Anthelmintic activity of *Annona crassiflora* leaves against *Haemonchus contortus*: part 1: *in vitro* inhibition of the hatchability and larval development

[*Atividade antihelmíntica de folhas de Annona crassiflora contra Haemonchus contortus: parte 1: inibição in vitro da eclodibilidade e do desenvolvimento larval*]

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Abstract
We evaluated the activity of *Annona crassiflora* leaves against *Haemonchus contortus* resistant to albendazol. Aqueous (AE), ethanolic (EE) and ethyl acetate (EAE) extracts were produced and the predominant presence of flavonoids was observed in HPLC-DAD chromatograms. Initially we evaluated the larval development inhibition (LDI) of dry *A. crassiflora* leaf powder or its AE directly in fecal quantitative cultures. The efficacies of the extracts, with or without tannins, on egg hatching inhibition (EHI) were investigated reveling that the EE was the most effective (LC_{90} = 8.96 mg/mL). However, after tannin removal, AE showed the highest activity (LC_{90} = 4.27 mg/mL). In the LDI test, the LC_{90} of AE was < 6.25 mg/g of fecal culture and to leaf powder of leaves was 69.14 mg/g. High efficacies of AE and EE for EHI were detected and the tannins were not the main active metabolites. The anthelmintic potential of this plant could be attributed to association between flavonoids and other metabolites.

Keywords: Cerrado; nematode; flavonoids; quantitative coproculture.

Resumo
Avaliou-se a atividade de folhas de *Annona crassiflora* contra *Haemonchus contortus* resistentes ao albendazol. Extratos aquosos (EA), etanólicos (EE) e acetato de etila (EAE) foram produzidos e a presença predominante de flavonoides foi observada em cromatogramas HPLC-DAD. Inicialmente avaliou-se a inibição do desenvolvimento larval (IDL) do pó seco de folhas de *A. crassiflora* e seu EA diretamente em coproculturas quantitativas. As eficácias dos extratos, com ou sem taninos, na inibição da eclosão dos ovos (IEO) foram investigadas revelando que o EE foi o mais efetivo (CL_{90} = 8,96 mg/mL). No entanto, após a remoção do tanino, o EA apresentou a maior atividade (CL_{90} = 4,27 mg/mL). No teste IDL, a CL_{90} de EA foi <6,25 mg/g na coprocultura e o pó desidratado das folhas foi de 69,14 mg/g. A alta eficácia do EA e EE para IEO foram detectados e os taninos não foram os principais metabólitos ativos. O potencial anti-helmíntico dessa planta poderia ser atribuído à associação entre flavonoides e outros metabólitos.

Palavras-chave: Cerrado; nematódeo; flavonoides; coprocultura quantitativa.

Introduction

Gastrointestinal nematodes (GN) reduce weight gain (WG), meat, wool, and milk production of sheep (Miller et al., 2012). *Haemonchus contortus* is the most common parasite in small ruminants reared in tropical and sub-tropical regions, promoting high mortality in lambs (Bastos et al., 2017).

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Most of the anthelmintics currently available on the market were developed after the 1960s and are essential in the control of *H. contortus* (Amarante and Sales, 2007). However, resistance to these products has been detected in GN of small ruminants on several continents (Raza et al., 2016; Bastos et al., 2017). The constant use of anthelmintics favors the selection of resistant populations, making it unfeasible to raise these animals in pastures (Santos et al., 2017).

Utilization of plants has provided alternatives for GN control (Githiori et al., 2005). The Cerrado biome occupies a large part of Central and South America and among its typical vegetal species, *Annona crassiflora* Mart. (Annonaceae), 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). However, lambs that received aqueous extract (AE) of these seed for anthelmintic treatment presented diarrhea and severe lesions followed by death (Oliveira et al., 2011).

The AE of the *Annona senegalensis* Pers. shells significantly reduced *H. contortus* hatchability (Alawa et al., 2003), while the 50% AE of *Annona muricata* leaves inhibited the hatching of this nematode with 84.91% efficacy (Ferreira et al., 2013). However, the anthelmintic effects of *A. crassiflora* extracts from leaves remain unknown. In this firth study, the aims were evaluated the effects of *A. crassiflora* leaves on the hatchability and larval development of *H. contortus*.

Materials and Methods

Collection of plant material

Young leaves were collected from eight individual adult *A. crassiflora* plants measuring a height of approximately 5 m, in March 2016 in a rural community of Montes Claros, Minas Gerais, Brazil (16°54′11″ S, 43°52′02″ W). The climate of this region is marked by a dry season from May to September and a rainy period in January and February and is classified as tropical wet with dry summer (As) according to the Köppen classification (Alvares et al., 2013).

Healthy leaves were selected and dried to constant weight in a forced air circulating dryer (TE 394/4, Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil) at 40 °C for 72 h. Dried leaves were ground in a Wiley mill (CE-430/Macro, Cienlab, SP, Brazil) and stored in paper bags in darkness (Morais-Costa et al., 2015). Plant samples were deposited in the Montes Claros Herbarium of Universidade Estadual de Montes Claros, as voucher specimen 3475.

Extract production

The aqueous extract (AE), ethanolic extract (EE) and ethyl acetate extract (EAE) were produced according to Morais-Costa et al. (2015). Sub-samples of all extracts were submitted to condensed tannin extraction according to the method proposed by Nyman et al. (1998). The supernatants were used to assess the effects of tannin-free extracts on egg hatching inhibition (EHI).

Extract characterization

We used a Waters Alliance 2695 HPLC system composed of a quaternary pump, an auto-sampler, a photodiode array detector (DAD) 2996, and a Waters Empower Pro data handling system (Waters Corporation, Milford, Connecticut, USA). The chromatograms were obtained at 210 nm, and the UV spectra were recorded on-line from 190 to 400 nm.

Total condensed tannin (proanthocyanidins) was according to the method described by Hiermann et al. (1986).

Egg hatching inhibition

A modified methodology of the hatchability inhibition test, proposed by the World Association for the Advancement of Veterinary Parasitology (Coles et al., 1992), was used to select the type of extract with better anthelmintic effect as described by Morais-Costa et al. (2016). Three Santa Inês 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%. Only *H. contortus* L3 produced in fecal cultures made with samples obtained from those treated sheep, were used to infect worm-free lamb (Duarte et al. 2012). After 22 days, the lambs showed a mean fecal egg count (FEC) >1500/g, determined using the modified McMaster technique (Gordon and Whitlock, 1939). Sedimentation in water, filtration, and flotation in saturated saline were conducted to obtain nematode eggs from lamb feces (Coles et al., 1992).

The experiments were performed in five replicates. Positive controls were exposed to levamisole phosphate (0.3 mg/mL) and the negative control was treated with sterile purified

water. Experimental treatments using *A. crassiflora* extracts with tannins were performed at 168.5, 84.3, 42.1, and 21.6 mg/mL, and without tannins at 10.0, 5.0, 2.5, 1.3, and 0.6 mg/mL.

In microwell plates (96 wells), we placed the mixtures comprising 100 µL of egg suspension containing ±80 fresh eggs and 100 µL of the extracts or controls (Nogueira et al., 2014, Morais-Costa et al., 2016).

The number of L1 relative to the initial number of eggs (remaining eggs plus L1) was determined for each replicate and subjected to variance analysis. Means were compared using Duncan's test ($P < 0.05$). Probit regression was used to estimate concentrations which inhibited 90% (LC_{90}) of egg hatching, using SAEG 9.1 software. The formula of Coles et al. (1992) was used to determine the % EHI:

% EHI = 100 × (1 - L1/initial number of eggs).

Larval development inhibition in quantitative cultures

We used the three lambs as described above which showed 2300 ± 250 FEC and 100% of *Haemonchus* spp. larvae after fecal cultures (Keith, 1953). Pooled feces were and immediately used in the quantitative coproculture test (Borges, 2003; Morais-Costa et al., 2016). The anthelmintic activity of the dried *A. crassiflora* leaf powder was evaluated at final concentrations of 83.3-333.3 mg/g of fecal culture. The final concentration of *A. crassiflora* leaf powder was achieved by replacing the vermiculite. The AE was evaluated at 6.25-50.0 mg/g of fecal culture. A solution of levamisole phosphate was used as a positive control (0.3mg/g) and sterile distilled water was the negative control.

The feces were collected directly from the rectal ampulla and all concentrations and the controls were evaluated in five replicates, which were incubated in a refrigerated chamber at 28 °C for 7 days (Nery et al, 2010; Morais-Costa et al., 2016).

The number of LPGF (L3/feces) was counted in a Sedgewick chamber with an optical microscope at 100x. The percent LDI was calculated according to the formula adapted from Borges (2003):

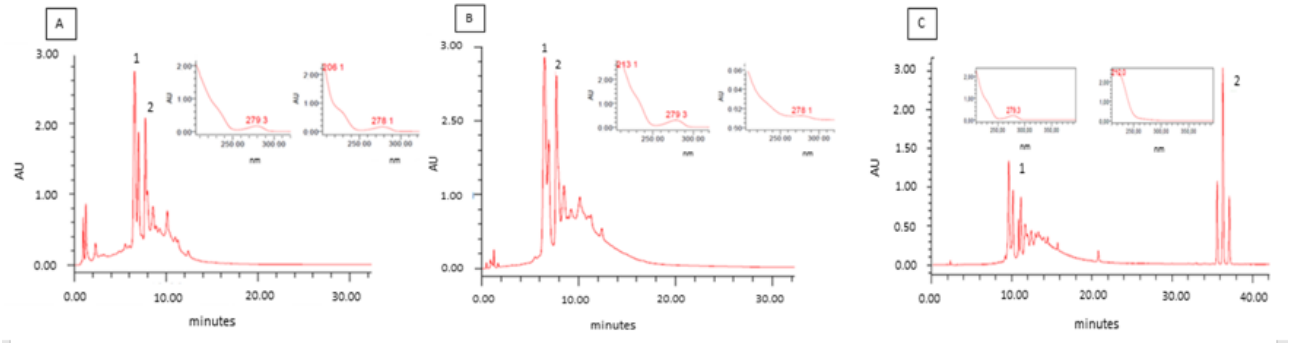
% LDI = 100 × (1 - LPGF of the treated group/LPGF of the untreated group).

The values of LPGF were transformed into $\log_{10}$ (x + 10) and subjected to analysis of variance (ANOVA). Means were compared with Duncan's test and *probit* regression was used in the estimation of LC_{90} at 5% significance using SAEG 9.1 software.

Results

Secondary metabolites of *Annona crassiflora* leaves

Chromatograms recorded by HPLC-DAD showed a similarity in the profiles of AE, EE and EAE, with a predominance of peaks corresponding to polar compounds, and UV spectra compatible with polyphenols (Figure 1A, B, and C). The two highest peaks observed indicated retention times of 6.980 and 7.731, 6.484 and 7.704, and 9.644 and 36.323 min for EA (Figure 1A), EE (Figure 1B), and EAE (Figure IC), respectively.



Data from the UV spectra were compatible with flavonoids (ʎ 279.3 and 278.1 nm for AE (Figure 1A) and EE (Figure1 B), respectively, and ʎ 279.3 and 212.0 nm for EAE (Figure 1C). The condensed tannin concentrations of AE, EE and EAE were 6.93%, 12.05%, and 0.64%, respectively.

Egg hatching inhibition

Egg hatching inhibition increased along with the concentration of the all extracts. For the AE with and without tannin, concentrations ≥ 10.53

and 2.5 mg/mL, respectively, and presented significantly lower average number of hatched larvae than the negative controls (Table 1). The LC_{90} for the extract with tannin was 85.48 mg/mL (95% CI: confidence interval 76.03-97.85) and 4.27 mg/mL (95% CI: 4.05-4.53) for the extract without tannin.

Table 1. Averages of blastomered eggs, embryonated eggs, *Haemonchus contortus* L1 Larvae and efficacy of aqueous extract of *Annona crassiflora* Mart. (Annonaceae) leaves with and without tannins.

Concentration mg/ml	Blastomered eggs	Embryonated eggs	Larvae L1	Eggs + Larvae	Efficacy (%)*
With tannin					
168.5	21.2 ^{c}	4.2 ^{cde}	2.0 ^{f}	27.4	97.2
84.3	33.2 ^{b}	2.2 ^{ef}	9.5 ^{e}	45.0	86.6
42.1	36.8 ^{b}	2.5 ^{de}	12.5 ^{e}	51.8	82.6
21.0	25.3 ^{c}	6.0 ^{c}	35.7 ^{d}	67.0	49.6
10.53	12.8 ^{d}	4.0 ^{cde}	46.5 ^{c}	63.3	34.5
Without tannins					
10.0	3.5 ^{e}	44.0 ^{b}	0.0 ^{f}	47.5	100.0
5.0	2.5 ^{e}	55.2 ^{a}	0.0 ^{f}	57.7	100.0
2.5	1.2 ^{e}	2.0 ^{ef}	49.5 ^{c}	52.7	30.3
1.3	0.7 ^{e}	5.3 ^{cd}	67.0 ^{b}	73.0	5.6
0.6	1.0 ^{e}	3.0 ^{de}	73.2 ^{a}	77.2	---
Levamisol	85.0 ^{a}	0.0 ^{f}	0.0 ^{f}	85.0	100.0
Untreated	0 ^{e}	0 ^{f}	71.0 ^{a}	71.0	---
CV (%)	23.1	18.2	9.8		

Different lower case letters indicate significant differences ($p<0.05$) by Duncan'test
(CV) Coefficient of variation,
* % efficacy = 100 x (1 - mean of L1/ mean eggs + L1)

For EE and EAE, with and without tannin, concentrations ≥ 21.6 and 1.3 mg/mL, respectively, and showed lower ($P < 0.05$) hatched larvae averages than the negative controls (Table 2 and 3). For EE with and without tannin, the LC_{90} was 8.95 (95% CI: 1.49-1.32) and 7.98 mg/mL (95% CI: 7.28-8.86), respectively. However, EAE showed a greater variation in LC_{90} values, with concentrations of 81.83 mg/mL (95% CI: 73.30-92-75) with tannin and 4.84 mg/mL (95% CI: 4.51-5.22) without tannin. There was a linear reduction in the number of eggs and larvae detected in the EHI test, which was inversely related to the concentration of the extracts.

Table 2. Averages of blastomered eggs, embryonated eggs, *Haemonchus contortus* L1 Larve and efficacy of ethanolic extract of *Annona crassiflora* Mart. (Annonaceae) leaves with and without tannins.

Concentration mg/ml	Blastomered eggs	Embryonated eggs	Larvae L1	Eggs + Larvae	Efficacy (%)*
With tannin					
337.0	0.0 ^{e}	0.0 ^{ef}	0.0 ^{e}	0.0	100.0
168.5	31.8 ^{b}	4.0 ^{d}	1.5 ^{d}	37.3	97.4
84.3	3.5 ^{e}	4.0 ^{d}	2.5 ^{d}	10.0	95.7
42.1	3.3 ^{e}	4.5 ^{cd}	3.0 ^{d}	10.8	94.8
21.6	3.3 ^{e}	3.5 ^{d}	3.8 ^{d}	10.6	93.5
Without tannins					
10.0	11.5 ^{c}	37.8 ^{a}	4.0 ^{d}	53.3	93.1
5.0	3.0 ^{e}	6.3 ^{c}	11.8 ^{d}	21.1	79.8
2.5	1.0 ^{f}	1.0 ^{e}	29.0 ^{c}	31.0	40.5
1.3	0.0 ^{f}	0.0 ^{e}	44.5 ^{b}	44.5	23.3
0.6	0.0 ^{d}	0.0 ^{e}	56.5 ^{a}	56.5	2.5
Levamisol	50.3 ^{a}	0.0 ^{e}	0.0 ^{d}	50.3	100
Untreated	0 ^{f}	0 ^{e}	58.0 ^{a}	58.0	---
CV (%)	10.5	16.2	15.7		

Different lower case letters indicate significant differences ($p<0.05$) by Duncan'test
(CV) Coefficient of variation,
* % efficacy = 100 x (1 - mean of L1/ mean eggs + L1)

Inhibition of larval development

The treatment with dehydrated *A. crassiflora* leaves or its AE reduced the mean number of infective larvae when compared to the negative control ($P < 0.05$, table 4). For AE, the LC_{90} was not estimated because all concentrations evaluated showed 100% of efficacy to reduce the larval development. The anthelmintic efficacy increased with leaf concentration of power leaves and the estimated LC_{90} was 69.14 mg/g of fecal culture.

Table 3. Averages of blastomered eggs, embryonated eggs, *Haemonchus contortus* L1 Larve and efficacy of ethyl acetate extract of *Annona crassiflora* Mart. (Annonaceae) leaves with and without tannins.

Concentration mg/ml	Blastomered eggs	Embryonated eggs	Larvae L1	Eggs + Larvae	Efficacy (%)*
With tannin					
337.0	0 ^{e}	0 ^{ef}	0 ^{e}	0	100.00
168.5	0 ^{e}	0 ^{ef}	1.6 ^{e}	1.6	95.43
84.3	5.8 ^{b}	0.8 ^{ef}	3.0 ^{e}	9.6	91.43
42.1	1.8 ^{d}	1.8 ^{d}	9.6 ^{d}	13.2	72.57
21.0	3.4 ^{c}	3.4 ^{c}	10.8 ^{cd}	17.6	69.14
Without tannins					
10.0	2.0 ^{d}	10.2 ^{b}	0.6 ^{e}	12.8	98.28
5.0	1.8 ^{d}	1.4 ^{de}	2.6 ^{e}	5.8	92.57
2.5	2.4 ^{cd}	1.4 ^{de}	13.0 ^{c}	16.8	62.85
1.3	2.8 ^{cd}	0.4 ^{def}	2.6 ^{b}	5.8	25.71
0.6	2.0 ^{d}	1.0 ^{def}	33.4 ^{a}	36.4	4.57
Levamisol	13.8 ^{a}	22.0 ^{a}	0 ^{e}	35.8	100.00
Untreated	0 ^{e}	0 ^{f}	35 ^{a}	35.0	---
CV (%)	32.84	28.60	18.93		

Different lower-case letters indicate significant differences ($p<0.05$) by Duncan'test
(CV) Coefficient of variation,
* % efficacy = 100 x (1 - mean of L1/ mean eggs + L1)

Table 4. Mean values *Haemonchus contortus* infecting larvae from treated coprocultures with different concentrations of the dehydrated leaf or aqueous extract of *Annona crassiflora* Mart. (Annonaceae), with levamisol (0.025mg/g) or untreated.

Treatments (mg/g)	LDGF*	Efficacy (%)**
333.3	12.0 ±1.8 ^{d}	99.72
250.0	10.0 ±-1.5^{d}	99.76
166.6	38.0 ±-5.32^{c}	99.12
83.3	90.0 ±-14.4^{b}	97.91
Untreated	4325.0 ±-646.75 ^{a}	---
Levamisol (0.025 mg/g)	0.00 ±- 0.0 ^{d}	100.00

* LDGF: Number of infective larvae (L3) /g feces.
Different letters in the columns indicate significant differences by the Duncan test with $P < 0.05$.
**Efficacy (%) = 100 x (1 – LPGF of the treated group/LPGF of the untreated group), adapted from Borges (2003).

Discussion

Haemonchus contortus infections in sheep herds can lead to several negative effects, such as an increase in production cost (Miller et al., 2012). The emergence of multi-drug resistant nematode populations has prompted a significant effort to find alternatives to conventional anthelmintic medicines. In this study, the leaf extracts of *A. crassiflora*, a tree common to the Cerrado, showed significant EHI and LDI efficacy against *H. contortus*. HPLC analysis revealed the presence of flavonoids in the leaf extracts, which mantained EHI activity, even after tannin removal.

The extracts containing tannins showed differences in EHI efficacy, with EE that showed the most being the most effective (LC_{90} 8.96 mg/mL). However, after tannin removal, AE was the more effective extract (LC_{90} 4.27 mg/mL). For AE and EAE without the presence of tannins, substantial reduction of LC_{90} was verified, increasing the EHI efficacy, which suggests the presence of other metabolites effective in the inhibition of embryogenesis and hatchability.

These results suggest that flavonoids, the presence of which was confirmed in the chromatograms, were the metabolites primarily responsible for this anthelmintic effect. In other

Annonaceae plants evaluated, the most frequent flavonoids were the O-glycosides of apigenin, kaempferol, quercetin and luteolin (Santos and Salatino, 2000). Other investigations involving species of the *Annona* genus have shown lower efficacies than those observed for *A. crassiflora* extracts. The methanolic extract of *Annona squamosa* bark at 6.25 mg/mL demonstrated an EHI efficacy of 77.4% (Kamaraj and Rahuman, 2011) and Ferreira et al. (2013) reported an efficacy of 84% for AE of *A. muricata* leaves at 50% final dilution.

Our results clearly demonstrated elevation of EHI efficacy after tannin removal from EA and EAE of *A. crassiflora* leaves, signified by reductions of their LC_{90} values. This observation was also verified in the study of other plants of the Brazilian Cerrado. The hydroalcoholic extracts of *Turnera ulmifolia* leaves showed the highest EHI. The inhibitory effects this extract was blocked by tannin removal (Oliveira et al., 2017).

The study of AE and EE derived from *Piptadenia viridiflora* leaves, a common tree of the Cerrado, showed the presence of flavonoids in HPLC chromatograms. In EHI tests, the LC_{90} of AE was 2.4 mg/mL, and of EE was 2.1 mg/mL. Similarly, after tannin extraction, higher EHI was observed (Morais-Costa et al., 2016).

In analyzing the larval exsheathment inhibition (LEI) activity of plant extracts, the flavonoids naringenin, quercetin, and luteolin showed high efficacy at 250 µM. These metabolites presented synergistic effects enhancing the LEI activity of procyanidin tannins (Klongsiriwet et al., 2015). African browse plant extracts showed high LEI activity, resulting from the presence of both phenolic and non-phenolic compounds. The concentration of condensed tannins was not necessarily related to the anthelmintic properties of the extracts against L3 *H. contortus* (Mengistu et al., 2017).

The flavonoid's anthelminitic effects and their synergism with other plant compounds have not yet been fully understood. However, their action may be attributed to their effects on enzyme activity and metabolic processes in parasites (Kerboeuf et al., 2008). In this research were verified the reduction of parasitic structures, which were inversely proportional to the concentration of the extracts of *A. crassiflora* leaves. This was also observed with the AE extract of *Genipa americana* leaves (Nogueira et al., 2014), the reduction of eggs and larvae that possibly was action of the enzymes as proteases.

The *A. crassiflora* leaf powder demonstrated notable LDI activity at 69.14 mg/g of fecal culture. However, the AE showed better efficacy because its LC_{90} was < 6.25 mg/g of fecal culture. These results indicated the metabolites of these leaves exhibiting the anthelmintic efficacy under natural conditions, because these substances were evaluated together with feces, where naturally occur the hatching and the larval development of GN as reported by Nery et al. 2010 and Morais-Costa et al. 2016.

Among other Cerrado species, LDI efficacies have been reported in fecal culture. Aqueous extract of *Caryocar brasiliense* peel at 200 mg/mL inhibited 94.8% of *H. contortus* larval development in fecal cultures, and phytochemical tests indicated the presence of catechins, steroids, flavonoids, saponins, xanthones, and tannins (Nogueira et al., 2012). *Ximenia americana* has low condensed tannin content (0.3%) and the major peaks of UV spectra in HPLC chromatograms are indicative of flavonoids in this species' leaf extracts. The AE of this plant at 333.3 mg dw/g of fecal culture demonstrated 99.8% LDI (Morais-Costa et al., 2015). The LC_{90} of AE of *Pipadenia viridiflora* leaves for LDI was only 13.6 mg/g and this extract showed low condensed tannin content (0.2%) and the UV data in HPLC analyze compatible with flavonoids (Morais-Costa et al., 2016), reinforcing the hypothesis that flavonoids may also act as inhibitors of nematode larval development.

Conclusion

Leaf extracts of *A. crassiflora* show the presence of flavonoids and high EHI and LDI activity against *H. contortus* resistant to albendazol. Tannins were not shown to be the principal components affecting EHI, hence it is necessary to isolate and characterize the principal active *A. crassiflora* compounds, and to assess their possible synergism

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 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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