OVICIDAL AND LARVICIDAL ACTIVITY OF Cocos nucifera L. EXTRACTS ON
Haemonchus contortus

(Atividade ovicida e larvicida de extratos de Cocos nucifera L. sobre Haemonchus contortus)

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ABSTRACT

The nematodes control is carried out with anthelmintics, but the development of resistance has required the search for alternatives such as medicinal plants. There are popular reports of antiparasitic activity of Cocos nucifera fruit. The purpose of this study was to evaluate the ovicidal and larvicidal activity of the liquid of green coconut husk fiber (LGCHF) and butanol extract obtained from LGCHF on Haemonchus contortus and investigate the chemical compounds present in these extracts. The in vitro assay was based on egg hatching (EHT) and larval development tests (LDT). The LGCHF concentrations tested in EHT ranged from 0.15 to 2.5 mg/mL, while in the LDT varied from 2.5 to 40 mg/mL. The LGCHF butanol extract concentrations used in EHT ranged from 4.06 to 65 mg/mL, whilst in the LDT varied from 5 to 80 mg/mL. The LGCHF and butanol extract showed 100% ovicidal efficacy at 2.5 and 10 mg/mL, respectively. In higher concentrations, the larvicidal effect of LGCHF and butanol extract was 81.30 and 99.80%, respectively. Phytochemical tests revealed the presence of triterpenes, saponins and condensed tannins. These results suggest the coconut extracts can be useful in gastrointestinal nematodes control of small ruminants. However, toxicological evaluation and in vivo studies are still necessary.

KEYWORDS: coconut, in vitro, gastrointestinal nematodes, sheep, goats.

RESUMO

O controle de nematoides é realizado com anti-helmínticos, mas o desenvolvimento da resistência tem exigido a busca por alternativas como as plantas medicinais. Existem relatos populares da atividade antiparasitária do fruto de Cocos nucifera. O objetivo desse estudo foi avaliar a atividade ovicida e larvicida do líquido da casca do coco verde (LCCV) e do extrato butanólico obtido a partir do LCCV sobre Haemonchus contortus e investigar os compostos químicos presentes nesses extratos. A avaliação in vitro foi baseada nos testes de eclosão de ovos (TEO) e desenvolvimento larvar (TDL). As concentrações de LCCV avaliadas no TEO variaram de 0,15 a 2,5 mg/mL, enquanto no TDL foram de 2,5 a 40 mg/mL. As concentrações do extrato butanólico do LCCV testadas no TEO variaram de 4,06 a 65 mg/mL, enquanto no TDL foram de 5 a 80 mg/mL. O LCCV e o extrato butanólico apresentaram 100% de eficácia ovicida nas concentrações de 2,5 e 10 mg/mL, respectivamente. Nas maiores concentrações, o efeito larvicida do LCCV e do extrato butanólico...
foi de 81,30 e 99,80%, respectivamente. Os testes fitoquímicos revelaram a presença de triterpenos, sapôninas e taninos condensados. Esses resultados sugerem que extratos do coco podem ser úteis no controle de nematóides gastrintestinais de pequenos ruminantes. Entretanto, estudos toxicológicos e uma avaliação anti-helmíntica in vivo ainda são necessários.

PALAVRAS-CHAVE: coco, in vitro, nematóides gastrintestinais, ovinos, caprinos.

INTRODUCTION

Gastrointestinal nematode parasites are the main cause of mortality in sheep and goats in northeastern Brazil. The nematode of greater prevalence in this region is *Haemonchus contortus* (Arosemena et al., 1999). This parasite infection may result in high losses in yielding profit because this nematode leads to a depression of appetite, changes in protein, energy and mineral metabolism. However, the main symptom of *H. contortus* infection is anemia due to hematophagy. It is estimated that the average blood loss by this parasite is 0.05 ml a day (Soulsby, 1987).

Sheep and goats gastrointestinal nematodes control is usually accomplished with anthelmintic treatment. However the development of resistant gastrointestinal nematode populations (Melo et al., 2003) is one of the most important and current problem in animal husbandry. The anthelmintics available in the trade have some limitations, such as high costs, residues in food (Herd, 1995), risk of environmental pollution and reduced efficacy in the production of sheep and goats due to low effectiveness. Considering these problems, alternatives such as phytotherapy can be used to decrease the use of synthetic products.

*Cocos nucifera* L. is a plant commonly found along the northeastern coast of Brazil, whose fruit is the coconut. The main use of coconut is the extraction of water that is well appreciated by the flavor and nutritional qualities (Senhoras, 2003). Moreover, an extensive range of popular medicinal uses of this plant has been reported. The decoction of the coconut husk ber has been used in northeastern Brazil as a traditional medicine to treat diarrhea, arthritis, bleeding and hemorrhages (Esquenazi et al., 2002; Mendonça-Filho et al., 2004). The liquid extracted from the coconut husk ber has *in vitro* antiproliferative, analgesic and antioxidant characteristics (Kirsberg et al., 2003; Alviano et al., 2004). The ethyl acetate extract obtained from the liquid of coconut husk ber showed *in vitro* antibacterial, antiviral, leishmanicidal (Esquenazi et al., 2002; Mendonça-Filho et al., 2004) and anthelmintic (Oliveira et al., 2009) activities. The objective of this study was to evaluate the ovicidal and larvicidal activity of the liquid of green coconut husk fiber (LGCHF) and butanol extract obtained from LGCHF on *H. contortus* and investigate the chemical compounds present in these extracts.

MATERIALS AND METHODS

**Plant material and extract preparation**

The LGCHF provided by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)/Agroindústria Tropical was obtained from coconut collected in fruit industry localized in Fortaleza, Ceará, Brazil. The green coconut husk fiber was crushed, pressed and filtered to obtain the LGCHF. To obtain the butanol extract, the LGCHF was filtered and subjected to three washes with solvents in 10:1 proportion using a decantation funnel. Initially, ethyl acetate was used and then butyl alcohol (butanol). The butanol solvent was eliminated in a rotary evaporator to produce the LGCHF butanol extract. To perform the tests, the LGCHF and butanol extract were diluted in distilled water and 3% dimethylsulfoxide (DMSO), respectively.
**Phytochemical study**

Phytochemical tests to detect the presence of phenols, tannins, leucoantocianidins, flavonoids, steroids, triterpenes and alkaloids were performed in LGCHF and butanol extract according to Matos (1997) methodology. These tests are based on visual observation of color modification or formation of precipitates after specific reagents addition.

In the Lieberman-Burchard test, a chloroform solution of the extracts (2 mL) is mixed with acetic anhydride (1 mL) and three drops of concentrated sulfuric acid. The development of a blue to green color indicates the presence of steroids and a red to brown color is indicative of triterpenoids. Alcoholic extracts (3 mL) in presence of an alcoholic FeCl$_3$ solution produce a dark blue precipitate in the presence of hydrolysable tannins and a green precipitate in the presence of condensed tannins or catechins. To evaluate the presence of alkaloids, the extracts were mixed with NH$_4$OH until pH 11 and the bases were extracted three times with diethyl ether–chloroform solution (3 + 1). In a separation funnel the organic layer was washed three times with HCl solution (0.1 M). The aqueous acid solution was divided into three portions and transferred to test tubes for adding the reagents which precipitate alkaloids: Hager (saturated solution of picric acid), Mayer (aqueous solution of mercuric chloride and potassium iodide) and Dragendorff (aqueous solution of bismuth-carbonate, hydrochloric acid and potassium iodide).

**Total tannins quantification**

The quantification of total tannins was performed in LGCHF and butanol extract according to Pansera et al. (2003). We used 5 mg of LGCHF and butanol extract diluted in 50 mL of distilled water. At a rate of 2 mL was added 2 mL of the Folin-Denis reagent and the resulting solution was shaken vigorously, left at rest for 3 minutes. After, 2 mL 8% sodium carbonate was added to the solution, agitated and left at rest for 2 hours, followed by centrifugation at 2000 rpm for 10 minutes. Quantification total tannins was performed using as standard the tannic acid. Thus prepared to be a pattern curve of tannic acid using 4, 6, 8, 10, 12 and 14 ppm of tannic acid diluted with water. The absorbance was measured at 720 nm, and a negative control was used for each reading. From the results the calibration curve was built and used for calculating total tannins. The readings were made in a spectrophotometer Spekol 1100. Three replicates were performed for each sample.

**In vitro assays**

One sheep was maintained in a metabolic cage and treated with three anthelmintics of different active principles on alternate days. After complete natural infection elimination, 5,000 *H. contortus* third-stage larvae (L3) were orally administered. This animal was used as a source of *H. contortus* eggs and larvae. For egg recovery approximately 10 g of feces were collected directly from the rectum of this animal and processed according to the technique described by Hubert & Kerboeuf (1992). The recovered eggs were dispersed in distilled water. To obtain the first-stage larvae (L1), an aliquot of egg suspension was incubated during 24 h at 37°C.

The egg hatching test (EHT) was based on the method described by Coles et al. (1992). A 0.25 mL of suspension containing approximately 100 fresh eggs was distributed in 5 mL tubes and incubated with the same volume of LGCHF or butanol extract at room temperature. The LGCHF concentrations used were 0.15, 0.31, 0.62, 1.25 and 2.5 mg/mL, while the butanol extract concentrations were 2.5, 5, 10, 20 and 40 mg/mL. After 48 h, lugol drops were added to stop the egg hatching. All eggs and L1 were counted under a microscope. This test was accompanied by two controls: a
negative, containing distilled water to the LGCHF or containing 3% DMSO to the butanol extract, and a positive with 0.025 mg/mL thiabendazole. Three repetitions with five replicates were performed for each extract concentration and control.

The larval development test (LDT) was based on the method described by Camurça-Vasconcelos et al. (2007). A 1 mL suspension containing approximately 300 L1 and the same volume of LGCHF or butanol extract were incubated with 2 g of feces from a sheep free of gastrointestinal nematodes at room temperature. The final concentrations of LGCHF used were 4.06, 8.12, 16.25, 32.5 and 65 mg/mL, while the butanol extract concentrations were 5, 10, 20, 40 and 80 mg/mL. After an incubation period of 6 days the L3 were recovered (Roberts & O’Sullivan, 1950) and counted under a microscope. This test was accompanied by two controls: a negative, containing distilled water to the LGCHF or containing 3% DMSO to the butanol extract, and a positive with 0.025 mg/mL thiabendazole. Three repetitions with five replicates were performed for each extract concentration and control.

Table 1. Mean efficacy (percentage ± S.D.) of the liquid of green coconut husk fiber (LGCHF) on H. contortus egg hatching.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Mean efficacy ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGCHF</td>
<td>2.5 mg/mL</td>
<td>100.00 ± 0.0a</td>
</tr>
<tr>
<td>LGCHF</td>
<td>1.25 mg/mL</td>
<td>86.22 ± 10.05a</td>
</tr>
<tr>
<td>LGCHF</td>
<td>0.62 mg/mL</td>
<td>19.30 ± 4.46b</td>
</tr>
<tr>
<td>LGCHF</td>
<td>0.31 mg/mL</td>
<td>11.24 ± 4.12b</td>
</tr>
<tr>
<td>LGCHF</td>
<td>0.15 mg/mL</td>
<td>10.87 ± 5.39b</td>
</tr>
<tr>
<td>Thiaabendazole</td>
<td>0.025 mg/mL</td>
<td>99.71 ± 0.88a</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>11.95 ± 6.00b</td>
</tr>
</tbody>
</table>

Different letters indicate significantly difference (P<0.05).

Statistical analysis

In the ETH, the efficacy of each treatment was calculated by the equation: L1/(eggs + L1) x 100, while in the LDT the formula used was: (L3 in the negative control group - L3 in the treated group)/ L3 in the negative control group x 100. The results were analyzed by ANOVA and Tukey test (P<0.05) using the Graph Pad Prism 3.0. Program and expressed as mean efficacy percentage of egg hatching or larval development inhibition ± standard deviation (S.D.). The effective concentration to inhibit 50% (EC50) of egg hatching and larval development was calculated by probit method (SPSS 8.0 for Windows).

RESULTS

Phytochemical tests revealed the presence of triterpenes, saponins and condensed tannins in LGCHF and butanol extract. The percentage of total tannins in the LGCHF and butanol extract was 19.65 and 18.08%, respectively.

The mean efficacy of LGCHF and butanol extract in the EHT is shown in tables 1 and 2. The LGCHF effectiveness at 2.5 mg/mL
and 1.25 mg/mL were 100 and 86.22%, similar to 0.025 mg/mL thiabendazole. However, the other concentrations showed efficiency statistically similar to the negative control. The ovicidal efficacy of the butanol extract was 100% in the three higher concentrations tested. In the 5 and 2.5 mg/mL concentrations, the butanol extract showed different activity of the positive and negative controls (P<0.05). The EC$_{50}$ of LGCHF and butanol extract in this test were 1.02 (0.94-1.09) mg/mL and 3.65 (3.39-3.92) mg/mL, respectively.

The mean efficacy of LGCHF and butanol extract in the LDT is demonstrated in tables 3 and 4. Only 65 mg/mL LGCHF and 80 mg/mL butanol extract had been efficacy statistically similar to 0.64 μl/mL ivermectin. The other concentrations of both extracts showed negligible efficiency. The EC$_{50}$ of LGCHF and butanol extract in this test were 49.04 (45.73-50.28) mg/mL and 3.65 (3.39-3.92) mg/mL respectively.

Table 2. Mean efficacy (percentage ± S.D.) of butanol extract obtained from the liquid of green coconut husk fiber (LGCHF) on *H. contortus* egg hatching.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Mean efficacy ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGCHF butanol extract</td>
<td>40 mg/mL</td>
<td>100.00 ± 0.0$^a$</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>20 mg/mL</td>
<td>100.00 ± 0.0$^a$</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>10 mg/mL</td>
<td>100.00 ± 0.0$^a$</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>5 mg/mL</td>
<td>81.43 ± 7.90$^b$</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>2.5 mg/mL</td>
<td>13.93 ± 4.49$^c$</td>
</tr>
<tr>
<td>Thiaabendazole</td>
<td>0.025 mg/mL</td>
<td>99.48 ± 0.66$^a$</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>3%</td>
<td>4.63 ± 1.73$^d$</td>
</tr>
</tbody>
</table>

Different letters indicate significantly difference (P<0.05).

Table 3. Mean efficacy (percentage ± S.D.) of the liquid of green coconut husk fiber (LGCHF) on *H. contortus* larval development.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Mean efficacy ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGCHF</td>
<td>65 mg/mL</td>
<td>81.30 ± 5.56$^a$</td>
</tr>
<tr>
<td>LGCHF</td>
<td>32.5 mg/mL</td>
<td>9.68 ± 5.35$^b$</td>
</tr>
<tr>
<td>LGCHF</td>
<td>16.25 mg/mL</td>
<td>0.04 ± 0.13$^c$</td>
</tr>
<tr>
<td>LGCHF</td>
<td>8.12 mg/mL</td>
<td>0.00 ± 0.00$^c$</td>
</tr>
<tr>
<td>LGCHF</td>
<td>4.06 mg/mL</td>
<td>0.00 ± 0.00$^c$</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>0.64 μl/mL</td>
<td>99.98 ± 0.06$^d$</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>0.00 ± 0.02$^c$</td>
</tr>
</tbody>
</table>

Different letters indicate significantly difference (P<0.05).
52.63) mg/mL and 32.52 (18.94-60.20) mg/mL, respectively.

DISCUSSION

This work evaluated in vitro the activity of the LGCHF trying to select a substance that can be used in gastrointestinal nematodes control. LGCHF and butanol extract presented ovicidal and larvicidal activity demonstrating the existence of chemical constituents with effectiveness on H. contortus. The 100% ovicidal effect of LGCHF in a low concentration (2.5 mg/mL) was better than butanol and ethyl acetate extracts which in the same concentration were only 13.93% and 68.45% effectiveness (Oliveira et al., 2009). LGCHF also was more effective than other plants extracts with anthelmintic effect as seed hexane extract of Mangifera indica (Costa et al., 2002), leaf hexane, chloroform, ethyl acetate and methanol extracts of Spigelia anthelminia (Assis et al., 2003), Acacia mearnsii (Minho, 2006), leaf hexane and ethanol extracts of Melia azedarach (Maciel et al., 2006) and leaf ethyl acetate and ethanol extracts of Azadirachta indica (Costa et al., 2008). This result can be attributed to combination of active compounds resulting in a synergistic effect (Rates, 2001). But the action mechanism involved in ovicidal effect is unknown.

The tannins found in LGCHF and butanol extract are associated with natural defense of plants against insects, and classified according to their properties and chemical structure in hydrolysable and condensed tannins (Athanasiadou et al., 2001). Several results suggest that condensed tannins, among the various secondary metabolic present in plants and fodder, are compounds with potential anthelmintic activity. The use of quebracho (Schinops sp) extract, containing 73% of condensed tannins (Athanasiadou et al., 2001) and acacia-negra (Acacia mearnsii) extract with 15% of condensed tannins (Minho, 2006) inhibited in vitro the larvae viability of H. contortus, Teladorsagia circumcincta and Trichostrongylus vitrinus. Moreover, it was observed that monomers of condensed tannins were responsible for decrease of the egg hatching, larval development and viability of infective larvae.

Table 4. Mean efficacy (percentage ± S.D.) of butanol extract obtained from the liquid of green coconut husk fiber (LGCHF) on H. contortus larval development.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Mean efficacy ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGCHF butanol extract</td>
<td>80 mg/mL</td>
<td>99.80 ± 0.29a</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>40 mg/mL</td>
<td>62.33 ± 10.64b</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>20 mg/mL</td>
<td>13.19 ± 2.97c</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>10 mg/mL</td>
<td>2.39 ± 2.42d</td>
</tr>
<tr>
<td>LGCHF butanol extract</td>
<td>5 mg/mL</td>
<td>0.95 ± 1.30d</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>0.64 μl/mL</td>
<td>99.98 ± 0.06a</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>3%</td>
<td>1.10 ± 1.20d</td>
</tr>
</tbody>
</table>

Different letters indicate significantly difference (P<0.05).
larvae of *Trichostrongylus colubriformis* (Molan et al., 2003). The antiparasitic activity of these metabolites is attributed to its ability to bind to the free protein available for larvae nutrition; reduced nutrient availability could have resulted in larvae starvation and death (Athanasiadou et al., 2001). Schultz (1989) reported that in insects and insect larvae that ingest the condensed tannins occurs connection of these compounds with intestinal mucosa causing autolysis, leading to an inability to use nutrients by the larva. This mechanism could be condensed tannins mode of action on nematodes larvae. Furthermore, these metabolites have the ability to link protein, as proline and hydroxyproline, in the cuticle, oral cavity, esophagus, cloaca and vulva of the nematode that would result in a structural parasite change causing its death (Hoste et al., 2006).

The phytochemical tests revealed the presence of chemical constituents that may be responsible for anthelmintic activity found. Among those constituents, it was detected the presence of triterpenoids and saponins but no relevant anthelmintic effect was reported.

Though the LGCHF ethyl acetate extract had showed *in vivo* no activity against sheep gastrointestinal nematodes (Oliveira et al., 2009), the results found in this study demonstrate the possibility of using LGCHF and LGCHF butanol extract against gastrointestinal parasites of small ruminants, because although the extracts have the same origin, the composition is different. However, more detailed studies are needed to verify the toxicity, and to evaluate the effects *in vivo* and establish the adequate doses for sheep and goats.

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