REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

ORIGINAL ARTICLE

http://doi.org/10.1590/S1678-9946201961059

In vitro ovicidal and larvicidal activity of *Carica papaya* seed hexane extract against *Strongyloides venezuelensis*

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ABSTRACT

Strongyloidiasis is a human parasitic disease caused by the helminth *Strongyloides stercoralis* whose treatment is particularly difficult in immunosuppressed patients due to their low responsiveness to conventional therapy. *Carica papaya* and its isolated compounds benzyl isothiocyanate, carpaine and carpasemine are promising compound for the treatment of *Strongyloides* infections due to their anthelmintic action. This study aims to examine the *in vitro* ovicidal and larvicidal activity of *C. papaya* seed hexane extract against *Strongyloides venezuelensis*, using egg hatching tests and larval motility tests as efficiency markers. The crude extract at the concentrations of 566 – 0.0566 mg/mL or the control with albendazole (0.025 mg/mL) and negative controls (water and PBS) were incubated with an equal volume of egg suspension (± 50 specimens) followed by counting of the specimens after 48 h. The same extract and dilutions were added to L3 larvae suspensions (±50 specimens) followed by analysis of larvae viability after 24, 48, and 72 h. The extract inhibited egg hatching with high efficiency at concentrations of 56.6 mg/mL (95.74%) and 5.66 mg/mL (92.16%). At the concentrations of 566 mg/mL (100%) and 56.66 mg/mL (97.32%), the extract inhibited larval motility as effectively as ivermectin (0.316 mg/mL; 100%), and more effectively than the other dilutions and the negative controls. The larvicidal effect depended on the extract concentration, but not on the treatment period. Therefore, *C. papaya* seed hexane extract has anthelmintic potential against *S. venezuelensis* and is a promising compound for the development of phytotherapies to treat strongyloidiasis.

KEYWORDS: *Carica papaya.* Seed extract. Egg hatching test. Larval motility test. Anthelmintic activity. Strongyloidiasis. *Strongyloides stercoralis. Strongyloides venezuelensis.*

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Received: 23 April 2019

Accepted: 25 September 2019

INTRODUCTION

Strongyloides sp. is an intestinal parasite that can cause potentially fatal disseminated infections in immunocompromised patients, such as those under corticosteroids therapy, malnourished patients, or those presenting with concomitant underlying diseases caused by the human immunodeficiency virus (HIV) and the human lymphotropic T-virus (HTLV)[1]. The current synthetic drugs prescribed for the treatment of strongyloidiasis are albendazole, thiabendazole, mebendazole and ivermectin[2,3]. The low efficacy in long-term treatments, malabsorption in cases of disseminated disease and undesirable, sometimes serious adverse effects of these drugs have reduced their effectiveness[2-5].

In general, parasite resistance to medications routinely used in clinical practice has prompted the search for new alternatives, including the use of plant-derived extracts containing antiparasitic compounds[6,7]. *C. papaya* stands out among the plants used for the production of folk medicines due to its anthelmintic action, as established in a preliminary survey about the control of intestinal parasites in Africa[8]. The anthelmintic activity of this plant has been attributed to carpasemine, carpaine-like alkaloids and seed glucosinolates that yield benzyl isothiocyanate, which exert ovicidal and larvicidal effects[9,10]. *C. papaya* latex and purified papain are effective against *S. venezuelensis* eggs and larvae and can be used as therapeutic alternatives for the control of strongyloidiasis[7]. Considering the anthelmintic potential of *C. papaya* against other gastrointestinal nematodes, the present study aims to examine the *in vitro* ovicidal and larvicidal activity of *C. papaya* seeds hexane extract against *S. venezuelensis*.

MATERIALS AND METHODS

S. venezuelensis eggs and larvae

The *S. venezuelensis* strain used in this study was maintained by serial passages of infective larvae (L3) in Wistar rats (*Rattus novergicus*) by subcutaneous inoculation. The Ethics Committee for Animal Use of the Universidade Federal de Uberlandia (Uberlandia, MG, Brazil) approved the study (protocol Nº 075/2008). Faeces from infected animals were used for egg collection and charcoal culture (72 h at 28 ºC) to obtain infective larvae (L3) through the Rugai method[11].

Preparation of *C. papaya* seed extract

The Botany Laboratory of the Universidade Federal de Goias (Jatai, GO, Brazil) confirmed the identity of the commercially available mature *C. papaya* fruits used in this study. They corresponded to the *C. papaya* species deposited at the *Herbario Jataiense* (Jatai, GO, Brazil, voucher Nº 981). Approximately 80 g of *C. papaya* seeds were removed from mature fruits, washed with water to remove pulp residues and dried at room temperature for five days. Then, about 76 g of dry seeds were crushed in a crucible and submitted to extraction in a Soxhlet apparatus for 24 h at 68 °C, using hexane as a solvent. After filtration through filter paper, the filtrate was transferred to a rotary evaporator at 50 °C to remove the solvent (hexane). The crude seed extract was serially diluted in phosphate buffered saline (PBS) to prepare concentrations ranging from 566 mg/mL to 0.0566 mg/mL.

Egg hatching test

The ovicidal activity of the crude extract was analysed using the egg hatching test reported by Coles et *al.*[12], with some modifications. Faeces from rats infected with *S. venezuelensis* were homogenized in water and filtered through a 50 µm sieve. After 15 min of sedimentation, the number of eggs was counted using an optical microscope and the concentration of the suspension was adjusted to 1 egg per µL of filtrate. Then, 50 µL of the suspension were incubated in Eppendorf tubes with 50 µL of the crude seed extract and its dilutions in PBS, for 48 h at 28 ºC. An albendazole solution (0.025 mg/mL)[13] was used as the positive control, while filtered water or PBS were used as negative controls. A 10% formaldehyde solution was added to the mixture and the number of eggs and larvae were counted using an optical microscope at 100 and 400× magnification. To analyse the integrity of the specimens, images were captured at 400× magnification using a Leica® camera, model DS750. All the tests were performed in triplicate for each concentration.

Larval motility test

The larval motility test was performed as reported by Cordeiro *et al*.[14], with some modifications. *S. venezuelensis* infective larvae (L3) were recovered from charcoal culture[11], and the concentration of the suspension was adjusted to 1 larvae per µL of PBS. Then, 100 µL of the suspension were transferred to Eppendorf tubes and mixed with 100 µL of the crude seed extract and its dilutions in PBS. The ivermectin solution (0.316 mg/mL) was used as the positive control[15] and filtered water and PBS were used as negative controls. The samples were incubated in triplicate for 24, 48, and 72 h, at 28 ºC, and larval motility was analysed using an optical microscope at 100× magnification.

Data analysis

The efficiency of inhibition of egg hatching (EHT) by the extract was calculated using the formula reported by Wood *et al*.[16]:

EHT (%) = [(number of eggs/(number of eggs + number of larvae)] × 100

The percentage of inhibition of larval motility (LMT) by each extract dilution was calculated using the formula adapted from Al-Rofaai *et al*.[17]:

LMT (%) = [(%) motility in the treatment group – (%) motility in the negative control)/(100 – (%) motility in the negative control)] × 100.

Data from both tests were submitted to logarithmic and non-linear regression analyses to determine the extract half-maximal inhibitory concentration (DL_{50}). Significant differences among the extract concentrations, the negative controls and the positive control were analysed using the Fisher's exact test. One-way analysis of variance (ANOVA) combined with the Kruskal-Wallis test was used to compare the mobile and total larvae counts after treatment for different periods and with different extract concentrations. Data were analysed using the statistical package of the GraphPad Prism software, version 5.0 for Windows (GraphPad Software Inc. California, USA). Differences among experimental outcomes were considered as statistically significant when $p < 0.05$. All the experiments were carried out in triplicate.

RESULTS

Analysis of the percentage of *S. venezuelensis* egg hatching indicated that *C. papaya* seed extract at 56.6 mg/mL and 5.66 mg/mL had the strongest inhibitory effects on egg hatching: 95.74% (± 1.77) and 92.16% (± 2.18), respectively; these effects were similar to that achieved after treatment with 0.025 mg/mL albendazole (positive control) (Figure 1A). Treatment with the extract at these dilutions or albendazole inhibited egg hatching more effectively than the negative controls containing water and PBS ($p < 0.01$).

The extract exerted a concentration-dependent inhibitory effect on egg hatching (Figure 1B), and resulted in a $DL_{50} = 78.14$ mg/mL. The *C. papaya* seed extract in its crude form and at a concentration of 566 mg/mL, lysed all

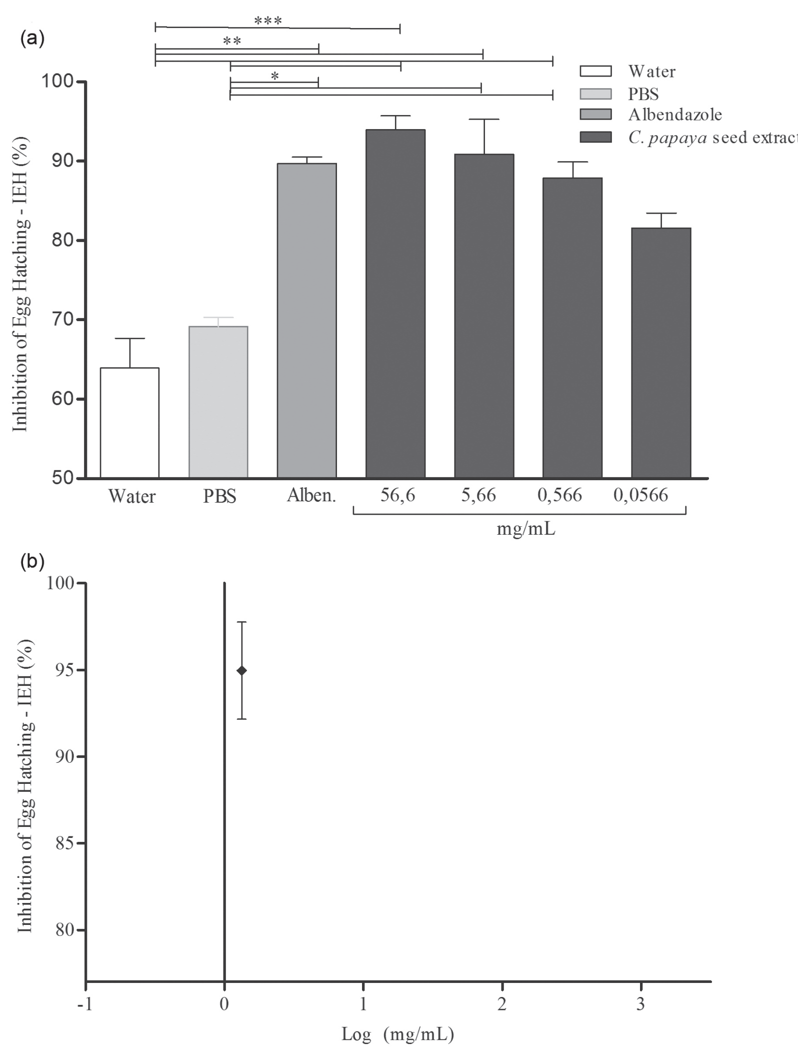

Figure 1 - Inhibition of *Strongyloides venezuelensis* eggs hatching by *Carica papaya* seed hexane extract. (A) Percentage of inhibition of eggs hatching determined after treatment of 50 specimens with different extract concentrations (566-0.0566 mg/mL) for 48 h, at 28 °C. Negative controls: water and PBS. Positive control: albendazole at 0.025 mg/mL. The analysis of TEO efficiency between the concentrations tested and the positive and negative controls was performed by the Fisher's exact test. (B) Non-linear regression curve for inhibition of eggs hatching as a function of extract concentration. Results are expressed as mean ± standard error of triplicate measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

or most of the *S. venezuelensis* specimens when compared with the other dilutions (Figure 2). The inhibitory effects of the extract at concentrations lower than 0.566 mg/mL were not significantly different from each other ($p > 0.05$).

Larval motility of *S. venezuelensis* L3 larvae was analysed *in vitro* after 24, 48, and 72 h of treatment with *C. papaya* seed extract (Figure 3). The extract at 566 mg/mL and 56.6 mg/mL suppressed larval motility as effectively as 0.316 mg/mL of ivermectin (positive control), resulting in 100% (± 0.0), 97.32% (± 2.68) and 100% (± 0.0), respectively, and was a little more effectively than the extract at 5.66 mg/mL ($p < 0.05$) (Figure 3A). Treatment with the extract at 0.566 mg/mL and 0.0566 mg/mL inhibited larval motility at percentages similar to those found for the negative controls with water and PBS ($p > 0.05$): 3.76% (± 0.5) and 5.38% (± 0.38), respectively; these values were significantly lower than that found for the positive control ($p < 0.0001$) (Figure 3A). Hence, high dilutions of the extract did not inhibit *S. venezuelensis* larval motility. It was not possible to count the number of specimens after treatment with the undiluted crude extract.

Figure 3B depicts the concentration-response curve of inhibition of *S. venezuelensis* larval motility as a function of the concentration of *C. papaya* seed extract, after 24 h of treatment. The extract exerted a statistically significant concentration-dependent effect ($p < 0.0001$), the intensity of which did not vary across the treatment periods; hence, the inhibitory action of the extract on larval motility was not time-dependent. The DL_{50} value determined at 24 h of treatment was 20.02 mg/mL. The crude extract lysed all the *S. venezuelensis* larvae added to the reaction tube, while the 566 mg/mL and 56.6 mg/mL concentrations partially degraded the larvae cuticle, as compared with the negative control containing water (data not shown).

DISCUSSION

Infections caused by soil-transmitted helminths represent an important public health problem, especially in developing countries[18]. In these locations, the interest in plant-based drugs for the treatment of parasitic diseases has increased progressively for many reasons, including serious side effects caused by the abuse and misuse of synthetic drugs and the difficultly of accessing pharmacological treatment[19,20].

C. papaya seed extract has demonstrated promising anthelmintic potential against nematodes such as *Ascaridia galli*, *Caenorhabditis elegans*, *Heterakis gallinarum*, *Meloidogyne incognita* and *Pheretima posthuma*[21,22]. However, additional studies need to be performed with other human pathogenic parasite species that cause endemic infections in tropical areas throughout the world, such as *Strongyloides stercoralis*.

The present study demonstrated the *in vitro* anthelmintic activity of *C. papaya* seeds hexane extract against *S. venezuelensis.* We found that the extract has effectively suppressed egg hatching and larval motility, in accordance with the standards recommended by the World Association for the Advancement of Veterinary Parasitology[16]. The current *in vitro* findings with the *C. papaya* seed extract

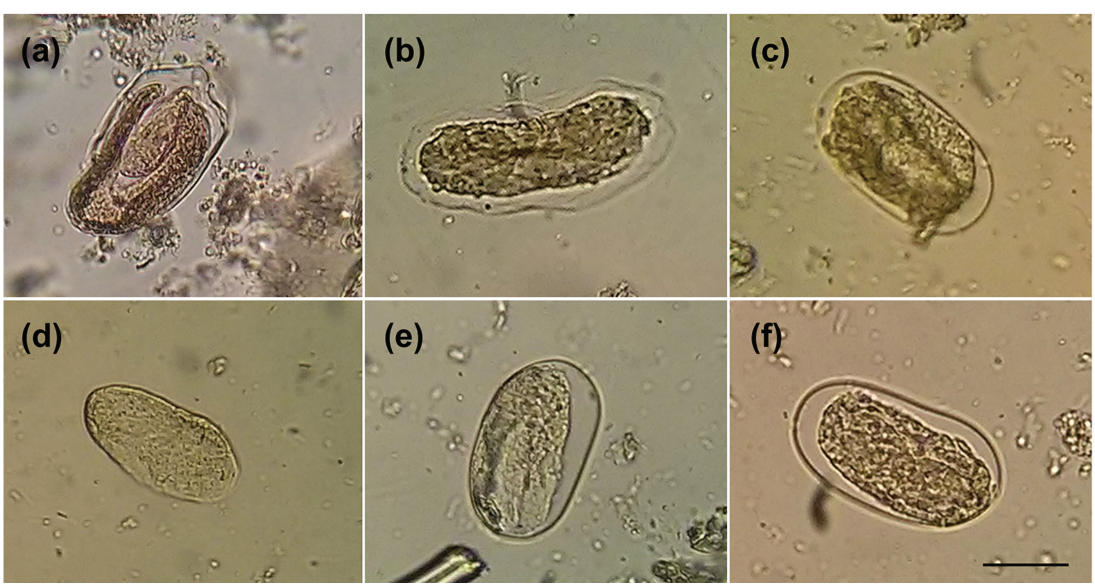

Figure 2 - Effect of *Carica papaya* seed hexane extract on the development and cuticle integrity of *Strongyloides venezuelensis* eggs. Fifty specimens were treated with the crude extract (a) or its 566 mg/mL concentration, (b) 5.66 mg/mL (c) and 5.66 mg/mL (d) dilutions, water (e), or PBS (f), for 48 h, at 28 ºC. Water and PBS were used as the negative controls. Images were captured at 40x magnification, scale bar 40 µm.

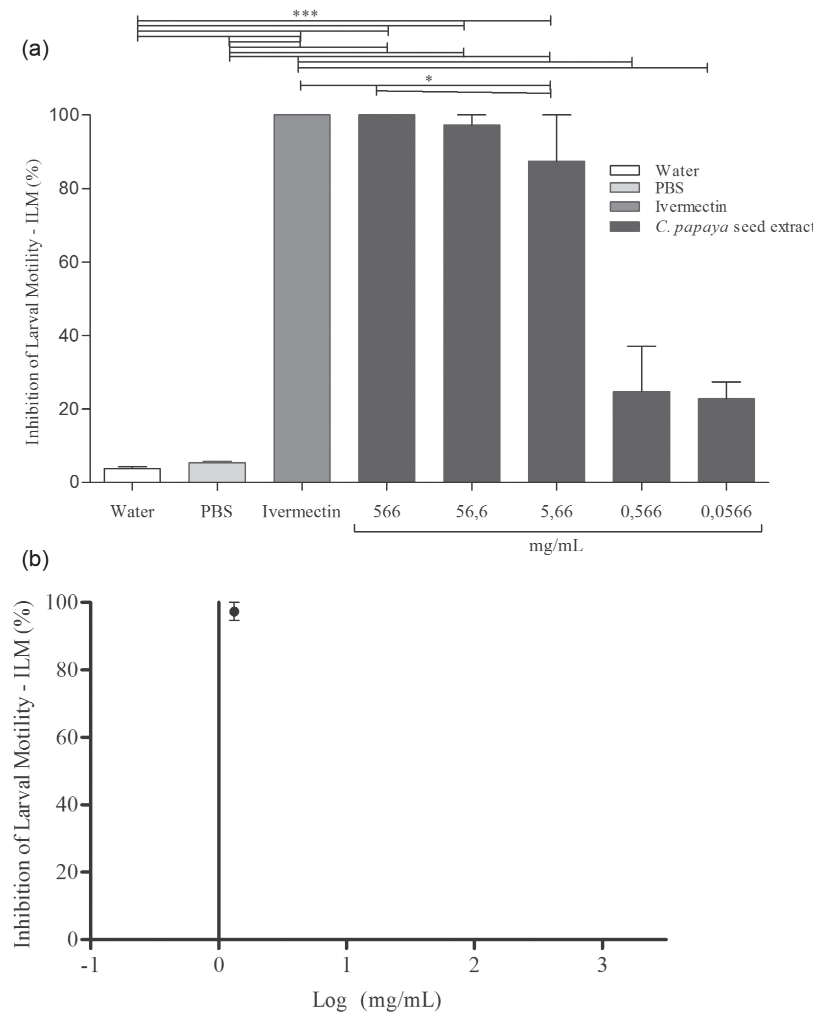

Figure 3 - Inhibition of *Strongyloides venezuelensis* larvae motility by *Carica papaya* seed hexane extract. (a) Percentage of inhibition of larval motility determined after treatment of 50 specimens with different extract dilutions for 24 h, at 28 °C. Larval motility was determined by counting mobile and immobile larvae from a total of 50 specimens per sample. Concentrations: 566-0.0566 mg/mL. Negative controls: water and PBS. Positive control: ivermectin at 0.316 mg/mL. One-way analysis of variance (ANOVA) for parametric data and the Kruskal-Wallis test for nonparametric data were used to compare the counts of mobile and total larvae. (B) Non-linear regression curve for inhibition of larval motility as a function of extract concentration. Results are expressed as mean ± standard error of triplicate measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

are corroborated by literature data on the *in vivo* effect of this preparation[8]. The aqueous extract of *C. papaya* seeds (i) suppress egg hatching and reduces the number of eggs produced by the nematodes *A.galli*, *H. gallinarum* and *Trichostrongylus tenuis* in infected birds[23]; and (ii) decreases the number of eggs per gram of faeces until the third day after sheep infection with endoparasites, presenting with 72% efficiency in reducing the parasite load[24]. The 24 h treatment with oil from dry and fresh *C. papaya* seeds is highly efficient in controlling and reducing the lethality of the nematodes *C. elegans* and *M. incognita*; this nematicidal effect seems to be mediated by the oil component benzyl isothiocyanate[21].

Many anthelmintic glycosides and alkaloids present in *C. papaya* seeds may interfere with the helminth homeostasis by inhibiting glucose uptake, sucrose transfer into the small intestine, nitrate generation, digestion and removal from the cuticle[25]. As helminths cannot store energy, damage to their cuticle leads to muscular paralysis and impairment of larval motility, resulting in parasite death in less than 24 h due to food deprivation[26]. It is possible that the alkaloid carpaine and the benzyl isothiocyanate present in *C. papaya* seeds exert the anthelmintic effect against the *S. venezuelensis* specimens, but it is necessary to isolate these compounds for new *in vitro* tests to validate this hypothesis. In any case, the anthelmintic effect of the hexane extract from *C. papaya* seeds against *S. venezuelensis* observed in this study makes it a potential candidate for the development of phytotherapeutic drugs to treat strongyloidiasis.

ACKNOWLEDGMENTS

We are grateful to Prof. Dr. Ricardo de Mattos Santa-Rita from the Laboratorio de Microscopia, Universidade Federal de Goias, Regional Jatai, for his technical skills in gathering microscope images.

AUTHORS' CONTRIBUTIONS

All authors have made significant contributions to the design, execution, analysis and writing of the study.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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