = -\frac{0{,}059}{n} pH \quad \text{(Eq. 2)}$$

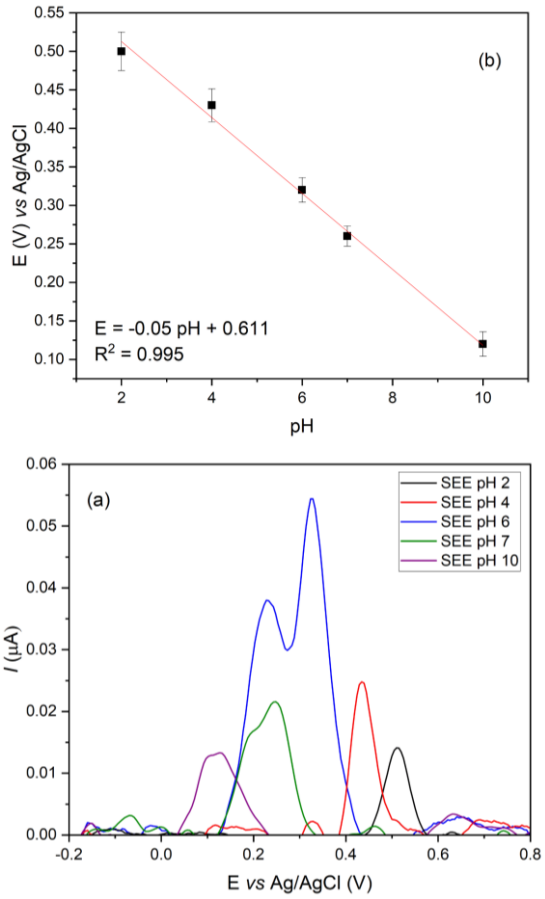

Fig. 6. (a) Differential pulse voltammetry of *G. cochinchinensis* seed extract in ethyl acetate at different pH values; (b) linear relation between pH and potential.

These results using electroanalytical methods, CV and DPV, are in agreement with antioxidant activity results *in vitro* described in this study. At the best of our knowledge, this is the first report about antioxidant activity of *G. cochinchinensis* seeds in *in vitro* methods, DPPH• for example. The choosing of adequate solvents is very important to obtain high amounts of antioxidant compounds, as can be observed from the differences between SDE and SEE (results shown in Table 3). Chosen ethyl acetate solvent was efficient in obtaining target secondary metabolites, phenolic compounds with antioxidant activity.

3.2. Analysis of composition of EA12, EM12 and PE by GC-MS

The extracts EA12, EM12 and PE from *G. cochinchinensis* were submitted to GC-MS analysis. The identified compounds and its retention time, and percentual area (%) are shown in Table 5.

The fraction EA12.1 is constituted by the following majority components: phenylethyl alcohol (22.18%), followed by 2,3-butanediol (5.59%), methyl 2-hydroxy-2-methylpropanoate (5.56%). On the other side, only one component was identified in the E12.1 fraction, 2-chloro-2,3,3-trimethylnorbornene. The major constituents identified in PEO were the fatty esters ethyl oleate (12.95%), ethyl stearate (11.87%) and ethyl palmitate (8.87%). It was analyzed just theses three samples in GC-MS, because the others extracts don't show peaks on chromatogram or don't were soluble in hexane analytical grade for this analysis.

Martins et al. [55] analyzed the essential oil from *G. brasiliensis* fruit peel and identified some oxygenated sesquiterpenes. The essential oil showed low antioxidant activity front DPPH scavenging and reducing power. The study of Martins et al. [55] about essential oil and this present study are in agreement with the Amorati et al. [56] that discuss the mechanism's antioxidant reaction of constituents of essential oils, such as α-pinene and α-terpineol, the last one is present in EA12.1. Among the constituents present in PEO, the use of methyl palmitate can be highlighted for its anti-inflammatory and anti-fibrotic biological activities, as well as the use of ethyl palmitate as a chemical marker for chronic alcohol consumption, being applied in clinical analysis and forensic sciences [57, 58]. The methods of obtaining volatile constituents and essential oil are in agreement with [59] that extract essential oil from hexane extract of *G. bancana* stem bark and with Périno-Issartier et al. [60] that discuss the possibilities of uses of ultrasound for essential oils extraction comparing the other techniques.

Table 5. Main constituents found in fractions of *G. cochinchinensis* by GC-MS.

Source	Compound	Retention time (min)	Area (%)
EA12	2,3-Butanediol	4.32	5.59
	(E)-But-2-en-1-yl propyl carbonate	4.36	2.86
	2-hydroxy-2-methyl-ethyl propanoate	4.79	5.60
	3-Furaldehyde	4.97	4.34
	cis linalool oxide	12.00	2.45
	Phenylethyl Alcohol	13.47	22.18
	Myrcenol	13.66	1.69
	Benzoic acid	15.10	2.81
	diethyl succinate	15.68	13.37
	α-terpineol	16.42	2.02
EM12	2-chloro-2,3,3-trimethylnorbornene	22.58	14.45
PEO	Methyl palmitate	49.551	4.58
	Ethyl palmitate	51.744	8.87
	Methyl linoleate	54.732	1.20
	Methyl elaidate	54.961	5.68
	Ethyl oleate	56.956	12.95
	Ethyl stearate	57.793	11.87

4. Conclusions

The antioxidant activity has a relation with the total phenolics and total flavonoids content. The higher total phenolic content found in the extracts EA8, EA4, E8.A and E4.A justify the effective antioxidant activities in all methods studied, mainly ethyl acetate pH 8 in the two extractions and ethyl acetate pH 4 obtained from ultrasound extractions. In this study, for pulp, ethyl acetate fractions indicated that this solvent is great for obtaining rich extractions and samples of phenolic and flavonoids contents. This fact repeats when carried out partition extraction for the seeds, which can be observed in antioxidant values for SEE (seed ethyl acetate

extract). It was found that pH 6 is an optimal antioxidant activity role of SEE in DPV technique. It can be observed between the antioxidant activity's values from both extraction methods that the use of ultrasound in extraction can be a good alternative, improving and increasing the yield, this is justified because of the difference of mass used and being minor mass in ultrasound extraction it was obtained near values of antioxidant activity. It was identified via GC-MS the main constituents of EA12, EM12 and PEO being terpenes, organic acids, esters and fatty esters. For future perspectives and studies, it pretends to investigate the effects of antioxidant activity *in vivo* models of *G. cochinchinensis* pulp and seeds and isolate news compounds in this species and compounds yet not reported.

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Author Contributions

Conceptualization: Kazmierczak, E.; Bassani, A. V.; Pereira, R. P.; Magalhães, C. G. Data curation: Kazmierczak, E.; Bassani, A. V.; Contador, M. H.; Grzygorczyk, S. Formal analysis: Kazmierczak, E.; Bassani, A. V.; Contador, M. H.; Grzygorczyk, S. Funding acquisition: Not applicable. Investigation: Kazmierczak, E.; Bassani, A. V.; Contador, M. H. Methodology: Kazmierczak, E.; Bassani, A. V.; Pereira, R. P.; Magalhães, C. G. Project administration: Kazmierczak, E.; Pereira, R. P.; Magalhães, C. G. Resources: Kazmierczak, E. Software: Metrohm® NOVA 2.1.7 and OriginLab® Origin 2022. Supervision: Pessôa, C. A.; Pereira, R. P.; Magalhães, C. G. Validation: Pessôa, C. A.; Pereira, R. P.; Magalhães, C. G. Visualization: Kazmierczak, E.; Pereira, R. P.; Magalhães, C. G. Writing – original draft: Kazmierczak, E.; Bassani, A. V. Writing – review & editing: Pessôa, C. A.; Pereira, R. P.; Magalhães, C. G.

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