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In vitro efficacy of latex and purified papain from *Carica papaya* against *Strongyloides venezuelensis* eggs and larvae

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ABSTRACT

Latex from *Carica papaya* is rich in bioactive compounds, especially papain, which may help to control parasitic diseases. This study evaluated the efficacy of latex from *C. papaya* and purified papain against *Strongyloides venezuelensis*. The Egg Hatching Test (EHT) and the Larval Motility Test (LMT) using fresh and frozen latex (250mg/mL), lyophilized latex (34mg/mL), and purified papain (2.8 mg/mL) were performed. Albendazole (0.025 mg/mL) and ivermectin (316 *ppm*) were used as positive controls. EHT and LMT were carried out through the incubation of each solution with *S. venezuelensis* eggs or larvae (± 100 specimens), and results were analyzed after 48h (EHT) or 24, 48, and 72h (LMT). EHT showed that latex preparations at higher concentrations (1:10 to 1:100) resulted in partial or complete destruction of eggs and larvae inside the eggs. The result from the 1:1,000 dilution was similar to the positive control. LMT showed effectiveness in all the tested dilutions compared to negative controls. Purified papain showed a dose-dependent response in the EHT. Purified papain (2.8 mg/ mL) showed similar results to lyophilized latex at 1:1,000 in the EHT. Latex and purified papain from *C. papaya* were effective against *S. venezuelensis* eggs and larvae *in vitro*, suggesting their potential use as an alternative treatment for strongyloidiasis.

KEYWORDS: Anthelmintic. *Carica papaya.* Latex. Papain. Strongyloidiasis.

INTRODUCTION

Strongyloidiasis is a global emerging infectious disease with highest prevalence in Southern, Eastern, and Central Europe, the Caribbean Islands, Southeast Asia, Latin America, and sub-Saharan Africa[1]. This parasitic infection is caused by *Strongyloides* species and can be life threatening, particularly in immunossupressed hosts due to chronicity or hyper infection and dissemination of the parasite to other parts of the body[2,3].

Frequently used drug therapies such as thiabendazole have not been effective in cases of disseminated *Strongyloides* sp. infection[4]. Recent reports have also indicated that these parasites have developed some level of resistance to most drug therapies[5,6], and that many current therapies have high toxicity, making treatment difficult to maintain[6,7].

Latex from *Carica papaya* is effective in controlling helminthic infections in animals[8,9,10]. Papain, another compound from this plant has anthelmintic activity through cuticle damage leading to high internal hydrostatic pressure and consequently, rupture of the parasite body[10,11,12].

New studies searching for biologically and functionally active plant compounds are essential for the development and synthesis of new drugs with less toxicity

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and less prone to drug resistance. *S. venezuelensis* can be maintained in laboratory animals to produce a large number of eggs and larvae for experiments such as the ones set up to investigate *S. stercoralis*[13].The aim of this study was to analyze the *in vitro* activity of latex and purified papain from *C. papaya* against *Strongyloides venezuelensis* eggs and larvae.

MATERIALS AND METHODS

Strongyloides venezuelensis eggs and larvae

Strongyloides venezuelensis strain was maintained through serial passages of infective larvae (L3) in Wistar rats (*Rattusnovergicus*) by subcutaneous inoculation. Feces from infected animals were used for egg collection and charcoal culture (72 h at 28 ºC) to obtain infective (L3) larvae[14].

Collection and preparation of latex and purified papain

Latex from *C. papaya* was collected from the green papaya fruit according to the procedure described by Monti *et al.*[15] with some modifications. Three to four incisions of 2 to 3 mm were made in the green fruit using a stainless steel blade. The latex was collected after 1 to 2 min. in a Falcon tube, and was used fresh, frozen (after storage at -20 ºC) or lyophilized. The quantity of purified papain used was calculated according to Azarkan*et al.*[16]. Fresh and frozen latex were diluted in phosphate buffered saline (PBS, pH 7.2) to acquire initial concentrations of 250 mg/mL, lyophilized latex and purified papain were diluted to 34 mg/mL and 2.8 mg/mL, respectively. Then 1:10 to 1:100,000 dilutions of all preparations were performed.

Egg Hatching Test (EHT) and Larval Motility Test (LMT)

The EHT was performed according to Coles *et al.*[17] with some modifications. Feces from *S. venezuelensis*-infected rats were previously homogenized in water and filtered using a 50 µm sieve. One hundred microliters of this solution containing 100 eggs were added to 100 µL of each preparation, then incubated at 28 ºC for 48 h, after which egg development was analyzed. Egg hatching and larvae from hatching were quantified using an optical microscope. Albendazole solution (0.025 mg/ mL) was used as the positive control[18].

The LMT was performed according to Cordeiro *et al.*[19] with some modifications. One hundred microliters of a solution containing 100 infective larvae (L3) were added to 100 µL of each preparation to be tested in a 24-well plate. The solution was incubated at 28 ºC, and larval motility was analyzed and quantified after 24, 48 and 72h using an optical microscope. Ivermectin solution (316 *ppm*) was used as the positive control[20].

Water and PBS were used as negative controls in both tests. Microscope images were obtained using the Leica Microsystems DM750 model camera.

Data analysis

The Inhibition of Eggs Hatching (IEH) was carried out according to Wood *et al.*[21] and to the Guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP). Results were calculated using the following formula:

IEH (%) = [(number of eggs) / (number of eggs + number of larvae)] x 100.

The Inhibition of Larval Motility (ILM) was carried out according to Al-Rofaai *et al.*[22] by using the following formula:

ILM (%) = [(%) motilityin the treatment group – (%) motilityin negative controls)/ (100 - (%) motilityin the negative control)] x 100.

Data were analyzed using the GraphPad Prism 5.0 software by calculating the half-maximal inhibitory concentration (IC_{50}). The Fisher's exact test was used to compare the experimental groups, positive and negative controls. One-way analysis of variance (ANOVA) for parametric data and the Kruskal-Wallis tests for non-parametric data were used to compare the groups. The Mann-Whitney test was used to compare data from purified papain and lyophilized latex with the same dilutions. It was considered statistically significant when $p < 0.05$.

RESULTS

Morphological changes in the development of *S. venezuelensis* eggs after treatment with latex and purified papain from *C. papaya*

High concentrations of latex preparations such as pure and 1:10 to 1:100 dilutions were not analyzed because they caused total or partial destruction of eggs represented by scarce or condensed material inside the egg, and damaged or fragmented larvae. Treatment with latex preparations and purified papain resulted in a small number of embryonated eggs compared to the negative controls (Figure 1).

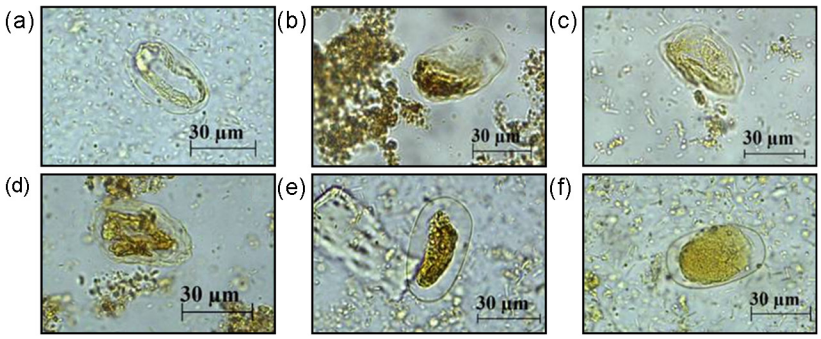

Figure 1 - Morphological changes in *Strongyloides venezuelensis* eggs after treatment with lyophilized latex from (a) *Carica papaya* at 1:1,000 and (b and c) purified papain at 2.8 mg/mL. Positive control: (d) Albendazole (0.025 mg/mL). Negative controls: (e) PBS and (f) water.

Inhibition of Egg Hatching (IEH) after treatment with latex and purified papain from *C. papaya*

Latex preparations from *C. papaya* had variable results in the EHT (Table 1). The 1:1,000 dilutions from fresh ($p < 0.05$), frozen ($p < 0.01$) and lyophilized ($p < 0.001$) latex has efficiently inhibited eggs hatching, a similar effect observed in the positive control (Albendazole, 0.025 mg/mL). There were no statistical differences among groups with different latex storage methods at the same concentrations.

Table 1 - Effects of different dilutions of fresh, frozen and lyophilized latex on Innibition of Egg Hatching (IEH) of *S. venezuelensis* 48h post-treatment

Tests	Number of eggs	Number of larvae	Inhibition of Eggs Hatching (%)
Fresh latex (250mg/mL)			
1:1000	90	13	87.38 ± 5.0^{7}A
1:10000	82	17	82.83 ± 1.01^{AC}
1:100000	79	31	71.82 ± 4.24^{BC}
Frozen latex (250mg/mL)			
1:1000	86	11	88.66 ± 0.51^{A}
1:10000	94	14	87.04 ± 4.62^{A}
1:100000	78	19	80.41 ± 4.80^{AB}
Lyophilized latex (34mg/mL)			
1:1000	90	9	90.91 ± 1.69^{A}
1:10000	80	14	85.11 ± 2.15^{A}
1:100000	80	13	86.02 ± 2.04^{A}
Water	73	30	70.87 ± 4.55^{B}
PBS	64	25	71.91 ± 1.29^{B}
Albendazole (0.025mg/mL)	88	9	90.72 ± 1.29^{A}

Different letters in the lines indicate statistically significant difference ($P<0.05$); PBS: phosphate buffered saline

Purified papain presented a dose-dependent response in the IEH (Figure 2). At 2.8 mg/ mL it was able to inhibit 91.18% (± 1.24) of eggs hatching. Purified papain was also effective by using 1:10 dilution compared to negative controls ($p < 0.05$). It was observed that the inhibition gradually decreased to 67.71% ± 2.05 (28 ηg/mL purified papain). Purified papain IC_{50} was 12.34 µg/mL.

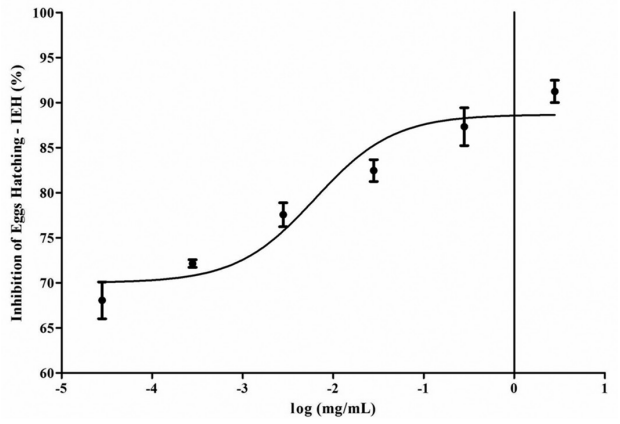

Figure 2 - Dose-dependent response of purified papain from *Carica papaya* on Inhibition of Egg Hatching (IEH) of *Strongyloides venezuelensis*. Results are presented as mean values ± standard error.

It was observed that, at the same dilutions, the activity of lyophilized latex and purified papain was different. Purified papain at 2.8 mg/mL showed similar efficacy to the lyophilized latex at 1:1,000 dilutions.

Inhibition of Larval Motility (ILM) after treatment with latex and purified papain from *C. papaya*

There were no statistical differences among groups by using the three latex preparation methods or dilutions at 24, 48 and 72 h post-treatment. It was shown that high concentrations of latex preparations inhibited larval motility at 24 h post-treatment. Lyophilized latex showed

to be effective in the ILM compared to negative controls (Table 2).

Table 2 - Effects of different dilutions of lyophilized latex on Inhibition of Larvas Motility (ILM) of *S. venezuelensis* 24h post-treatment

Tests	Immobility (%)	Efficacy (%)
Lyophilized latex (34mg/mL)		
1:10	76.74 ± 1.47^{AD}	73.98
1:100	75.26 ± 1.33^{A}	72.31
1:1000	67.71 ± 2.22^{A}	63.86
1:10000	60.00 ± 2.97^{AD}	55.24
1:100000	74.07 ± 1.48^{A}	70.99
Water	10.64 ± 3.58^{B}	0.00
PBS	14.55 ± 0.57^{B}	4.37
Ivermectin (316*ppm*)	94.17 ± 0.46^{C}	93.48

Different letters in the lines indicate statistically significant difference ($p < 0.05$); *ppm: parts per million.*

As IEH result, the purified papain presented a dose-dependent response in the ILM, with stronger reduction in motility at higher concentrations (Figure 3). There were no statistical differences among groups at 24, 48, and 72 h post-treatment and no differences between purified papain and lyophilized latex. The IC_{50} for purified papain at 24 h was 0.6839 µg/mL.

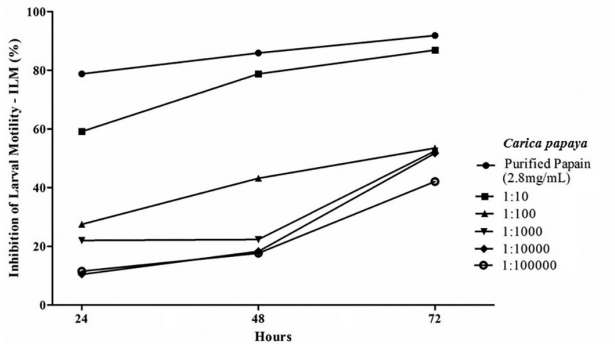

Figure 3 - Dose-dependent response of different dilutions of purified papain from *Carica papaya* on Inhibition of Larval Motility (ILM) in *Strongyloides venezuelensis*. Results are presented as mean values ± standard error.

DISCUSSION

Strongyloidiasis is a serious public health problem with worldwide distribution, and is particularly frequent in developing countries[23,24]. Helminthes control has been limited mainly by the high toxicity of drug therapies and also by the development of parasite resistance to anthelmintics[25].

The present study demonstrates for the first time that latex and purified papain from *C. papaya* had potential activity against *S. venezuelensis* eggs and larvae. These results are consistent with previous studies suggesting anthelmintic potential of *C. papaya* latex against other nematodes. Other studies report a significant reduction in egg production and gastrointestinal nematode burden in various animals, including rodents[26,27], pigs[28], birds[29], sheep[12], and humans[30].

Pure latex was prepared and stored using different procedures to test whether methods such as lyophilization and freezing may interfere with efficacy. None of the processes has affected the activity of latex compounds, as no statistical differences were observed among fresh, frozen or lyophilized latex. High concentrations of latex showed some toxicity, revealed by the total or partial destruction of parasitic forms.

Fresh, frozen and lyophilized latex at 1:1,000 dilutions were very effective in inhibiting egg hatching and larval motility (i.e., activity was similar to the commercial anthelmintic). Other concentrations have also shown inhibitory effects compared to negative controls, and have demonstrated moderate effectiveness in accordance with the standards recommended by the WAAVP[31]. The WAAVP describes an anthelmintic as highly effective when it presents an inhibitory effect greater than 90% regarding the parasite activity, and moderately effective at 80-90% inhibition.

Latex from *C. papaya* contains cysteine proteinases, such as papain and chymopapain which have demonstrated anthelmintic potential[8,9,10,12,28,32]. In the present study EHT results differed when the same concentrations of purified papain and lyophilized latex were tested, suggesting that the effect of latex is not only due to the presence of papain. It can be infered that there are other compounds in the pure latex that promote parasite destruction and the observed synergistic effects.

An anthelmintic treatment is considered effective in the EHT if it inhibits egg development and hatching of larvae, or if it results in a high number of non-embryonated eggs[33]. According to Camurça-Vasconcelos *et al.*[34], eggs embryonation is typically complete within 48 h after elimination in feces, and at that time, the development and eclosion of eggs may be observed under a microscope. In the current study, all latex treatments and purified papain have inhibited the eggs development 24 h post-treatment, and resulted in a high number of non-embryonated eggs.

The mechanism by which cysteine proteinases from plants such as *C. papaya* decrease the motility in larval nematodes

is via impairment and degradation of the parasite cuticle due to the papain catalytic activity on disulfide bonds[26,27]. The reduction in larval motility observed at 24 h is consistent with the observations of Stepek *et al.*[26], who reported papain-associated cuticle digestion in *Meleidogine* sp. and decreased numbers of larvae after treatment. *C. papaya* latex has also been found to cause paralysis in *Pheritimaposthuma in vitro*[32], and is effective in the control of *Haemonchus contortus*[10] and *Trichuris muris*[9], both *in vivo*.

The IC50 discrepancy regarding the treatment of eggs and larvae indicates the difference between the larval cuticle and the eggs shell. In addition, emphasizes the effectiveness of another compound, that is not papain, against eggs. Papain acts by releasing the internal structures of larvaes, leading to the death of parasites[26]. In this sense, papain inhibits the action of the larvae, preventing reinfection cycles in disseminated strongyloidiasis and could possibly interfere with parthenogenetic females by preventing eggs posture.

This study has demonstrated for the first time that *C. papaya* latex and purified papain were effective against *S. venezuelensis* eggs and larvae. Our results suggest that these bioactive compounds may be used, in the future, as alternative therapies for the control of gastrointestinal nematodes such as *Strongyloides stercoralis*. Further studies should be conducted to evaluate the toxicity and *in vivo,* as well as side effects, prior to clinical trials for the treatment of strongyloidiasis.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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