

Antimicrobial and Antidiarrheal Activities of Methanolic Fruit Peel Extract of *Pouteria caimito*

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ABSTRACT

Introduction: *Pouteria caimito* is a native Amazonian fruit specie, popularly known as "abiu", that belongs to the Sapotaceae family. It has a wide popular use to relieve diarrhea, cough, fever and inflammation, among other purposes. However, few reports in the scientific literature deal with its phytochemical composition and biological activity. This study aimed to evaluate the antidiarrheal and antimicrobial activity of *Pouteria caimito* fruit peel extract.

Methods: The antidiarrheal activity was evaluated in mice, using modulation models of normal defecation, cathartic agent-induced diarrhea and stimulated intestinal transit. Antimicrobial activity was, in turn, analyzed against strains of gram-positive and gram-negative bacteria (*E. coli*, *S. epidermidis*, *S. aureus*, *Klebsiella pneumoniae*, *E. faecalis* and *Salmonella tify*) through the determination of the minimum inhibitory concentration (MIC) by the microdilution in broth method. **Results:** This study showed that there was no antidiarrheal activity in the experimental models analyzed and that the methanolic extract of *P. caimito* (MEPC) can positively modulate normal defecation. Regarding the antimicrobial activity, the crude extract and the ethyl acetate fraction presented a satisfactory action against most of the strains evaluated except for *Enterococcus faecalis* and *Salmonella tify*. **Conclusions:** The MEPC did not present any measurable antidiarrheal activity, but more possibly a laxative effect. The extract and acetate fraction showed antimicrobial activity against strains of gram-positive and gram-negative bacteria. The activity of the crude extract was more potent than the results obtained by the use of acetate fractions; the fractions with lower polarity did not present any relevant antimicrobial activity.

Key words: Abiu, Antidiarrheal activity, Antimicrobial activity, Sapotaceae.

INTRODUCTION

Numerous plants used in folk medicine as "antidiarrheal" contain chemical substances that aid in the control of diarrhea associated with increased secretion and intestinal motility due to their astringent, antimicrobial or antiparasitic actions.¹⁻³ Inhibition of bacterial growth by a plant extract may be related to the action of secondary metabolites or a group of active compounds, the phytocomplex, present in the plant, which may be responsible for the biological effects of a medicinal plant.⁴

The genus *Pouteria* (Sapotaceae family) has as its main constituents the triterpenoids and flavonoids⁵, and such compounds have proven anti-inflammatory, antiviral, analgesic, cardiovascular, antitumor and anti-HIV⁶ activity, and are also active against gram-positive and gram-negative bacteria, and fungi.⁷ Studies with fruit residues have been growing, demonstrating that nutrients such as phenolic compounds and flavonoids are mainly concentrated in fruit peels and seeds.⁸

The *P. caimito* species can be found throughout the Amazon, cultivated in farms, orchards and backyards, as well as part of the urban afforestation of some regions in the north of Brazil.⁹ The presence of lupeol, α -amirin, erythrodiol and Dammarendiol II was revealed in the phytochemical investigation

of the fruit benzene extract, and from their peels were isolated: taraxerol, taraxene-3-one and β -sitosterol.¹⁰ For the species are attributed several therapeutic uses, evidenced in ethnopharmacological surveys. In particular, its antidiarrheal and anti-dysentery activities, and against other pathologies whose genesis are related to the presence of microorganism.¹¹

Due to sanitary and population health issues, researches are continuously carried out with the aim of developing antimicrobials, agents that kills microorganisms or stops their growth, as the main objective to prevent or treat an infection, reducing or eliminating the pathogenic organisms.

Since biological activities for several species of the Sapotacea family and for the *Pouteria* genus have already been reported, it was important to investigate the fruit of the *P. caimito* species.

Despite its widespread ethnopharmacological use in the treatment of gastrointestinal disorders, there is no report in the scientific literature of studies validating the action of this *P. caimito* on gastrointestinal motility. The present work describes an investigation of the possible antidiarrheal and antimicrobial properties activities of the methanolic extract of *P. caimito* fruit peels, which is used empirically for this purpose in several areas of the Amapá state, Brazil. The present study shows the modifications produced by the crude extract of the species in the intestinal

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transit, both in normal conditions and in cathartic agent-induced diarrhea.

MATERIAL AND METHODS

Collection and botanical identification

The mature fruits of *P. caimito* species were collected in the Municipality of Santana, Amapá, Brazil (latitude: 0°3'9.24"S / longitude: 51°9'57.99" W), by the dusk, in the months of april and may, in a residential area, where other different species of plants were also cultivated. After, they were sent to the Animal Experimentation Laboratory (LEA) of the Federal University of Amapá (UNIFAP) and an exsiccata was deposited in the *Amapaense* Herbarium (HAMAB) for specimen identification, under the number 019138.

Preparation of crude extract

According to Brazilian Pharmacopoeia,¹² to obtain the methanolic extract of *P. caimito* (MEPC), the fruit peels (736 g) were dried in an oven (EL 1.4, Odontobrás, São Paulo, SP, Brazil) at 40°C (104°F) for 72 h and milled in a knife mill (031, Valinhos, São Paulo, Brazil). The powder obtained (544 g) was macerated in 3 L of methanol (CH₃OH), under stirring, every 24 h for 7 days. The resulting extractive solution was filtered and concentrated in a rotary evaporator (FST 801, Fisatom, São Paulo, Brazil) at a temperature of 40°C (104°F) until obtaining a pasty extract.¹³

Obtaining the fractions (partition: liquid-liquid)

From the crude extract, the liquid-liquid partition was performed, using a system of solvents in increasing order of polarity. Initially the crude extract was resuspended in methanol/water (4:6) solution, then the serial partition was performed with 200 mL of the following solvents: n-hexane (C₆H₁₄), chloroform (CHCl₃) and ethyl acetate (C₄H₈O₂), respectively. Subsequently, all fractions obtained were concentrated on a rotary evaporator and the final product was weighed for yield calculation. To calculate the yield, the following formula was used.¹⁴

$$\text{Yield} = \frac{\text{weight of the fraction (g)}}{\text{weight of extract (g)}} \times 100\%$$

Animals

Male Swiss albino mice (*Mus musculus*), from the Laboratory of Animal Experimentation of UNIFAP, weighing between 25 and 35 g, were used for antidiarrheal activity assays. The animals were kept under controlled temperature conditions (25°C/ 77°F) and a light-dark cycle of 12 h (6:00 a.m. - 6 p.m.), with free access to feed and water.

Evaluation of antidiarrheal activity

For each experimental protocol (modulation of normal defecation, cathartic agent-induced diarrhea and stimulated intestinal transit) five groups of five animals (n = 5) were used, to which were administered, respectively: positive control (loperamide 5 mg/kg), vehicle (5% Tween80) and MEPC at doses of 30 mg/kg, 100 mg/kg and 300 mg/kg, following the study proposed by Medeiros,¹⁶ with modifications.

Before each experiment, the animals were submitted to an 18 h fasting with free access to water. They were placed separately in appropriate cages, with metal grating at the bottom to avoid coprophagy, as well as the ingestion of wood shavings.¹⁵

Modulation of normal defecation in mice

After the fasting period, each group of animals received their respective treatments: positive control (loperamide 5 mg/kg), the control (vehicle: 5% Tween80) and MEPC at doses of 30 mg/kg, 100 mg/kg and 300

mg/kg. Subsequently, they were then placed in individual cages with filter paper at the bottom for feces visualization and after one hour of administration, the total number of feces was counted for each individual animal, every hour, up to four hours. The total number of feces excreted per group was registered and compared with the groups receiving the extract and the control groups.¹⁶

Cathartic agent-induced diarrhea in mice

Following the model of Izzo *et al.*¹⁷ and Galvez *et al.*¹⁵, with modifications, diarrhea was induced by oral administration of castor oil (0.3 mL/ animal). After 30 minutes the treatments were administered: loperamide (5 mg/kg), vehicle (Tween80® a 5%), MEPC 30 mg/kg, 100 mg/kg e 300 mg/kg. The animals were then placed in individual polyethylene cages, with filter paper in the bottom (changed every hour). The severity of diarrhea was evaluated for four hours on an arbitrary scale, and a score was assigned to each animal, taking into account the consistency of the feces present in each paper, as follows: 0 = no feces; 1 = normal feces; 2 = diarrheal (soft or aqueous feces). The number of normal and diarrheal feces were noted and compared to the respective control groups counts.

Stimulated intestinal transit in mice

The respective groups of animals were initially treated with the extract (MEPC 30, 100 and 300 mg/kg), loperamide (5 mg/kg) and the vehicle. After 60 minutes, castor oil (0.3 mL/animal, v.o) was administered and, after another 60 minutes, a suspension of activated charcoal (5.0%) in Agar (1.5%) was also administered (0.3 mL/animal, v.o) to the animals. They were euthanized 40 minutes after administration of the activated Agar-charcoal suspension, having their intestines dissected to verify the distance covered by the marker suspension. The total length of the small intestine and the length of the distance covered by the marker suspension were measured.¹⁸ The results were expressed as a percentage of the distance covered by the suspension in function of the total length of the small intestine, calculated by the formula below¹⁹:

$$\% \text{ intestinal transit} = \frac{\text{distance traveled by charcoal}}{\text{total small intestine length}} \times 100\%$$

Antimicrobial activity of the extract and fractions

Microorganisms and culture medium

Bacteria were obtained from the National Institutes of Health Quality Control (INCQS)/ Oswaldo Cruz Foundation (FIOCRUZ), three gram-positive strains: *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228) and *Enterococcus faecalis* (ATCC 9083); and three gram-negative strains: *Escherichia coli* (ATCC 8789), *Klebsiella pneumoniae* (ATCC 4382), *Salmonella typhi* (ATCC 259). They were kept at the Quality Control, Bacteriology and Microbiology Laboratory of UNIFAP, on nutrient agar. The culture medium used in the microbiological assays were Müller-Hinton Agar (Merck®), prepared and used according to the manufacturer's instructions.

Bacterial inoculum

The bacterial inoculum of the used strains was obtained from 24 h culture at 35°C (95 °F) and the suspensions prepared in test tubes (16 x 16 mm) containing 10 mL of sterile saline (NaCl 0.9%). Then, these suspensions were shaken for two minutes with the aid of Vortex apparatus, the turbidity being compared and adjusted to that presented by the 0.5 standard of McFarland scale, equivalent to 10⁸ CFU/mL^{20,21,22}. The bacterial suspensions were diluted 1:9 v/v in saline solution to obtain a final inoculum containing, approximately, 10³ CFU/mL as described by the Clinical and Laboratory Standards Institute.²³

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MIC and MBC assays were performed using the method of microdilution in broth, in the Mueller-Hinton broth, as described by the Clinical and Laboratory Standards Institute.²³

For the microdilution test, 100 µl of the nutrient broth and then 100 µl of the natural product (crude extract, hexane fraction, chloroform fraction and ethyl acetate fraction) were added to each of the 96 wells of the plate, at the initial concentration of 5 mg/mL. Serial dilution at the ratio of 2 was performed (5 mg/mL to 0.16 mg/mL) for the extract and fractions. After the dilutions, 100 µl of the inoculum suspensions were inoculated into all wells. The plate was incubated at 35°C (95°F) for 24h and cloraphenicol (50 µg/mL) was used as a positive control. Control of the culture medium and the negative control (vehicle) were also performed.

In order to determine the MIC of extract and fractions against the bacterial species tested, a solution of resazurin sodium in sterilized distilled water (0.01% w/v) was used. After the incubation period, 20 µL of this indicator solution was dispensed into each well, and then incubated again for 3h at 35°C (95 °F) in an oven. The interpretation of the result related to MIC was seen more securely, defined as the lower concentration of the natural product capable of inhibiting bacterial growth, evidenced by the blue color of the indicator used, where the change from blue to pink indicate reduction of resazurin by growth of bacterial strains.²⁴

To determine MBC, 10 µL of the contents of each well showing bacterial growth were seed and incubated on Mueller-Hinton agar at 37 °C (98.6 °F) for 24h. The MBC was defined as the lowest concentration of extracts or fractions that resulted in no growth or less than three colonies (99.9% death), as described by Quadros *et al.*²⁵

Statistical analysis

Results were expressed as mean ± standard error of the mean (s.e.m.) and analyzed using one-way variance analysis (one-way ANOVA / ANOVA) followed by the Tukey's test. The software used was GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, U.S.A.). The level of significance was set at $p < 0.05$.

Ethical and legal aspects

All the experimental protocols were duly appreciated by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Amapá (UNIFAP) receiving approval number 0017/2016.

RESULTS

Yield of crude extract and fractions

After concentration of the methanolic extract of the fruit peel *P. caimito* (MEPC), the hexane (FH), chloroform (FC) and ethyl acetate (FA) fractions, the percent yields were: 9.00%, 3.58, 1.74% and 0.80%, respectively.

Evaluation of MEPC on modulation of normal defecation

Regarding the effect of MEPC on modulation of normal defecation in mice, it was evidenced that, 4 h after the treatments, the increasing doses of the extract (30 mg/kg, 100 mg/kg, 300 mg/kg) induced a mean evacuation of 0.35 ± 0.18 , 2.7 ± 0.67 and 0.4 ± 0.08 , respectively. These effects were different in relation to those observed in the negative control group (vehicle) and positive control group (loperamide 5 mg/kg), both with mean evacuation of 0.01 ± 0.07 (Table 1).

The number of evacuations throughout the 4h after the treatment with MEPC at the dose of 100 mg/kg was significantly higher when compared to the other doses (300 mg/kg and 30 mg/kg) and to the vehicle. In all groups, except for the one which received extract at the dose of 100 mg/kg, it's observed that the values of evacuations reach zero at the end of 4 h. The positive control group (loperamide) had the results expected.

Evaluation of the effects of MEPC on cathartic agent-induced diarrhea in mice

The total evacuation means of the extract at doses of 30, 100 and 300 mg/kg were 1.4 ± 0.45 , 1.05 ± 0.32 and 0.65 ± 0.29 , respectively. Compared to vehicle group (1.65 ± 0.60), therefore, even though the mean evacuation were dose-proportionally inhibited, this effect is not statistically significant (Figure 1).

When compared to the vehicle group, the diarrheal evacuations of the extract groups also did not present significant difference between the means (Figure 1).

Effect of MEPC on stimulated intestinal transit in mice

Oral administration of the extract at the doses tested (30, 100 and 300 mg/kg) did not show a significant reduction of the distance covered by the marker suspension when compared to the vehicle group, suggesting that the extract did not interfere in the intestinal transit (Figure 2). The mean inhibition was $74,66\% \pm 8$, $72\% \pm 8,62$ e $77,30\% \pm 6,2$ for the MEPC (dose 30 mg/kg, 100 mg/kg and 300 mg/kg, respectively), demonstrating no significant difference between means.

Evaluation of the antimicrobial activity of the extract and fractions

With regard to antimicrobial activity, it can be observed in Table 2 that the crude extract was more effective against the strains of *S. epidermidis* and *E. coli*, with MIC of 0.6 mg/mL for both strains. The crude extract also presented relevant MIC values for the strains of *S. aureus* (1.2 mg/mL) and *K. pneumoniae* (1.2 mg/mL). Considering the fractions (hexane, chloroform and ethyl acetate), the ethyl acetate fraction presented better results, with MIC of 2.5 mg/mL for *S. aureus* and 1.2 mg/mL for *S. epidermidis*, *E. coli* and *K. pneumoniae*. The hexane and chloroform fractions presented MIC above 5 mg/mL for *S. aureus*, *S. epidermidis*, *E. coli* and *K. pneumoniae* strains. There was no antimicrobial activity against strains of *E. faecalis* and *S. tiphy*.

Table 1: Mean of 4-hour evacuations of MEPC-treated groups at 30, 100 and 300 mg/kg and control groups.

Treatment	1 ^a hour	2 ^a hour	3 ^a hour	4 ^a hour	Mean ± e.p.m of 4h
Loperamide	0.4 ± 0.24	0	0	0	0.01 ± 0.07
Vehicle	0	0.4 ± 0.24	0	0	0.01 ± 0.07
MEPC 30 mg/kg	1.4 ± 0.5	0	0	0	0.35 ± 0.18
MEPC 100 mg/kg	4.6 ± 1.54	4.6 ± 1.43	1 ± 0.55	0.6 ± 0.6	$2.7 \pm 0.67^*$
MEPC 300 mg/kg	1.2 ± 0.58	0.4 ± 0.25	0	0	0.4 ± 0.08

* $p < 0.05$ vs loperamide, vehicle, MEPC 30 mg/kg e 300 mg/kg

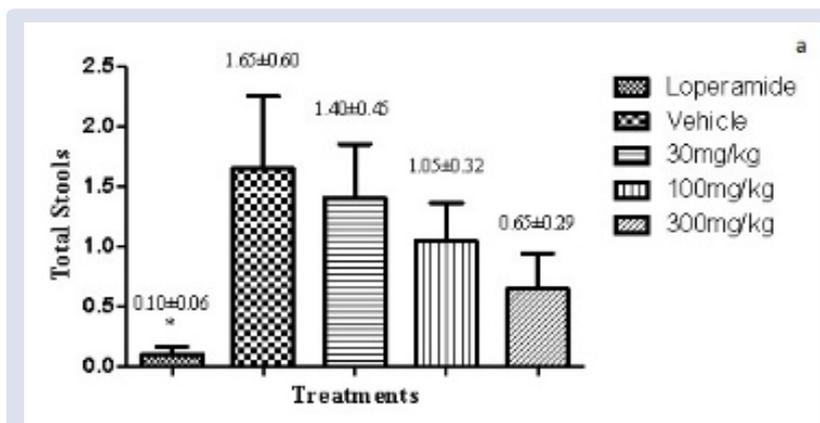


Figure 1: Effect of different doses of MEPC (30, 100 and 300 mg/kg), vehicle and loperamide on diarrhea induced by castor oil. In (A) total stools and (B) diarrheal stools.

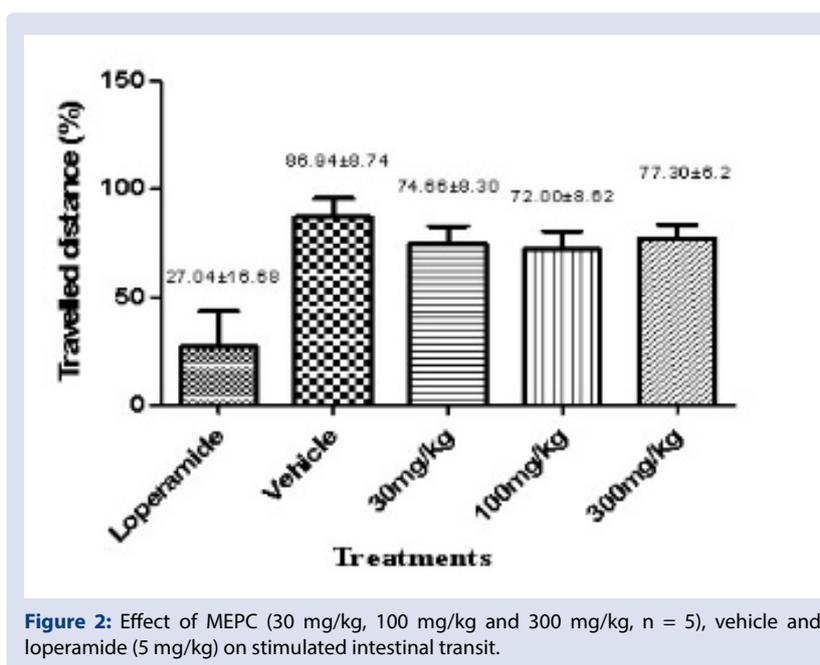


Figure 2: Effect of MEPC (30 mg/kg, 100 mg/kg and 300 mg/kg, n = 5), vehicle and loperamide (5 mg/kg) on stimulated intestinal transit.

Table 2: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *P. caimito* extract and fractions against gram positive and negative bacteria.

BACTERIA	CE		HF		AF		CF	
	CIM	CBM	CIM	CBM	CIM	CBM	CIM	CBM
<i>Staphylococcus aureus</i>	1.2	> 5	> 5	> 5	2.5	> 5	> 5	> 5
<i>Staphylococcus epidermidis</i>	0.6	5	5	> 5	1.2	2.5	5	5
<i>Enterococcus faecalis</i>	-	-	-	-	-	-	-	-
<i>Escherichia coli</i>	0.6	> 5	> 5	> 5	1.2	2.5	> 5	> 5
<i>Klebsiella pneumoniae</i>	1.2	> 5	> 5	> 5	1.2	1.2	5	> 5
<i>Salmonella tiphy</i>	-	-	-	-	-	-	-	-

CE= crude extract; HF = hexane fraction; AF = acetate fraction; CF = chloroform fraction

DISCUSSION

Many plant compounds, such as the triterpenes and flavonoids found in the genus *Pouteria*⁵, are responsible for proven biological activities, such as antimicrobial, fungicidal, anti-inflammatory and antioxidant.²⁶⁻²⁸ Plants used in folk medicine as antidiarrheal have in their composition chemicals with astringent, antimicrobial or antiparasitic actions, helping to control diarrhea, in addition to reducing intestinal transit and increasing water absorption.²⁹

Others researches, with similar methodology used in the present study, have demonstrated the potential antidiarrheal activity of plant extracts (*Psidium guajava*, *Stachytarpheta cayenensis*, *Polygonum punctatum*, *Eugenia uniflora*, *Aster squamatus*, *Byrsonima cinera* and *Cymbopogon citratus*)²⁹⁻³² and according to a study by Di Carlo *et al.*³³, plants with flavonoids present an inhibitory activity on the intestinal transit of mice and inhibit the water and sodium secretion stimulated by castor oil in rats. In addition, plants that contain tannins in their composition may have an antidiarrheal effect.²⁹

Considering its popular use and assuming that terpenes, phenols and flavonoids, reported for *Pouteria* genus e *P. caimito* species⁵, could contribute to a possible antidiarrheal activity of this plant, we investigated whether the extract of the fruit peels would have an influence on normal defecation, stimulated diarrhea and intestinal transit. However, the extract was not able to significantly inhibit these responses in mice, as demonstrated in the studies above. The MEPC, had, instead, a positive modulation effect on this parameter, but it was not possible to determine the reason in the present study.

The presence of certain phytochemicals constituents in plants can be influenced by some factors such as season, time of harvesting, handling and packaging of the plant, or degradation of constituents by environmental factors.³⁴ That may be one of the reasons why the studied species do not demonstrate the activity expected.

Assuming that diarrhea may be infectious, antimicrobial drugs could be used on the treatment, but as a result of the generalized resistance of these pathogens to medication, pharmacotherapeutic possibilities have become increasingly restricted.³⁵ Then, due to the presence of several studies with the *Pouteria* genus and aiming to investigate the antidiarrheal effect attributed to the species under study, we attempted to identify the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) against the bacterial species tested.

Others species of *Pouteria* genus showed action against bacterial strains, including those tested in this study. The species *P. venosa*³⁶, *P. torta*^{37,38}, *P. pallida* and *P. gardnerii*^{39,40} showed activity against *S. aureus*. The *P. venosa*³⁶ has an effect on *S. epidermidis*, and *P. torta*³⁷, *P. ramiflora*, *P. glomerata*, *P. psamophila* and *P. grandiflora*⁴¹ had antimicrobial action for *E. coli*. A recent study showed that strains of *Klebsiella pneumoniae* were the target of the inhibitory action of the *P. caimito* leaf extract.⁴² There were no studies, to our knowledge, demonstrating the action of the genus on *Enterococcus faecalis* and *Salmonella tiphy*.

In the present study, both the crude extract and the ethyl acetate fraction showed action against gram-positive bacteria (*S. aureus* and *S. epidermidis*), and gram-negative (*E. coli* and *K. pneumoniae*), being the activity of the crude extract more potent. However, there was no action of MEPC and fractions against strains of *Enterococcus faecalis* and *Salmonella tiphy*, such data corroborating with the literature data for the genus. According to Fabry et al.⁴³, for an extract to be considered as potentially useful in therapeutics, the MIC value should be lower than 8 mg/mL and in the evaluation of the antimicrobial activity of *P. caimito*, as the results were better for the crude extract and then for ethyl acetate fraction, the results obtained in this study suggest that the active compounds have more polar characteristics, since the hexane and chloroform fractions were not efficient. Moreover, the antimicrobial effect may be due to the synergism of substances that, when fractionated, tend to decrease efficacy.

CONCLUSIONS

In view of the obtained results, we can conclude that the methanolic extract of *P. caimito* (MEPC), at the doses tested, did not present direct antidiarrheal activity on the gastrointestinal tract, either altering the normal defecation frequency, inhibiting induced diarrhea or stimulated intestinal transit. However, MEPC and the acetate fraction showed antimicrobial activity against strains of Gram-positive and Gram-negative bacteria. The activity of the extract was the most potent and the fractions with less polarity did not present any relevant activity.

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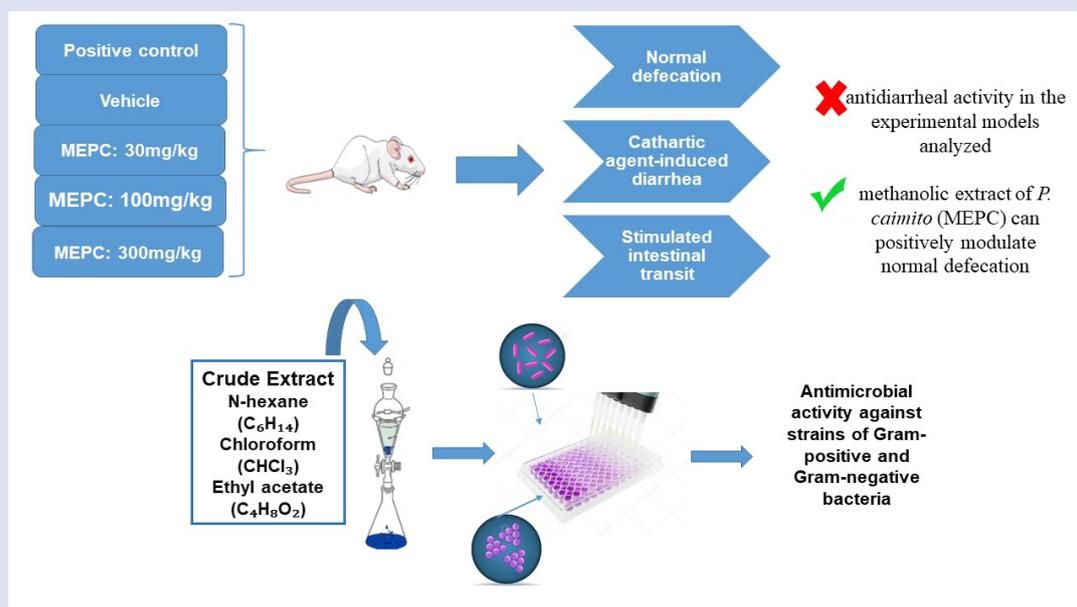
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GRAPHICAL ABSTRACT



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