Anthelmintic potential and in vivo acute toxicity study of Piptadenia gonoachanta leaf aqueous extract

Mariana Costa Fausto1, Idelvânia dos Anjos Nonato1, Guilherme Costa Fausto1, Rogério Pinto2, Fabrício Luciani Valente1, Lorendane Millena de Carvalho1, Artur Kanadani Campos1, Marcelo Barcellos da Rosa3, Jackson Vitor de Araújo1, Camilo Amaro de Carvalho4

1Department of Veterinary Medicine, Federal University of Viçosa, Viçosa, MG, Brazil.
2Department of Pharmacy, Univiçosa, Viçosa, MG, Brazil.
3Department of Chemistry, Federal University of Santa Maria, Santa Maria, RS, Brazil.
4Department of Medical and Nursing, Federal University of Viçosa, Viçosa, MG, Brazil.

ABSTRACT

Piptadenia gonoacantha (Leguminosae) is an arboreal species found in southern and southeastern Brazil. Several compounds have been identified in their leaves, such as anthraquinones, phenolics, tannins, saponins, coumarins and flavonoids. The presence of these metabolites is associated with antioxidant, anti-inflammatory, antibiotic and anthelmintic properties. This study evaluated the phytochemical profile of in vitro anthelmintic activity and in vivo toxicity of the aqueous extract of P. gonoacantha leaves. The extracts were distinctly evaluated by extraction method: maceration (EAM), ultrasonication (EAU) and lyophilization (EAL). Anthelmintic activity analysis was carried out for all preparations at 25, 50 and 100 mg/mL against Panagrellus sp. in an in vitro assay. In vivo toxicity was assessed by biochemical, hematological and histological evaluation of Balb/C mice treated orally with EAU (100, 200 and 400 mg.Kg\(^{-1}\)) for 10 consecutive days. Phytochemical analysis showed the presence of tannins, flavonoids, coumarins, saponins, glycosides, phenolic compounds and anthraquinone. Anthelmintic potential showed significant results, with inhibition potential ranging from 93.93% and 99.88% among the different concentrations and preparations tested. In absolute values, the EAPG at 50 mg / mL showed a higher percentage of inhibition, reaching the following lute values, the EAPG at 50 mg / mL showed a higher percentage of inhibition, reaching the following lute values, the EAPG at 50 mg / mL showed a higher percentage of inhibition, reaching the following lute values. EAU has proved to be safe for in vivo use because it showed low toxicity, enabling it to future pre-clinical and clinical studies.

INTRODUCTION

The interest in the use of bioactive plant compounds responsible for therapeutic actions has been an area of growing research (Viegas et al., 2006; Sandoval-Castro et al., 2012). According to the World Health Organization (WHO), 85% of the population of developing countries uses medicinal plants in nature or herbal formulations. However, in order to be used for this purpose, it is important to identify which pharmacological principles are present in the vegetal species and whether they have toxic components that could create risks to animal and human health (Simões et al., 2004; Carvalho et al., 2014). Therefore in vivo and in vitro tests must be performed to evaluate their effects (Mellor, 2000). Although the chemical and pharmaceutical industries have produced a wide variety of drugs in recent years, there is an increase in microorganism resistance. This fact encourages the search for alternative sources such as herbal substances with antimicrobial and antiparasitic activity (Ribeiro, 2008; Padilha et al., 2010).
Scarce knowledge about parasite survival mechanisms and the decrease of investments in innovative anti-parasitic molecules diminish the development of new effective chemical groups in parasitosis control (Geary and Thompson, 2003).

_Piptadenia gonoacantha_ (Leguminosae-Mimosoideae) is an arboREAL species found predominantly the south and southeast Brazil. It has also been described in some northern and northeastern states. This species is easily identified by its thorny longitudinal woody wings along the trunk and branches. This gives its popular names like alligator wood, alligator, alligator hull, among others (Carvalho et al., 2010). Phytochemical studies have identified several compounds in the leaves of this plant as anthraquinones, phenolics, tannins, saponins, coumarins and flavonoids. The presence of these compounds has been associated with antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial and antiparasitic properties.

This study aimed to determine the phytochemical profile of _P. gonoacantha_ leaves and evaluate their _in vitro_ anthelmintic activity against _Panagrellus_ sp. and _in vivo_ toxicity on Balb/C mice biochemical, hematological and histological parameters after oral administration of aqueous extract daily for ten consecutive days.

**MATERIAL AND METHODS**

**Plant material**

One kilogram of fresh _P. gonoacantha_ leaves was harvested in Viçosa, located in the state of Minas Gerais, Brazil, 20°45'14''S and 42°52'55''W, in November 2014, in the spring season. The material was identified and deposited at the Federal University of Viçosa Botanical Garden, voucher specimen #35,530. Leaves were selected, cleaned and dried at 40°C with forced air circulation until dehydration. The dried material was then ground in a cutting mill.

**Extracts preparation**

Aqueous extracts of _P. gonoacantha_ leaves were prepared at the concentration of 20g.200mL⁻¹ (w/v) by maceration (EAM), ultrasonication (EAU) and lyophilization (EAL) in order to validate the best extraction method. EAM was obtained after 1 h at room temperature, and the EAU, obtained under the same conditions, however, subjected to ultrasound (Unique® - MaxiClean 1400), following methodology used by Carvalho et al. (2011). Extracts were filtered separately under vacuum to remove completely dry matter, obtaining maximum solution volume. After filtration, 200 mL of EAU was lyophilized to obtain EAL.

**Phytochemical tests**

Phytochemical evaluation was carried out in all extracts produced from _P. gonoacantha_ leaves. They were subjected to a series of chromatographic and precipitation reactions, according to methods described by Simões et al. (1999) and to characterize the secondary compounds classes, such as naphthoquinone (acid/base reaction), flavonoids (cyanidin and sulfuric acid reaction), tannins (reaction with iron salts, precipitation of proteins), coumarins (observation under ultraviolet light), phenolic compounds (Folin-Ciocalteu reaction), triterpenes and steroids (Liebemann-Burchard reaction), glycosides cardiotonics identification (Baljet test and Kedde test), characterization of saponins (Liebemann-Burchard reaction and foam index) and alkaloids presence identification (Mayer, Hager and Gragendorff reagents). The phytochemical screening was performed in triplicate.

**Helminths mobility assay**

Motility test was conducted according to Cunha et al. (2003). _Panagrellus_ sp. were incubated in oat-water medium culture in Petri plates. Then they were removed from the plate, wrapped in surgical gauze and placed in Griffin beakers containing the water at room temperature. After 12 h, the _Panagrellus_ sp. pellet was recovered from the beaker and washed three times by re-suspension in distilled water followed by centrifugation at 250 g and 20°C for 5 min. Active and inactive _Panagrellus_ sp. were identified and counted under light microscopy according to Barçante et al. (2003) with modifications. A volume of 0.5 mL of the nematoid suspension (2000 _Panagrellus_ sp.) and 1.5 mL of the _P. gonoacantha_ extract were added to each well of a 24-well culture plate.

Three concentrations (25, 50 and 100 mg.mL⁻¹) of each preparation (EAM, EAU, and EAL). As positive and negative controls, 2% fenbendazole and distilled water were used, respectively. Six replicates were performed for each preparation. Living and dead larve counts in 100 µL aliquots were performed after 24, 48 and 72 h of incubation. Dead individuals were identified by the lack of motility. The results were plotted as percentages.

**Animals**

Sixty-day-old female Balb/C mice (25-35 g) were obtained from the Central Animal Facility of UFV. The animals were kept under controlled environmental conditions at 23±2°C, 12h of light/dark cycle, commercial food (Presence®) and water _ad libitum_. The methods used in this study was approved by the Ethics Committee for the Use of Animals of the School of Biological and Health Sciences - CEPEUA/FACISA #106/2014-II. The animals were randomly distributed into five groups with five animals each and received 0.5 mg.kg⁻¹ PO/day of the flowing compounds per group: Ivermectin (IVE) 1% (w/v) (positive control); distilled water (negative control); and _P. gonoacantha_ leaf aqueous extract (EAU) at 100, 200 or 400mg.kg⁻¹. After ten days, the animals were euthanized by an anesthetic overdose of sodium thiopental. Blood, intestine, liver and kidney were harvested for analysis.

**Haematological and biochemical analysis**

For the evaluation of parameters hematological and biochemical characteristics of blood animals, the methodology described by Vasconcelos et al. (2007) with modifications. Blood samples were obtained by cardiac puncture and placed in tubes
with HB anticoagulant (Laborlab®) for hematologic analysis and without anticoagulant for biochemical analysis. The values for erythrocytes, leukocytes, platelets, hemoglobin, hematocrit, erythrocyte indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined using an automated analyzer of hematologic cells, Human Count (Human do Brasil LTDA®). Leukocyte count was performed on smears stained with Romanowsky stain under light microscope. In each slide, 100 cells were analyzed and counted. By biochemical analysis, the following parameters were obtained: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, total cholesterol, triglycerides, and urea. The tests were carried in automatic machine Huma Star 300 (Human do Brasil LTDA®) with Labtest® commercial systems.

Histopathology

After necropsy, intestine, liver and kidney were removed, washed with saline (0.9%) and fixed in formalin buffered at 10% for 24 hours. Subsequently, the material was subjected to histological techniques, including dehydration in ascending series of alcohols, clearing in xylol and embedding in paraffin (58±2°C). Then, 5µm-thick sections were obtained in a manual rotation microtome (American Optical Company®, United States of America) and subsequently stained with hematoxylin-eosin (HE) (Grimaldi Son, 1981). The following parameters were evaluated: Liver –inflammatory infiltrate, vacuolation, hyperemia, fibrosis, degeneration, and necrosis; Kidney –sclerosis, glomerular shrinkage, inflammatory infiltrate, tubular degeneration and necrosis, fibrosis, and hyperemia; Intestine– edema, hyperemia and inflammatory infiltrate. A score scale from 0 to 3 were considered to all parameters: 0 – absence of injury; 1 – mild injury (up to 25%); 2 – moderate injury (25 to 50%); and 3 – severe lesions (more than 50%) (Biondo-Simoes et al., 2006). Histological interpretations were performed at Pathology Laboratory of the Department of Veterinary/UFV by two examiners without prior knowledge of the study groups. Micrographs were obtained in the Laboratory of Pathology of the Medicine and Nursing Department/UFV, using a light microscope BX 41 (Olympus®, Tokyo, Japan).

Data analysis

ANOVA was performed to analyze parametric data and Kruskal-Wallis for nonparametric data. As a post-hoc analysis, Student-Newman-Keuls test was used. A 5% level of significance was considered for all statistical tests.

RESULTS AND DISCUSSION

Phytochemical profile of the three preparations of *P. gonoacantha* leaf aqueous extract is described in Table 1. The same chemical compounds were detected in all three preparations. Thus, the phytochemical analysis revealed the presence of flavonoids, phenolics, tannins, anthraquinone, saponins, coumarins, and cardiotonic glycosides. Carvalho et al. (2014) evaluated hydroalcoholic compounds at 80% (v/v) extracted from *P. gonoacantha* leaves and found the same compounds, corroborating results presented here. According to Leão (2007), such compounds are called secondary metabolites and are related to the chemical plant defense, guaranteeing their survival in their natural habitat. Furthermore, those compounds present a wide diversity and their presence in a given solution will depend on the extraction process and extraction solution used (Alawa et al., 2003).

Typically, alcoholic solutions are more effective than aqueous solutions in the extraction process, being able to solubilize compounds with low and high polarity (Chaicouski et al., 2014). Aqueous preparations only allow obtainment of high solubility compounds such as glycosides, tannins, and alkaloids (Fonseca et al., 2005). However, alkaloids were not found in this study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Method</th>
<th>EAM</th>
<th>EAU</th>
<th>EAL</th>
</tr>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>Dragendorf</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>Hager</td>
<td>-</td>
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<td></td>
<td>Mayer</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Borntrager</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Magnesium acetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>Potassium hydroxide</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>Folin-ciocalteau</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Aluminum chloride</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ferric chloride</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cardiotonich eterosides</td>
<td>Baljet</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>Lead acetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Lohmann-burchard</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

EAM: Aqueous extract, macerated; EAU: Aqueous extract, ultrasonicated; EAL: Aqueous extract, lyophilized. (+) Present; (-) Absent.

To evaluate nematicidal properties, toxic activity on *Panagrellus* sp. of different *P. gonoacantha* leaf preparations was evaluated by mobility assay. Although they are not parasitic, they are used as an experimental model because they can be maintained and multiplied in laboratory conditions (Katiki et al., 2011). Thompson et al. (1996) reported that the effectiveness of preparations evaluated in low concentrations against this species, it is possible that they have anthelmintic activity against other nematodes.

In this study, after 24 hours of incubation, all preparations of the extract (EAM, EAU, EAL) showed nematicidal action on *Panagrellus* sp. with the same efficacy as the control groups (Figure 1 and 2). In absolute terms, EAU at 50 mg/mL showed the highest inhibition percentage, reaching 99.88%. However, all the concentrations showed inhibition above 93.0%, while in control groups there was no larvae inhibition during the first 24 hours.
Among the extraction methods evaluated, EAM presented as an efficient and affordable alternative to be used in helminthes control. The preparation of medicinal species in water is a commonly used practice in folk medicine and its use as a natural vermifuge is becoming more popular as an alternative method, sustainable and adaptable for rural communities (Alawa et al., 2003; Fonseca et al., 2005; Bizimenyera et al., 2006). However, aiming to obtain a formulation for large-scale industrial production, lyophilized extract is presented as a more acceptable formulation because it provides greater physical, chemical and microbial stability, and facilitates homogenization and standardization of the active ingredients (Runha et al., 2001; Chaicouski et al., 2014).

At light microscopy, most of the larvae *Panagrellus* sp. in the negative control wells showed to be viable throughout the evaluation period, i.e., after 72 hours (Figure 2a). In contrast, *P. gonoacantha* leaf extract led to helminth membrane perforation, resulting in occasional leakage of internal contents and causing lysis after 24 h of incubation (Figure 2b). After 48 h of incubation with the extract, the outer membrane of the larvae showed fragility along all its extension (Figure 2c). All preparations and concentrations showed 100% of the larvae lysed. However, after 48h of incubation, *Panagrellus* sp. larvae used in the positive control become unfeasible. Incubation with fenbendazole, positive control, led to the dissolution of the cuticle of the larvae, making it impossible to evaluate and score (Figure 2d).

In the present study, the extracts were able to effectively inhibit the larvae of *Panagrellus* sp. Cunha et al. (2003) studied the action of the methanolic extract obtained from the leaves of *Leucaena leucocephala*, that belongs to the same family of *P. gonoacantha* (Leguminosae - Mimosoideae), and verified the induction of mortality in 96.69% of *Panvillus rdivius*. In the same work, the extract was submitted to a phytochemical identification, which indicated that the nematidal substance produced by *L. leucocephala* belongs to the class of alkaloids. Silva et al. (2003) also associated the presence of alkaloids with the destruction of intestinal parasites larvae in the environment. Thus, although this compound was not found in the phytochemical analysis performed in this work, its presence in the leaves of *P. gonoacantha* cannot be ruled out, since its absence may be due to the extraction process used. On the other hand, it can be inferred that the presence of other secondary metabolites is related to the lysis verified under light microscopy. According to Oliveira et al. (2011), the active compounds of plants have been widely studied for the control of intestinal parasites, emphasizing the tannins, which are the secondary metabolites most found in plants. In their condensed form, tannins have the ability to bind and alter physicochemical properties of proteins (Hoste et al., 2006). Therefore, plants in which phytochemical composition contains tannins have a high potential for larval motility inhibition and sheath destruction (Hoste et al., 2006; Nery et al., 2009).

Santos et al. (2013) have shown that the parasites cuticle is rich in proline and hydroxyproline, not only on its external face but also in the oral cavity, esophagus, cloaca, and vulva. These authors emphasize that cuticular changes can be observed under electron microscopy after incubation of nematodes with extracts containing tannins, which corroborates the findings of this research. The nematidal action of tannins is closely related to the ability to bind and inhibit proteins and enzymes involved in secretory, excretory and metabolic activities, essential for the life of nematodes (Athanasiadou et al., 2001). However, according to Githiori et al. (2005), this action may vary according to the plant species, parasite and host involved.
As with tannins, flavonoids may also be related to the antihelmintic activity (Silva et al., 2009; Bastos et al., 2014). These are heteroside compounds and constitute a large group of plant pigments widely distributed in nature (Arantes et al., 2005). They have the capacity to form complexes with soluble extracellular proteins and, due to their lipophilic character, promote the rupture in the membrane of several microorganisms, among them the parasites (Sartori, 2005). Therefore, the evaluation of the nematicidal effect of the leaves of P. gonoachanta should still be the object of future research, aiming to fractionate and quantify the chemical compounds obtained in different forms of extraction and to verify if this activity depends on a single active substance or a combination of various substances and their concentrations. In addition, works using electron microscopy may help in understanding the mechanism of action of these compounds, especially on the membrane of these organisms. The toxicity of P. gonoachanta was also evaluated in vivo. However, no significant changes were observed (p<0.05) (Table 2). Among transaminases, ALT is considered the most specific to assess liver damage (Giannini et al., 2005). Since the liver is the main organ of degradation of the drug, it can cause damage to the membrane of the hepatocytes promoting the increase in the levels of the enzyme (Rocha et al., 2012). However, according to Qureshi et al. (2013), biochemical changes induced by ivermectin are difficult to explain and are associated with the mechanisms responsible for their antiparasitic activity. The quantitative and qualitative evaluations of blood elements, a significant increase (p <0.05) was observed in red blood counts of animals treated with EAU at 200 and 400mg. Kg⁻¹, respectively, when compared to animals from the other groups. Comparing the values of mean corpuscular hematocrit (HCM), the difference was between the animals belonging to the negative control group and the group treated with ivermectin 1%, compared to the animals from the other treatments (Table 3). Significant difference (p<0.05) was also observed in the percentage of lymphocytes when compared to the animals belonging to the group treated with ivermectin 1% and with EAU 400mg.Kg⁻¹. Animals treated with the drug had a higher amount of these cells. According to Bonamin and Paulino (1996), drugs such as levamizol and ivermectin can be used as immunomodulators because they are capable of promoting an increase in the induction of T lymphocyte proliferation, increased chemotaxis and phagocytic activity. On the other hand, the decrease of these cells in the group treated with the highest dose of the extract can be explained, according to Melo et al. (2008), due to the greater amount of tannins ingested by the animals, since this compound is known to induce a significant increase (p <0.05) were found only in animals treated with ivermectin 1%. Kruskal-Wallis and Student-Newman-Keuls Method as post-hoc test, α = 5%.a. Values followed by distinct letters on the line differ from each other. Kruskal-Wallis test and Student-Newman-Keuls as post-hoc test, α = 5%.
In the small intestine, although not significant (p>0.05), it was possible to verify submucosal layer rupture and muscular tunica detachment. Hepatic and intestinal lesions can be justified because ivermectin is mainly metabolized in these organs (Arise et al., 2012). In a study conducted by Arise et al. (2012), rats treated with an oral combination of ivermectin and albendazole also showed an increase in the polymorphonuclear leukocytes population in the serum of these animals. The liver is the main site of drug detoxification, ivermectin degradation can lead to free radicals production and concentration. Those radicals, when bound to oxygen, can lead to reactive oxygen species formation, capable of causing damage to the membrane and proteins, resulting in a process of cell vacuolization and death (Filho et al., 2007). In the kidneys, ivermectin 1% may cause inhibition of renal excretion of various substances (Fricker et al., 1999). According to Whelton et al. (2004), the malfunctioning of glomerular filtration, promotes the retention of substances such as urea, justifying the increase of their levels in the serum of these animals. On the other hand, animals treated with different *P. gonoacantha* EAU doses showed no significant lesions on the liver, kidney, and intestine after acute administration. It may suggest an anthelmintic action without toxicity on those organs. In agreement with this action, the presence of secondary metabolites confer to the EAU antioxidant and anti-inflammatory activity, as demonstrated in previous studies (Pereira et al., 2010; Carvalho et al., 2014). However, studies involving the chronic administration of EAU produced from *P. gonoacantha* leaves should be performed so that future clinical trials with target species can be conducted.

**CONCLUSION**

Aqueous extracts of *P. gonoacantha* leaves have secondary metabolites with anthelmintic activity, supporting their use in the control of gastrointestinal parasitic diseases of animals. Extracts obtained by ultrasonography prove to be safe due to low toxicity after acute exposure, allowing progress in clinical studies to prove possible benefits in other target species.

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