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In vitro activity of neem (*Azadirachta indica*) and cassava (*Manihot esculenta*) on three pre-parasitic stages of susceptible and resistant strains of *Teladorsagia* (*Ostertagia*) *circumcincta*

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ABSTRACT

Anthelmintic resistance of gastrointestinal nematodes is considered as one of the main limiting factors causing significant economic losses to the small ruminant industry. The anthelmintic properties of some plants are among the suggested alternative solutions to control these parasitic worms. The present study investigated the anthelmintic activity of neem (*Azadirachta indica*) and cassava (*Manihot esculenta*) leaf extracts against the susceptible and resistant strains of one of the most important nematodes in small ruminants, *Teladorsagia* (*Ostertagia*) *circumcincta*. Three different *in vitro* tests: egg hatch test, larval development assay, and larval paralysis assay were used to determine the efficiency of neem and cassava extracts on three pre-parasitic stages of *T. circumcincta*. The LC_{50} was determined for the most potent extract in each plant as well as the phytochemical tests, total tannin quantification and cytotoxicity on peripheral blood mononuclear cells of goats. The results revealed a high anthelmintic activity of neem methanol extract (NME) and cassava methanol extract (CME) on both strains of *T. circumcincta* without significant differences between the strains. The first stage larvae were more sensitive with the lowest LC_{50} at 7.15 mg/ml and 10.72 mg/ml for NME and CME, respectively, compared with 44.20 mg/ml and 56.68 mg/ml on eggs and 24.91 mg/ml and 71.96 mg/ml on infective stage larvae.

1. Introduction

Livestock production is one of the important economic sources in many countries especially in developing regions (Bellaver and Bellaver, 1999; Gura, 2008; Timon and Hanrahan, 1985). Anthelmintic resistance of gastrointestinal nematodes considered as one of the limiting factors, is a serious problem worldwide, affecting livestock production (Jabbar et al., 2006; Kusiluka and Kambarage, 1996). *Teladorsagia* (*Ostertagia*) *circumcincta* is one of the most economically important parasitic nematodes of goats and sheep in warm subtropical regions, and it is a dominant species of trichostrongylid nematodes in cooler parts of the world (Akhter et al., 2011; Bishop et al., 1996; Chartier and Reche, 1992; Eckert and Hertzberg, 1994; Nabavi et al., 2011). Adult *T. circumcincta* infests the abomasum of ruminants, particularly sheep, goats, and cattle, causing severe symptoms that lead to their deaths, such as increase in abomasal pH, elevation of plasma pepsinogen levels, decreased appetite, watery diarrhoea, and changes in gastrointestinal function (Al-Saqur et al., 1981; Fox, 1997; Simpson et al., 2009).

Single and multiple anthelmintic resistance of *T. circumcincta* were reported worldwide such as in Denmark, Britain, Scotland, New Zealand, Netherlands, Iran, and Australia (Bjørn et al., 1991; Cawthorne and Whitehead, 1983; Eysker et al., 2006; Gopal et al., 1999; Jackson et al., 1992; Suter et al., 2004).

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Recently, some studies investigated the activity of plant extracts as alternative means to control *T. circumcincta* (Al-Shaibani et al., 2008, 2009; Minho et al., 2008). Neem (*Azadirachta indica*: Meliaceae) and cassava (*Manihot esculenta*: Euphorbiaceae) were found to have good activity against some microorganisms (Mishra et al., 2005; Islam et al., 2009; Udeinya et al., 2004; Melo et al., 2009; Punthanara et al., 2009; Zakaria et al., 2006), while also showing high activity against *Haemonchus contortus*, which infects the abomasum of small ruminants besides *T. circumcincta* (Costa et al., 2008; López et al., 2007; Marie-Magdeleine et al., 2010; Rahman et al., 2011). However, no previous work has been reported using neem or cassava against *T. circumcincta*.

The present work aimed to study the direct effect of neem and cassava leaf extracts against eggs, first stage larvae (L_1), and infective stage larvae (L_3) of susceptible and resistant strains of *T. circumcincta*.

2. Materials and methods

2.1. Plant materials and extraction

Neem and cassava leaves were collected in Pulau Pinang, northwest Peninsular Malaysia in February 2009. Voucher specimens of neem and cassava were identified by a plant taxonomist and deposited at the herbarium of the School of Biological Sciences, Universiti Sains Malaysia, under reference numbers 11181 and 11182, respectively. The leaves were cleaned, milled to powder, and extracted using hexane, chloroform, ethyl acetate and 80% methanol sequentially in a Soxhlet apparatus. All extracts were filtered using Whatman #1 filter paper, and the filtrates were concentrated in a rotary evaporator (EYELA model N-1000), and then dried in a vacuum oven (45 °C). The extracts separately were kept in a sealed dark glass vial at 4 °C until use. A stock solution of plant extract at 100 mg/ml was prepared in distilled water and 3% of Tween 20 was used first to increase the solubility in the distilled water.

Five serial concentrations (50, 25, 12.5, 6.2, and 3.1 mg/ml) used in three different *in vitro* tests for each plant extract were prepared by diluting the stock solution of the extracts further with distilled water.

As negative control, 3% Tween 20 was used, and 5% albendazole was used as positive control.

2.2. Parasite isolates and experimental infection

Two isolates of *T. circumcincta* were used: *McMaster*, isolated prior to 1960, is susceptible to all anthelmintics and *Wamiro*, isolated in 2002, is resistant to benzimidazoles, levamisole, macrocyclic lactones and naphthalophos. The parasite isolates were provided by the Commonwealth Scientific and Industrial Research Organisation (CSIRO), McMaster Laboratories, Chiswick Research Station, Armidale, NSW, Australia.

Two nematode-free goats over six months old were kept indoors in an experimental farm in Pinang, Malaysia. These goats were used as experimental animals to serve as a source of *T. circumcincta* eggs for further *in vitro* tests. Each animal was infected orally with 6000 L_3 of one strain according to the guidelines of the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH): specific recommendations for ovines, 1999 (Vercruysse et al., 2001).

2.3. Egg recovery and purification

Eggs from each parasite strain were obtained six weeks post-infection. The eggs were recovered from the faeces of experimental animals according to Schürmann et al. (2007) and adjusted to approximately 1000/ml in distilled water.

2.4. Egg hatch test (EHT)

This test aimed to evaluate the ovicidal activity of neem and cassava leaf extracts on susceptible and resistant strains of *T. circumcincta*. EHT was conducted according to the standard procedure of World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles et al., 1992, 2006). 100 μl of the egg suspension, containing approximately 100 fresh eggs, was added to the wells of sterilized 96-well microtitre plates. A 100 μl extract of neem and cassava leaf in hexane, chloroform, ethyl acetate, and 80% methanol was separately added in triplicate to the wells in serial concentrations of 50, 25, 12.5, 6.2, and 3.1 mg/ml in five independent trials. Albendazole, at a concentration of 0.1% (Ademola and Eloff, 2010; Kamaraj and Rahuman, 2011), was used as the positive control, and 3% aqueous solution of Tween 20 (Acros Organics, USA) was used as the negative control. All plates were incubated at room temperature (25–27 °C) for 48 h, and then one drop of Lugol's iodine was added to each well to stop the development of any egg or larva. All unhatched eggs and developing larvae were counted.

2.5. Larval development assay (LDA)

This assay aimed to evaluate the activities of neem and cassava leaf on the L_1 development of the susceptible and resistant isolates of *T. circumcincta*.

LDA was performed as described by Ademola and Eloff (2010). Briefly, 70 μl of egg suspension, recovered as mentioned above and containing approximately 100 eggs, was pipetted into each well of the sterilized 96-well microtitre plates with 20 μl of nutritive medium. This medium consisted of 1:9 (v/v) Earle's balanced salt solution and yeast extract in saline (1 g of yeast extract in 90 ml of saline solution) (Sigma–Aldrich, Germany). Then, 10 μl of amphotericin B (Sigma–Aldrich, USA) at 5 μg/ml was added to prevent the growth of fungi. The plates were incubated at 27 °C for 48 h to allow hatching of L_1. The presence of L_1 was verified after this incubation period. A total of 100 μl of plant extract at 50, 25, 12.5, 6.2, and 3.1 mg/ml was added in triplicate to the wells, and the trial was independently repeated five times. For positive control, some wells were treated with 100 μl of 0.5% albendazole, while in negative controls, the wells were treated with 100 μl of 3% Tween 20. The plates were incubated for 6 days at room temperature (25–27 °C) and then one drop of Lugol's iodine solution was added to each well to kill the larvae and stop their