



Anthelmintic activity of *Annona crassiflora* leaves against *Haemonchus contortus*: part 2: efficacy *in vivo* and blood parameters

[Atividade antihelmíntica de folhas de *Annona crassiflora* contra *Haemonchus contortus*: parte: eficácia *in vivo* e parâmetros sanguíneos]

"Scientific Article/Artigo Científico"

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Abstract

In this study we evaluated the oral toxicity of leaf extracts of *Annona crassiflora* for mice and the blood and parasitological parameters of lambs experimentally infected with *Haemonchus contortus* and treated with leaves of this plant. The highest dose of AE (aqueous extract) administered to mice (203.0 mg/kg bw) was well tolerated, suggesting low toxicity. At necropsy, macroscopic examination revealed no abnormalities of the evaluated viscera. Lambs infected with the nematode were divided into one group treated with leaf powder and a control group that did not treat. Split-plot design analysis was performed where the treatments were defined as plots and three periods of collection were defined as subplots. Similar performances to weight gain were observed among the lamb groups. The oral administration of leaf powder at 2.75g /Kg bw did not alter the physiological blood parameters in comparison to untreated lambs; however, this dose was not efficient for fecal egg reduction. We consider that other formulations and administration protocols should be evaluated to promote an effective alternative control using the leaves of this plant.

Keywords: Alternative control; haemonchosis; oral toxicity; blood tests; Cerrado.

Resumo

Neste estudo avaliou-se a toxicidade oral de extratos de folhas de *Annona crassiflora* em camundongos e os parâmetros parasitológicos e sanguíneos de cordeiros experimentalmente infectados com *Haemonchus contortus* e tratados com folhas dessa planta. A dose mais elevada do extrato aquoso (EA), administrada aos camundongos (203,0 mg / kg de peso corporal (pc)) foi bem tolerada, sugerindo baixa toxicidade. Na necropsia, o exame macroscópico não revelou anormalidades nas vísceras avaliadas. Cordeiros infectados com o nematódeo foram divididos em um grupo tratado com pó das folhas e um grupo controle não tratado. A análise do delineamento foi em parcelas subdivididas, os tratamentos foram definidos como parcelas e três períodos de coleta como subparcelas. O ganho de peso médio similar entre os grupos de borregos avaliados durante o período avaliado. A administração oral do pó das a 2,75g / kg pc não alterou os parâmetros fisiológicos do sangue em comparação aos cordeiros não tratados; no entanto, essa dose não foi eficiente para a redução fecal de ovos. Outras formulações e protocolos de administração devem ser avaliados para promover um controle alternativo e eficaz usando as folhas dessa planta.

Palavras-chave: Controle alternativo; haemonchose; toxicidade oral; exames de sangue; Cerrado.

Introduction

Haemonchus contortus is the most common parasite in small ruminants reared in tropical and subtropical areas (Miller et al., 2012). Sheep with haemonchosis show anemia and submandibular swelling, with high mortality in young lambs and ewes in the peripartum period (Bastos et al., 2017). This parasitosis results in production losses, including decreased weight gain and feed conversion efficiency; reduced meat, wool, and milk production (Miller et al. 2012).

The constant use of anthelmintics has favored the selection of resistant populations, making it unfeasible to raise these animals in pastures (Santos et al., 2017). Additionally, the resistance to anthelmintics has been detected in nematodes from small ruminants on several continents (Raza et al., 2016; Bastos et al., 2017). However, the use of plant extracts to reduce populations of multiresistant nematodes may be a key strategy for integrated control programs against gastrointestinal nematodes from ruminants (Githiori et al., 2005; Tariq et al., 2017).

Annonacea has showed high *in vitro* efficacies against *H. contortus* (Alawa et al., 2003; Ferreira et al., 2013). *Annona crassiflora* Mart. popularly known as "panã" or "araticum" is a tree species widely used by the human population for the nutritional value of its fruits (Araya, 2004). Ethanolic extracts of leaves and peel of this plant have also demonstrated excellent antioxidant activity (Roesler et al., 2007). The oil from the its seeds is used to treat scalp infections, whereas an infusion of its seeds and leaves is used in folk medicine as an antidiarrheal (Silva Júnior, 2005). The aqueous extract (AE) of these seed has showed notably antihelmintic effect; however, lambs orally treated with extract presented diarrhea and severe lesions followed by death (Oliveira et al., 2011). In the previous study, we observed high efficacy of leaf extract of *A. crassiflora* to *in vitro* inhibition of the hatchability and larval development of *H. contortus* resistant to albendazol.

The seeds and fruits of this plant were not produced during all periods of year; differently, abundant leaf masses are available during all seasons. However, the *in vivo* anthelmintic effects and its potential benefits or toxicity for the treatment with leaves of this Annonacea in lambs with haemonchosis is not known. In this study, the aims were to evaluate the oral toxicity of AE of *A. crassiflora* leaves in mince and the blood and parasitological parameters of lambs with a high

infection of resistant *H. contortus* and treated with leaf powder of this plant.

Materials and Methods

Collection of plant material

Young leaves were collected and samples were deposited in the Montes Claros Herbarium of Universidade Estadual de Montes Claros, as voucher specimen 3475. The aqueous extract (AE), were produced according to Morais-Costa et al. (2015) as wrote in the previous manuscript (part 1).

Toxicity in mice

To determine the maximum tolerated dose in adult mice, tests were performed according to Walum (1998). On days 1 and 2, this extract was administrated at 2.03 mg/kg and 20.3 mg/kg, respectively. Subsequently, the extract was administered at 203.0 mg/kg on days 3 and 4. The mice were euthanized by cervical dislocation on day 5 after AE administration and internal organs and mucosa were clinically examined at necropsy. These Experimental procedures were carried out in accordance with the Animal Experience Ethical Committee of Minas Gerais Federal University (CETEA – UFMG) and approved by this committee, under protocol number 042/2008.

In vivo test in lambs

Sixteen 4-6-month-old Santa Inês x Dorper lambs of mean bw 25.5 kg (eight male, eight female) were subjected to a fecal egg reduction test (Coles et al., 1992). These lambs were infected with 2000 L3 of a strain albendazole-resistant *H. contortus*. This strain was obtained from sheep raised on a farm located in Montes Claros, Minas Gerais State, Brazil, which was treated with albendazole whit efficacies < 70% (Duarte et al., 2012).

A group of untreated lambs served as negative control; a second group, was administered a dose of 2.75g (dm)/kg bw) in a same volume of concentrate during three days. The dose was based on the LC₉₀ estimated by the LDI test reported in the previous study (part 1), considering the volumes of pre-stomachs and abomasums of lambs (Nogueira et al., 2012). Treatment was conducted in the morning, following 12 h fasting. Lambs were monitored for clinical signs and weighed in the morning before feeding on the day of treatment and on days 7 and 14 after treatment. The mean weight gain of groups was compared by Duncan's test at 5% significance level.

Fecal egg counts were recorded on the two days prior to treatment and on the day of treatment (initial period, before treatment), with the mean used to standardize levels for each lamb group. Subsequently, mean FEC was calculated on days 7, 8, and 9 (second period); days 14, 15, and 16 (third period). Each period covered an average of 3 days, obtaining two counts each day (Morais-Costa et al., 2016).

The modified McMaster technique was performed with saturated NaCl with minimum sensitivity of 25 eggs/g of feces (Gordon and Whitlock, 1939). The morphological identification, according to Keith (1953), for L3 from fecal cultures showed 100% of larvae of the genus *Haemonchus*. The efficacies of treatments were calculated by formula adapted from Coles et al. (1992):

The efficacy of treatments was calculated by formula adapted from Coles et al. (1992):

$$\% \text{ FEC reduction} = 100 \times [1 - (\text{FEC mean of treated group} / \text{FEC mean of untreated group})]$$

The FEC data obtained were transformed to $\log_{10}(x + 10)$ and subjected to ANOVA in a split plot design with respect to the four evaluated periods. Means were compared by the Duncan' test ($P < 0.05$) using SAEG 9.1 software.

Blood parameters

Blood samples were collected from the jugular vein into tubes containing ethylene diamine tetra-acetic acid (EDTA) and stored at 4 °C (Morais-Costa et al., 2016). The blood cell

numbers and hematocrit were evaluated in an electronic automatic analyzer (2.800 BC Vet®, Mindray Medical International Ltd. Shenzhen, China). Data were subjected to ANOVA and Pearson's correlation with split plots, considering 5% significance and, using SAEG 9.1 software.

Both *in vivo* parasitological test and blood exams were approved by the Ethics Committee on the use of animals (CEUA) of the Federal University of Minas Gerais, Brazil, under number 275/2013.

Results

Toxicity in mice

In the mice toxicity tests, no clinical alterations in mucosal tissue, changes in animal behavior, or mortality were observed in the mice during the 4 days of extract administration. The maximum dose of 203.0 mg/kg bw was tolerated by both male and female mice. At necropsy, macroscopic examination revealed no abnormalities of the kidney, spleen, lung, liver, or other viscera.

In vivo at sheep

The sheep initially presented mean FEC 1167 ± 45 , and after treatment with *A. crassiflora* leaves no FEC reduction was observed in comparison with the untreated group (Table 1). However, for both groups, significant FEC reduction was observed in the second week post-treatment (648.85 ± 29.91).

Table 1. Mean fecal egg count in lambs after feed of *Annona crassiflora* leaves at 2.75mg (dm)/kg bw and untreated control

Groups of lambs	Begin	First week	Second week
Untreated	1273.60 ± 137.5 ^a	1123.30 ± 123.5 ^a	627.7 ± 94.1 ^b
<i>A. crassiflora</i>	1061.30 ± 95.5 ^a	1406.70 ± 140.0 ^a	670.0 ± 60.3 ^b

Different uppercase letters in rows and lowercase letters in columns indicate significant differences by Duncan' test ($P < 0.05$). Efficacy = $100 \times (1 - \text{mean FEC of treated group} / \text{mean FEC of control group})$.

After the third week post-treatment, both groups presented greater weight gain as compared to the other evaluated periods ($P < 0.05$). Animals treated with *A. crassiflora* showed no behavioral changes, submandibular edema, weakness, or loss of appetite during the experiment (Table 2). Five untreated animals presented diarrhea after the first week, which lasted approximately 4 days which was not observed for the treated lambs.

Blood profile

Average erythrocyte number, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and corpuscular hemoglobin concentration differed significantly depending on the evaluated periods ($P < 0.05$), but were not influenced by the treatment (Table 3). In the third period, segmented neutrophil concentrations were higher than the other analyzed periods (Table 4; $P < 0.05$).

A negative Pearson's correlation was observed between the FEC means and red cell distribution width (RDW) ($-0.43, P < 0.05$), bw and corpuscular hemoglobin concentration mean (CHCM) ($-0.53, P < 0.05$) and between bw and

monocyte concentration ($-0.72, P < 0.01$). Positive correlations were detected between FEC mean and platelet values ($0.55, P < 0.01$), bw and hemoglobin concentration ($0.51, P < 0.05$) and between bw and hematocrit ($0.58, P < 0.01$).

Table 2. Effects of oral administration of *Annona crassiflora* Mart. leaves (2.75g / kg) on body weight (bw) and weight gain (gw) in kg for lambs infected with *Haemonchus contortus*

Groups	Begin	First week		Second week		Third week	
	bw	bw	gw	bw	gw	bw	gw
<i>A. crassiflora</i>	26.91	26.85	-0.06 ^b	26.59	-0.26 ^b	27.72	1.13 ^a
Untreated	25.22	24.45	-0.70 ^b	24.58	0.13 ^b	25.63	1.05 ^a

Different letters in the lines indicate significant differences with the Duncan's test ($P < 0.05$). Coefficient of variation of body weight = 30.64% and Coefficient of variation of weight gain = 32.93%

Table 3. Red blood profile in lambs infected with *Haemonchus contortus* and treated orally with *Annona crassiflora* leaves (2.75g / kg bw) or untreated.

Groups	Uninfected	Day of treatment	Second week post-treatment	Reference values	CV (%)
Erythrocytes ($\times 10^6 / \mu\text{L}$)					
Untreated	5.82	6.02	15.31		
<i>A. crassiflora</i>	5.69	5.69	14.77	9- 15	13.06
Average	5.75 ^B	5.8 ^B	15.04 ^A		
Hemoglobin (g/dL)					
Untreated	14.38	15.08	15.12		
<i>A. crassiflora</i>	14.63	14.93	14.63	9- 15	5.94
Hematocrit (%)					
Untreated	41.73	43.73	44.98		
<i>A. crassiflora</i>	31.45	43.00	42.65	27 - 45	17.66
Average	36.58 ^B	43.36 ^A	43.81 ^A		
Mean corpuscular volume(fl)					
Untreated	71.64	73.45	29.39		
<i>A. crassiflora</i>	75.66	77.91	30.14	28 - 40	12.34
Average	73.65 ^A	75.68 ^A	29.76 ^B		
Mean corpuscular hemoglobin concentration (g/l)*					
Untreated	34.44	34.65	33.66		
<i>A. crassiflora</i>	34.32	34.84	33.04	31 - 34	3.37
Average	34.38 ^A	34.74 ^A	33.34 ^B		
Amplitude distribution erythrocytes (%)					
Untreated	17.15	16.55	16.42		
<i>A. crassiflora</i>	16.70	16.93	16.48	17.8-19.3	4.029
Platelet /mm ³					
Untreated	276500.0	435500.0	435166.7		
<i>A. crassiflora</i>	352250.0	531000.0	527666.7		1.83

Values with different letters differ significantly by Duncan's test ($P < 0.05$). * = Reference range for sheep (Kahn and Line, 2010). CV = Coefficient of variation.

Discussion

In this study, despite a low toxicity in orally treated mice, the *in vivo* administration of *A. crassiflora* leaves at 2.75 g (ms)/kg bw for 3 days showed no FEC activity. These differences between *in vitro* and *in vivo* anthelmintic efficacies in plant treatments have been previously reported (Peneluc et al., 2009; Nogueira et al., 2012) and could be associated with bioavailability of plant compounds in different segments of the ruminant gastrointestinal tract (Athanasiadou et al., 2007; Eguale et al., 2006). Adult nematodes may also be

more tolerant to the vegetal active components, or rumen microbiota may reduce metabolite activity (Nogueira et al., 2012).

The significant reduction in FEC mean observed in the second week post-treatment could be related to an improved immune response of the animals with increasing age (Ciarlini et al., 2002). The weight gain observed in lambs treated with *A. crassiflora* leaves was similar those of control group; which was significantly higher and correlated with FEC reduction in the last week of the experiment.

No physiological changes were observed in blood parameters after 15 days of *A. crassiflora* administration or in the control group. The hematocrit mean was > 35% for all lambs treated with *A. crassiflora* leaves representing a normal value according to Harvey (2012). The reductions in hematocrit and erythrocyte counts are indicative of anemia and are important parameters for

evaluation of the infection and pathogenesis of hematophagous nematodes (Bowman, 2010). The lambs fed *A. crassiflora* leaves presented blood values within the reference standards 2 weeks after treatment (Table 4), indicating that plant metabolites did not promote hematopoiesis inhibition.

Table 4. Mean values for leukocytes, neutrophils, lymphocytes, monocytes and eosinophils in sheep treated with *Annona crassiflora* leaves (2.75g / kg bw) or untreated.

Groups	Uninfected	Day of treatment	Second week pos-treatment	Reference values *	CV (%)
Leukocytes / μ L					
Untreated	8950.0	8883.33	9666.67	4.000 a 12.000	1.9
<i>A. crassiflora</i>	7900.0	10416.7	10366.7		
Neutrophils / μ L					
Untreated	5878.75	6004.50	6870.83	3000 a 15000/ μ L	2.11
<i>A. crassiflora</i>	5144.50	6792.83	6969.50		
Banded neutrophils / μ L					
Untreated	1	1	1	0 a 300 / μ L	150.59
<i>A. crassiflora</i>	1	3	1		
Segmented neutrophils / μ L**					
Untreated	5833.5	5940.3	6802.5	3.000 a 11.500/ μ L	2.07
<i>A. crassiflora</i>	5097.8	6585.2	6905.5		
Média	5455.6 ^B	6262.7 ^B	6854.0 ^A		
Eosinophils / μ L					
Untreated	354.50	340.50	247.17	100 a 1259/ μ L	47.0
<i>A. crassiflora</i>	270.75	286.67	287.0		
Lymphocytes/ μ L					
Untreated	2448.25	2251.66	2229.33	2000 a 9000/ μ L	2.84
<i>A. crassiflora</i>	2201.75	2926.17	2835.67		
Monocytes / μ L					
Untreated	268.5	286.0	319.17	0 a 750	1.83
<i>A. crassiflora</i>	283.0	411.0	274.50		

Values with different letters differ significantly by Kruskal-Wallis's test ($P < 0.05$). * = Reference range for sheep (Kahn and Line, 2010). CV = Coefficient of variation.

In acute haemonchosis, anemia is characterized by a progressive and rapid decrease in erythrocyte count (Harvey, 2012) and hematocrit values below 15% are concomitant with weakness and indicate poor prognosis (Bowman, 2010). In this study, lambs presented anemia with reduction in erythrocyte concentrations prior to treatment due to nematode spoliation. However, a normal erythrocyte count was observed 2 weeks after treatment for both groups, suggesting a reduction in blood spoliation owing to improved tolerance of the animals to the parasitism, as evidenced by the negative correlation with FEC average in the penultimate week of the study. The proportion of segmented neutrophils increased in the last period, which could indicate recovery of the inflammatory process in the abomasum mucosa promoted by the nematode.

Conclusion

Despite the low toxicity in orally treated mice and no alterations to the normal blood parameters of lambs, the administration of dry leaves of *A. crassiflora* to the lambs with haemonchosis did not reduce the FEC in comparison to the untreated group. Further research using higher dosages, with more frequent administration and new formulations is needed to evaluate the potential of utilizing this plant as an alternative anthelmintic control or as a food source for ruminants.

Conflict of Interest

The authors of this manuscript have no financial or personal relationship with individuals or organizations that could influence or bias the content of the paper.

Ethics Committee

The research project was approved by Animal Experience Ethical Committee of Minas Gerais Federal University (CETEA – UFMG) and approved by this committee, under protocol number 042/2008 and by the ethics committee of the Ethics Committee on the use of animals (CEUA) of the Federal University of Minas Gerais, Brazil, under number 275/2013.

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