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HEALTH SCIENCES

Schinopsis brasiliensis Engl. to combat the biofilm-dependents diseases *in vitro*

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Abstract: Dental caries and periodontal disease are the most prevalent of the biofilm-dependent diseases. With numerous side effects on the use of chlorhexidine, the search for new safe therapeutic alternatives for microorganisms involved with these diseases increases every day. This study aimed to evaluate the antimicrobial activity and cytotoxicity of extracts made from the bark of *Schinopsis brasiliensis* Engl. against five oral microorganisms and analyze their phytochemical and thermal degradation profile. The liquid-liquid partition was performed with hexane, chloroform and ethyl acetate. The identification and quantification of the chemical marker was done. Antimicrobial activity was evaluated based on the minimum inhibitory concentration. The cytotoxicity was analyzed based on the hemolysing potential of the samples. The thermal degradation profile was performed by two different methods. Gallic acid was identified as the main compound of the samples and showed the highest amount in the chloroform fraction. All samples were able to inhibit the growth of the microorganisms tested and showed no cytotoxicity. The ethanol extract absorbs less heat than the fractions. All samples exhibited exothermic peak consistent with degradation of gallic acid. Based on the results, the samples used are potential candidates for use in dental formulations for biofilm control.

Key words: antibacterial agents, natural products, oral diseases, *Schinopsis brasiliensis*.

INTRODUCTION

The resistance of microorganisms to antibiotics has become a big problem for medicine, seeking to develop new antimicrobial therapies with high resistance to microorganisms (Pereira et al. 2011). Therefore, research on herbal products is growing gradually. In dentistry, these studies mainly examine the effect of bacteria and fungi on the most prevalent oral diseases such as dental caries and periodontal disease, which are considered biofilm-dependent (Lima et al. 2014, Santamaria Junior et al. 2014, Sponchiado et al. 2014).

Dental caries is a widely prevalent disease problem globally (Bagramian et al. 2009). It is a multifactorial and prevalent chronic infectious resulting from tooth-adherent specific bacteria, primarily Streptococcus spp, that metabolize sugars to produce acid, which over time, demineralizes tooth structure (Abranches et al. 2018). *Streptococcus mitis, S. oralis,* and *S. salivarius* are associated with an initial bacteria tooth-adhesion, which can be used as a bridge to other oral microbiota members such as other streptococci and periodontopathogens (Butler et al. 2017). Furthermore, there are reports of meningitis caused by *S. salivarius* (Falomir et al.

2014, Suy et al. 2013, Vignier et al. 2014); whereas *S. oralis* and *S. mitis* can increase virulence factors of *Candida albicans* (Palma et al. 2019) and cause bloodstream infections in pediatric patients (Basaranoglu et al. 2019). The *S. mutans* is the most frequently associated microorganism with caries development, being responsible for initiated tooth demineralization acidifying microenvironment and tooth surface (Abranches et al. 2018; Baker et al. 2016). Moreover, *S. mutans* is also associated with bacteremia and endocarditis (Nakano et al. 2005, 2009). So, it is crucial to research solutions to minimize these infections.

Therefore, many studies using bioactive compounds and plants have been done (Lima et al. 2014, Macedo-Costa et al. 2017, Veloso et al. 2020, Sette-de-Souza et al. 2014, Silva et al. 2012). In this context, the *Schinopsis brasiliensis* Engl., is used in folk medicine to combat various diseases, including toothache, diarrhea, flu, and inflammations (Agra et al. 2006, Albuquerque 2006, Albuquerque et al. 2007, 2012). The bark of *S. brasiliensis* presents polyphenols, flavonoids, tannins, and saponins (Chaves et al. 2015, Fernandes et al. 2015) and a lot of compounds with biological activities have been isolated (Cardoso et al. 2005, 2015, Santos et al. 2017). In this way, Santos et al (2014) detected flavonoids and tannins in the hydroalcoholic extract of the bark of *S. brasiliensis*, along with the absence of toxicity. These phenolic compounds in the bark of this plant are degraded in temperatures above 125 °C (Fernandes et al. 2013).

The objective of this study was to evaluate the antimicrobial activity against oral bacteria and the cytotoxicity of extract and fractions produced from the bark of *S. brasiliensis* Engl. and analyze its phytochemical and thermal degradation profile.

MATERIALS AND METHODS

Collection of Plant material and obtaining of the extract

The bark of *Schinopsis brasiliensis* Engl. was collected from the semi-arid region in the State of Paraíba, Brazil. A voucher specimen was prepared and identified in the herbarium of Professor Jayme Coelho de Morais, at the Federal University of Paraíba and given the number EAN-14049.

The plant material was dried at 40 ± 1 °C. The ethanolic extract (EE) was obtained by percolating bark powder, using ethanol (Merck - Darmstadt, Hessen, Germany) as a solvent for five days in three cycles at 30 °C, and concentrated in a vacuum rotary evaporator (40 °C, 55 rpm) until complete solvent removal was achieved.

Partition of the ethanolic extract

The EE was subjected to liquid-liquid partition. The EE was then solubilized in a solvent system of ethanol:water (80:20, v/v) in a 1:1 (v/v). EE solution was put in a separatory funnel and the solvents [hexane - Hex, chloroform – Chlo, and ethyl acetate – EtAc - (Merck - Darmstadt, Hessen, Germany)] were sequentially added in a 1:1 (v/v) and stirred. The funnel was allowed to stand for about 10 minutes so that there was phase separation. The process was repeated three times for each solvent to reach the appropriate amount of each fraction. Subsequently, each fraction was concentrated in a vacuum rotary evaporator (40 °C, 55 rpm) until complete solvent removal was achieved.

Minimum Inhibitory Concentration

For this stage the standard strains used were the American Type Culture Collection (ATCC) *Streptococcus mutans* (25175), *Streptococcus oralis* (10557), *Streptococcus mitis* (903) and *Streptococcus salivarius* (7073), which were

provided by the Oswaldo Cruz Foundation (FIOCRUZ-RJ).

The minimum inhibitory concentration (MIC) determination was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI 2009). The inoculum was standardized in tubes containing 5 mL of 0.9% sterile saline solution. The microbial suspension was adjusted using a spectrophotometer (Shimadzu UV-mini 1240 – São Paulo, Brazil) at a wavelength of 625 nm, equivalent to 10^6 Colony Forming Units per mL (CFU/mL). For this step, the extract and the fractions were solubilized in 10% dimethyl sulfoxide (DMSO - Merck - Darmstadt, Hessen, Germany). One hundred microliters of each fraction or extract at a concentration of 500 mg/mL was serially diluted in brain heart infusion broth (BHI – Difco - Detroit, MI, USA) in a 96-well plate (TPP - Trasadingen, Switzerland) along with the positive control (0.12% Chlorhexidine - Sigma-Aldrich - St. Louis, MO, USA). Ten microliters of inoculation with the microorganism were added to the wells. The plates were incubated at 37 ± 0.5 °C for 24 hours. The bacterial growth was indicated by the addition of 20 µL of aqueous resazurin (Sigma-Aldrich - St. Louis, MO, USA) at 0.01% with subsequent incubation at 37 ± 0.5 °C for two hours. Viable bacteria reduce the dye, changing its color to blue, and the MIC was defined as the lowest concentration of test substance that inhibited the change of color of resazurin.

Cytotoxicity and Selectivity Index

The red blood cells' preparation for the cytotoxicity assay followed the method described by Cruz-Silva et al. (2000). Whole blood from a voluntary individual working in our laboratory, type O+, was collected and placed in a tube containing heparin. The plasma was removed after centrifugation at 2500 rpm for 5 minutes. Subsequently, the suspension of erythrocytes was washed three times with 1% saline solution. The erythrocytes were re-suspended in the same solution, and the volume was adjusted to 5%. Then 1.5 mL of 5% erythrocyte suspension was added together with 1.5 mL of the test solution at concentrations of 1.0 mg/mL, 2.5 mg/mL, and 5.0 mg/mL in tubes, remaining for 1 hour at room temperature for the hemolysis to occur. After this period, each tube was centrifuged at 2500 rpm for 10 minutes, and the supernatant was removed for reading in a spectrophotometer (Shimadzu UV-mini - 1240) at a wavelength of 540 nm (Cruz-Silva et al. 2001). The positive control used was Turk's solution 1% negative and 1% saline solution. The analysis was performed in triplicate. The calculation of the potential hemolyzing substances followed the following equation:

$$Hp = \frac{Ae - Ab}{At} x\,100 \quad (1)$$

Where:

Hp = Hemolyzing potential (in percentage)

Ae = Absorbance of the test sample

Ab = Absorbance of the Negative Control

At = Absorbance of the Positive Control

From the data generated from each sample's linearity, it was possible to determine the appropriate concentration of each extract that would cause hemolysis of red blood cells by 50% (IC_{50}). These data were used to determine the selectivity index (SI) of each extract for the bacteria tested, and enabled the calculation according to the methodology proposed by Protopopova et al. (2005) using the equation:

$$SI = \frac{IC_{50}}{MIC} \quad (2)$$

Where:

SI = Selectivity Index

IC_{50} = concentration of each extract that would cause hemolysis of red blood cells by 50%

MIC = Minimum Inhibitory Concentration against the test microorganism

Phytochemical screening

For the determination of total flavonoid content, the method proposed by Meda et al. (2005) was followed. Aluminum chloride ($AlCl_3$) was added to the extract and diluted in 2% methanol. After waiting 10 minutes, the sample was taken to a spectrophotometer (Shimadzu UV mini - 1240) at an absorbance of 415 nm. The calibration curve was obtained using solutions of quercetin. The concentration of flavonoids was expressed in milligrams equivalent of quercetin.

The quantification of total tannins was completed by the methodology proposed by Makkar & Becker (1993). A vanillin solution of 4% (w/v) in methanol was added to the extract. Then 1.5 mL of concentrated HCl (37%) was added. The absorbance was read at 500 nm in a spectrophotometer (Shimadzu UV mini - 1240). The result was determined by interpolating the absorbance of the samples against a calibration curve constructed from standards of catechin and expressed as mg catechin equivalents per g extract.

Finally, to determine the content of total polyphenols, the method described by Chandra & Mejia (2004) was used, adding Folin-Ciocalteu 1N to the aqueous extract. After 2 minutes, the aqueous solution of 20% sodium carbonate (Na^2CO^3) was added, remaining there for 10 minutes. Soon, the absorbance at 757 nm in a spectrophotometer (Shimadzu UV mini - 1240) was performed. The calibration curve was obtained from gallic acid solutions. The concentration of polyphenols was measured in equivalent milligrams of gallic acid.

All analyzes were performed in triplicate.

Chemical Marker Identification and Quantification

Each sample was analyzed by High Performance Liquid Chromatography (HPLC) for quantification of gallic acid. The analysis followed the method described by Schieber et al. (2001). The chromatographic test was conducted on Shimatzu (Shimadzu Corporation, Kyoto, Japan) equipment with a diode array detector (model SPD-M10A) in a C-18 column (Phenomenex Gemine - 4.6 mm x 250 mm x i.d. 3 µm) at a flow rate of 1.0 mL/min. The analysis was conducted using a wavelength of 272 nm. The column was operated at a temperature of 30 °C. The mobile phase used was 1% formic acid: methanol - 90:10 (v/v). The sample injection volume was 10 µL.

Thermal Analysis

The thermogravimetric curve (TG) and differential thermal analysis (DTA) were performed using a DTG-60 (Shimadzu Corporation, Kyoto, Japan). Samples were heated at a heating rate of 10 °C/min from 25 °C to 900 °C in a nitrogen atmosphere in an alumina crucible containing approximately 7.0 mg of sample (Sette-de-Souza et al. 2018).

RESULTS

Minimum Inhibitory Concentration, Cytotoxicity and Selectivity Index

In table I, it is possible to see that all tested samples showed a minimum concentration capable of inhibiting the growth of microorganisms and the absence of cytotoxicity was also present in all samples.

Phytochemical screening

Table II shows the quantitative phytochemical characterization results obtained with the extract and fractions of *S. brasiliensis* Engl. It is possible to observe no tannins in all fractions, while there are tannins in Ethanolic Extract.

Table I. Hemolyzing Potential, IC_{50}, Minimum Inhibitory Concentration (MIC – mg/mL) and Selectivity Index (SI) of tested solutions.

Sample	Hemolyzing Potential			IC_{50} (mg/mL)	S.mu		S.o		S.mi		S.s	
	1.0 mg/mL	2.5 mg/mL	5.0 mg/mL		MIC	SI	MIC	SI	MIC	SI	MIC	SI
Hex	0.0%	9.0%	13.0%	15.77	0.250	63	0.500	31	0.500	31	0.500	31
Chlo	0.0%	5.0%	10.0%	22.04	0.500	44	1.000	22	0.250	88	0.250	88
EtAc	1.0%	8.0%	14.0%	15.40	0.500	30	1.000	15	0.500	30	0.500	30
EE	1.0%	2.0%	4.0%	50.27	0.500	100	0.500	100	0.500	100	0.250	201
CHX	*	*	*	0.38	0.150	2	0.300	1	0.060	6	0.150	2

Hex: Hexane fraction; Chlo: Chloroform fraction; EtAc: Ethyl Acetate fraction; EE: Ethanolic Extract; CHX: Chlorhexidine; S.mu: *Streptococcus Mutans*; S.o: *Streptococcus oralis*; S.mi: *Streptococcus mitis*; S.s: *Streptococcus salivarius*. *Chlorhexidine concentration was determinated by 0.06, 0.12 and 0.20 mg/mL to the hemolyzing potential.

Table II. Content of total tannins, total flavonoids and total polyphenols of *S. brasiliensis* Engl. extract obtained by spectroscopy in the visible region.

Sample	Total Tannins (µg/mg)	Total Flavonoids (µg/mg)	Total Polyphenols (µg/mg)
Hex	-	8.40	75.38
Chlo	-	25.71	592.01
EtAc	-	17.69	559.33
EE	15.83	6.94	598.55

Hex: Hexane fraction; Chlo: Chloroform fraction; EtAc: Ethyl Acetate fraction; EE: Ethanolic Extract.

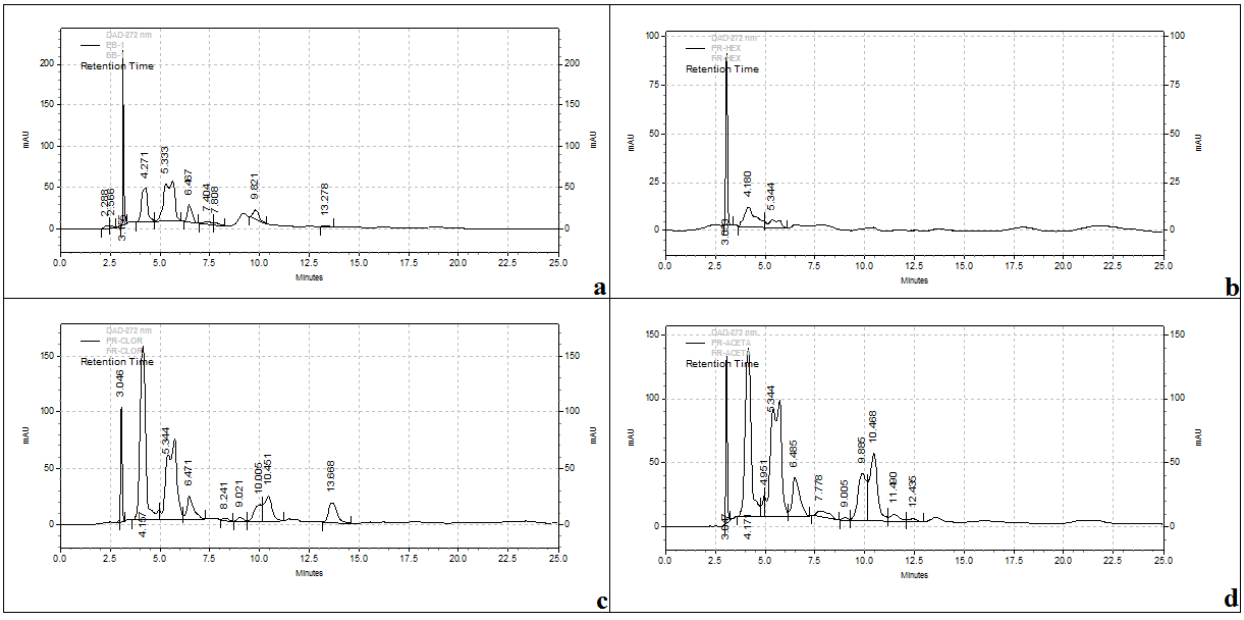

Figure 1. Chromatogram of the sample. a: ethanol extract; b: Hexane fraction; c: Chloroform Fraction; d: ethyl acetate fraction.

Chemical Marker Identification and Quantification

The quantitative determination of phytochemical extract and fractions of *S. brasiliensis* Engl. detected the presence of large quantities of polyphenols. Based on this result, gallic acid was chosen as a marker, and we proceeded to quantify the samples. In all samples analyzed a predominant presence of gallic acid is observed, which has a close retention time of 3.0 minutes (Figure 1).

According to the standard curve (y = 61635x - 11502; with R^2 0.9957), the chloroform fraction had the most significant amount of gallic acid (52.02 mg/g), followed by ethyl acetate fraction (50 39 mg/g) and the ethanol extract (15.56 mg/g). The sample which had the lowest amount of gallic acid was the hexane fraction (6.51 mg/g).

Thermal Analysis

The curves of TG and DTA of the ethanol extract, fractions, and gallic acid can be viewed in Figure 2 and 3. The peaks of each event are shown in Table III.

DISCUSSION

All extracts tested had higher MIC values on the bacteria tested than the positive control (0.12% chlorhexidine). According to Castro et al. (2009), this can be explained by the fact that the natural products contain biological compounds diluted in the sample, while chlorhexidine is a synthetic drug. However, it is important to note that chlorhexidine has adverse effects that limit its period of use. In a systematic review, Haas et al. (2014), showed that chlorhexidine showed good results in reducing biofilm and gingivitis. However, it must be used for short treatments due to its adverse effects, and the natural agent-based products are more suitable for everyday use. Therefore, the hexane fraction of the EE of *S. brasiliensis* Engl is a potential agent that could be incorporated into preventive and therapeutic products in dentistry, due to its activity against *Streptococcus mitis* and *S. oralis*, which are responsible for the initial colonization of the tooth surface, and against *S. mutans*, which is primarily responsible for the initiation / progression of dental caries and other diseases. Moreover, Duarte et al. (2006) have reported the activity of phenolic compounds against *S. mutans* biofilm and, therefore, a further justification for the use of the extract in this area.

The samples showed no cytotoxicity at the concentration tested, which was also observed by Santos et al. (2014). According to Bézivin et al. (2003), SI indicates how many more times the test product is effective against specific target cells than human cells. For the compound to be considered an ideal prototype of a new drug, it is expected that the presented SI is greater than or equal to 10 (Protopopova et al. 2005). According to Reimão et al. (2010), the increase in the numerical value of the SI compound indicates that it is more toxic to microorganisms and less toxic to human cells. Therefore, as the IS of all tested samples were higher than that stated by the authors, the findings of this research are important in the search for the development of natural products. The low toxicity is an interesting feature along with the satisfactory antimicrobial activity displayed.

Other studies with *S. brasiliensis* Engl. showed a separation of phytocompounds when the polarity is increased (Santos et al. 2014). No fraction studied presented tannins equivalent to catechin, but the EE showed 15.83 mg/g of tannins. This may be related to the polarity of each solvent used, which possibly resulted in the lack of extraction of compounds with a polarity similar to catechin. This does not mean that the samples produced with bark

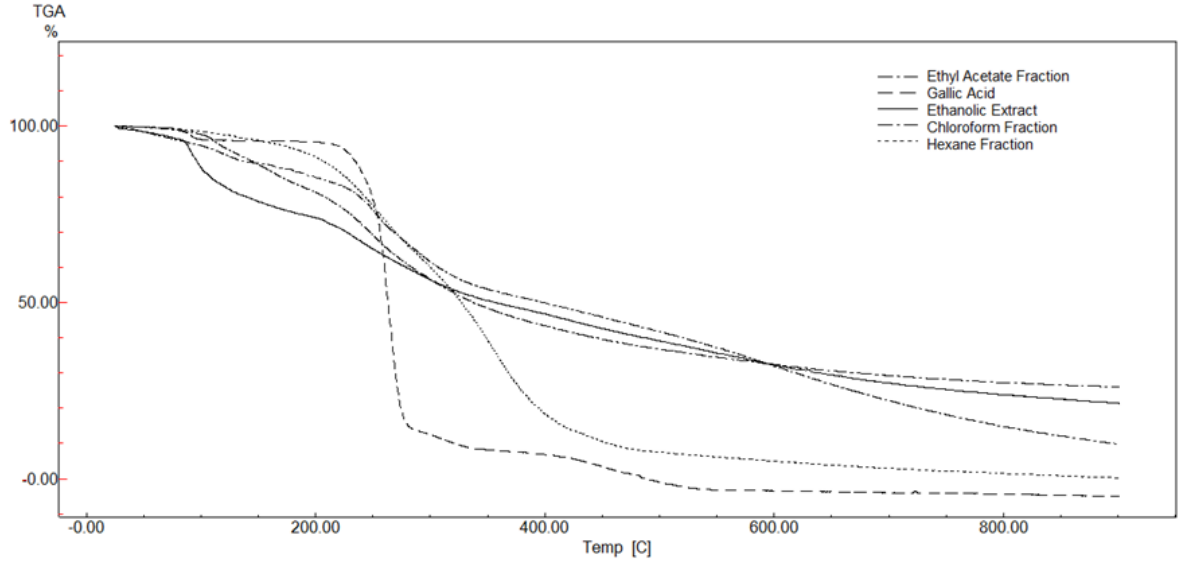

Figure 2. TG curve of gallic acid and the samples produced from the extract of S. *brasiliensis* Engl.

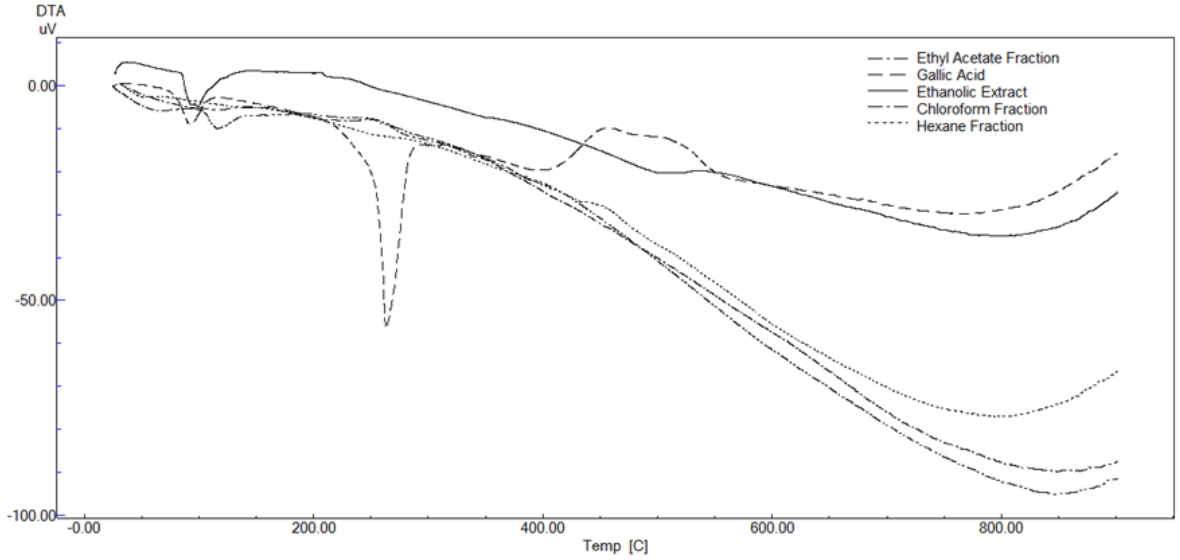

Figure 3. DTA curve of gallic acid and the samples produced from the extract of S. *brasiliensis* Engl.

Table III. Data from TG curves of the extract and fractions *S. brasiliensis* Engl.

Sample	Step 1		Step 2		Step 3	
	Onset - Endset (°C)	Mass loss (%)	Onset - Endset (°C)	Mass loss (%)	Onset - Endset (°C)	Mass loss (%)
Hex	29.8–201.5	9.72	201.5–479.8	81.97	-	-
Chlo	29.5–108.7	3.50	108.9–219.8	18.90	223.9-478.6	38.74
EtAc	29.4–225.2	16.97	225.3–327.8	26.42	328.0–553.2	46.23
EE	30.0–86.9	4.55	87.0–205.8	21.54	205.8–360.4	51.33
GA	29.8–205. 9	4.59	205.9–334.6	86.58	335.4–535.4	8.63

Hex: Hexane fraction; Chlo: Chloroform fraction; EtAc: Ethyl Acetate fraction; EE: Ethanolic Extract; GA: Gallic Acid.

of *S. brasiliensis* Engl. do not contain tannins. However, the amount of flavonoid found in each fraction was higher than that found in the EE, this is probably because the fractions of the compounds are more concentrated than in the EE and therefore tend to be higher. In their study, Santos et al. (2014) observed qualitatively the presence of auronas, catechins, chalcones, flavanones, saponins and tannins in the ethyl acetate fraction, while hexane fraction and chloroform only steroids and triterpenes were identified, respectively. Differently of our study, these authors did not quantify the compounds; they only observed the full presence or absence, without using a standard compound, which may have generated these different results from the fraction of ethyl acetate. In addition, when working with natural products and compounds, one must take into account factors that influence the production of secondary metabolites by plant, such as the period in which the plant was collected, rainfall, seasonality, potential natural predators, etc (Gouvea et al. 2012, Li et al. 2014, Moore et al. 2014). Thus, it is possible to explain these differences by the fact that those authors collected parts of *Schinopsis brasiliensis* in another region and another period compared to our collection.

It was observed in the DTA curve that the EE absorbs less heat than the fractions. All samples show an endothermic peak in the range of 85.0 to 130.0 °C, consistent with the humidity loss, water and/or volatile solvent used in each system. The TG curve of the EE has three thermal decomposition events. The first, which is set in the range of 30.00 to 86.96 °C is related to loss of water and solvent from the extract, as the mass loss was approximately 4.55% of the total sample. The second event (87.01 to 205.75 °C) may be associated with the degradation of phytochemical compounds found in the sample, such as tannins and flavonoids, with an equivalent mass loss of 21.54% (1.33 mg). The third event (from 205.85 °C) could be related to the degradation of gallic acid present in the sample, considering that this temperature coincides with the second step where the degradation of phenol lost reaches 51.33% of total sample mass (3.17 mg). The fraction chloroform curve is very similar to the EE and is even the sample with the closest profile to the EE, in respect to both thermal decomposition peaks and mass percentage loss in each step.

The TG curve of ethyl acetate fraction also shows three different stages of thermal decomposition. The first is between 29.35 to 225.23 °C, similar to that found in EE. However, besides the loss of moisture and residual solvent fraction, there is probably some loss of another compound, as this loss was equivalent to 16.97% of the total (1,13mg). The second event, which happened between 225.30 and 327.87 °C, may be related to the degradation of flavonoids found in the sample, as well as the degradation of gallic acid present, equivalent to 26.42% of mass loss (1.76 mg), since the peak gallic acid degradation occurs at about 263 ºC in an exothermic event. Finally, the degradation starts at 328.00 ºC, and possibly the degradation of carbonaceous residues comes at the penultimate stage, which does not reach complete degradation (46.23% mass loss).

The TG curve of the hexane fraction is the only one that shows two degradation phenomena. The first occurs between 29.78 to 201.52 °C, which can be related to water loss and residual solvent fraction, equivalent to 9.72% of its total mass. The second from 201.53 to 479.82 °C, has a mass loss of 81.97%, corresponding to 5.80 mg of the fraction, practically degrading almost all the present compounds.

The endothermic peaks of the DTA events coincide with the degradation temperature ranges found in the sample TG curves. No

events found were exothermic. The more polar fractions have discrete endothermic peaks when compared to the peaks of the EE and the fraction of ethyl acetate. Therefore, it is possible to deduce that both the hexane fraction as well as the chloroform fraction require less heat to begin their degradation.

CONCLUSIONS

Due to the satisfactory antimicrobial activity found against bacteria involved in the initial colonization of the tooth surface and at the start of caries, along with the low cytotoxicity and high selectivity, all samples of the EE of the bark from *Schinopsis brasiliensis* Engl, are potential ingredients that could be used in dental formulations to control oral biofilm.

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Sette-de-Souza PH participated in all stages. Santana CP contributed to sample preparation to phytochemical screening and thermal analysis and interpreting the results. Sousa IMO and Foglio MA contributed to sample preparation to HPLC, performed the analysis and interpreting the results. Medeiros FD and Medeiros ACD conceived of the presented idea, interpreting the results and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript and revisions.

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