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ORIGINAL ARTICLE

# EVALUATION OF THE EFFECTS OF *Spondias mombin* EXTRACT AS AN ALTERNATIVE ANTIMICROBIAL DRUG

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## ABSTRACT

The extract of *Spondias mombin* has constituents which may improve psychiatric disorders, in addition to having antiviral, antifungal, and antimicrobial activity. But despite having several benefits, it is necessary to assess whether the extract may interfere with cell metabolism so furthermore its microbicide potential can be explored. Fifteen Wistar rats were used, divided into four groups (control group; control with extract; hyperlipidemic diet; hyperlipidemic diet and extract). For 12 weeks, the animals were weighed and their blood glucose was assessed. Afterwards, they were euthanized, and the biological material was collected. The evaluation confirmed the efficacy of the extract of *S. mombin* against cell metabolism of rats, without negatively altering cell viability; the group of rats with an hyperlipidemic diet showed an increase in body weight; however, in the individual assessment of the organs, there were no significant changes. The glycemic index, liver parameters, lipids, and mineral ions did not show changes. Furthermore, the antimicrobial potential of *S. mombin* extract was observed against *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* BLACC. The results suggest that *S. mombin* extract did not interfere with cell viability, did not show cytotoxicity to cells that were exposed to it, nor did it interfere with the metabolism, organs, and biochemical indices of rats with a standard or hyperlipidemic diet. Considering such characteristics and the potential activities observed in this present study, additional evaluation should be conducted to further assess the role of *S. mombin* extract as a source of new alternative antimicrobial drug as well as its possible beneficial activity to the cardiovascular system.

KEY WORDS: *Spondias mombin*; hyperlipidemic diet; antimicrobial inhibition; obesity; *Staphylococcus aureus*.

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## INTRODUCTION

*Spondias mombin*, popularly known as Cajá, Cajazeira, and Taperebá is a fruit species that belongs to the *Anacardiceae* family and it is found mainly in the North and Northeast of Brazil (Silva, 2015a; Ayoka et al., 2008). All tree´s parts are used in traditional medicine because its bioactive compounds, such as flavonoids, carotenoids, phenolic compounds, and ß-cryptoxanthin, and they play essential roles in the body such as preventing oxidative stress and degenerative diseases (Canuto et al., 2010; Yahia et al., 2010a; Yahia et al., 2010b; Cabral et al., 2016; Pereira, 2017; Tiburski et al., 2011).

Biochemical analysis showed that *S. mombin* is a food with high nutritional and functional content, with considerable amounts of vitamin C, provitamin A, and low energy content since it is poor in starches and rich in insoluble fibers (Silvino et al., 2017).

Diet supplementation with *S. mombin* has potential to attenuate cardiac remodeling after myocardial infarction, as it helps to reduce fibrosis, myocardial hypertrophy, oxidative stress cascades, energy metabolism, and inflammatory processes, such as those that occur during the infarction, thus denoting its cardioprotective effect (Pereira et al., 2020; Sameh et al., 2018).

The ability of *S. mombin* compounds, especially flavonoids, to prevent oxidative stress is due to the ability of reducing inflammation markers, and leading to an improvement in the lipid profile, which alters the synthesis, accumulation, and storage lipids pathways in hepatocytes and adipocytes, as well as making it an alternative to attenuate and even reduce the number of overweight and/or obese individuals (Pereira, 2015; Assini et al., 2013).

It was also found that the extract of *S. mombin* has constituents that improve psychiatric disorders, in addition to having antiviral, antifungal, and antimicrobial activity against selected microorganisms, such as strains of *Staphylococcus aureus* (Ayoka et al., 2005; Ayoka et al., 2006; Ayoka et al., 2008; Cristofoli et al., 2019; Corthout et al., 1994); Maduka et al., 2014; Temitope et al., 2017; Lima et al., 2021; Wena et al., 2011; Kouadio et al., 2020; Siqueira et al., 2020).

Along with the facts mentioned earlier, it is necessary to assess whether the extract, despite having several benefits, interferes with cell metabolism so that its microbicide potential can be explored. Thus, this study, which is an experimental research with *S. mombin* extract in an animal model, verified its effects on Wistar rats submitted or not to a hyperlipidemic diet.

## MATERIAL AND METHODS

### *Cell culture*

RAW 264.7 cells (murine macrophages) were used from the Institute of Tropical Pathology and Public Health at the Federal University of Goiás (UFG) in Brazil. These cells were cultured in RPMI 1640 (Sigma Chemical Co.® St Louis, MO, EUA) supplemented with 10% fetal bovine serum (FBS, Cripion®, São Paulo, Brazil), inactivated at 56°C for 30 min, 2 mM L-glutamine (Sigma Chemical Co®), 50µM of 2 mercaptoethanol (Sigma-Aldrich®), 10U/mL penicillin and 100 µg mL streptomycin (Sigma-Aldrich®) and 10 mM Hepes (Sigma-Aldrich®). Cell concentration was $2x10^5$ cells in 2 mL of complete RPMI medium by well on 6-well culture plates (Costar®, Nova York, EUA), incubated in an oven with 5% $CO_2$ and 35°C at the Cell Cultivation Laboratory of the Pontifical Catholic University of Goiás (ECMV/PUC-GO).

### *Mitochondrial metabolic activity (MTT)*

The mitochondrial metabolic activity (MTT) was verified by tetrazolium dye uptake assay, whose principle of action is the [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide salt] by the viable cells, which are reduced, within the mitochondria, to a product called formazan, through the enzyme succinate-dehydrogenase and form insoluble purple crystals (Mosmann, 1983).

Raw 264.7 cells ($1x10^5$ cells/well) were seeded, in triplicate, in 96 well flat-bottom microplates in RPMI 1640 medium and exposed to five concentrations from 5 to 60 µg/mL (5, 10, 20, 40 and 60 µg/mL) of the studied extract. After 48 hours of incubation in an incubator at 37° C with 5% $CO_2$, 5 µL of MTT was added to each well and incubated for another 3 hours. With this process, the reading was performed in an ELISA reader (Spectrophotometer) (Biolisa Reader, Bioclin®, Belo Horizonte, MG) with a double filter of 450 and 630 nm.

Cytotoxicity was calculated according to the equation: %Viability = (Average of Absorbance of each concentration of the extract – control basal / Absorbance mean of controls – control basal) x 100

Cell viability was expressed, when applicable, as IC50 (concentration that inhibited cell growth by 50% compared to the untreated group). Therefore, the IC50 was defined as it was applied in the experiment. The results were analyzed with calculations and expressed in graphs using the statistical tool GraphPad Prism version 7.0.

*Animals*

Fifteen male Wistar rats were used. These were obtained in Sectorial Animal Facility of the Pontifical Catholic University of Goiás (PUC-Goiás). After 21 days of weaning, the animals were kept in polypropylene boxes, with a maximum capacity of 4 animals per box, which were lined with wood shavings and changed every three days. The environment in which they stayed was controlled; the temperature was 21°C, there was a light-dark cycle of 12-12h, with water and industrialized feed or hyperlipidemic diet *ad libitum*.

The procedures performed in this study were submitted and approved by CEUA/PUC-Goiás protocol 8820121018, following National Council for The Control of Animal Experimentation (CONCEA) (11.794/08).

*Diet protocol and administration of* Spondias mombin *extract*

Two types of diets were available to animals: standard diet consisting of commercial feed for small rodents and the hyperlipidemic diet. The hyperlipidemic diet comprises a mixture of standard feed, milk chocolate, peanuts, and cornstarch biscuit, in the respective ratio of 3:2:2:1. After the ingredients were mixed, the hyperlipidemic food was molded and stored in clear plastic bags identified in a refrigerated -4°C environment until it was distributed to the animals.

Concentrated *S. mombin* extract was dissolved at 0,15 % in filtered drinking water and administered orally. The other animals received filtered water. Both were offered *ad libitum*. The amount of water and extract ingested were recorded daily using a graduated glass beaker.

The animals were divided into four groups, namely:

Group 1: Standard diet with filtered water intake (CT);

Group 2: Standard diet with ingestion of *S. mombin* extract (CT+EX);

Group 3: Hyperlipidic diet with ingestion of filtered water (HL);

Group 4: Hyperlipidic diet with ingestion of *S. mombin* extract (HL+EX).

*Blood glucose and body weight*

During experimental period, rats were weighed on a scale (Vonder) to assess body mass (grams), and the blood glucose (mg/dL) was measured using a high-precision digital glucometer (Accu-Chek Active). Both procedures were performed twice a week on standardized days.

*Biochemical analysis*

After 12 weeks, the animals were anesthetized (thiopental, 80mg in a single dose). The rib was exposed for blood collection through the cardiac puncture, and 2mL of blood was collected for biochemical evaluation. Samples were collected in a sterile test tube without anticoagulant, centrifuged (1,000g for 10min) and serum was stored in Eppendorf tubes at less than -20°C until the analysis. Biochemical dosages were performed using Biosystems® kits, through kinetic, enzymatic, and colorimetric methodologies, automated A15 (Biosystems).

Albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), calcium, HDL cholesterol, total cholesterol, creatinine, glycemia, phosphorus, total protein, triglycerides and urea were analyzed.

*Organ weight assessment*

At the moment of the euthanasia, animals had their heart, kidneys, brain, and liver removed. These organs were weighted (semi-analytical scale; BK400, GEHAKA®, Brazil, Sao Paulo) and stored in formalin.

*Bacterial susceptibility test against* Spondias mombin *extract*

Experiments were carried out in the Microbiology Section of the Clinical Laboratory of PUC-Goiás. The technique of diffusing extract in wells on Mueller agar was chosen to carry out bacterial susceptibility tests with hydroalcoholic vegetable extracts (Michelon et al., 2016; Silveira et al., 2009).

A flat-bottom flask graduated in 1,000 mL, 22.8 grams of OXOID® brand Mueller-Hinton agar, lot: 1485367 and 600 mL of deionized water were added, the flask was sealed with tape for autoclave (15 minutes at 121°C). The agar was distributed in Petri dishes measuring 150 mm in diameter and 15 mm in depth. Soon after the agar solidified, the plates were exposed to germicidal (UV) light in a biological safety cabinet for 30 minutes.

Strains of *Escherichia coli* ATCC 25923, *Klebsiella pneumoniae* ATCC 700603 *Staphylococcus aureus* ATCC 29213, and a clinical isolate of *Staphylococcus aureus* BLAC that were stored and made available by the Microbiology sector of the Laboratory of Clinical Analysis at PUC Goiás were also used.

The bacterial strains used in the study were seeded on selective culture media (MacConkey agar for *E. coli* and *K. pneumoniae*; Mannitol Salt agar for *S. aureus*) and incubated at 36.5°C for 24 hours. These colonies grown on the selective culture media, were used to prepare bacterial suspensions in sterile 0,85% NaCl solution, corresponding to the 0,5 MacFarland scale.

Subsequently, the bacterial suspensions were seeding on the surface of Mueller Hinton agar by the sweeping swab method.

Next, circular cavities with a diameter of 10mm were made on Mueller Hinton agar, using sterile test tubes, where different volumes of *S. mombin* extract were deposited, namely: 50 µL, 100 µL, and 200 µL. The tests were carried out in triplicate on different dates.

*Statistical analysis*

For data analysis, the ANOVA analysis of variance was applied, followed by Bonferroni, to analyze whether the variance would determine whether mean of the groups used in the experiments was different. In this way, for each evaluated parameter, mean, standard deviation, and variance data were expressed. The collected data was relocated to the Graph Prism®, version 7, to tabulate and graph the data obtained in the experiment. The results regarding blood glucose and weight were analyzed by the analysis of variance with two factors, two-way ANOVA. The result of organ weight was passed through ANOVA, Brown-Forsythe and Bartlett's tests to verify the equality of variances, and biochemical analyses were carried out with Tukey's multiple comparison test or t test when relevant, in which all possible averages are compared, based on the reduced difference, considering the percentiles of the groups used.

## RESULTS

*Evaluation of the cytotoxicity of* Spondias mombin L*. fruit extract*

RAW 264.7 cells exposed to *S. mombin L*. alcoholic extract at concentrations of 5, 10, 20, 40, and 60 µg/mL showed no impairment in viability indicating absence of citotoxicity until 60 µg/mL *in vitro*.

*Weight and blood glucose assessment*

Figure 1 shows the variation in weight (in grams) in animals during the three months of the experiment. A significant weight difference was observed from the sixth week on between the hyperlipidemic group (HL group) and the other groups. The HL group in the twelfth week show to be heavier than observed in the other groups, followed by the CT + EX group, then with the HL + EX group, and lastly, the CT group. The average weight variation was 49.75g (± 22.77) in the HL group, 35.75g (± 29.,97) in the CT + EX group, 33.25g (± 14.77) HL + EX group and 31.33g (± 0.57) in the CT group.

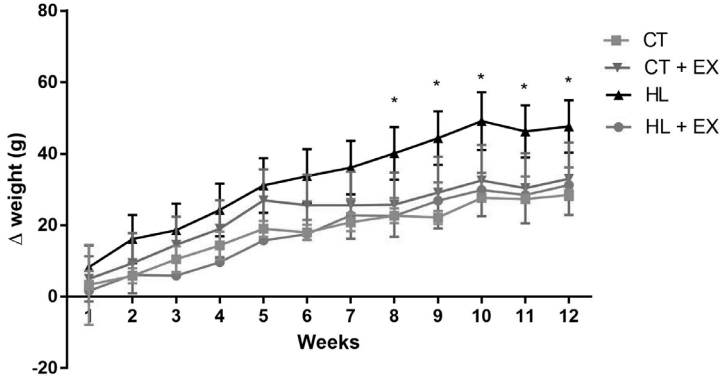


*Figure 1.* Wistar rats body mass gain curve. Each curve represents a group of animals (CT, CT+EX, HL, HL+EX), with a mean ± SD. * shows statistic differences between groups $p < 0.05$ (HL vs HL+EX).

Blood glucose was measured from a sample collected from animal tail. Figure 2 shows the mean (± SD) values of blood glucose during twelve weeks experiment. The mean blood glucose at the final week was 259.33 mg/dL (± 65.39) in CT group, 237.5 mg/dL (± 203.61) in CT + EX group, 232.5 mg/dL (± 76.42) in HL group, then 204.25 mg/dL (± 43.23) in HL + EX group. There was no significant difference in glycemic index among the groups in the experiment, regardless the diet they were given. Therefore, the addition of *S. mombin* extract did not alter the glycemic metabolism of rats.

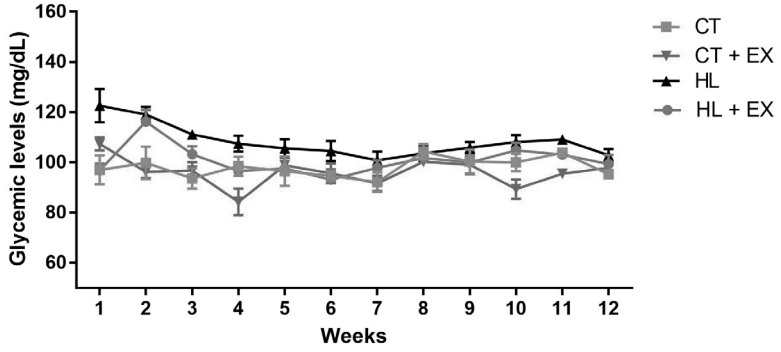


*Figure 2.* Wistar rats tall glycemia curve. Wistar rats were kept for 12 weeks in different diets. Blood was collected weekly from the tail and glucose measurement was performed. Each curve represents a group of animals (CT, CT+EX, HL, HL+EX), with mean ± SD at each point.

*Evaluation of biochemical parameters*

Hepatic parameters (ALT, AST, total protein and albumin), renal (urea and creatinine), lipids (HDL cholesterol, total and triglycerides), glycemic and mineral ions (calcium and phosphorus) were measured at the end of the experiment, after 12 weeks. The parameters above were analyzed and when compared, it was noticed that *S. mombin* extract did not interfere in biochemical parameters between groups without extract when compared with those who consumed the extract. As expected, there was a statistically significant difference in the urea of animals with an hyperlipidemic diet ($p<0.0001$) (Table 1).

*Table 1.* Means and standard deviation of the biochemical parameters.

| Parameters | CT | CT EX | HL | HL EX |
|---|---|---|---|---|
| Albumin-SER (g/dL) | 2.9466 ± 0.0141 | 3.2075 ± 0.2828 | 2.6975 ± 0.6151 | 2.7025 ± 0.3323 |
| ALT-SER (U/L) | 111.0333 ± 16.6170 | 145.15 ± 59.1848 | 51.625 ± 24.1123 | 70.775 ± 32.5976 |
| AST-SER (U/L) | 52.6333 ± 27.7366 | 22.375 ± 5.9918 | 42 ± 37.3759 | 22.5333 ± 22.2336 |
| Calcium Arsenazo-SER (mg/dL) | 11.1933 ± 0.3271 | 10.745 ± 1.4012 | 8.7825 ± 2.3874 | 9.7025 ± 2.1742 |
| CHOL HDL Direct-SER (mg/dL) | 40.3666 ± 26.6503 | 32.875 ± 9.6160 | 21 ± 5.2933 | 24.7 ± 7.1610 |
| Cholesterol-SER (mg/dL) | 58.6666 ± 10.2632 | 57.25 ± 12.4733 | 54.25 ± 8.9209 | 51.5 ± 15.0665 |
| Creatinine-SER (mg/dL) | 0.41 ± 0.02 | 0.4525 ± 0.1575 | 0.3475 ± 0.0340 | 0.285 ± 0.0869 |
| Glucose-SER (mg/dL) | 259.3333 ± 65.3936 | 237.5 ± 203.6164 | 232.5 ± 76.4264 | 204.25 ± 43.2309 |
| Phosphorus-SER (mg/dL) | 9.36 ± 0.9101 | 11.53 ± 3.2343 | 8.6625 ± 1.0190 | 9.0425 ± 1.4365 |
| Protein Total -SER (g/dL) | 5.98 ± 0.1352 | 6.18 ± 0.5742 | 5.265 ± 1.0170 | 5.3125 ± 1.3064 |
| Triglycerides -SER (mg/dL) | 109.3333 ± 51.1598 | 94.25 ± 7.8475 | 71.25 ± 22.3811 | 86.5 ± 62.4633 |
| Urea UV-SER (mg/dL) * | 41 ± 2 | 50.25 ± 2.8722 | 28.25 ± 0.9574 | 28.75 ± 3.5939 |

Subtitles: CT (Control group); CT EX (Control group + extract); HL (Hyperlipidic), HL EX (Hyperlipidic + extract); SER (Serum); UV (enzymatic system for detecting serum urea by photometry); CHOL (Cholesterol); AST (Aspartate aminotransferase; ALT (Alanine aminotransferase); g/dL (Gram per deciliter); U/L (International unit per liter); mg/dL (Miligram per deciliter).

*Organ weight assessment*

After 12 weeks of the dietary protocol, the animals had their heart, kidneys, brain, and liver extracted, weighed and placed in a solution containing formaldehyde. It was noted that there was no statistically difference in organ weight among the groups. Such protocol was adopted since the extract with different dietary protocols could promote histological changes in these target organs. Such modifications would come to be verified both with the naked eye and microscopically when present (Table 2).

*Table 2.* Means and standard deviation of the organs weight

| Organs | CT | CT EX | HL | HL EX |
|---|---|---|---|---|
| Heart | 1.231 ± 0.2507 | 1.3777 ± 0.2435 | 1.2142 ± 0.1403 | 1.178 ± 0.0884 |
| Kidney | 2.8063 ± 0.1395 | 3.023 ± 0.4129 | 2.5417 ± 0.1403 | 2.5805 ± 0.1625 |
| Brain | 1.887 ± 0.0582 | 1.9682 ± 0.2386 | 1.8175 ± 0.1218 | 1.8522 ± 0.1335 |
| Liver | 10.506 ± 2.2.0886 | 11.001 ± 1.0829 | 10.536 ± 0.8330 | 9.4145 ± 1.1056 |

Subtitles: CT (Control group); CT EX (Control group + extract); HL (Hyperlipidic), HL EX (Hyperlipidic + extract)

*Antimicrobial Evaluation*

The *S. mombin* extract did not inhibit the growth of *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25923 in any of tested volumes. However, antimicrobial potential of the extract in the volume of 200µL was observed, inhibiting the growth of *S. aureus* ATCC 29213 and *S. aureus* BLAC, as shown in Table 3.

*Table 3.* Diameter of bacterial inhibition by extracts in different concentration.

| | *Spondias mombin* | | |
|---|---|---|---|
| | 50 µL | 100 µL | 200 µL |
| *Escherichia coli* ATCC 25923 | - | - | - |
| *Staphylococcus aureus* ATCC 29213 | - | - | 01 mm |
| *Staphylococcus aureus* BLAC | - | - | 01 mm |
| *Klebsiella pneumoniae* ATCC 700603 | - | - | - |

Subtitles: Antimicrobial activity of the studied extract in volumes of 50 µL, 100 µL e 200 µL in *Escherichia coli* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* BLAC and *Klebsiella pneumoniae* ATCC 700603.

## DISCUSSION

Results of this study suggest that *S. mombin* extract did not interfere with the cellular metabolic activity in the animals investigated. Therefore, the application of *S. mombin* extract in animal cells did not show any change, being considered non-toxic to the cellular conditions and consequently to animal organs, since the body homeostasis of rats and their physiological process remained stable.

In addition to the assessment of cytotoxicity, this study suggests that an hyperlipidemic diet induces obesity. During the 12 weeks of the experiment, the group with hyperlipidemic diet (HL group) showed the greatest weight gain compared to other groups so, contributing to various chronic diseases associated with obesity, such as hypertension and diabetes (Lima et al., 2013). The current data show that administration of *S. mombin* extract may contribute to lower the carbohydrates and lipids absorption, since the mass gain indexes were lower in HL+EX group (Pereira et al., 2015; Assini et al., 2013).

However, the potential role of *S. mombin* extract as a weight gain and blood glucose reducer needs to be further evaluated. This statement is valid when differences in weight and blood glucose reduction between CT and CT+EX group were analyzed. The administration of *S. mombin* extract in the diet of Wistar rats plus a control diet did not show significant difference in mass gain and blood glucose reduction, requiring further studies to corroborate if the mass-reducing and glycemic-reducing are effects of *S. mombin* extract. This absence in difference of glycemic and weight measurement may corroborate an evidence that *S. mombin* extract did not contribute to alteration in rats' metabolism, as well as all other biochemical parameters evaluated.

*S. mombin* is composed of flavonoids, carotenoids, phenolic compounds, and ß-cryptoxanthin (Canuto et al., 2010). Among these, flavonoids, which are polyphenolic molecules, have been listed in several studies because their consumption can prevent some diseases such as some types of cancer, type 2 diabetes, neurodegenerative disorders, osteoporosis, and decrease lipid levels. This could contribute to reduce the overweight and obesity index, as evaluated in our study, since the mass gain index of the HL+EX group was lower than in other groups (Weng et al., 2012; Wedick et al., 2012; Hwang et al., 2012; Hardcastle et al., 2011; Hooper et al., 2008; Cassidy et al., 2012; Pereira et al., 2015; Assini et al., 2013).

In addition to reducing lipid indices, supplementation with *S. mombin* contributes to the cardiovascular system reducing the fibrosis process, myocardial hypertrophy and acting in oxidative stress cascade and inflammatory processes(Pereira et al., 2020; Sameh et al., 2018).

Studies with leaves aqueous extract of *S. mombin*, in different protocols and doses, demonstrated that the extract did not induce signs of toxicity in rats, in terms of biochemical and behavioral, histological, and hematological

aspects (Silva, 2015b). This fact agrees with results showed in our experiment, demonstrating that the extract, in addition to not alter markers such as kidney and liver function, is not cytotoxic and have antimicrobial activity.

*S. mombin* extract inhibited *S. aureus* ATCC 29213 and *S. aureus* BLACC growth. The inhibition diameter in both assays was 1 mm at concentrations of 200 µL, thus confirming the antimicrobial activity shown by previous studies and other experiments (Ayoka et al., 2005; Corthout et al., 1994; Maduka et al., 2014; Temitope et al., 2017). However, the antimicrobial effect of *S. mombin* extract did not show bacterial inhibition at concentrations of 50 µL and 100 µL for the assays of the respective bacteria. Furthermore, no inhibitory diameter was observed against *E. coli* ATCC 25923 and *K. pneumoniae* ATCC 700603 in any of the concentrations performed.

In conclusion, the results obtained are promising as they suggest that *S. mombin* extract does not interfere with cell viability and did not show cytotoxicity to the cells to which they were exposed, not interfering with the body homeostasis of the rats in the experiment. Furthermore, the absence of changes in glycemic indices and mineral ions, in the lipid profile and liver parameters corroborate the data indicating the lack of physiological change in rats when subjected to administration of *S. mombin* extract. Further evaluations of this extract including larger numbers of MDR *S. aureus* isolates, as well as other microorganisms, will be necessary. Overall, the results of this present study may serve as a basis for further assessment of the potential of *S. mombin* extract as a source of new alternative antimicrobial drug, as well as its possible beneficial activity to the cardiovascular system.

## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest do disclose.

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