



# *In vivo* Hemostatic Activity of *Jatropha mollissima*: A Triple-Blinded, Randomized, Controlled Trial in an Animal Model

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## Abstract

**Objective** The objective of this study was to evaluate the hemostatic activity of the sap from *Jatropha mollissima* (Pohl) Baill. in rats.

**Materials and Methods** Twenty-four Wistar rats were randomized into four groups ( $n = 6$ ): the JM25 and JM40 groups were treated with ethanolic extract from the sap of *J. mollissima*, in a concentration of 25 and 40 mg·mL<sup>-1</sup>, respectively; the MO group was treated with Monsel's solution and the control group SC with a 0.9% sodium chloride solution.

**Statistical Analysis** Data were submitted to the Kurskal–Wallis' test, followed by Dunn's post hoc ( $p < 0.05$ ).

**Results** There was a significant reduction in the bleeding time of the group from the JM25 extract ( $p = 0.001$ ) when compared with MO and SC. There were no statistically significant differences between groups JM25 and JM40 ( $p > 0.05$ ). The JM25 group did not present rebleeding, a result significantly different from the MO group ( $p = 0.001$ ). Monsel's solution showed significant bleeding, six times greater than the control group SC.

**Conclusion** The *J. mollissima* extract, in the concentration of 25 mg·mL<sup>-1</sup>, showed the highest hemostatic efficiency and was found to be a promising biomaterial for the elaboration of a hemostatic product.

## Keywords

- hemostasis
- phytotherapy
- biomaterial

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## Introduction

Hemostasis is a natural process that takes place after a lesion, in which blood loss is hampered.<sup>1</sup>

The basic treatment for bleeding wounds consists the application of pressure using gauze, in an effort to stop the blood flow, which may also need the use of suture materials.<sup>2</sup> As a result, hemostasis will occur in minutes, with the exception of cases involving serious wounds or bleeding disorders, in which hemostatic materials can be used to induce or accelerate coagulation.<sup>1</sup> The hemostatic materials should be able to curb the bleeding rapidly, be biocompatible and if possible biodegradable.<sup>2</sup>

Biomaterials have contributed to the health research field, especially in association with phytotherapy,<sup>3</sup> employing different medicinal plants with known or unknown biological activities.<sup>4,5</sup> As a result, quality control to ascertain the safety of these phytotherapy is necessary to guarantee safety against toxicity.<sup>6</sup>

The specie *Jatropha mollissima* (Pohl) Baill., popularly known as “pinhão-bravo” is a plant that is present in the entire Brazilian semi-arid regions and part of the caatinga vegetation<sup>7,8</sup> with different medicinal applications.<sup>9,10</sup> It has antiophidic properties, acting against the effects caused by snake bites, such as the formation of edemas and local hemorrhage, and it possesses antibacterial activity against pathogens such as *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella typhimurium*, and *Listeria monocytogenes*.<sup>11,12</sup>

However, it is popularly used as a healing plant, with its latex and sap being used to treat hemorrhages, cuts, and injuries,<sup>7,8,13</sup> despite the lack of studies that consolidates its hemostatic and healing action.<sup>13,14</sup>

Considering the above, the objective of this study was to evaluate *in vivo* the hemostatic action of the *J. mollissima* (Pohl) Baill. sap, aiming to propose a hemostatic product for medical–odontological use.

## Materials and Methods

### Sample Size

This study was approved by the Animals Research Ethics Committee of the CSTR: Centro de Saúde e Tecnologia Rural (Center of Health and Rural Technology) UFCE: Universidade Federal de Campina Grande (Federal University of Campina Grande) CSTR/UFCE, under approval No.06/2020. For a 12-second standard deviation and a minimal intergroup difference of 34 seconds for the time of bleeding, a sample of five animals was needed to reach an 85% statistical power, with 0.05 alpha. The size of the sample was similar to that used in the previous studies.<sup>15,16</sup>

### Animal Selection

This research used 24 male adult Wistar rats of around 250 g, from the UFCE vivarium. The animals were randomized into four groups ( $n = 6$ ): JM25 and JM40, both treated with the ethanolic extract of the *J. mollissima* sap, in concentrations of 25 and 40 mg·mL<sup>-1</sup>, respectively; the MO group was treated with Monsel's solution (Lenzafarm, Minas Gerais, Brazil), and

the control group SC was treated with a 0.9% sodium chloride (NaCl) solution (Sorimax, Farmax Ltd., Minas Gerais, Brazil).

The animals were placed in EB 273C (Insight Ltd, Sao Paulo, Brazil) vivarium cabinets, with temperature control at  $23 \pm 1^\circ\text{C}$ , 41% humidity, and were fed, during the entire period of the experiment, a solid diet of animal ration and water “*ad libitum*.”<sup>17-19</sup>

### Biomaterial

The *J. mollissima* sap was collected in the city of Boa Vista, Paraíba, Brazil. Three incisions were made on the stem of the plant and the dripping sap was collected. Later, it was filtered through a 100-mesh filter and stored in disposable 20 mL syringes (Descarpack Ltd., Sao Paulo, Brazil), in which they were transported to the *Northeast Laboratory for the Evaluation and Development of Biomaterials*—Laboratório de Avaliação e Desenvolvimento de Biomateriais do Nordeste (Northeast Laboratory for the Evaluation and Development of Biomaterials) CERTBIO/UFCE, whereupon they were transferred to glass recipients, identified, and stored in freezers at  $4^\circ\text{C}$ , protected from light throughout the process.<sup>13</sup>

The ethanolic extract of the sap was obtained through a preparation with 1:1 ratio of sap to solvent, that is, 1 L of sap to 1 L of ethanol. The mixture was left to rest at room temperature and protected from light for 3 days, so the substances could be extracted. After this period, the extract was filtered through a vacuum filtration and concentrated using a rotary evaporator (Heidolph Laborota 4000) until the ethanol was entirely eliminated. Subsequently, it was frozen in the ultra-low temperature freezer for 48 hours and submitted to a lyophilization process, to completely remove the water via sublimation. The result was a dry, reddish orange extract.

Finally, the crude ethanolic extract from the *J. mollissima* sap was diluted in a saline solution NaCl 0.9%, to obtain the concentrations of 25 and 40 mg·mL<sup>-1</sup>.

### Surgical Procedure

For the surgical intervention, the animals were anesthetized using an intraperitoneal injection<sup>20</sup> of a mixture of 10% ketamine hydrochloride (Vetnil, Vetecia, Sao Paulo, Brazil) (75 mg/kg), 2% xylazine hydrochloride (União Química, Sao Paulo, Brazil) (10 mg/kg), and 0.15 mL of 0.9% NaCl.

Later, the surgical area was cleaned using a 2% chlorhexidine solution (Riohex, Rioquímica Ltd., Sao Paulo, Brazil), and a distal segment of 10 mm (► **Fig. 1**) was amputated from the tip of the tail of the rat, in a crosscut with a size-15 scalpel blade.<sup>21,22</sup>

Then, 1 mL of the solution was applied using a 1-mL insulin syringe attached to a 25/7 needle. The application lasted 20 seconds (► **Fig. 2**), followed by soft compression with a sterile gauze for 1 minute. A chronometer was used to measure how long it took for hemostasis to be attained. The time count started at the moment of incision and stopped at the moment the bleeding at the site stopped completely. To determine the amount of blood lost, each sterile gauze was weighted before and after the procedure using a precision laboratory scale (Shimadzu AY220; Shimadzu, Sao Paulo, Brazil), and the difference in grams, after subtracting the weight of



**Fig. 1** Marking (10 mm), with the aid of a sterile ruler with millimeters, for the amputation of the tip of the tail of the animal.

the corresponding solution applied, was used as a measure for the amount of blood lost. After the bleeding had stopped, each animal was monitored for 20 minutes, to detect any cases of rebleeding. The evaluation of hemostatic analyzes were performed by previously calibrated researchers ( $\kappa = 0.90$ ).

After this procedure, the animals were sedated using intraperitoneal ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg). Then, the rats were euthanized, using the cervical dislocation technique<sup>23</sup> recommended by the American Veterinary Medical Association Guidelines for the Euthanasia of Animals.<sup>24</sup>

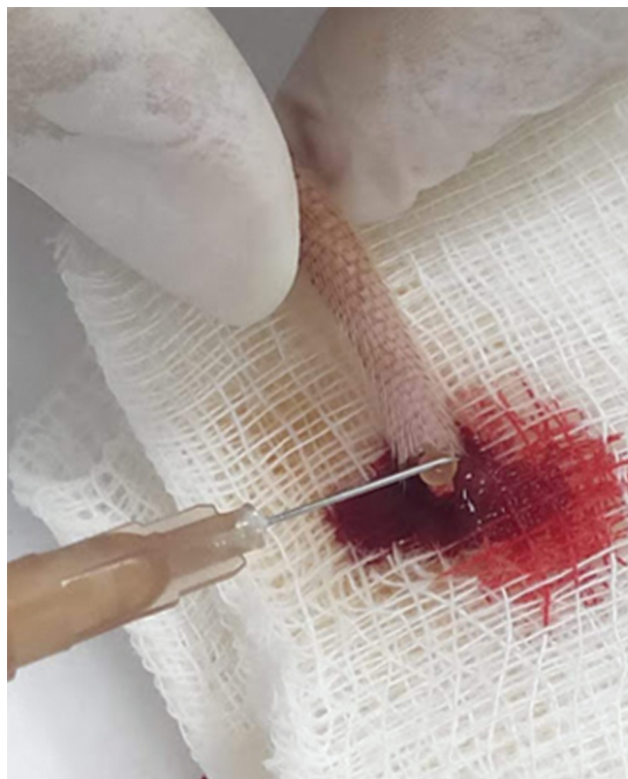
This study was randomized and triple-blind; each experimental material used in the animals was inserted in groups I to IV,<sup>25</sup> in such a way that the examiner and the statistical evaluator had no knowledge of the materials used.

### Statistical Analysis

Data were analyzed using the software GraphPad Prism, version 5.0 (San Diego, California, United States). The statistical method was chosen based on the model of distribution and variance of data, which was determined using the Kolmogorov–Smirnov's and Levene's tests, respectively. The results of the hemostatic analysis did not have a normal distribution, and as a result, they were submitted to the non-parametric Kruskal–Wallis' test, followed by the Dunn's test to determine the differences between the groups ( $p < 0.05$ ).

### Results

The bleeding time was longer and similar for the Monsel's solution group—MO (2 minutes and 74 seconds) and for the saline control group—SC (2 minutes and 59 seconds).



**Fig. 2** Application of the ethanolic extract from the *Jatropha mollissima* extract in a 25 mg·mL<sup>-1</sup> concentration.

The duration of the bleeding was significantly lower in the hemostatic group that used the 25 mg·mL<sup>-1</sup> *J. mollissima* extract—JM25 ( $p = 0.001$ ), when compared with MO and SC. There were no statistical differences between the 40 mg·mL<sup>-1</sup> *J. mollissima* extract—JM40—and the others.

The rebleeding lasted longer in the MO group (1 minute and 7 seconds), but there were no statistical differences when compared with JM40 and control group SC ( $p > 0.05$ ). The JM25 group was the only one that showed no rebleeding after the first hemostasis, with a statistically significant difference from the MO group ( $p = 0.001$ ).

The weight of the blood up to the first hemostasis was similar for the JM25 group and for the control SC, but significantly lower than the MO group ( $p = 0.002$ ). The MO group displayed six times more bleeding than the SC control group (► **Table 1**).

### Discussion

The pharmacological properties and chemical composition of the genus *Jatropha* have shown correlations between secondary metabolites and the different bioactivities, such as the hemostatic, healing, and antimicrobial action, especially in the case of *Jatropha curcas* and *Jatropha gossypifolia*, *J. mollissima* is still a seldom-studied species.<sup>26</sup>

The latex/sap of some *Jatropha* species has been described as hemostatic and healing when applied topically on injuries.<sup>27,28</sup> Hemostasis is a process in which the blood flow diminishes, and clots are formed to avoid further blood loss. It happens in two stages: the primary stage, in which blood

**Table 1** Hemostatic analysis of the amount of blood, time of bleeding, and rebleeding, for the different groups evaluated

Analysis	Groups				p-Value <sup>a</sup>
	JM25	JM40	MO	SC	
Time of bleeding (s)	95.66 (5.46) <sup>A</sup>	121.01 (12.26) <sup>AB</sup>	164.50 (26.33) <sup>B</sup>	155.66 (22.93) <sup>B</sup>	0.001
Time of rebleeding (s)	0.0 (0.0) <sup>A</sup>	17.16 (4.44) <sup>AB</sup>	64.66 (28.04) <sup>B</sup>	16.83 (8.51) <sup>AB</sup>	0.001
Weight of the blood (g)	0.13 (0.03) <sup>A</sup>	0.27 (0.13) <sup>AB</sup>	0.78 (0.33) <sup>B</sup>	0.12 (0.07) <sup>A</sup>	0.002

Abbreviations: JM25, *Jatropha mollissima* 25 mg·mL<sup>-1</sup>; JM40, *Jatropha mollissima* 40 mg·mL<sup>-1</sup>; MO, Monsel's solution; SC, sodium chloride 0.9%.

Note: The values represent the mean values and the standard deviation for the six animals evaluated in each group, Different superscript capital letters (A,B) expressed statistically significant differences in the lines.

<sup>a</sup>Means followed by distinct letters express significant differences ( $p < 0.05$ ), according to the Kruskal–Wallis' nonparametric test, followed by Dunn's multiple comparisons test.

vessels contract and the platelet plug is formed, and the secondary stage, where the plug is reinforced with a fibrin strands to maintain the clot in its place through the activation of the coagulation cascade.<sup>1</sup>

Therefore, the topic hemostasis is established as a process that works with the objective of stopping the bleeding of damaged health vessels, to accelerate one or all stages of hemostasis and stop the hemorrhage.<sup>29</sup>

The procoagulation activity of the *J. gossypifolia* latex was verified in different studies<sup>30,31</sup> that showed the protein precipitation as its mechanism of action, which induced coagulation and later hemostasis. The extract significantly diminished the time of *in vitro* human blood coagulation in healthy individuals treated with a drop of latex at the site of the lesion.<sup>31</sup>

In this study, the hemostatic action of the *J. mollissima* sap was evaluated using the technique of amputating the extremity of the tail of rats, which is a standard and reliable practice to analyze the hemostatic properties of biomaterials.<sup>32</sup> The JM25 group, to which an extract of a concentration of 25 mg·mL<sup>-1</sup> was applied, presented a significantly higher diminution ( $p = 0.001$ ) in the time of bleeding, which was 1 minute and 35 seconds, when compared with controls SC and MO, in which bleeding lasted 2 minutes and 35 seconds and 2 minutes and 44 seconds, respectively. Although the 25 mg·mL<sup>-1</sup> concentration led to a notably shorter time of bleeding than the 40 mg·mL<sup>-1</sup> concentration, there was no significant difference between these groups. This study suggests that, at 40 mg·mL<sup>-1</sup>, the sap may be provoking toxic side effects that are stronger than its hemostatic powers at the injured site, which were not presented in the 25 mg·mL<sup>-1</sup> (w/v) concentration.

These results are in accordance with studies that investigated the therapeutic properties of the *J. mollissima* latex and found phenolic compounds,<sup>11,13,27,33</sup> saponins,<sup>13,27,33</sup> tannins, flavonoids,<sup>11,13,27,33</sup> coumarins, alkaloids, and steroids.<sup>11</sup> The tannins, expressively found in the latex, have shown important antimicrobial, anti-inflammatory, and healing powers, being capable of controlling bleeding through their astringent effect over the contraction of damaged vessels and tissues, precipitating the proteins of blood and favoring hemostasis.<sup>13,34,35</sup>

In this study, the animals were observed for 20 minutes after the initial hemostasis. Only the JM25 group, with a concentration of 25 mg·mL<sup>-1</sup> of the extract, did not present rebleeding, with a significant difference from the MO group ( $p = 0.001$ ), which presented the longest rebleeding, with

1 minute and 4 seconds. Monsel's solution has ferric subsulfate, sulfuric acid, and nitric acid in its composition, and has been used as a topical hemostatic in small surgical, dermatological, gynecological, and odontological procedures<sup>36</sup>; when it gets in touch with the bleeding injury, it provokes a reaction triggered by the high pH of the ferric subsulfate, which denatures and agglutinates the blood proteins, stimulating the obstruction of the blood vessels by blood clots.<sup>37</sup>

However, in spite of the benefits from the ferric subsulfate, the MO group presented the longest time of rebleeding (1 minute and 4 seconds) when compared with the SC and JM40, and a significant amount of rebleeding, six times higher than the SC control group. These results are in accordance with studies which showed that Monsel's solution is not sufficiently efficient, since it has toxic agents from the sulfate group in its composition, which promote several significant side effects, such as erythema, postinflammatory hyperpigmentation, and increased risk of infection.<sup>36,38</sup>

According to the results, the ethanolic extract from the *J. mollissima* (Pohl) Baill. sap in a 25 mg·mL<sup>-1</sup> concentration was found to be a promising biomaterial, presenting significant hemorrhage action with therapeutic potential, to be used in the clinical treatment of local hemorrhage. Clinical trials in humans<sup>39</sup> could be used to confirm the hemostatic potential of the *J. mollissima* sap showed in this study, in addition to verifying whether smaller concentration could maintain the same hemostatic power and its mechanisms.

## Conclusion

The *J. mollissima* sap extract:

- Is an effective biomaterial to elaborate a hemostatic material for clinical use;
- Has a hemostatic effect on the time of bleeding and rebleeding;
- At 25 mg·mL<sup>-1</sup>, the hemostatic efficacy is higher.

## Conflict of Interest

None declared.

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