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Bioactive compounds from *Syzygium malaccense* leaves: optimization of the extraction process, biological and chemical characterization

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ABSTRACT: *Syzygium malaccense* belongs to the family Myrtaceae and it is popularly recognised as 'jambo' in Brazil. It is poorly studied regarding the bioactive compounds of fruits and leaves. This study aimed at optimizing the extraction and characterization of bioactives compounds from *S. malaccense* leaves. Extraction was optimized using a 23 full factorial design to evaluate the effect of ethanol concentration, time and temperature on polyphenols and antioxidants contents. Analyses of phenolic compounds content (Folin-Ciocalteau and HPLC-DAD), *in vitro* antioxidant capacities (DPPH, ABTS and FRAP), antimicrobial activity against bacteria and yeasts of ethanolic extract was carried out in optimal conditions of extraction. The optimized extraction condition was 45 min., 80°C and 40:60 (v v^{-1}) ethanol:water. The extract showed high total phenolic content (0.074 mg GAE kg^{-1}), DPPH (0.666 µmol TE kg^{-1}) and ABTS (0.853 µmol TE kg^{-1}) free radical scavenging ability and appreciable ferric reducing power (1.267 µmol Fe^{2+} kg^{-1}). HPLC methodology allowed the identification of four bioactive compounds: gallic acid (0.00036 mg kg^{-1}), catechin (0.00021 mg kg^{-1}), rutin (0.00027 mg kg^{-1}) and quercetin (0.00003 mg kg^{-1}). The extract showed inhibitory activity against *S. aureus* (1 µg L^{-1}); *S. bongori* (8 µg L^{-1}), *C. albicans* and *C. tropicalis* (2 µg L^{-1}). It also showed microbicidal potential against *S. aureus*; *C. albicans* and *C. tropicalis*.

Keywords: antifungal agents; antioxidant; flavonoids; factorial design; total phenolic content.

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Introduction

Syzygium malaccense (formely, Eugenia malaccensis) belongs to the family of Myrtaceae and was originally found in Malaysia and India (Arumugam, Manaharan, Heng, Kuppusamy, & Palanisamy, 2014). In Brazil, it is commonly known as jambo, and it has adapted to the climate of the northern and northeastern regions, and various parts of the plant have been applied in popular medicine (Batista et al., 2017). Some research has described the composition and properties of the fruits and stems of the plant; however its leaves have been poorly studied. Reports in recent scientific literature have shown that the leaves of *S. malaccense* are rich in phenolic compounds and flavonoids and present antioxidant and antiglycemic activities (Augusta, Resende, Borges, Maia, & Couto, 2010; Arumugam et al., 2014; Batista et al., 2017).

Compounds with antioxidant abilities have attracted the interest of researchers as well as pharmaceutical and food companies because they can offer human health benefits. The benefits include free radical scavenging capacity and interruption of oxidative processes, which can contribute to preventing human disease conditions (Khan & Wang, 2018). In addition to the interest in natural antioxidants, numerous research studies are being developed around the world seeking new antimicrobial agents derived from natural sources for applications in food products and in the formulation of new antibiotics (Saucedo-Pompa et al., 2018).

It should be noted that for adequate study of the biological properties of any natural product, it is essential to assess the conditions of extraction of their bioactive compounds. The process conditions employed in the extraction of bioactives from natural sources can influence the content and composition of phenolic compounds, antioxidants and antimicrobial activity of the resulting extract (Ortiz et al., 2019). In this sense, the experimental design methodology allows a multivariate evaluation of the influence of variables that can affect the response of the experiment in an efficient and economical way, with fewer experiments (Zordi et al., 2014).

Within this context, the objective of the present study was to optimize the extraction of bioactive compounds from *S. malaccense* leaves using experimental planning and response surface methodology. Total phenolic content, antioxidant and antimicrobial potentials against bacteria and yeasts were also evaluated. In addition, some phenolic compounds were identified by high-performance liquid chromatography with diode array detection (HPLC-DAD).

Material and methods

Reagents

The reagents 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7 (TPTZ), Folin-Ciocalteu, 2,3,5-triphenyltetrazolium chloride (TTC) and ethanol were obtained from Sigma-Aldrich; Müller-Hinton broth was obtained from Kasvi and broth infusion brain heart (BHI) was obtained from Acumedia. Authentic standards (HPLC grade, with purity ⩾ 99%) of gallic acid, catechin, vanillic acid, caffeic acid, epicatechin, p-coumaric acid, ferulic acid, rutin, salicylic acid and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO). The antibiotics used were Fluconazole obtained from the Cimed Group, in addition to Norfloxacin, Tetracycline and Natamicina.

Microorganisms and raw material

Strains of *Salmonella bongori* ATCC 43975, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 118804 and *Candida tropicalis* ATCC 13803 were used in the assays of antimicrobial activity evaluation.

The leaves of *S. malaccense* were collected in the town of Vilhena in the state of Rondônia, Brazil, in March 2014. The leaves were dried at 35°C in a convection oven for approximately five days, and were subsequently crushed in a knife mill (Willye TE-650, Tecnal) and stored in a freezer for later analyses.

Ethanolic extraction of bioactive compounds

A 2^3 two-level full factorial design and response surface methodology (RSM) were used to evaluate the influence of the parameters: extraction temperature (30 – 80ºC/ X_1), extraction time (15 - 45 min./ X_2) and ethanol concentration (40 – 80 %/ X_3) on the factors: DPPH free radical scavenging capacity (Y_1) and total phenolic content (Y_2). The experimental design was composed of 8 runs in triplicate at the central point, corresponding to a total of 11 assays as described in Table 1. Experimental data was analyzed using Environment R (version 3.1.1) as the statistical software. The dataset presented was adjusted according to the following first-order polynomial Equation 1:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_{12} + \beta_{13} X_{13} + \beta_{23} X_{23} \quad (1)$$

where:

Y is the expected response; β_0 represents the intersection; β_1, β_2 and β_3 are the linear coefficients, β_{12}, β_{13} and β_{23} are the coefficients of interaction, and X_1, X_2 and X_3 are independent variables, extraction temperature, extraction time and ethanol concentration, respectively.

Table 1. Variable levels and responses based on 2^3 factorial design matrix.

Assay	X_1	X_2	X_3	Y_1	Y_2
1	30 (-1)	15 (-1)	40 (-1)	0.563	0.054
2	80 (+1)	15 (-1)	40 (-1)	0.596	0.058
3	30 (-1)	45 (+1)	40 (-1)	0.519	0.054
4	80 (+1)	45 (+1)	40 (-1)	0.666	0.074
5	30 (-1)	15 (-1)	80 (+1)	0.546	0.052
6	80 (+1)	15 (-1)	80 (+1)	0.591	0.054
7	30 (-1)	45 (+1)	80 (+1)	0.652	0.060
8	80 (+1)	45 (+1)	80 (+1)	0.655	0.068
9	55 (0)	30 (0)	60 (0)	0.608	0.059
10	55 (0)	30 (0)	60 (0)	0.594	0.061
11	55 (0)	30 (0)	60 (0)	0.617	0.065

X1: temperature (ºC). X2: time (min.). X3: ethanol concentration (% v v-1). Y1: DPPH scavenging capacity (µmol TE kg-1). Y2: total phenolics (mg GAE kg-1). Values are mean ± SD (n = 3).

Total phenolic content (TPC)

Total phenolic content was determined by using the Folin-Ciocalteu assay. Volumes of 0.5 mL of the hydroalcoholic extract of leaves of *S. malaccense* (320 mg L^{-1}), 2.5 mL of the Folin-Ciocalteu (100 mL L^{-1}) and 2.0 mL of Na_2CO_3 solution (40 g L^{-1}) were used in a test tube. The mixture was held without light for 2 hours at room temperature and then absorbance was determined at 740 nm on a spectrophotometer. A blank mixture containing 0.5 mL of distilled water, 2.5 mL of Folin-Ciocalteu and 2 mL of Na_2CO_3 was used. The results were found based on a calibration curve using gallic acid as a standard (R^2 = 0.984) and expressed as gallic acid equivalent per gram of leaves (mg GAE kg^{-1}) (Singleton, Orthofer, & Lamuela-Raventos, 1999).

Antioxidant activity assay using the DPPH free radical scavenging method

DPPH free radical scavenging capacity was assessed using the DPPH reagent. In a test tube, the following substances were added: 0.5 mL of the extract of *S. malaccense* leaves (133 mg L^{-1}), 3 mL of ethanol and 0.3 mL of DPPH radical solution in ethanol (0.5 mmol L^{-1}). The mixture was held in darkness at ambient temperature for 45 min. Then, absorbance of the mixture was measured on a spectrophotometer at 517 nm. The control sample was prepared with 3.5 mL of ethanol:water (80:20 v v^{-1}) and 300 µL of the DPPH solution (0.5 mmol L^{-1}). A blank mixture containing 0.5 mL of the extracts of jambo leaves (133 mg L^{-1}) and 3.3 mL of ethanol ethanol:water (80:20 v v^{-1}) was used. DPPH scavenging was measured using a calibration curve with trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as standard (R^2 = 0.973). The results were expressed as µmol of trolox equivalents per gram of leaves (µmol TE kg^{-1}) (Brand-Williams, Cuvelier, & Berset, 1995).

Antioxidant activity assay using the ABTS cation radical decolorization method

The ABTS radical was obtained from the reaction of 5 mL of ABTS (7 mmol L^{-1}) with 88 µL of potassium persulfate solution (140 mmol L^{-1}), in the absence of light for 16 hrs. The ABTS radical solution was diluted in ethanol by absorbance of 0.700 at 734 nm. In a test tube, 30 µL of diluted sample (1.6 g L^{-1}) and 3 mL of solution containing the radical ABTS were added. Absorbance was measured in a spectrophotometer, at 734 nm after 6 min of reaction, and ethanol was used as blank control. Quantification was performed by using the standard curve of Trolox and results were expressed as mmol Trolox equivalents per gram of leaves (µmol of TE kg^{-1})(Re et al., 1999).

Antioxidant activity assay using the ferric reducing antioxidant power (FRAP) method

The FRAP reagent was obtained from a mixture of 25 mL of acetate buffer (0.3 mol L^{-1}), 2.5 mL of solution TPTZ [2,4,6-tris(2-pyridyl)-s-triazine; 10 mmol L^{-1}) and 2.5 mL of aqueous solution of ferric chloride (20 mmol L^{-1}). In a test tube, 100 µL of the extract of *S. malaccense* leaves (200 mg L^{-1}) and 3 mL of reagent FRAP were added. The mixture was maintained in a water bath at 37°C for 30 min. and then absorbance was measured in a spectrophotometer at 595 nm and the FRAP was used as blank control. Fe (III) to Fe (II) reducing power was expressed as mmol $FeSO_4$ per gram of leaves (mmol Fe^{2+} kg^{-1}) (Benzie & Strain, 1996).

HPLC-PDA analysis

Analysis was performed using a 920 LC system (Varian Inc., Walnut Creek, CA, USA). The software Galaxie was used to control the autosampler, gradient settings, DAD and data acquisition. A C18 RP column (250 x 4.6 mm, 5µm) (Eclipse Plus, Agilent Technologies, Wilmington, DE, USA) was employed in the analysis and maintained at 30°C. Volumes of 10 µL of extracts at concentration of 80 g L^{-1} were injected. The mobile phase consisted of a gradient mixture of solvent A (2% aqueous acetic acid solution) and solvent B (acetonitrile 40% acidified with 2% aqueous acetic acid solution), with a 1 mL flow-rate. The gradient was started with 5% solvent B and adjusted for 20% at 2 min.; 25% B at 15 min.; 85% B at 25 min. maintained for 5 min.; 20% B at 33 min.; 5% B at 36 with 8 min. of a conditioning step. Peak areas were determined at 280 nm for gallic acid, vanillic acid and flavanols: catechin and epicatechin; 300 nm for coumaric acid and salicylic acid; 320 nm for caffeic and ferulic acid and 360 nm for flavonoids: rutin and quercetin.

Evaluation of antimicrobial potential

Antimicrobial activity was evaluated by the broth microdilution method to determine minimal inhibitory concentration (MIC) as described by the National Committee for Clinical Laboratory Standards with subtle

adjustments (NCCLS, 2003). The extract from *S. malaccense* leaves obtained in optimal extraction conditions was concentrated in a rotary evaporator and freeze dried; then, dilutions with final concentrations ranging from 25 to 1400 μg mL^{-1} were prepared. The antimicrobial potential of the extract was tested against the bacteria *Salmonella bongori* (Gram negative) and *Staphylococcus aureus* (Gram positive), and also against the yeasts *Candida tropicalis* and *Candida albicans*. As a positive control (1000 μg mL^{-1}), norfloxacin and tetracycline were used for bacteria and fluconazole and natamicyn for yeasts. Saline solution 0.9 % (w v^{-1}) was used as a negative control.

Assays were carried out in Elisa 96 well-plates containing 100 μL of Müller-Hinton broth (bacteria) or Sabouraud broth (yeast), 100 μL of different concentrations of the extract of *S. malaccense* leaves and 5 μL of microbial strains previously standardized on the MacFarland scale 0.5 (1.5 x 10^8 CFU mL^{-1}). The plates were incubated for 24 hour at 37 (bacteria) or 28°C (yeast) and then 20 μL of 2,3,5-triphenyltetrazolium chloride (TTC) dye at 0.5 mg mL^{-1} was added to all plate wells, followed by two-hour re-incubation. Positive results for microbial inhibition were observed in the absence of alteration in the culture medium coloration (inactive cells). The presence of viable cells was indicated by the development of pink staining. Positive samples of the MIC assay were inoculated in plates containing (Brain-heart infusion) BHI Agar and incubated for 24 hours at 37°C to determine minimum bactericidal concentration (MBC) or at 28°C to determine minimum fungicidal concentration (MFC). Positive results for bactericidal and fungicidal activities were verified by an absence of microbial colony development on the BHI agar surface.

Results and discussion

Optimization of extraction conditions of bioactive compounds

Optimization by RSM was carried out to verify that the independent variables - temperature (X_1), time (X_2) and ethanol concentration (X_3) - significantly influenced the process of extraction of phenolic compounds and antioxidant activity in *S. malaccense* leaves. The total phenolic content and antioxidant activity ranged from 0.052 mg GAE kg^{-1} (assay 5) to 0.074 mg GAE kg^{-1} (assay 4) and from 0.519 μmol trolox kg^{-1} (assay 3) to 0.666 μmol trolox kg^{-1} (assay 4), respectively (Table 1). The assay 4, corresponding to the combination of higher levels of temperature and time and lower level of ethanol concentration showed the highest responses for antioxidant activity and TPC (Table 1).

The main effect, extraction time had a positive and significant linear effect ($p < 0.05$) on phenolic content (Table 2). TPC increased more than 29,98% when extraction temperature and extraction time was increased from 30ºC and 15 min. (assay 5: 0.052 mg GAE kg^{-1}) to 80ºC and 45 min. (assay 4: 0.074 mg GAE kg^{-1}). It is possible to suggest that mass transfer increases over time until a maximum level of extraction has been achieved and that after the optimum extraction time and temperature occurred degradation of some thermolabile phenolic compounds, thus leading to a lower concentration of phenolic compounds. On the other hand, none of the study variables showed a significant effect ($p < 0.05$) on the DPPH radical scavenging ability.

Equation 2 represents the mathematical model that describes the influence of extraction time and temperature on total phenolic content (TPC). The model was statistically significant and predictive where the calculated F value (5.12) was 8.1 times higher than the tabulated F value (28.5) at the 95% confidence level (Table 3). The coefficient of determination (R^2) was 0.94, indicating that 94% of data variability can be explained by the model and p value for lack of fit was not significant.

$$\mathrm{TPC(mg\ GAE\ g^{-1})} = 62.31 + 8.32\ X_1 + 9.62\ X_2 \qquad (2)$$

Table 2. Analysis of variance (ANOVA), regression coefficients for the response content of total phenolics in the extracts from jambo leaves.

Terms	Regression coefficients	Std. error	t ratio	Prob > t
Intercept (β_0)	62.31	1.73	35.96	> 0.001
Temperature (β_1) (L)	8.32*[a]	2.12	3.92	0.029
Temperature (β_1) (Q)	-5.51[b]	4.06	-1.35	0.268
Time (β_2) (L)	9.62*[a]	2.12	4.53	0.020
Ethanol concentration (β_3) (L)	-1.81[b]	2.12	-0.85	0.455
Temperature X time (β_{12}) (L)	5.71[b]	2.12	2.69	0.074
Temperature X ethanol concentration (β_{13}) (L)	-3.30[b]	2.12	-1.56	0.217
Time X ethanol concentration (β_{23}) (L)	1.53[b]	2.12	0.72	0.523

(L) Linear; (Q) Quadratic; *[a] significant at $p < 0.05$; [b] not significant.

Table 3. ANOVA table for the effect of temperature, time and ethanol concentration on TPC extraction.

Terms	DF	Sum of Squares	F ratio	Prob > F
Temperature (L)	1	138.74	19.92	0.047
Temperature (Q)	1	16.57	2.38	0.26
Time (L)	1	185.10	26.57	0.036
Ethanol concentration (L)	1	6.61	0.94	0.43
Temperature X time (L)	1	65.36	9.38	0.09
Temperature X ethanol concentration (L)	1	21.86	3.14	0.22
Time X ethanol concentration (L)	1	4.69	0.67	0.50
Model	1	438.96	28.5	
Lack of fit	1	13.10	1.88	0.30
Error	2	13.93		
Total	10	465.99		
R^2	0.94			
F value	146.13			
F statistic tablec	5.12			

Figure 1 shows the response surface and contour graphic (B) for total phenolic content response, concerning the interaction between the variables of temperature (X_1) and time (X_2). As can be seen, the increase in extraction time and temperature actually contribute to greater extraction of phenolic compounds. These results suggest that the extracted bioactive compounds feature a chemical structure that has an affinity with both polar solvents tested, and there were no significant differences in regards to ethanol:water proportions used in the extraction. In this sense, the use of less ethanol in extraction solutions may be more attractive in the economic aspect of the process.

The assessment by linear regression and response surface of results regarding the content of phenolic compounds indicates that the maximum TPC (0.074 mg GAE kg^{-1}) was found when using a time of 45 min., temperature of 80°C and 40:60 ethanol:water (v v^{-1}) as solvent extractor. The methanolic extract of *S. malaccense* evaluated by Batista et al. (2017) showed the same total phenolic compounds (0.053 mg GAE kg^{-1}), indicating that there is no significant difference in extracts produced with ethanol or methanol. Nunes, Aquino, Rockenbach, and Stamford (2016) when using methanol:water (8:2 v v^{-1}) as an extracting solvent, found lower total phenolic content and antioxidant activity in fruits of *S. malaccense* grown in Brazil, namely 0.007 mg EAG kg^{-1} in the edible portion of the fruit and 0.012 mg EAG kg^{-1} in the peel fraction. These results suggest that the distribution of bioactive components is different in each part of the plant, hence each one of them has to be assessed individually.

Scavenging of synthethic free radicals and characterization by HPLC of the *S malaccense* leaves in optimum extraction condition

The antioxidant activity of leaf extract produced in optimum extraction condition was assessed by in vitro methods. The FRAP assay is a indirect method based on a reduction of complex Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine) to Fe^{2+} form by an antioxidant under acidic conditions and an intense blue color product is developed with an absorption maximum at 593 nm. The extract showed a high capacity of ferric ion reduction (1.267 ± 0.005 µmol de Fe^{2+} kg^{-1}). Studies investigating the reduction capacity of an *S. malaccense* methanolic extract conducted by Batista et al. (2017), showed that the methanolic extract of leaves has a very similar antioxidant potential.

The ABTS method is used to evaluate the antioxidant activity of lipophilic and hydrophilic compounds while DPPH method would be better for lipophilic constituents and they are characterized by their simplicity and speed, allowing its application in routine analyses. The extract was able to scavenge ABTS and DPPH radicals with values of 0.853 ± 0.001 and 0.666 ± 0.004 µmol trolox kg^{-1} respectively. Interestingly, Arumugam et al. (2014) have reported that *S. malaccense* from Malaysia displayed good free-radical scavenging properties, as determined by DPPH and ABTS radical-scavenging assays.

In addition, the use of high performance liquid chromatography-diode array detection (HPLC-DAD) at a wavelength of 280 nm (Figure 2A) and 320 nm (Figure 2B) enabled identification and quantification of gallic acid and flavonoids: catechin, rutin and quercetin in the extract of *S. malaccense* leaves (Table 4).

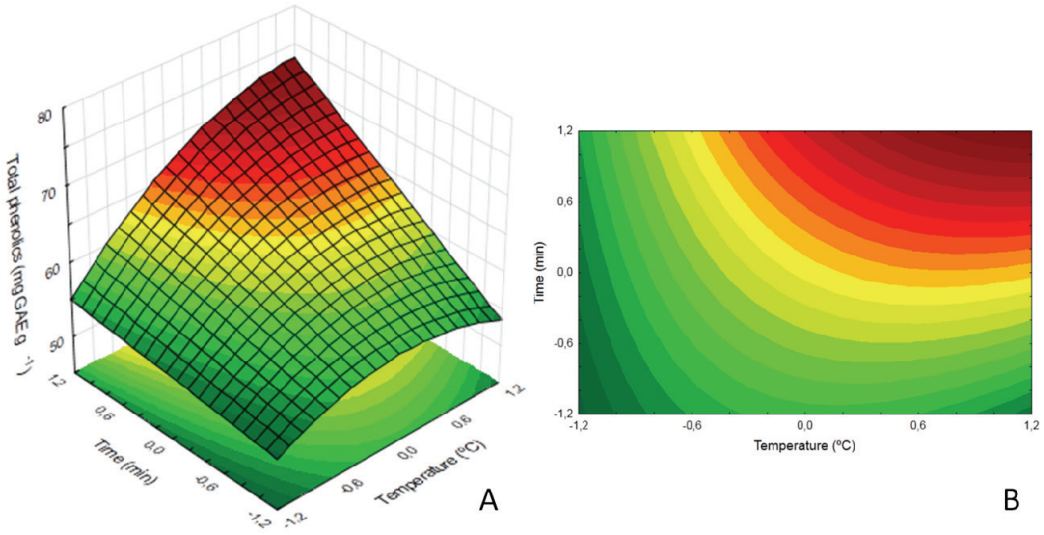

Figure 1. Response surface (A) and contour graphics (B) showing the influence of the variables temperature (X_1) and time (X_2) on the content of total phenolics extracted from jambo leaves.

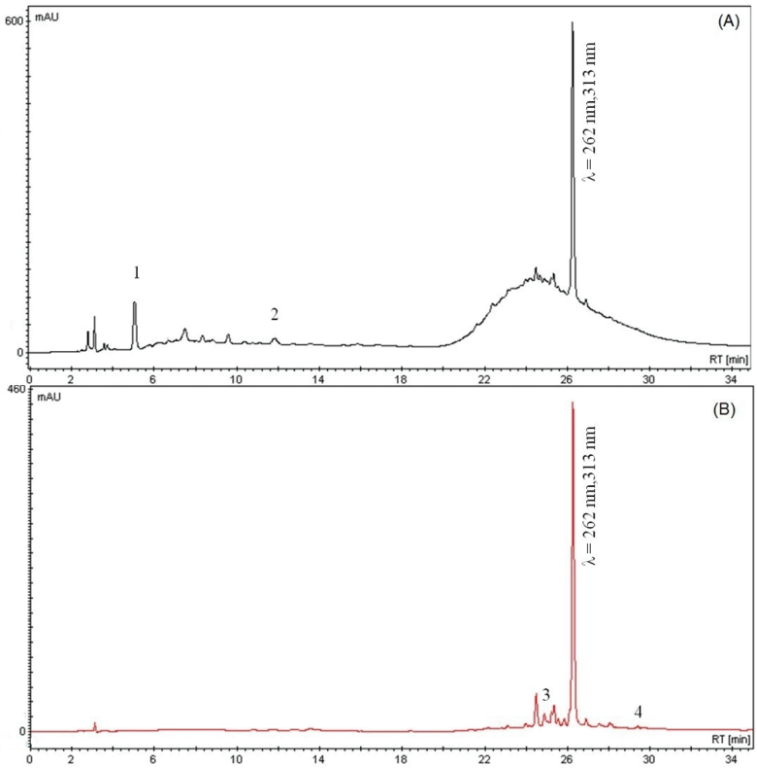

Figure 2. Chromatogram of the leaf extract of *S. malaccense* (A) at 280 nm and (B) 320 nm (1 - gallic acid; 2 - catechin; 3 - rutin; 4 - quercetin).

Table 4. Chromatographic parameters of phenolic compounds analyzed by HPLC-DAD.

Phenolic compound	Retention time (min.)	Band UV (nm)	R^2	Concentration (mg kg^{-1})
Gallic acid	5.0	271	0.9868	0.00036
(+)-Catechin	11.8	276	0.9965	0.00021
Vanillic acid	14.4	280	0.9979	n.d
Caffeic acid	15.2	320	0.995	n.d
(-)- Epicatechin	18.3	280	0.9779	n.d
p-Coumaric acid	22.6	300	0.995	n.d
Ferulic acid	24.4	320	0.9992	n.d
Rutin	23.9	348	0.9917	0.00027
Salicylic acid	26.5	300	0.9996	n.d
Quercetin	28.5	370	0.9838	0.00003

n.d: not detected.

As can be seen in the chromatograms in Figure 2, there is an intense signal with retention time of approximately 47 min. and maximum absorption in the UV region at two wavelengths (262 and 313 nm). This signal was possibly generated by a compound from the class of flavonoids, since this class of compounds absorbs in the ultraviolet (UV) region of the electromagnetic spectrum and features two bands of absorption characteristics (Nuutila, Kammiovirta, & Oksman-Caldentey, 2002). Myricitrin has been considered as a chemosystemic indicator of the genus *Syzygium*, in order to be able to suggest that the main signal may have been generated by myricitrin or their derivatives (Tian et al., 2011).

The functional molecules present in the extract were identified to be flavanols and flavonoids in addition to gallic acid. The relationships among the TPC and the antioxidant properties have been well described in previous studies (Nanda, Sultana, & Radhakrishnan, 2014; Priya, Prakasan, & Purushothaman, 2017). The antioxidant activity of these compounds is closely associated with their chemical structure and depends on such structure, mainly because of its several electron-donating groups (Gülçin, Emastaş, & Aboul-Enein, 2012), which can stabilize radicals after deprotonation. The identification of gallic acid in the sample corroborates with the study of Arumugam et al. (2014) that also identified this phenolic acid in the *S. malaccense* leaves collected in Malaysia.

Hidayati, Ersam, Shimizu, and Fatmawati (2017) and Sheela and Cheenickal (2017) studied extracts produced from leaves of genus *Syzygium* and concluded that they are sources of phenolics and flavonoids and presents a great antioxidant potential, which corroborate with our study.

Antimicrobial activity of the leaf extract of *S. Malaccense*

The leaf extract of *S. malaccense* obtained under optimized operating conditions showed an inhibitory effect against all microorganisms assessed, as shown in Table 5. Greater inhibitory activity was checked against the bacterium *Staphylococcus aureus*, with minimum inhibitory concentration (MIC) of 1 μg L^{-1}. *S. aureus* was the most sensitive microorganism to the extract of *S. malaccense*, as it showed the lowest minimum bactericidal concentration (MBC; 5 μg L^{-1}) compared with the other evaluated microorganisms.

Gram-positive bacteria, (i.e., *S. aureus*) have greater susceptibility to antimicrobial agents (Cheng, Bekhit, McConnell, Mros, & Zhao, 2012). Such behavior may be due to the structural conformation of the cell wall, which is more permeable to antimicrobial agents and, unlike Gram-negative bacteria, Gram-positive bacteria do not have periplasmic space capable of storing enzymes involved in resistance mechanisms, such as efflux pumps or inactivation of the antimicrobial agent (Allen et al., 2010).

On the other hand, there was lower susceptibility with *Salmonella bongori* bacteria; there was only a bacteriostatic effect at the concentration of 8 μg L^{-1}. This higher resistance to the inhibitory activity of the leaf extract of *S. malaccense* could be due to hydrophobic characteristics of the cell wall of Gram-negative bacteria (Allen et al., 2010).

The yeasts *C. albicans* and *C. tropicalis*, had MIC of 2 μg L^{-1} and minimum fungicidal concentration of 8 μg L^{-1} (Table 5). Antimicrobial activity of methanolic extract of *S. malaccense* leaves (cultivated in the Ernakulum district, Kerala, India) was reported against *C. albicans*. Through the disc diffusion method, these authors found that the zone of inhibition ranged from 0.4 to 0.9 mm and by the MIC assay they found minimum inhibitory concentration of 0.01 μg L^{-1}. They also found anthelmintic dose dependent activity in the assay performed in vitro using *Haemonchus contortus* (Nematoda) (Gayen, Hossain, Saifuzzaman & Faroque, 2016).

The results found in our study show that the leaves extract of *S .malaccense* grown in the city of Vilhena, state Rondônia, Brazil, has a moderate degree of antimicrobial activity both against bacteria and yeasts and these activities could be related to phenolic compounds identified in *S. malaccense* extract. The relationships among the phenolic compounds and the antimicrobial properties have been well described in previous studies (Tohma et al., 2016; Ghimire, Seong, Yu, Kim, & Chung, 2017).

Table 5. Minimum inhibitory concentration of the leaf extract of *S. malaccense* compared with different microorganisms.

Microorganism	MIC (μg L^{-1})	MBC or MFC (μg L^{-1})
Staphylococcus aureus (ATCC 25923)	1	5
Salmonella bongori (ATCC 4397)	8	>14
Candida albicans (ATCC 118804)	2	8
Candida tropicalis (ATCC 13803)	2	8

MIC: Minimum inhibitory concentration; MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration.

Conclusion

The experimental design methodology and response surface analysis demonstrated that ideal conditions to maximize extraction of bioactive compounds were: temperature of 80°C, a time of 45 min. and an ethanol:water (40:60 v v^{-1}) solution as extracting agent. *S. malaccense* leaves proved to be rich in phenolic compounds and have high antioxidant ability. The resulting extract showed minimum inhibitory concentration and bactericidal effect against *Staphylococcus aureus*. A concentration of 8 μg L^{-1} showed bacteriostatic effect against *Salmonella bongori* and fungicidal effect against the yeasts *Candida albicans* and *Candida tropicalis*. Gallic acid and flavonoids catechin, rutin and quercetin were found in significant concentrations.

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