Phytochemical Profile and Investigation of the Spasmolytic Activity of Hydroalcoholic Extract of Syzygium cumini (L.) Skeels Seeds

F. S. Monteiro¹*, A. F. S. Carvalho², R. M. Ribeiro¹, A. C. R. Borges¹ and M. O. R. Borges¹

¹Federal University of Maranhão (UFMA), São Luis, MA, Brazil.
²Clinical Hospital of Federal University of Minas Gerais (HU-UFGM), Belo Horizonte, MG, Brazil.

Authors’ contributions

This work was carried out in collaboration among all authors. Authors FSM and AFSC performed the experiments and wrote the first draft of the manuscript. Authors RMR, ACRB and MORB managed the study analyzes and bibliographic research. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Perform the phytochemical analysis and investigate the spasmolytic activity of the hydroalcoholic extract obtained from S. cumini seeds (EHS-SC).

Study Design: Qualitative phytochemical analysis and test of the EHS-SC on isolated smooth muscles (aorta, trachea, jejunum and uterus) of rat, to value effect relaxant and/or inhibitor.

Place and Duration of Study: Pharmacognosy Laboratory II (Pharmacy course) and Pharmacology Research and Post-Graduate Laboratory (Department of Physiological Sciences) of the Federal University of Maranhão, between January 2017 and July 2018.

Methodology: EHS-SC was submitted to phytochemical analysis and changes in color, fluorescence and absence or presence of precipitate were observed. The smooth muscle segments were suspended (tension of 1 g) in glass vats containing specific saline solution, at an appropriate temperature and after stabilization period, was stimulated by a suitable contractile agent to observe the effect of EHS-SC in the phasic and/or tonic component.
**Results:** EHS-SC showed the majority presence of phenols, steroids, alkaloids and flavonoids (flavones, xanthones, flavonols) and was more potent in inhibiting phasic contractions induced by $10^{-6}$ M carbachol (CCh) in isolated rat jejunum ($E_{\text{max}}$: 83.5 ± 6.7%; $n = 3$). In addition, the EHS-SC (81.0; 243.0 and 729 µg/mL) antagonized the CCh effect ($n = 4$), increasing the $EC_{50}$ (6.5 ± 1.3 x $10^{-7}$ M) of the CCh to 8.5 ± 1.4; 18.5 ± 3.4 and 40.5 ± 7.4 $x$ $10^{-7}$ M and reducing the $E_{\text{max}}$ (100%) of the CCh to 82.9 ± 10.5; 67.6 ± 6.0 and 10.1 ± 8.3%.

**Conclusion:** Spasmolytic activity may be combined with antimicrobial and antidiarrheal activity according to literature data, where they show that the seeds have the same secondary metabolites, signaling the therapeutic potential for the treatment of colic and/or diarrhea.

Keywords: *Syzygium cumini*; phytochemistry; spasmolytic activity; Jamun; seeds.

1. **INTRODUCTION**

*Syzygium cumini* (L.) Skeels (Synonym: *Eugenia jambolana* Lam.), belonging to the family Myrtaceae and to the genus *Syzygium* [1], is a tree introduced in Brazil [2], popularly known as jamun, being found in several states in the Southeast, Northeast and North regions, where the population makes use of the various parts of this plant for different purposes [3].

For example, the seeds of jamun are often used in folk medicine to treat Diabetes Mellitus, blisters in the mouth, cramps, diarrhea, digestive complaints, dysentery and stomach pain [4]. These medicinal properties can be explained by the presence of secondary metabolites produced as a defense system for the plant itself, but of great medicinal interest for humanity [5].

Some secondary metabolites of *S. cumini* seeds are described in the literature such as alkaloids (jambosine) and glycosides (jambolin or antimellin) [6]; tannins and phenols (ellagic, gallic, caffeic and ferulic acids); monoterpenes and flavonoids (rutin, quercetin, and β-sitosterol) [7,8]; saponins, triterpenes [9], among others.

To justify the use in popular medicine, the seeds of *S. cumini* have been studied in pre-clinical trials, presenting activities such as antibacterial [10], anti-HIV [11], antioxidant and antilipemic [12,13], radio and chemoprotective [14,15], central nervous system depressant [16], antibiotics [17], anti-inflammatory [18], anti-ulcer [19], antianemic [20] and antidiarrheal [21,22].

In addition to these, spasmolytic activity and the probable mechanism of action in smooth muscle, have been investigated with extracts from the seeds, fruits and leaves of the species *S. cumini* [23-27]. In this context, this work aimed to continue the investigation of spasmolytic activity, presenting unpublished results of the effect of the hydroalcoholic extract obtained from the seeds of *S. cumini* on segments of the smooth muscle (uterus, jejunum, aorta and trachea) isolated of rat, as well as hypothesis of a possible spasmolytic mechanism of action.

2. **MATERIALS AND METHODS**

2.1 **Materials**

2.1.1 **Botanical material**

*Syzygium cumini* seeds were collected in the Federal University of Maranhão (UFMA), University City Dom Delgado, municipality de São Luís, Maranhão - Brazil (2°33'09.8 "S; 44°18'20.5" W), in December 2017. A sample of the plant was sent to the Herbarium Ático Seabra of the Faculty of Pharmacy of the UFMA whose exsiccate has the identification number 1485.

2.1.2 **Animals**

Were used rats of the species *Rattus norvegicus*, Wistar lineage, adults, male and female, healthy at the clinical examination, approximately 80 days old, provided by Central Biotery of the UFMA. The animals were kept in polyethylene cages, lined with xylan, with food and water ad libitum and under a 12-hour light / dark cycle, at a temperature of 22°C.

2.1.3 **Devices and saline solutions**

Force transducers (Model nº: MLTF0202) for measuring tension (0.0 and 25.0 g) coupled to an amplifier (ADInstruments Powerlab, Australia) which in turn was connected to a computer. Kymograph with soot-covered cylinder to record isotonic contractions using a lever with frontal inscription. (DTF, Brazil).
According to the experiments carried out, solutions were used Locke Ringer (maintained at 32°C for rat uterus), Tyrode Normal (maintained at 37°C for jejunum), both aerated with oxygen; and Normal Krebs (maintained at 37°C for trachea and aorta), aerated with a carbogenic mixture. The compositions are described below respectively: Normal Krebs Solution (mM): NaCl (118.0); KCl (4.6); KH₂PO₄ (1.1); MgSO₄ (5.7); CaCl₂ (2.5); NaHCO₃ (25.0); Glucose (11.0) [28]. Locke Ringer's solution (mM): NaCl (154.0); KCl (5.6); CaCl₂ (2.16); MgCl₂ (2.1); NaHCO₃ (5.95); Glucose (5.55) [29]. Normal Tyrode's Solution (mM): NaCl (135.0); KCl (5.0); CaCl₂ (2.0); MgCl₂ (1.0); NaHCO₃ (15.0); Na₂HPO₄ (1.0); Glucose (11.1) [30].

2.2 Methods

2.2.1 Preparation of crude extract

After collection, the fruits were peeled and the seeds were dried at room temperature and, subsequently, pulverized in a mill. The respective powder (920 mg) was subjected to an extraction process (maceration), for 8 days, with 70% ethanol, in the proportion of 1:9 w/v, and filtered. From the filtered solution, extract was concentrated at a temperature of 50°C and with the aid of the rotary evaporator, in order to remove all the solvent. Soon after, the concentrate was subjected to the lyophilization process [31]. The product (150 mg) obtained received the name "hydroalcoholic extract of the seeds of S. cumini (EHS-SC)" with a yield of 16.3% and was solubilized in distilled water to a concentration of 10 mg/mL (stock solution), preserved at 0°C and diluted in distilled water according to the need for each experimental protocol on the day of the experiment. EHS-SC concentrations were used in multiples of three, with the maximum concentration being 729 µg/mL since it is the maximum concentration used in experiments with isolated organs [32]. When in this concentration the observed effect was greater than 50%, the concentration that will give 0% effect was sought.

2.2.2 Qualitative phytochemical analysis

The lyophilized form EHS-SC was used to perform the phytochemical analysis [33]. To investigate the presence of phenols and tannins, a test tube was used, where 3-4 mL of the hydroalcoholic extract was placed; 3 drops of alcoholic ferric chloride solution were added; any color variation or precipitate formation was observed; the color change without precipitation will be positive for phenols; the development of dark blue precipitate will indicate the presence of hydrolyzable tannins and the formation of dark green precipitate will be positive for condensed tannins.

Regarding the flavonoids, there were 3 test tubes numbered 1 to 3 with 4 mL of extract in each; tube 1 was acidified with an acid solution (4 drops of 1N HCl) at pH 3, tube 2 was alkalized to pH 8.5 (20 drops of base) and tube 3 was alkalized to pH 11 (24 drops of base), any color change was observed. To coumarins were investigated with the aid of a capillary tube; two strong stains approximately 1.5 cm in diameter were made on a piece of non-fluorescent filter paper; a drop of 1N potassium hydroxide (KOH) alcoholic solution was applied to one of the spots and about these were covered with an opaque non-fluorescent card and exposed to the action of UV light for about 2-3 minutes; the development of progressive and strong greenish fluorescence, clearly visible in the unobstructed half of the alkaline spot, will indicate the presence of coumarins.

For the presence of alkaloids, the dry residue of the extract was dissolved with 20 mL of 0.1 N sulfuric acid (H₂SO₄) and subsequently heated for 2 minutes; after filtering and cooling the filtrate, it was divided into three test tubes; soon after, the specific reagents of Mayer, Hager, and Dragendorff were dripped; the formation or not of precipitate was observed. In relation to triterpenes and steroids, the dry residue was extracted with 10 mL of chloroform, taking care to crush the residue well with the solvent; the chloroform solution was filtered, drop by drop, into a closed funnel with cotton covered with a few decigrams of anhydrous sodium sulfate, into a clean and dry test tube; 1 mL of acetic anhydride was added, and it was gently stirred, adding three drops of concentrated sulfuric acid; it was stirred again and color development was observed; the blue color followed by permanent green is positive for free steroids; brown to red color indicates the presence of free triterpenes.

In the saponin test, the dry residue insoluble in chloroform from the previous test was dissolved in 10 mL of distilled water and the solution was filtered in a test tube; the tube with the solution was shaken for 3 minutes, in order to observe the formation or not of foam; persistent and abundant foam (collar) indicates the presence of saponins.
2.2.3 Preliminary pharmacological screening

2.2.3.1 Initial procedure

All rats were euthanized with CO₂ gas following the principles of laboratory animal care based on the guidelines of the bioethics committee. The uterus was isolated from rats 24 h before treated with diethylstilbestrol (1 mg/kg) subcutaneously, to induce estrus. The jejunum, trachea, and aorta were isolated from rats that remained fasting for 18 h, with water at will. The isolated tissues were cleaned, under a Petri dish containing adequate nutrient solution and aerated with oxygen. After removing the fat, each tissue was sectioned (1.5 cm from the jejunum and uterus; 4 mm from the aorta and trachea) and suspended (tension of 1 g) in glass vats (05 or 10 mL) containing physiological solution, at an appropriate temperature. The uterus remained 45 min under stabilization and the other tissues remained 1 h, with intervals of 15 min of washing with nutrient solution to avoid the interference of metabolites [34].

2.2.3.2 Effect of EHS-SC from the phasic component of smooth muscle

After the initial procedures, as described in item 2.2.3.1, the segments of visceral tissues (uterus and jejunum) were tensioned (1 g) through a frontal registration lever in a smoked cylinder of a kymograph, in the evaluation of the phasic component of the contraction. After the stabilization period, two curves of similar amplitudes were induced by oxytocin (10⁻² IU/mL) or carbachol (CCh) 10⁻⁵ M in rat uterus, and CCh 10⁻⁶ M in rat jejunum. In the presence of different concentrations of the EHS-SC extract, a third contraction was induced to assess the inhibitory effect. The maximum effect inhibitory (Eₘₐₓ) was expressed with mean ± S.E.M. [35].

2.2.3.3 Effect of EHS-SC on the tonic component of smooth muscle

After the initial procedures, as described in item 2.2.3.1, to evaluate the tonic component of the contraction, isometric force transducers were used to measure tension. The tissues of rat uterus and rat jejunum were contracted by high potassium (60 and 75 mM, respectively); the isolated rat aorta was contracted by phenylephrine (PHE) 10⁻⁶ M and the isolated rat trachea was contracted by CCh 10⁻⁵ M; and when a stable contraction was attained (15-20 min), EHS-SC (243 and 729 µg/mL) was cumulatively added in an attempt to obtain dose-relaxation curves. The relaxant effect induced by EHS-SC was expressed as the reverse percentage of the initial contraction force elicited by the contracting agent and maximum effect relaxant (Eₘₐₓ) expressed with mean ± S.E.M. [36].

2.2.4 Investigation of the possible spasmolytic mechanism of action of the EHS-SC

The investigation of the mechanism of action was carried out by observing the effect of the EHS-SC on the two similar concentration curves - cumulative responses with the use of CCh (10⁻⁹ up until 3 x 10⁻⁵ M) in rat jejunum; then, in the absence of CCh, the EHS-SC (81, 243 and 729 µg/mL) was incubated for 15 min in different tests and preparations. After that time, a new cumulative CCh response curve was displayed in the presence of the EHS-SC. The type of antagonism was evaluated by comparing it with the associated powers and the maximum precision of the CCh curves in the absence (control) and in the presence of the EHS-SC [37].

2.2.5 Statistical analysis

All the results obtained were expressed as the percentage of the mean ± standard error of the mean (S.E.M.) and analyzed statistically using the “t” test or analysis of variance (ANOVA) “one-way” followed by the Bonferroni test, where P values less than 0.05 were considered significant. The values of EC₅₀ (concentration of a substance that produces 50% of its maximum effect) and Eₘₐₓ (maximum effect) were calculated by non-linear regression for all experiments performed [38]. All data were analyzed using the Graphpad Prism® program version 5.01 (Graphpad Software Inc., San Diego CA, USA).

3. RESULTS

3.1 Qualitative Phytochemical Analysis

The result of the phytochemical analysis carried out with the EHS-SC is described in Table 1. It was possible to observe the majority presence of phenols, steroids, alkaloids, and flavonoids (flavones, xanthones, flavonols).
3.2 Preliminary Pharmacological Screening

3.2.1 Effect of EHS-SC front the phasic component of smooth muscle

EHS-SC inhibit phasic contractions induced by oxytocin $10^{-2}$ IU/mL ($E_{\text{max}} = 9.6 \pm 1.3\%$) or CCh $10^{-5}$ M ($E_{\text{max}} = 12.9 \pm 2.1\%$) equipotent way in rat uterus (Fig. 1A and B, n = 3); on the other hand, was more potent in isolated rat jejunum contracted by CCh $10^{-6}$ M ($E_{\text{max}} = 83.5 \pm 6.7\%; n = 3$) (Fig. 2).

3.2.2 Effect of EHS-SC on the tonic component of smooth muscle

EHS-SC had no relaxing effect on isolated rat uterus pre-contracted by KCl 60 mM, rat trachea pre-contracted by CCh $10^{-5}$ M or jejunum pre-contracted by phenylephrine $10^{-6}$ M ($E_{\text{max}} = 19.5 \pm 6.9\%$) (Figs. 3, 4, 5 and 6, n = 3).

3.3 Investigation of the Spasmyolytic Mechanism of Action of the EHS-SC

EHS-SC (81, 243 and 729 µg/mL) antagonized the cumulative concentration-response curves to CCh ($10^{-9}$ up until $3 \times 10^{-5}$ M), shifting to the right, in parallel with an increase in the $EC_{50}$ ($6.5 \pm 1.3 \times 10^{-7}$ M) of the CCh to $8.5 \pm 1.1; 18.5 \pm 3.4$ and $40.5 \pm 7.4 \times 10^{-7}$ M and reducing the $E_{\text{max}}$ (100%) of the CCh to $82.9 \pm 10.5; 67.6 \pm 6.0$ and $10.1 \pm 8.3\%$ (Fig. 7).

4. DISCUSSION

The study of phytochemical compounds is justified by the fact that secondary metabolites may undergo qualitative or quantitative variations influenced by the main factors: hereditary (genetic composition), ontogenetic (stage of development) and environmental [39]; in addition to requiring compliance with the ideal conditions for culture, harvesting, drying, stabilization, manufacturing, conservation and storage [40].

<table>
<thead>
<tr>
<th>Secondary metabolites</th>
<th>EHS-SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenols</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
</tr>
<tr>
<td>Coumarins</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>+++</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+++</td>
</tr>
</tbody>
</table>

**Flavonoids:**
- Anthocyanidins/Anthocyanins -
- Flavones, xanthones, flavonols +++

'+++ = present in high quantity, '-' = absent

Fig. 1A and B. Effect of EHS-SC against phasic contractions induced by oxytocin (A) $10^{-2}$ IU/mL or CCh (B) $10^{-6}$ M in isolated rat uterus

The vertical columns and bars represent the mean and standard error of the mean of three experiments, respectively. One-way ANOVA followed by Bonferroni’s post-test, **$p < 0.01$, ***$p < 0.001$ (control vs. EHS-SC)
Fig. 2. Effect of EHS-SC against phasic contractions induced by CCh \(10^{-6}\) M in isolated rat jejunum

The vertical columns and bars represent the mean and standard error of the mean of three experiments, respectively. One-way ANOVA followed by Bonferroni's post-test, *** p <0.001 (control vs. EHS-SC).

Fig. 3. Original representative record of one of the three experiments observed with the EHS-SC on tonic contraction induced by KCl (60 mM) in isolated rat uterus

The upward arrow represents the addition of PHE (phenylephrine). The down arrows represent the concentrations of the EHS-SC (243-729 µg/mL, respectively); g (grams of tension); min (minutes); W = wash.

Fig. 4. Original representative record of one of the three experiments observed with the EHS-SC on tonic contraction induced by CCh (10^{-5} M) in isolated rat trachea

The upward arrow represents the addition of CCh (carbachol). The down arrows represent the concentrations of the EHS-SC (243-729 µg/mL, respectively); g (grams of tension); min (minutes); W = wash.
In this study, it is possible to prove the presence of the same secondary metabolites found in phytochemical studies carried out with S. cumini seeds by other authors. [9,41-44]. Although the role of secondary metabolites is the defense of the plant, they are extremely important for human health [45,5], for example, polyphenols can play an important role in preventing gastrointestinal disorders [46]; also, many alkaloids have been shown to have antidiarrheal or antihypertensive effects [47].
To investigate whether the secondary metabolites present in the EHS-SC extract have any pharmacological activity, a preliminary screening was carried out to assess the presence of spasmolytic activity and a possible mechanism of action of EHS-SC in isolated smooth muscle tissues.

The isolated tissues are easy to handle preparations and the presence or absence of the spasmolytic effect can be fully assessed. Smooth muscle is present in several hollow organs in the body systems of animals or humans controlling various physiological processes [48], such as basal tone of the arteries and trachea, labor or postpartum, intestinal peristalsis, among others, whose deregulations are implicated in diseases such as hypertension, asthma, preterm birth, uterine colic, dysentery and intestinal colic [49].

The contraction in intestinal and uterine smooth muscle in response to various agents is often composed of two phases: a phasic, fast and unsustainable component, followed by a tonic, slow and sustained component. The mechanism responsible for the phasic component is related to the activation of a metabotropic receptor coupled to protein G. On the other hand, smooth vascular muscles and tracheas are tonics and therefore do not have a phasic component characteristic of rhythmic muscles [50,51].

Through preliminary pharmacological screening, a selective and more efficient action of EHS-SC is observed in the isolated rat jejunum (Fig. 2), which directed the research to investigate the mechanism of action in this muscle tissue. In the literature, it is observed that scopolamine, as well as atropine, have spasmolytic activity because they competitively inhibit muscarinic metabotropic M3 type receptors [52].

In isolated rat jejunum the M3 type metabotropic receptors are principally responsible for the phase component of the contraction. Then the following came up question: would the EHS-SC be acting out of competitive antagonism as well such as scopolamine and atropine in isolated rat jejunum? To answer this question, we investigated the type of antagonism by which the EHS-SC acts. The result shows, at the functional level, non-competitive antagonism (Fig. 7).

The non-competitive antagonism can be explained by the blocking of channels of calcium-dependent voltage (CaV) or by activating the potassium channels, which are present in the plasma membrane of intestinal smooth muscle [53]. The activation of the CaV is responsible for the sustained tonic component of the contraction, while the regulation of the contractile process is done through the activation of the potassium channels. The evaluation of the participation of the CaV or the potassium channel in the relaxing action mechanism of the EHS-SC can be done by analyzing Figs. 2, 3, 5 and 7, where, it can be seen that the EHS-SC did not significantly relax the rat uterus and the rat jejunum when pre-contracted by high concentrations of extracellular potassium (electromechanical coupling), but inhibited when contracted by CCh (pharmacomechanical coupling) [54].

As the main mechanism by which KCl induces contraction is the opening of the CaV by depolarization of the membrane [55], the CaV block hypothesis is ruled out, corroborating the hypothesis of the participation of K+ channels in the mechanism of action of EHS-SC in rat jejunum.

Moreover, Archana et al. [56] showed that the ethanol extract of S.cumini seeds relaxed 55% of the contraction induced by low concentrations of potassium chloride (22.36 mM), suggesting activation of potassium channels [56]. Besides that, Mehmood et al. [57] with the methanolic extract of Matricaria chamomilla L., and this mechanism was associated with antidiarrheal, antisecretory and antispasmodic activity [57]. In addition, Syzygium cumini seeds are used as a therapy for the treatment of diarrhea [58]; and Shamkuwar; Pawar; Chauhan [22] showed that the aqueous extract of Syzygium cumini seeds has antidiarrheal activity in vivo [22].

Diarrhea is a major public health problem worldwide, being one of the major causes of infant morbidity and mortality, representing the second leading cause of death in children under five years of age [59]. About a third of children living in developing countries, where unfavorable hygienic-sanitary conditions facilitate the spread of pathogens, are affected by enteric infections with or without diarrheal events [60].

5. CONCLUSION

In this study, the presence of the same secondary metabolites, found according to other studies carried out with the seeds of S. cumini, guarantees the quality of the botanical material. In addition, spasmylactic activity is selective for isolated rat jejunum, conferring an action to the intestinal system. Besides that, the antimicrobial
Effect shown against enteropathogenic bacteria [61], as well as the antidiarrheal activity in vivo [22], corroborates the hypothesis that EHS-SC could be a spasmyloytic potential with antidiarrheal and antimicrobial action and probably acts by activating potassium channels in rat jejunum. Further studies are needed to confirm this hypothesis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experiments have been approved by the ethics committee on the use of animals (CEUA) at UFMA (process No. 3584 / 2014-97).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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