



Separation of antibacterial biocompounds from *Hancornia speciosa* leaves by a sequential process of pressurized liquid extraction

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ABSTRACT

Hancornia speciosa has bioactive molecules such as phenolic compounds which exhibit antioxidant and antimicrobial properties. Nevertheless, the separation of these substances from a medicinal plant can be different according to the extraction process and solvent used. Thus, the present study aimed was to separate phenolic compounds from *Hancornia speciosa* leaves in three steps by using a sequential pressurized liquid extraction and evaluated their antioxidant and antibacterial activity. Results showed that the temperature increase from 25 to 60 °C provides a significative enhancement in the extraction yield. The raw ethanol extract (39.0 ± 0.2%) showed the highest overall extraction yield. However, fractionated ethanol extract (FEE) at 60 °C exhibited the highest amount of total phenolic compounds (347.0 ± 4.12 mg/g extract). Fractionated ethyl acetate extract (FAE) at 60 °C presented improved quantity of total flavonoids (230.4 ± 3.5 mg/g extract) and rutin content (16.6 ± 0.3 mg/mL extract). The antioxidant activity results for IC₅₀ of DPPH indicated that FEE (3.8 ± 0.1 µg/mL) was the best free radical scavenger. FAE and FEE were able to inhibit the growth of multidrug-resistant *E. coli*.

1. Introduction

Hancornia speciosa (Apocynaceae, family) is a plant commonly known as mangabeira, which has been used in the treatment of diabetes, hypertension, inflammatory, and infectious illnesses. The mangabeira medicinal properties are attributed to its secondary metabolites such as tannins, terpenes and phenolic compounds [1]. Flavonoids are an important class of phenolic constituents in natural products due to their beneficial effects on human health. Rutin is a flavonoid that has shown pharmacological properties such as antihypertensive, antioxidant activity, and also antibacterial effects [2,3].

The separation of these bioactive substances from plant medicinal is receiving increasing interest as an alternative treatment against grown of bacterial strains [4,5]. Mainly, due to bacterial multidrug resistance that has cause recurrent and chronic infections decrease the quality of life and increase the time of the patient in the hospitals. Moreover, the low efficiency of commercial antibiotics against bacteria causes relevant public health concerns worldwide [6].

Bioactive compounds found in medicinal plant extracts can exhibit antimicrobial activity against multidrug-resistant pathogens [4]. For this, the flavonoids and other phenolic compounds can be obtained by distinct extraction methods such as maceration, ultrasound, hydro-distillation, supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE). Nevertheless, PLE has been performed under temperature and pressure conditions which provides improved extraction yield with the attainment of high concentration of interest bioactive compounds such as the phenolic class [7–9].

The compressed liquid solvents present an improved capacity to solubilize bioactive compounds. Also, the selectivity of the solvent is deeply dependent on its physico-chemical properties and on the characteristics of the interaction between solvent and solute of interest. The sequential extraction of compounds from a vegetable sample using solvents of distinct polarity provides varied profiles of active natural substances, allowing better mass transfer of the bioactive compound of interest due to the purification of the samples [10–13].

Bioactive compounds from vegetable samples can be first extracted

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with nonpolar solvents, such as hexane or dichloromethane, to remove unwanted fatty acids or wax compounds, followed by other solvents with increased polarity [14,15]. The present study aimed to separate phenolic compounds from *Hancornia speciosa* leaves by using a sequential in three steps pressurized liquid extraction and evaluate their antioxidant and antibacterial activity.

2. Material and methods

2.1. Chemicals

N-hexane (Vetec, purity 99.5%), ethyl acetate (Vetec, purity 99.5%), ethyl alcohol (Vetec, purity 99.5%), (JT Baker), Sodium Carbonate Anhydrous PA (Dynamics), Dimethylsulfoxide PA (Synth), 2,2-diphenyl-1-picrylhydrazyl radical DPPH (Sigma), Folin Ciocalteu (Dynamic), Potassium Acetate (Synth), Aluminum Nitrate PA (Synth), Formic Acid $\geq 95\%$ (Sigma-Aldrich), Gallic Acid Monohydrate PA (Neon), Hydrated rutin $\geq 94\%$ (Sigma-Aldrich), Water Milli-Q, 2,3,5-Triphenyltetrazolium chloride $\geq 98\%$ (Sigma-Aldrich) were used as received.

2.2. Mangabeira leaves samples and pressurized liquid extraction process

The mangabeira leaves were collected from the Germplasm Bank of Embrapa Coastal Tablelands (Sergipe, Brazil) at “Campo do Caju” (11°06'59.7"S; 37°11'12.5"W). It was registered an exsiccata at the Herbarium of the University Tiradentes-Aju (Protocol: n°. 0838). All leaves used in the extractions were dried at 40 °C for 72 h in a hot-air circulation oven. The leaves were then crushed to the range size from 16 to 32 mesh using sieves of Tyler series. The humidity of samples was then measured ($7.8 \pm 0.6\%$), and the material was stored protected from light and room temperature in a refrigerator.

All extractions were performed in an extraction unit that consists of a solvent reservoir, two thermostatic baths, a syringe pump and a positive displacement pump, a stainless-steel extractor with a jacketed vessel, pressure transducer, universal process indicators, and valves complete the unit. Fig. 1 provides a description of the extraction unit. Details of the experimental unit can be found in Jesus et al [16].

The extractions were performed with 10 g of mangabeira leaves, using a solvent flow rate of 1 mL/min, the pressure of 10 MPa, and temperatures of 25 °C and 60 °C. The extractions were conducted at a

total time of 180 min (60 min for each solvent: hexane, ethyl acetate and, ethanol/water). Between each solvent, the extractor was pressurized and flushed with carbon dioxide in order to remove the initial residual solvent from the extractor/raw material. With this procedure, it was possible to conduct the extraction of the mangabeira leaves with the three distinct solvents: hexane in the first-step, ethyl acetate in the second step and ethanol/water in the third one. The results of the sequential extraction process were compared with those from the one-step extraction of the samples at same flow rate, pressure, temperature, and solvent volume experimental conditions, where the extractions were conducted for 180 min by each individual solvent.

The overall extraction yields from the mangabeira leaves were calculated according to the following equation [17]:

$$\text{Yield\%} = (\text{DME}/\text{IM}) \times 100$$

DME: dry mass extract (g); IM: initial sample mass before extraction (g).

2.3. Determination of total phenolic compounds

Total phenolics were determined by using the Folin-Ciocalteu method [18]. Briefly, 0.5 mL of the sample was diluted to methanol (250 ppm) and mixed with 2.25 mL of Folin-Ciocalteu solution, 1.75 mL of 7.5% sodium carbonate solution, and 0.5 mL of distilled water. After, the solution was incubated at 45 °C for 20 min. The absorbance of the mixture was measured at 765 nm using a UV-vis spectrophotometer (721 G visible spectrophotometer). The calibration curve was obtained from gallic acid standard ($y = 0.0108x + 0.021$), range of 5–140 $\mu\text{g}/\text{mL}$ ($R^2 = 0.9997$). Total phenolic values were expressed as mg of gallic acid equivalents by g of extract (mg GAE/g E).

2.4. Determination of flavonoids content

The flavonoid content in the extracts was determined using the aluminum nitrate colorimetric method. This reported method is based on the reaction of flavonoids with aluminum [19]. Briefly, 250 ppm of the sample diluted to 0.5 mL of methanol was mixed with 0.1 mL of aluminum nitrate 10%, 0.1 mL of potassium acetate 1 M and 4.3 mL of methanol. Then, the mixture was left to stand for 10 min at room temperature in the dark. Rutin was used as the standard for calibration curve ($y = 0.0039x + 0.0033$), range of 5–140 $\mu\text{g}/\text{mL}$ ($R^2 = 0.9986$). The absorbance at 425 nm (721 G visible spectrophotometer) was used for the determination of flavonoids content. The results were reported

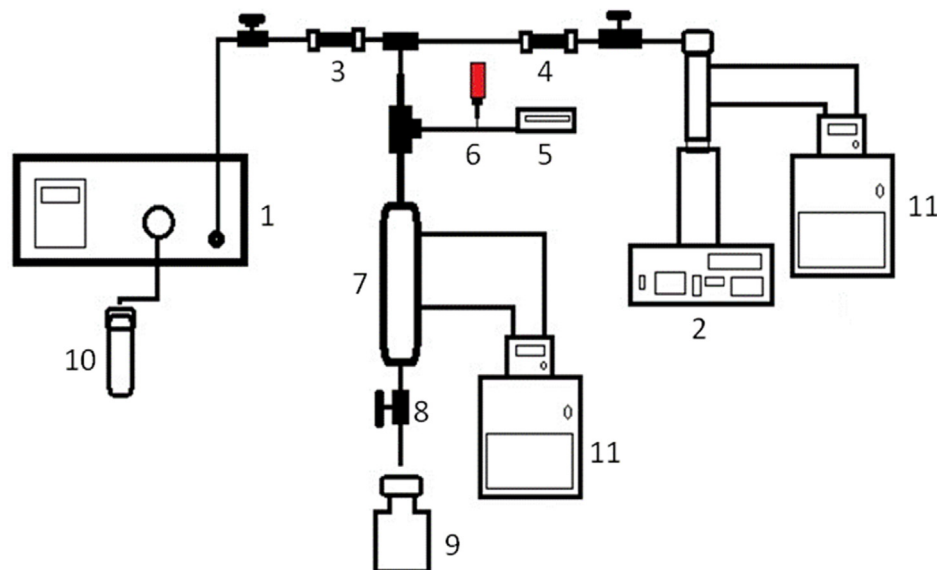


Fig. 1. Experimental apparatus for pressurized liquid extraction: (1) isocratic pump; (2) Syringe pump; (3–4) Check valves; (5) Pressure indicator; (6) Pressure transducer; (7) Extractor; (8) Needle valve; (9) Sample collector; (10) Liquid solvent reservoir; (11) Thermostatic bath.

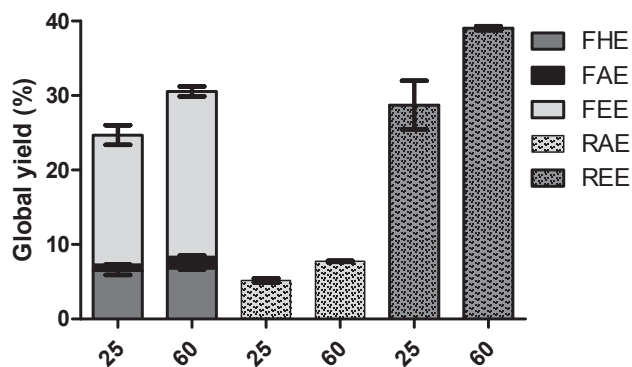


Fig. 2. Extraction overall yield obtained from mangabeira leaves using different solvents at 25 °C and 60 °C. FHE: fractionated hexane extract, FAE: fractionated acetate extract, FEE: fractionated ethanol extract, RAE: raw acetate extract and REE: Raw ethanolic extract. The values were presented as a mean \pm standard deviation.

as mg of rutin equivalents per g of extract (mg RE/g) [19,20].

2.5. High-performance liquid chromatography – HPLC

HPLC analysis was performed according to Santos et al. [21]. A liquid chromatograph (Shimadzu LC-20AT, with UV–vis detector) and an analytical Lichrospher RP18 column (250 \times 4.6 mm, 5 μ m particle size) was used. The elution mobile phase was composed by 60% of methanol and 40% of an aqueous solutions of formic acid (0.1%, v/v). The isocratic flow rate was 0.5 mL/min for 30 min and the detection wavelength was adjusted at 330 nm. All runs were performed at constant temperature of 30 °C. 20 μ L of the samples from solutions containing raw and fractionated extracts (100 ppm) dissolved in methanol (MeOH) were injected for the analyzes. The rutin identification in the mangabeira extracts was performed using an authentic standard of rutin (Sigma Aldrich, HPLC grade). A calibration curve ($y = 32027x - 23240$), ranging from 0.4 to 50 μ g/mL ($R^2 = 0.9967$) of rutin standard was used to its quantification in the extracts. The samples injected in the HPLC system were always filtered through a 0.45 μ m membrane.

2.6. Determination of antioxidant activity using DPPH

For the DPPH assay, each raw and fractionated extracts of mangabeira leaves (5 mg) were previously diluted in methanol (10 mL) to obtain a stock solution at concentration of 500 μ g/mL. 300 μ L of mangabeira extracts at distinct concentration (50, 25, 12.5, 6.2, 3.1, and 1.5 μ g/mL) were mixed with 2700 μ L of the DPPH solution (0.06 mM). The mixtures were homogenized and held for 10 min at ambient temperature without light incidence and the absorbance was measured at 517 nm (visible spectrophotometer of 721 G). The control was performed using only the DPPH solution.

The radical scavenging activity (RSA) was calculated as a percentage, using the following equation [22]:

$$\%RSA = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

The results were expressed as the IC_{50} , i.e. the amount of antioxidant needed to reduce the initial concentration of radicals by 50%. IC_{50} values were calculated using a nonlinear regression curve from Prism software 5.0 software.

2.7. Isolation and identification of bacteria

The microorganisms used were isolated from nosocomial infectious waste. Bacteria were initially seeded in a primary culture media such as

Blood Agar, Chocolate Agar, Mannitol Agar and Methylene Blue Eosin Agar (EMB). The inoculum was incubated in a bacteriological oven for 24 h at 35 °C. After this time, it was observed bacteria growing on Eosin Methylene Blue (EMB) agar, a medium used for the isolation and identification of gram-negative enteric bacteria. The isolated microorganisms were submitted to a subsequent phenotypic identification for negative gram bacillus using the criteria developed by [23,24], and according to results obtained, it was identified the *Escherichia coli* bacteria.

2.8. Determination of antimicrobial susceptibility test (AST)

Antibacterial activity of the extracts obtained and 14 commercial antibiotics (Ciprofloxacin, Levofloxacin, Amikacin, Gentamycin, Cephalotin, Cefoxitin, Cefuroxime, Cefotaxime, Ceftriaxone, Cefepime, Ertapenem, Meropenem, and Imipenem) was performed by the AST technique [25], with adaptations. The extracts at concentrations of 500 and 250 μ g/mL were diluted in water containing 0.9% sodium chloride and 1% of DMSO (vehicle) and subsequently added to the BHI (Brain Heart Infusion) culture medium containing the bacterial inoculum. The *Escherichia coli* inoculum was prepared according to the 0.5 McFarland scale (1.0×10^8 UFC mL⁻¹). After this period the plates were kept in a bacteriological oven at 37 °C for 24 h. The analysis was concluded by the change from colorless to red color using reagent 2,3,5-trifeniltetrazólio (TTC) 0.05%, indicating the presence of microorganisms.

2.9. Statistical analysis

All data are reported as a mean value \pm standard deviation (SD). Statistical analysis was conducted by the Prism 5.0 software using ANOVA followed by Tukey's test. The values are considered significantly different using $p < 0.05$.

3. Results and discussion.

3.1. Extraction overall yield

The extraction yields of mangabeira leaves are presented in Fig. 2. In this figure, the results are compiled using the sequential solvent extraction (FHE: Fractionated Hexane Extract, FAE: Fractionated Ethyl Acetate Extract, FEE: Fractionated Ethanol Extract) and using just one solvent per extraction (REA: Raw Ethyl Acetate Extract, and REE: Raw Ethanolic Extract).

Results in Fig. 2 indicated that the three-step extraction ($24.7 \pm 8.4\%$ and $30.5 \pm 10.8\%$ to 25 °C and 60 °C, respectively) by distinct solvents exhibited a slight lower ability for extract solubility compared to the one-step extraction ($28.7 \pm 3.2\%$ and $39.0 \pm 0.2\%$ to 25 °C and 60 °C, respectively for raw ethanolic extracts), suggesting that most parts of the extract is been removed from the vegetable matrix in the begging of the extraction with each solvent. It can also be observed that the ethanol/water mixture (80:20 v/v) largely improved the overall extraction yield of compounds from mangabeira leaves compared to other. These results were founded both in the one-step extraction and in the three-step extraction (Fig. 2), as this solvent presents a strong polarity and can be considered an efficient extractor for moderately polar and polar compounds [26–28].

The results presented in Fig. 2 also indicated that increasing the temperature from 25 °C to 60 °C leads to an enhancement in the overall extraction yield, independent of the solvent polarity. The increase of temperature provides weakening of the chemical interactions in the vegetable matrix and decreasing the viscosity of solvent and solute. As a consequence, the mixture presents better mass transfer properties, which helps the permeation of solvent in the vegetable matrix pores to solubilize the solute [29,30]. Thereby, the increase of temperature highlights the physicochemical and solubility properties of the solvent

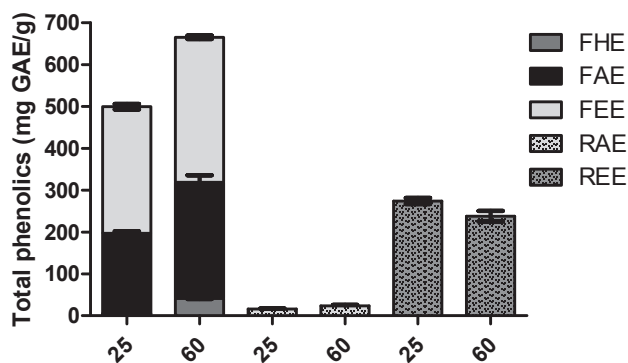


Fig. 3. Total phenolic compounds obtained at 25 °C and 60 °C from mangabeira leaves were expressed as mg of gallic acid equivalents g of dried extract (mg GAE/g): FHE-Fractionated Hexane Extract, FAE-Fractionated Acetate Extract, FEE-Fractionated Ethanol Extract, RAE-Raw Acetate Extract, and REE-Raw Ethanol Extract. The values were presented as a mean \pm standard deviation.

and compounds extracted, leading to the enhancement in the overall extraction yield [15,31].

3.2. Total phenolic content

In the present study, n-hexane was the solvent used in the extraction first step (FHE) to separate nonpolar substances from mangabeira leaves (Fig. 3). Therefore, we found that ethyl acetate solvent in the extraction second step showed a most significant amount of phenolics compounds (287.4 ± 16.9 mg/g extract at 60 °C, and 190.0 ± 5.0 mg/g extract at 25 °C) compared to the same solvent (REA) when used in the extraction one step (24.8 ± 2.6 mg/g extract at 60 °C, and 15.9 ± 0.8 mg/g extract at 25 °C). These finds suggest that the sequential extraction can be acting as a clean-up process of the sample, improving the concentration of specific compounds in each extract [32].

Fig. 3 also exhibited that the highest amount total phenolic compounds (347.0 ± 4.1 mg/g extract at 60 °C, and 302.5 ± 6.4 mg/g extract at 25 °C) were obtained in FEE, in the sequential extraction process in third-step at 60 °C and the lowest phenolic content (24.8 ± 2.6 mg/g extract at 60 °C, and 15.9 ± 0.8 mg/g extract at 25 °C) in the REA. The solvent type used for the extraction process is a determining factor for obtaining the bioactive compounds [27,28]. Moreover, the three steps extraction (overall yields of 499.3 and 675.6 mg/g extract at 25 and 60 °C, respectively) result in a better strategy for phenolic compounds separation compared to the one-step extraction.

3.3. Total flavonoid content

Fig. 4 presents the total flavonoid content obtained in the extracts of the mangabeira leaves by PLE process. It can be observed that the FAE fraction showed the highest quantity of flavonoids (145.3 ± 5.1 and 230.5 ± 3.5 mg/g extract at 25° and 60 °C, respectively). This result combined with Fig. 2 suggests that FAE was the most concentrated mangabeira leaves extract in flavonoids. The total flavonoid (371.4 mg) obtained in the three-step extraction was upper than the sum of the one-step extraction (97.1 mg), confirming that the sequential process of pressurized liquid extraction produced a higher content of flavonoid and phenolic compounds from mangabeira leaves compared to the one-step extraction.

Presented a study of flavonoid content from mangabeira leaves using a maceration technique with ethanol at room temperature as solvent [6]. Authors found 29 ± 1.1 mg/g extract of total flavonoids in their study, much lower than the results obtained in the present study. This found indicated that not only the solvent and its polarity scale is

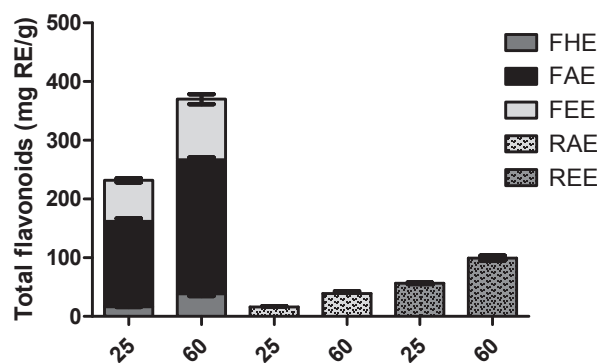


Fig. 4. Total flavonoid content obtained at 25 °C and 60 °C from mangabeira leaves values reported as mg of rutin equivalents g of dried extract (mg RE/g): FHE: fractionated hexane extract, FAE: fractionated ethyl acetate extract, FEE: fractionated ethanol extract; RAE, raw acetate extract, REE: Raw ethanol extract. The values were presented as a mean \pm standard deviation.

important during extraction, but also the extraction technique can largely influence on obtaining the compounds present in the extract [33,34].

3.4. High-performance liquid chromatography – HPLC

Mangabeira leaves extracts has shown rutin peak in HPLC analysis [35]. Other compounds also can be found in mangabeira extracts, such as L – (+) – bornesitol, quinic acid, chlorogenic acid, and kaempferol. However, rutin has been the main phenolic compound from the mangabeira leaves due to high biological properties [6]. Moreover, the rutin shows pharmacological properties as antioxidant and antibacterial which provides human health benefits [2]. Table 1 presents the rutin concentration in the raw and fractionated extracts of mangabeira leaves.

It can be observed that temperature was the determining factor to increase the rutin concentration, where FAE fraction obtained at 60 °C exhibited the highest quantity of rutin (16.6 ± 0.3) among all extracts obtained in the present study. Moreover, results in Table 1 suggest that the fractionation of the extracts by the sequential extraction corroborates to the concentration of rutin in the extracts. The quantification obtained by the HPLC and the results from Fig. 4, suggest that ethyl acetate in the second step of extraction showed the best capacity to separate flavonoids from mangabeira leaves.

In general, the results indicated that an increase in the solubility of flavonoids (Fig. 4), including rutin (Table 1), and other phenolic compounds (Fig. 3) in the three-step extractions compared to one-step extraction (raw extraction), and these finds can be attributed to a clean-up process of the mangabeira samples during the sequential extraction.

Table 1

Rutin concentration in the extracts of mangabeira leaves from the sequential pressurized liquid extraction process.

Samples	Rutin concentration ($\mu\text{g}/\text{mL}^{-1}$)	
	25 °C	60 °C
FHE	ND ^{b,c}	0.9 ± 0.1 ^{a,c}
FAE	4.9 ± 0.3 ^{b,A}	16.6 ± 0.3 ^{a,A}
FEE	2.4 ± 0.3 ^{b,B}	3.2 ± 0.1 ^{a,B}
REA	ND ^{b,c}	0.6 ± 0.1 ^{a,c}
REE	2.4 ± 0.1 ^{b,B}	3.1 ± 0.3 ^{a,B}

Values represented as a mean \pm standard deviation (n = 2); ND: not found. Distinct lowercase letters in the same line indicate significant differences among the temperature levels. Distinct uppercase letters in the same column indicate significant differences among the solvent/process extraction levels. All analysis considered p-level < 0.05.

Table 2

Antioxidant activity of raw and fractionated extracts from mangabeira leaves expressed as IC₅₀ of DPPH values.

Sample	µg/mL of extract	
	25 °C	60 °C
FHE	ND ^{a,D}	ND ^{a,D}
FAE	8.7 ± 0.9 ^{a,C}	6.2 ± 0.1 ^{b,C}
FEE	3.8 ± 0.1 ^{a,A}	4.1 ± 0.3 ^{a,A}
REA	ND ^{a,D}	ND ^{a,D}
REE	6.5 ± 0.5 ^{a,B}	5.0 ± 0.2 ^{b,B}

Values represented as a mean ± standard deviation (n = 3); ND: not determined.

Distinct lowercase letters in the same line indicate significant differences among the temperature levels. Distinct uppercase letters in the same column indicate significant differences among the solvent/process extraction levels. All analysis considered p-level < 0.05.

Table 3

Antimicrobial susceptibility test of mangabeira leaves extracts and commercial antibiotics.

Antibacterial agents	<i>E. coli</i>	<i>E. coli</i> KPC
Vehicle [#]	Resistant	Resistant
FAE (250 µg/mL)	Susceptible	Resistant
FAE (500 µg/mL)	Susceptible	Susceptible
FEE (250 µg/mL)	Susceptible	Resistant
FEE (500 µg/mL)	Susceptible	Susceptible
Cephalotin	Resistant	Resistant
Cefuroxime	Resistant	Resistant
Ertapenem	Susceptible	Resistant
Meropenem	Susceptible	Resistant
Imipenem	Susceptible	Resistant
Ciprofloxacin	Susceptible	Resistant
Levofloxacin	Susceptible	Resistant
Amikacin	Susceptible	Susceptible
Gentamycin	Susceptible	Resistant
Cefoxitin	Susceptible	Resistant
Ceftazidime	Susceptible	Resistant
Cefotaxime	Susceptible	Resistant
Ceftriaxone	Susceptible	Resistant
Cefepime	Susceptible	Resistant

Vehicle[#] (Water, 0.9% sodium chloride and 1% of DMSO, solution used for extracts dilution).

Moreover, the results were more significant for ethyl acetate in the second step of extraction (60 mL solvent) regarding the same solvent in the one-step extraction (180 mL solvent), where it had been obtained a lower quantity of the biocompounds studied.

3.5. Determination of antioxidant activity

Table 2 presents the results of the antioxidant activity study of the extracts from mangabeira leaves obtained by PLE. The results indicated that the FEE extract showed the best IC₅₀ (3.8 ± 0.1 µg/mL) of DPPH values. According to Table 2 and Fig. 3, it can be suggested that the concentration in the phenolic compounds in mangabeira leaves extracts are directly linked to the antioxidant activity. Table 2 also indicates that the sequential process of pressurized liquid extraction improves the antioxidant propriety for ethyl acetate fraction and also permit to obtain ethanol extracts with IC₅₀ of DPPH values (5.0 ± 0.2 and 4.1 ± 0.3) in lower time and solvent volumes.

The results of this study showed that the extracts obtained by ethyl acetate and ethanol at 60 °C in the sequential pressurized liquid extraction process produce extracts with higher antioxidant activity compared to IC₅₀ value (50 µg/mL) of the Santos et al. (2016) that used maceration ethanol extracts of mangabeira leaves. These finds again evidence the importance of extraction technique.

3.6. Antimicrobial susceptibility evaluation

The mangabeira leaves extract with the highest concentration of phenolic compounds (FEE, Fig. 3), and the highest concentration of flavonoids (FAE, Fig. 4) were tested against the growth of *E. coli* isolated from nosocomial wastes. It was also evaluated the susceptibility of *E. coli* nosocomial to commercial antibiotics.

The results presented in Table 3 showed that *E. coli* suggestive to KPC was multidrug-resistant to 13 commercial antibiotics from 14 tested, including resistance to the carbapenems class. The gram-negative bacterial cell wall is a strongly polar barrier and contains efflux pumps that act as a bacterial resistance mechanism, expelling compounds that pass through the outer membrane (Khan et al., 2009). However, the *E. coli* suggestive to KPC was susceptibles to a 500 µg/mL of FEE or FAE.

The high efficiency of vegetal extracts as an antibacterial agent is suggested to be related to their ability for cause in cell wall damage, enzymatic inactivation, suppression of oxidative phosphorylation, and restriction of protein synthesis [5]. Phenolic acids and flavonoids are suggested as the main responsible for their antibacterial properties [36]. The results presented in Table 3 indicate that a synergism among the bioactive compounds from mangabeira leaves extracts seems to be a potential alternative against the growth of multidrug-resistant *E. coli* that cause nosocomial infections.

4. Conclusions

In this work a sequential pressurized liquid extraction using n-hexane, ethyl acetate and ethanol was developed to obtain distinct extracts from mangabeira leaves. The increase in temperature enhanced the capacity of separation of bioactive compounds from mangabeira leaves. The extract fraction from ethanol at 60 °C exhibited the higher amount phenolic compounds, whereas the extract fraction from ethyl acetate (FAE) at 60 °C produced extracts with the highest quantity of flavonoids and rutin. The best free radical scavenger to IC₅₀ of DPPH was found to FEE at 60 °C. From the results it can be concluded that the sequential process improved the quality of the extraction, enhancing the concentration of the active compounds in the extracts. The most promising extract fractions (FAE and FEE) were able to inhibit the growth of nosocomial *E. coli*, independent of their multidrug-resistance. Thereby, these extracts seem to be a good alternative for the treatment of nosocomial *E. coli*.

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