



Cassia occidentalis: effect on healing skin wounds induced by *Bothrops moojeni* in mice

Maraísa B. Delmut^{1*}, Leila M. L. Parente^{2†}, José R. Paula^{2†}, Edemilson C. Conceição^{2†}, Adriana S. Santos^{3†} and Irmantraut A. H. Pfrimer^{4†}

*Correspondence: maraisadelmut@gmail.com

[†]These authors are equally contributed to this work.

¹Federal University of Mato Grosso do Araguaia UFMT. Campus University (Campus II), BR-070, Km 5. Bar Herons, Mato Grosso, MT, 78600-000, Brazil.

²Research Laboratory of Natural Products, Pharmacy School, Federal University of Goiás, Av. Universitária, esq. com 1ª Avenida, Setor Universitário, CEP: 74605-220, Goiânia/GO – Brazil.

³Department of Patology, Veterinary School, Federal University of Goiás, Campus Samambaia, Rodovia Goiânia-Nova Veneza, Caixa Postal 131, CEP 74001-970, Goiânia/GO, Brazil.

⁴Postgraduate in Environmental Science and Health, Catholic University of Goiás, Av. Universitária 1.440, Setor Universitário, Goiânia-GO, CEP: 74605-010, Goiânia/GO, Brazil.

Abstract

Objective: To evaluate the healing activity of an extract from the leaves of *Cassia occidentalis* on skin wounds induced by the venom of *Bothrops moojeni* in mice.

Methods: A total of 24 Swiss albino mice were divided into 4 groups: Group 1 animals were treated for 7 days with lanette cream; Group 2 animals were treated for 7 days with 10% leaf extract of *Cassia occidentalis* in lanette cream; Group 3 animals were treated for 14 days with lanette cream; and Group 4 animals were treated for 14 days with 10% leaf extract of *Cassia occidentalis* in lanette cream. To induce wounds, the animals were anesthetised intramuscularly after the dorsum cervical region was shaved and inoculated intradermally with 4 µg *Bothrops moojeni* venom. The gross and histological evaluations were assessed 7 and 14 days after inoculation of the poison.

Results: Extract from the leaves of *Cassia occidentalis* decreased inflammation and epidermal hyperplasia and increased the vascular proliferation and reepithelialisation of wounds induced by *Bothrops moojeni* venom.

Conclusions: These data suggest that the leaves of *Cassia occidentalis* stimulated the healing of wounds induced by the dermal venom of *Bothrops moojeni* in mice, and they can be considered an alternative product to treat wounds caused by this snakebites.

Keywords: Medicinal plants, angiogenesis, *bothrops*

Background

The genus *Bothrops* is found in the Americas and is characterised by its adaptation to different types of environments. It is found in diverse ecosystems, even in flooded regions, and is responsible for the majority of snakebites in Brazil [1,2]. *Bothrops moojeni* is a species that has been attracting increased medical attention because its venom is composed of a complex mixture of proteins with biological activity, and it is used as a model to evaluate new drugs [3].

Snake bites can result in severe pain, oedema, bruising, redness and haemorrhagic blisters, which can progress to necrosis, which affects the skin, muscles and tendons [4] and is a major cause of loss of organ function.

Studies have been performed with various substances and therapies to minimise the local effects caused by *Bothrops* poisoning. Of these treatments, medicinal plants, such as *Cassia occidentalis*, are popularly used for

snakebites [5].

C. occidentalis is an herbaceous species that is native to the Americas. It belongs to the family Fabaceae (Leguminosae) and subfamily Caesalpinioideae [6]. It is used in various traditional medicines for curing various diseases, with antibacterial, antifungal, anti-diabetic, anti-inflammatory, anticancer, antimutagenic, and hepatoprotective activities, and it has also been used to treat snakebites [7].

There are no data in the scientific literature on the action of medicinal plants on the side effects arising from snakebites. This study is aimed to evaluate the healing effect of extracts of leaves of *Cassia occidentalis* on skin wounds induced by the venom of *Bothrops moojeni* in mice.

Methods

Animals

Were 24 female Swiss albino mice, aged 60 days and

weighing 20 to 40 g from the Central Biotery of the UFG were used in the study.

The animals were acclimatised in the bioterium of the Centre of Studies and Research Toxic and Pharmacological (NEPET-UFG), Faculty of Pharmacy, UFG, for 15 days. They were kept in individual polyethylene cages lined with wood shavings under controlled environmental conditions (temperature 23 ± 2 °C, relative humidity between 50 and 70% and a 12 h light/dark photoperiod). Water and food were provided ad libitum. - The ethical principles in animal testing recommended by the Brazilian Society of Science in Laboratory Animals SBCAL – COBEA were followed.

Collection of plant material and the preparation of extract from the leaves of *Cassia occidentalis*

The leaves of *C. occidentalis* were collected in the Goiânia region in December 2010 ($15^{\circ} 01' 11.8''$ south and $49^{\circ} 52' 32.2''$ west) at an altitude of 756 meters.

The hydroalcoholic extract was obtained through percolation. - A glass percolator and 95% ethanol (p/v) was used as the extraction liquid. The process was monitored by the identification of glycosides anthraquinones with the Borntraeger reaction [8]. The extracts were concentrated with a rotary evaporator at a temperature below 40 °C at a 1:4 ratio, resulting in extracts of the leaves (EF) of *C. occidentalis*. To obtain the herbal formulation, 10% of the extract from the leaves of *C. occidentalis* was incorporated into lanette cream (cream base) and packed in a plastic pot.

Preparation of *Bothrops moojeni* venom

The crude venom of *Bothrops moojeni* was prepared according to the method proposed by [9]. The animals were intradermally inoculated with 4 µg of venom diluted in 10 ml of saline applied 0.1 ml in each mouse to cause necrosis with a 10 mm diameter. After 24 hours, the wound was treated with the topical application of 100 µL of ethanolic extract in the treated group and 100 µL of saline in case of untreated group.

Experimental design

The animals were weighed and randomly divided into four groups (n=6):

Group 1 – the animals were treated for seven days with lanette cream.

Group 2 – the animals were treated for seven days with lanette cream containing 10% of the leaf extract of *C. occidentalis* in (EF).

Group 3 – the animals were treated for fourteen days with lanette cream.

Group 4 – the animals were treated for fourteen days with lanette cream containing 10% of the leaf extract of *C. occidentalis* (EF).

Wound induction

To induce the wound, the animals were anesthetised

intramuscularly with a decloridrato combination of ketamine (70 mg kg^{-1}) and xylazine (10 mg kg^{-1}). After shaving the dorsal neck region, the mice were inoculated intradermally with 4 µg *B. moojeni* venom. Paracetamol was administered orally as an analgesic, and the dose was calculated by allometry. After 24 hours, topical treatments were initiated and performed daily at the same time.

Wound evaluation

Wound induction and treatment was performed without complications. There was no complication in relation to anaesthesia and the animals demonstrated good general motor activity and normal behaviour for the species after the procedures.

All animals were examined daily for general appearance. Besides, macroscopic evaluation was done through daily observation of the wound, and the data was recorded individually.

Euthanasia of animals

At the end of the experiment, the animals were weighed and euthanised by anaesthesia with a combination of ketamine hydrochloride (100 mg kg^{-1}) and xylazine hydrochloride (30 mg kg^{-1}).

Histological evaluation

For histological evaluation, a fragment of each wound was removed, fixed in 10% formalin, and processed and stained with haematoxylin and eosin (HE).

The histological examination was conducted by the Division of Pathology, Veterinary School, Federal University of Goiás (Brazil). The following variables were evaluated on day 7: fibrin, haemorrhage, oedema, hyperaemia, inflammatory infiltration of polymorphonuclear cells, the infiltration of fibroblasts and vascular proliferation. On day 14, the following parameters were evaluated: reepithelialisation, epithelial hyperplasia, collagen, vascular proliferation and inflammatory infiltrates. Adapted scores were used [10].

Statistical analysis

The results were submitted to statistical treatment by the use of GraphPad InStat software (Version 3.05 for Windows). From the Kolmogorov-Smirnov test for normality, histological analysis data were performed a semi-quantitative assessment using the Kruskal-Wallis test, and the least significant differences were determined using the Dunn test. The statistics were considered significant when the *p* value was less than 0.05 (*p* < 0.05).

Results

Animal weights

The results pertaining to the weight of the animals at the beginning and end of the assessment period are shown in **Table 1**. All of the experimental groups showed a decrease

Table 1. The average weight of the animals at the beginning and end of the evaluation period (7 and 14 days after the inoculation of *Bothrops moojeni* venom).

Groups	Average of Weights (g)		
	Initial	Final	Final averages
Group 1	30,66	27,66	28,44
Group 2	30,33	28,33	31,39
Group 3	30,83	29,33	30,22
Group 4	32,16	28,50	29,22

Group 1 - animals treated for seven days with lanette cream.
 Group 2 - animals treated for seven days with lanette cream with 10% leaf extract of *C. occidentalis* (EF).
 Group 3 - animals treated for fourteen days with lanette cream.
 Group 4 - animals treated for fourteen days with lanette cream with 10% leaf extract of *C. occidentalis* (EF).

Table 2. Median histological variables evaluated at day 7 after the inoculation of *Bothrops moojeni* venom in mice treated with 10% of leaf extracts of *Cassia occidentalis* in lanette cream (EF).

Histological variables	Lanette cream		P
	<i>C. occidentalis</i> (-)	<i>C. occidentalis</i> (+)	
Necrosis	3,0 ^a	3,0 ^a	0,28
Oedema	0,0 ^a	0,0 ^a	0,44
Hyperaemia	1,0 ^a	1,0 ^a	0,26
Haemorrhage	3,0 ^a	3,0 ^a	0,43
polymorphonuclear inflammatory infiltrate	2,5 ^a	3,0 ^a	0,14
Infiltration of fibroblasts	2,0 ^a	1,0 ^a	0,12
Vascular proliferation	1,0 ^a	2,0 ^b	0,032*

(p = level of significance. Different letters differ significantly (Kruskal-Wallis, Dunn's post-test, * p < 0.05)
 (-) Without plant extract
 (+) With plant extract

Table 3. Median histological variables assessed at day 14 after the inoculation of *Bothrops moojeni* venom in mice treated with 10% of the extract of leaves of *Cassia occidentalis* in Lanette cream (EF).

Histological variables	Not treated	Treated with EF	P
Collagen	2,0 ^a	2,0 ^a	0,57
Inflammatory infiltrate	2,0 ^a	2,0 ^a	0,50
Vascular proliferation	1,0 ^a	2,0 ^b	0,045*

(p = level of significance. Different letters differ significantly (Kruskal-Wallis, Dunn's post-test, * p < 0.05).

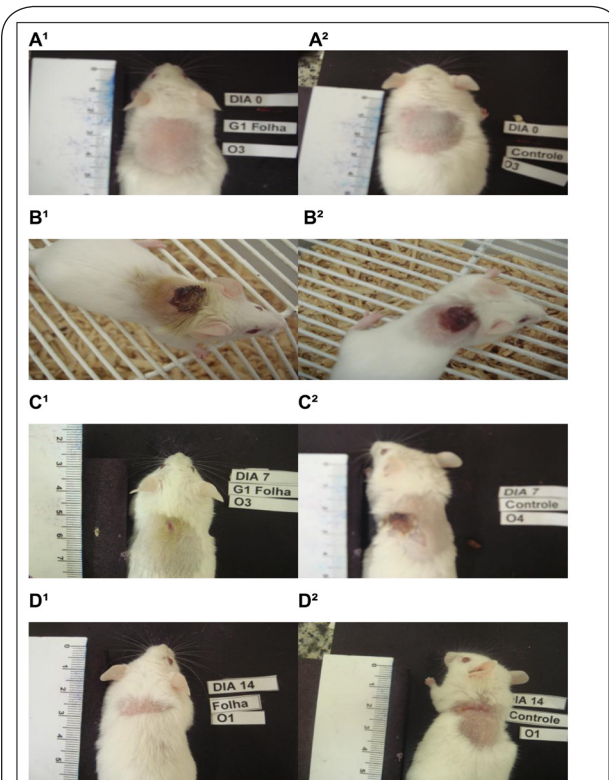


Figure 1. A- After inoculation with *B. moojeni* poison; **B** - 3 days following the inoculation of mice with *B. moojeni* venom and treatment with ¹10% leaf extract of *Cassia occidentalis* in lanette cream (EF) or ²lanette cream only (not untreated); **C** - 7 days after the inoculation of mice with *B. moojeni* venom and treatment with ¹10% leaf extract of *Cassia occidentalis* in lanette cream (EF) or ²lanette cream only (not untreated); **D** - 14 days after the inoculation of mice with *B. moojeni* venom and treatment with ¹10% leaf extract of *Cassia occidentalis* in lanette cream (EF) or ²lanette cream only (not untreated).

in the final weight.

Macroscopic analysis

In the macroscopic analysis, the wounds treated with leaf extract were dry, thinner and had few signs of inflammation on the 3rd day, and they disappeared on the 6th day after inoculation of the poison (Figure 1). In the groups that were not treated with the plant extract, the wounds appeared red. On the 3rd day, the animals showed a reaction of pain to the touch, and the wounds only disappeared on the 9th day after inoculation of the poison (Figure 1).

Area of skin wounds decreased during the healing process. In mice treated with the plant extract, all wounds were completely healed on the 14th day after inoculation of the poison. In mice not treated with the plant extract, only 60% of the animals showed complete healing on the 14th day (Figure 1).

The macroscopic evaluation of wounds in animals treated with leaf extracts revealed that 100% of the mice showed complete reepithelialisation of wounds on 14th day. In Mice not treated with plant extract, partial reepithelialisation was observed. 40% of the animals had open wounds even on the 14th day after inoculation of the poison (Figure 1).

Microscopic analysis

Results of histological analyses are presented in

Table 4. Median (mm) epidermal hyperplasia at 14 days after the inoculation of *Bothrops moojeni* venom in mice treated with 10% of the extract of leaves of *Cassia occidentalis* in Lanette cream (EF).

Groups	Hyperplasia
Lanette without EF	86,41 ^a
Lanette with EF	69,04 ^b
P	0,0076*

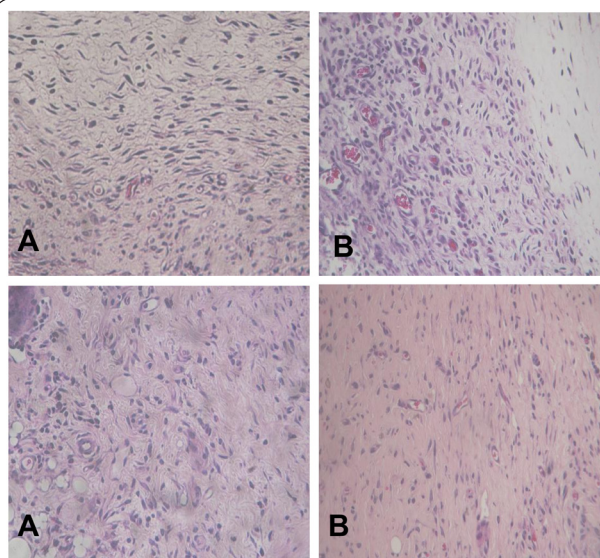


Figure 2. Photomicrographs of the skin wounds in mice at 7 (A) and 14 (B) days postoperatively demonstrating the presence of blood vessels. A1 and A2 are control mice, and B1 and B2 are mice treated with lanette cream with 10% leaf extract of *Cassia occidentalis*. Haematoxylin-eosin. 20x.

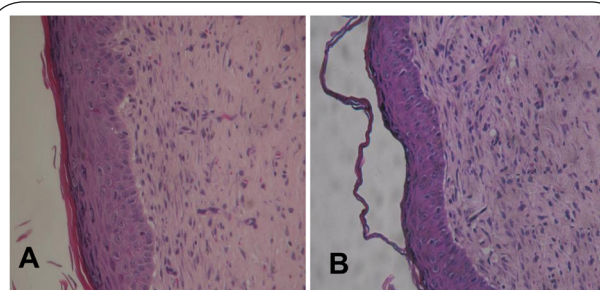


Figure 3. Photomicrograph of mouse skin wound showing epidermal hyperplasia. Group not treated with *Cassia occidentalis* (A1) and group treated with lanette cream with 10% leaf extract of *Cassia occidentalis* (B1) on the 14th day postoperatively. Haematoxylin-eosin. 20x.

Tables 2, 3 and 4 and Figures 2 and 3. In mice treated with plant extract, regardless of the time of treatment (7 or 14 days), a significant increase in vascular proliferation (**Tables 2 and 3 and Figure 2**) was observed. A significant reduction in epidermal hyperplasia was observed on 14th day in the animals treated with leaf extracts of *C. occidentalis* compared to the mice not treated with plant

extracts (**Table 4 and Figure 3**).

Discussion

In this study it was observed that leaf extract of *C. occidentalis* decreased inflammatory processes and epidermal hyperplasia and increased vascular proliferation and the reepithelialisation of wounds induced by *Bothrops moojeni* in mice.

Bothrops snake bites are an important public health problem in tropical regions around the world. The local reaction caused by bothropic venom has been the focus of several investigations. The use of substances with the therapeutic potential of minimising these reactions has been studied, with medicinal plant, such as *C. occidentalis*, which is popularly used for snakebites [11].

From this work, it is clear that the cutaneous wounds of the animals in the mice treated with plant extract were dry and had few signs of inflammation. Scientific studies have shown that *Cassia occidentalis* has an enormous biological potential and may have anti-inflammatory, antiplatelet, muscle relaxant, and anti-haemolytic activities and inhibit lipid peroxidation. These effects may occur probably as it is rich in derivatives like anthraquinones [5].

The reduction in epidermal hyperplasia observed in the groups treated with plant extract may be associated with a positive effect of *C. occidentalis* on the inflammatory phase. Moreover, the venom of snakes of the genus *Bothrops* has high proteolytic activity, which is responsible for the primary characteristic systemic effects observed after snakebites [12].

Wound healing occurs in overlapping and interdependent phases [13], including inflammation, repair, and maturation. Many processes occur during the proliferative phase of wound repair, including angiogenesis, fibroplasia and epithelialisation [14], highlighting that angiogenesis is one of the most important histological features in the granulation tissue in the healing process of the skin [15]. In this study, animals treated with plant extract showed complete epithelialisation and angiogenesis on the 7th and 14th day after wound induction by poison.

Angiogenesis is the growth or formation of new blood vessels from pre-existing vessels at the ends of the wound that were previously devoid of blood vessels [16]. One way to enhance the healing process is to stimulate angiogenesis, which can be established by removing debris and supplying essential nutrients and oxygen to the wound site. The results of this study corroborate other studies [10,17,18,19,20]. Similar effects have been observed in the study, which justifies the macroscopic findings of the best resolution of the healing process.

The microscopic analyses revealed a decrease in epidermal hyperplasia on the 14th day in the group treated with the extract of leaves of *C. occidentalis*. Hyperplasia corresponds to the increased cellularity of a tissue due to a higher functional demand or hyperstimulation [21]. During

the inflammatory process, hyperplasia can occur through phenomena that are intrinsic to inflammation, such as hyperaemia and the synthesis of substances that stimulate cell division. Thus, the more intense and long-lasting the inflammatory response, the greater the hyperplastic response of the cells involved in the process [22].

During the healing process, when there is a delay in the elimination of the initial cause of the lesion by the inflammatory response, there is a higher number of hyperplastic cells [23], and leaf extracts of *C. occidentalis* can act to minimise the inflammatory effects of *Bothrops moojeni* poison and accelerate the healing process.

Conclusion

These data suggest that extract of leaves of *C. occidentalis* stimulate the healing of wounds induced by the venom of *B. moojeni* in mice, and it can be considered an alternative product to treat snakebite wounds.

List of abbreviations

C. occidentalis: *Cassia occidentalis*

B. moojeni: *Bothrops moojeni*

HE: haematoxylin and eosin

EF: leaf extract of *C. occidentalis*

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MBD planning and execution of all the experimental stage, tabulation and analysis of data and writing of the manuscript. LMLP experimental design, planning and execution of all the experimental stage, and tabulation and analysis of data and writing of the manuscript. JRP collection and identified the plants species and analysis of extract. EMC cream preparation for the experiment. ASS microscopic analysis. IAHP orientation, experimental design and writing of the manuscript.

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