Original Article

Anthelmintic activity of *Leucaena leucocephala* protein extracts on *Haemonchus contortus*

Atividade anti-helmíntica de extratos proteicos de *Leucaena leucocephala* sobre *Haemonchus contortus*

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Abstract

The objective of this study was to evaluate the effects of protein extracts obtained from the plant *Leucaena leucocephala* on the nematode parasite *Haemonchus contortus*. The seeds, shell and cotyledon of *L. leucocephala* were separated and their proteins extracted using a sodium phosphate buffer, and named as TE (total seed extract), SE (shell extract) and CE (cotyledon extract). Soluble protein content, protease, protease inhibitory and chitinase activity assays were performed. Exsheathment inhibition of *H. contortus* larvae were performed at concentrations of 0.6 mg mL^{-1}, and egg hatch assays were conducted at protein concentrations of 0.8, 0.4, 0.2, 0.1 and 0.05 mg mL^{-1}. The effective concentration for 50% hatching inhibition (EC$_{50}$) was estimated by probit. Different proportions of soluble proteins, protease and chitinase were found in TE and CE. Protease inhibitory activity was detected in all extracts. The EC$_{50}$ of the CE and TE extracts were 0.48 and 0.33 mg mL^{-1}, respectively. No ovicidal effects on *H. contortus* were detected in SE extracts, and none of the protein extracts demonstrated larvicidal effects on *H. contortus*. We therefore conclude that protein extracts of *L. leucocephala* had a detrimental effect on nematode eggs, which can be correlated with the high protease and chitinase activity of these extracts.

Keywords: Plant proteins, protease, protease inhibitor, chitinase.

Resumo

O objetivo deste estudo foi avaliar a atividade anti-helmíntica de extratos proteicos de leucena (*Leucaena leucocephala*) sobre *Haemonchus contortus*. As sementes, as cascas e os cotilédones foram moídos separadamente e as proteínas extraídas com tampão fosfato de sódio e denominados: TE (extrato total), SE (extrato casca) e CE (extrato cotilédone). O teor de proteínas, atividade proteolítica, inibitória de protease e quitinolítica dos extratos foram verificados, além da ação sobre a eclosão de ovos e desembainhamento larvar de *H. contortus*. A concentração efetiva para inibição de 50% da eclosão dos ovos (EC$_{50}$) foi calculada através do *probit*. Foi demonstrado que TE e CE possuem, em diferentes proporções, proteínas solúveis, protease e quitinase. Atividade inibitória de protease foi encontrada em todos os extratos. A EC$_{50}$ dos extratos CE e TE foram 0,48 e 0,33 mg de proteína mL^{-1}, respectivamente. O extrato SE não apresentou atividade sobre a eclosão dos ovos. Os extratos proteicos não apresentaram efeito larvicida sobre *H. contortus*. Conclui-se que a ação de extratos proteicos de *L. leucocephala* afetam negativamente a eclodibilidade dos ovos, correlacionando-se com a alta atividade de protease e quitinase dos extratos testados.

Palavras-chaves: Proteínas vegetais, protease, inibidor de protease, quitinase.

Introduction

Infections with gastrointestinal nematodes are a primary cause of the loss of small ruminant livestock (HOUNZANGBE-ADOTE et al., 2005; CEZAR et al., 2008). The nematode *Haemonchus contortus* is considered the most important because of the extensive damage it has caused to livestock. Controlling these parasites consists primarily of using synthetic anthelmintics (OLIVEIRA et al., 2011), but inappropriate use of anthelmintics has led to an increase in resistant nematode strains (TORRES-ACOSTA & HOSTE, 2008). As such, certain plant compounds have been considered as an alternative for the control of these parasites (EGUALE et al., 2007; BUTTLE et al., 2011).

Plants have a variety of defense and protection response mechanisms (SHARMA et al., 2011). Seeds have proteins whose

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function is to both store nutrients for germination and growth and act as defense proteins against pathogens, especially proteases, protease inhibitors, and chitinases (CARLINI & GROSSI-DE-SÁ, 2002). It is thought that proteases and chitinases may be have potential nematicidal properties because of their ability to break down important macromolecules that constitute the cuticle of these parasites.

Nematodes feature a morphologically complex cuticle composed of resistant proteins such as collagen, which functions as a protective barrier between the parasite and the external environment (SHAMANSKY et al., 1989; RHOADS & FETTERER, 1990). The cuticle of *H. contortus* eggs has three layers: an internal layer composed of lipids with some associated proteins; an intermediate layer composed of chitin fibers surrounded by proteins; and an outer layer composed of proteins and lipids (MANSFIELD et al., 1992).

Different classes of proteases are known to be inactivated by plant proteins, called protease inhibitors (OLIVA et al., 2000). Gastrointestinal nematodes produce a variety of proteases that have different functions during development (RHOADS et al., 2000; WILLIAMSON et al., 2003). Although nematode parasites can utilize protease inhibitors to protect themselves from degradation by host proteases, to facilitate feeding and to manipulate the host response to the parasite (KNOX, 2007), protease inhibitors can also prevent or delay the development of the parasite (IZUHARA et al., 2008), representing a potential biotechnological approach to the study and development of new anthelmintic drugs.

Among the variety of plant species with potential anthelmintic properties, *Leucaena leucocephala* is prominent. Native to Central America, Leucaena can be found in many tropical and subtropical regions of the world (LIM, 2012; NEHDI et al., 2014) and is often used as forage for livestock (PRASAD et al., 2011; PANDEY & KUMAR, 2013). Leucaena exhibits anthelmintic properties against both *H. contortus* (OLIVEIRA et al., 2011) and *Trichostrongylus colubriformis* (CUNHA et al., 2003).

Many studies have demonstrated the effectiveness of plant extracts in controlling *H. contortus*, but relatively little research has been conducted using protein extracts. This study aims to examine the anthelmintic activity of protein extracts of *L. leucocephala* on the gastrointestinal nematode *H. contortus* and to verify the activities of some proteins that could be related to the bioactivity of the extracts against this parasite.

Materials and Methods

Plant material

The seeds of *L. leucocephala* were commercially acquired (Sementes Caiçara, Brejo Alegre, SP, Brazil) and mechanically ground in a mill. Lipid extraction from the resulting powder was performed with hexane (1:6, w/v) at 25 °C. After two solvent changes per day over two consecutive days, the hexane was removed and the powder was allowed to dry overnight at 25 °C under forced air circulation. The powder was subsequently stored in sealed vials at –4 °C. In addition, the shell and cotyledon were separated, ground and defatted as described for the whole seed.

Protein extraction and quantification

Soluble proteins in the powdered seeds of *L. leucocephala* were extracted using a sodium phosphate buffer (PBS, 100 mM, pH 7.0) (1:5 w/v) under constant stirring for 3 h at 4 °C. The suspensions were centrifuged at 12,000 x g for 30 minutes at 4 °C. The resulting supernatants were centrifuged under the same conditions, with the final supernatants classified as TE (total seed extract), SE (shell extract) and CE (cotyledon extract).

The soluble proteins in TE, SE and CE were quantified according to Bradford (1976), using bovine serum albumin (BSA) as the standard. The protein content was expressed as milligram of protein per gram fresh weigh and was calculated using the following equation:

$$\text{Protein content } (\text{mgP}/\text{gMF}) = \frac{\text{protein concentration } (\text{mg/mL})}{\text{buffer volume } (\text{mL}) * \text{seed weight } (\text{g})} \quad (1)$$

Protease, protease inhibitor and chitinase activity assays

The total proteolytic activity of *L. leucochephala* seed extracts was examined using azocasein as a nonspecific substrate (XAVIER-FILHO et al., 1989). One unit of activity (UA) was defined as the amount of enzyme capable of increasing absorbance by 420 nm at 0.01 mL^{-1} in 60 min.

Cysteine proteinase inhibitor activity was determined by measuring the inhibition of papain activity using benzoyl-DL-arginine-β-naphthylamide (BANA) as substrate (ABE et al., 1992). One unit of inhibitory activity (UI) was defined as the decrease of 0.01 absorbance units at 540 nm mL^{-1} min^{-1} compared with the control (papain activity in the absence of the inhibitor).

Determination of chitinase activity consisted of sample capacity in release of n-acetyl-d-glucosamine (NAG) from colloidal chitin by hydrolytic action (BOLLER et al., 1992), expressed in nkat.

Egg hatch assay

H. contortus eggs were recovered from the feces of experimentally infected goats (BIZIMENYERA et al., 2006). Approximately 100 eggs per well^{-1} were submitted to incubation with protein extracts from seeds of *L. leucocephala* in concentrations between 0.05 mg and 0.8 mg of protein mL^{-1} (mg P^{-1} mL^{-1}) in quadruplicate. Protein extracts were diluted in PBS (100 mM, pH 7.0). The assay contained two controls: (i) distilled water; and (ii) PBS (100 mM, pH 7.0) that was used to prepare and dilute protein extracts. The 24-well plates containing different extracts and controls were incubated for 48 h at 27 °C. Hatched larvae (dead or alive) and unhatched eggs were counted under a dissecting microscope at 40× magnification.

Larval exsheathment assay

Infective larvae (L$_3$) of *H. contortus* were obtained from the feces of an experimentally infected donor goat and incubated, as described by Roberts & O'Sullivan (1950). The larval exsheathment

assay was performed according to Bahuaud et al. (2006). Viable larvae of ages 2 to 3 months were used in the assays. The extracts of the *L. leucocephala* seeds were tested at the concentration of 0.6 mg P^{-1} mL^{-1} in PBS (100 mM, 50 mM NaCl, pH 7.2). The assay was performed in four replicates, with PBS used as a control. Larvae were incubated with protein extracts and PBS for 3 h at 22 °C. After this period of incubation, larvae were rinsed three times with PBS via centrifugation for 5 minutes at 1000 rcf. Exsheathment was induced by a solution of sodium hypochlorite (2% v/v) and sodium chloride (16.5% w/v) diluted in PBS. The kinetics of larval exsheathment in the different experimental treatments was then monitored by microscopic observation (at 40× magnification). The percentages of exsheathed larvae were identified at 10-minute intervals for 60 minutes.

Statistical analysis

Statistical comparisons of the *H. contortus* egg hatch assays were assessed using two-way ANOVA with Bonferroni post-tests. The effective concentration for 50% hatching inhibition (EC$_{50}$) was estimated by probit. Larval exsheathment rates were compared using the ANOVA test. The significance level used in all statistical tests was 0.05, and all analyses were performed using the GraphPad Prism software, v. 6.0 (GraphPad Software, 2007).

Results

Protein content, protease, protease inhibitor and chitinase assays

The CE had higher protein content (109.95 mgP gMF^{-1}) than did the TE (53.30 mgP gMF^{-1}), whereas only a minimal amount of protein (1.67 mgP gMF^{-1}) was detected in the SE (Figure 1a).

Protease activity was not detected in the SE. However, the enzyme was detected in both the TE (45 UA) and in higher levels in the CE (95 UA) (Figure 1b). Protease inhibitory activity was detected in all prepared extracts, with TE showing higher levels of activity (670 UI) (Figure 1c). Chitinase activities in the TE and CE were 0.16 and 0.11 nkat, respectively. No significant amounts of this enzyme (0.007 nkat) were found in the SE (Figure 1d).

Biological activity on H. contortus

Different protein extracts obtained from seeds of *L. leucocephala* exhibited distinct ovicidal effects on *H. contortus*. Ovicidal efficacy of the TE (99.2% and 56.6% at 0.8 and 0.4 mg mL^{-1}, respectively) was significantly higher than that of the CE (83.4% at 0.8 mg mL^{-1}). This difference in efficacy was reflected in the EC$_{50}$ values of the TE and CE. The EC$_{50}$ of CE (0.48 mg mL^{-1}, 95% CI: 0.40-0.57) was significantly greater than that of TE (0.33 mg mL^{-1}, 95% CI: 0.29-0.38). There were few signs of ovicidal effects against *H. contortus* in SE assays at the tested concentrations, however. Higher concentrations of TE and CE (0.6 mg mL^{-1}) were tested for effects on *H. contortus* larvae, but cuticular loss rates after

60 minutes did not differ among the control (buffer), TE and CE (98.8%, 98.2% and 95.3%, respectively).

Discussion

Plants have long been studied as potential sources of chemicals for controlling animal and human parasites due to their numerous medicinal and therapeutic properties (HERNÁNDEZ-VILLEGAS et al., 2011). The detrimental effects that *H. contortus* nematodes have on the development of goats and sheep have led to numerous studies examining the use of plant extracts as nematode control agents (ALONSO-DÍAZ et al., 2008; MARIE-MAGDELEINE et al., 2010; HERNÁNDEZ-VILLEGAS et al., 2011).

Although secondary metabolites have been extensively correlated with nematicidal effects, demonstrating their applicability to controlling the gastrointestinal nematodes of small ruminants, plant proteins represent a novel approach for controlling these parasites (SALLES et al., 2014). The use of therapeutic proteins is one of the fastest growing sectors in the pharmaceutical market and has undergone several generations of development to achieve increasingly viable commercial products. These proteins can be produced at relatively low cost and feature a reduced risk of side effects and high bioavailability (MARTIN, 2006). In the present work, although no soluble proteins were detected in SE, such proteins were detected in TE and CE at rates of 53.30 mgP gMF^{-1} and 109.95 mgP gMF^{-1}, respectively (Figure 1a).

Little is known about the potential anthelmintic properties of cysteine proteases, but some studies have shown that these enzymes may act on the cuticle of the nematode, causing severe damage and leading to the death of the nematode (PHIRI et al., 2013; LUOGA et al., 2015). Protease inhibitors may also play an important role in the control of nematode parasites by inhibiting the production of proteins necessary for nematode development (LAWRENCE & KOUNDAL, 2002). Both TE and CE displayed proteolytic activity (Figure 1b). Proteases extracted from other plants, including *Carica papaya*, *Ananas comosus* and *Ficus* sp., have previously been shown to be effective against the plant nematodes *Meloidogyne incognita*, *M. javanica* and *Globodera rostochiensis*, as well as nematodes infecting the rodents *Trichuris muris*, *Protospirura muricola* and *Heligmosomoides polygyrus* (STEPEK et al., 2006, 2007a, b, c).

The protein extracts used in this study exhibited clear signs of protease inhibitory activity (Figure 1c). Protease inhibitors are present in all living beings and have important protective functions. In nematodes, metalloproteinase enzymes play important roles in the formation of the cuticle, and protease inhibitors can thus be used to inhibit and prevent the development of the parasite by interfering with these enzymes (LAWRENCE & KOUNDAL, 2002). Moreover, the shells of *H. contortus* eggs have a protein layer that includes several proteases (MANSFIELD et al., 1992). Because proteases are present in all stages of nematode development, the use of protease inhibitors may represent a highly effective means of controlling these parasites. We did not, however, observe a correlation between the concentration of protease inhibitors and egg-hatching inhibition.
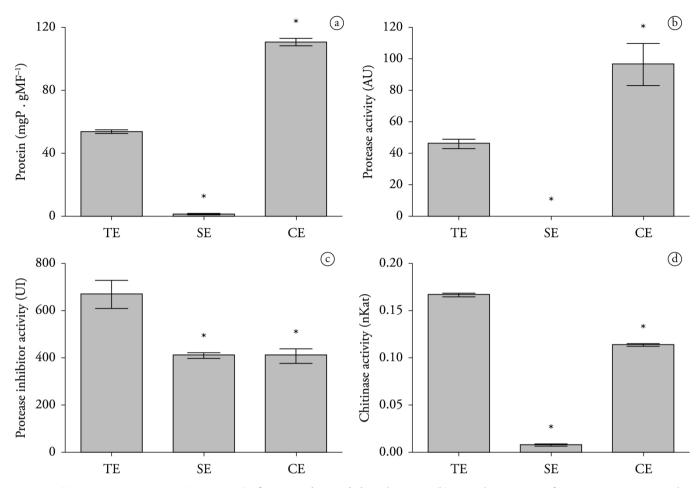
Figure 1. (a) Protein concentration (mgP gMF^{-1}) of *Leucaena leucocephala* seed extracts. (b) Proteolytic activity of protein extracts expressed in AU (Activity Unit). (c) Analysis of the protease inhibitor activity of protein extracts expressed in UI (Unit Inhibition). (d) Chitinase activity of protein extracts expressed as nkat (nanokatal). TE: total extract, SE: shell extract and CE: cotyledon extract. Data are mean ± SEM of three samples. Asterisks indicate significant (p<0.05) difference of SE and CE compared to TE.

Chitinase activity was observed primarily in TE and CE assays (Figure 1d). Chitin is an important component of egg shells, and acts as a protective barrier against the external environment (ROGERS & BROOKS, 1977). The main proteins of the cuticle of *H. contortus* eggs are degraded by the enzymes proteinase K and chitinase (MANSFIELD et al., 1992). Pathogenic fungi and some nematodes use chitinase to break through this protective barrier and penetrate the host (LEGER et al., 1993; HUBER et al., 1991; SHAHABUDDIN et al., 1993). We showed that the TE and CE interfere with *H. contortus* egg development. The efficiency of the TE and CE coincides with high protease and chitinase activity, which suggests that these enzymes could have potential ovicidal properties.

Still extracts of acetone and water from the aerial components of *L. leucocephala* showed 90% larvicidal effectiveness against *H. contortus* (OLIVEIRA et al., 2011). This method extracted compounds of secondary metabolites, consisting primarily of condensed tannins and other phenolic compounds (CORK & KROCKENBERGER, 1991). The protein extracts used in our study did not exhibit larvicidal effects.

In this study, we observed that protein extracts obtained from *L. leucocephala* detrimentally affected nematode eggs, which correlated with the high levels of protease and chitinase activity of these extracts. It is believed that the active mechanism may vary according to the stage of development of the nematode and, therefore, the differences in cuticle and proteins between the eggs and the larvae. Further research should be conducted to determine the ovicidal effects of plant proteins, as well as the active mechanism(s) of these proteins, to discover new approaches for treating gastrointestinal infection in small ruminants by nematodes.

Acknowledgements

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