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In vitro Anthelmintic Activity of *Croton tiglium* Seeds Extract on *Haemonchus contortus*

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ABSTRACT

Background: *Haemonchus contortus* is a blood sucking parasite whose habitat is the abomasum of ruminants, which poses a significant threat to the health and production of sheep and goats in warm temperate and tropical regions. *H. contortus* leads to anemia, hypoproteinemia, reduced exercise tolerance and edema subcutaneous tissue that can result in the death of animals. In acute infection, animals suddenly become anemic and many are found dead. Even in the chronic phase, the condition is accompanied by a mild but persistent parasitic burden and is characterized by weight loss, widespread malaise and in some individuals, anemia. The objective of this study was to identify the anthelmintic activity of *Croton tiglium* seeds extract against *Haemonchus contortus* and comparison with some drugs used against internal and external parasites.

Methods: A study was performed to evaluate the anthelmintic activity of the methanolic extract of *Croton tiglium* seeds against *Haemonchus contortus* using an adult worm motility test. Six graduated concentrations of extract (25, 50, 75, 100, 125 and 150 mg/ml), were tested at different periods and changes over time in the viability of worms were registered for 2, 4 and 6 hr. Normal RPMI-1640 medium and ivermectin 0.1% were used as negative and positive control, respectively. Also, a histological study was conducted on the the integumentary tissue of the *H. contortus* and the changes that occur in it were observed.

Result: At 2 and 4 h post treatment, the concentrations of *C. tiglium* seeds extract (100, 125 and 150 mg/ml) have caused significantly higher mortality compared to the other concentrations, while, similar to ivermectin that caused death for nematodes within 6 h. The lower concentration (25 mg/ml) was significantly more lethal than the negative control (RPMI-1640 medium) at 2, 4 and 6 h of exposure. Each concentrations damages the cuticle and muscles of *H. contortus*. The increase in concentration is proportional to the increase in damage to the integumentary. The present study indicated that all concentrations of methanolic extract of *C. tiglium* seeds produced anthelmintic activity.

Key words: Haemoncosis, Ivermectin, Nematode, Small ruminants.

INTRODUCTION

Haemoncosis is a disease caused by infection with *Haemonchus contortus*, a blood-sucking nematode parasite in the abomasum of small ruminants such as goats and sheep (Rahman and Hamid (2007). These parasites cause weight loss, anemia, hypo albumin anemia, lipoprotein anemia and sudden death, especially in cattle (Hellgren *et al*., 2003). Also, acute haemonchosis is characterized by anemia, submandibular edema, pale mucous membranes and ascites, the most easily recognized being lethargy, dark stools and hair loss, while the chronic form is characterized by weight loss and poor body (Besier *et al*., 2016). This parasitic infection results in direct economic losses related to decreased animal performance or death as well as indirect economic losses linked to the high cost of anthelmintic drugs and the labor and equipment required for the control of parasitosis (Qamar *et al*., 2011).

The control of *H. contortus* control is mainly based on the use of commercial anthelmintics; such as albendazole, benzimidazoles, levamisole, pyrantel, morantel, oxantel, monepantel, tribendimidine, piperazine and ivermectin (Gasser and Samson, 2016). The use of synthetic anthelmintics is thought to cause resistance if used for a long period with in appropriate doses (Haryuningtyas, 2008).

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The use of herbal anthelmintics can be an alternative that is cheap, safe and can overcome the problem of resistance with the presence of multitarget compounds.

Croton tiglium belongs to the family *Euphorbiaceae* in equatorial and moderate regions of the World (Hecker, 1968). It is widely used in folk medicine to treat certain cancers (Nath *et al*., 2013). The seeds, leaves, roots and bark of *C. tiglium* are used in conventional medicine to treat constipation, dyspepsia, dysentery, digestive disorders, enteritis, diarrhea, peptic ulcers, fever, alternative anti-worms and snake poisoning (Tsai *et al*., 2004). *Croton tiglium* seeds have been reported to be famous for their toxicity because

seed oil contains phorbol esters and crotonic acid in addition to fatty acids, also to the existence of active plant components (Hu *et al*., 2010).

The problem of resistance to anthelmintic drugs, their toxicity and growing concern about the presence of drug residues in animal products has led to a renewed interest in the use of herbal medicines. The in vitro tests using free-living stages of parasitic nematodes offer a means of evaluating the anthelmintic activity of new plant compounds (Asase *et al*., 2005). Considering the preceding rationale, the goal of this work was to determine the *in vitro* anthelmintic activity of *C. tiglium* seeds methanolic extract against *H. contortus*.

MATERIALS AND METHODS

Preparation of extracts

The *Croton tiglium* seeds were collected from a local market in Riyadh, Saudi Arabia. Powder totaling 500 g from the plant was extracted with 70% methanol as follows: 100 g of dry powder was added to 400 ml of 70% methanol and mixed gently for 1 h using a magnetic stirrer. The obtained solution was left at room temperature for 24 h, then stirred again and filtered. The solvent was then evaporated on a rotary evaporator (Inter world highway, LLC).

Adult worm collection

Parasites were obtained from the abomasum of sheep slaughtered in Al-Kharj abattoir, Saudi Arabia. Abomasum was handled by opening along the major curvature and the worms are collected using a small paint brush. Parasites were collected into containers with physiological saline solution (0.9%) and transferred to the laboratory of Parasitology (Department of Zoology, College of Science, King Saud University). After washing the worms several times with saline, healthy ones with normal microscopic structure and good motility were selected. They were kept in RPMI 1640 medium (nutritious and growth medium) until the experiment began (Sambodo *et al*., 2018).

Adult worm motility test

Solutions of *C. tiglium* seeds extract was prepared at six different concentrations (25, 50, 75, 100, 125 and 150 mg/ml). Five actively moving adult worms were then placed into each petri dish at room temperature. Normal RPMI-1640 medium (nutritious and growth medium) (Al-Genome International, Sharjah) and ivermectin 0.1% (Brovafarma, Ukraine) were also prepared and used as negative and positive controls. The test was repeated three times for all treatments. After treatment, observations were made by recording the death time for worms at the 2,4 and 6 h. Worms are considered dead if the worms do not move for 30 sec after the worm's body parts are touched using a surgical needle and shaking the petri dish. Dead worms were fixed in 10% formalin and stored in the refrigerator until used.

Histological preparation

For the histological study, the integumentary tissue of *H. contortus* was performed according to Jeyathilakan *et al*. (2012). In brief, integument tissues were fixed in 10% formalin for 24 h, dehydrated with graded alcohol concentrations and then cleared in Xylol. Worms were embedded in paraffin, then sections were sliced at 5-7 ìm in the transverse plane using a rotary microtome (Nauver company, South Korea). Tissue staining was performed with hematoxylin and eosin (H and E) stain.

Statistical analysis

Data were analyzed *via* the Statistical Package for the Social Sciences (SPSS for Windows (IBM), version 22, Chicago, USA) and presented as averages and $P<0.05$ considered a significant value.

RESULTS AND DISCUSSION

A significant elevation in mean inhibition of adult worm motility beginning 2 h post-exposure with 150 mg/ml of *C. tiglium* seeds extract and ivermectin 0.1%. After 4 hours of the exposure time, ivermectin 0.1% and concentrations of 100, 125 and 150 mg/ml of the extract resulted in substantially higher inhibited motility compared to the remaining concentrations below 75 mg/ml. The lower concentration (25 mg/ml) was substantially more lethal than the negative control (RPMI-1640 medium) at 4 h of exposure. Within 6 h after exposure, concentrations of 100, 125 and 150 mg/ml of *C. tiglium* seed extract caused death for all parasites ($P<0.05$) (Fig 1, Table 1).

The problem of resistance to anthelmintic drugs, their toxicity and growing concern about the presence of drug residues in animal products has led to a renewed interest in the use of herbal medicines. The *in vitro* tests using free-living stages of parasitic nematodes offer a means of evaluating the anthelmintic activity of new plant compounds

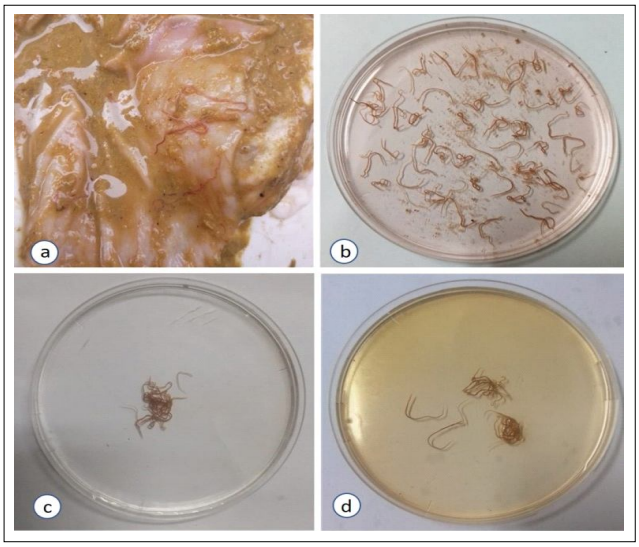

Fig 1: (a): *H. contortus* in abomasum; (b): Worms with RPMI 1640 medium (Nutritious and growth medium); (c): Worms treated with ivermectin 0.1% after 6 h; (d): Worms treated with *C. tiglium* seeds extract (150 mg/ml) after 6 h.

(Asase *et al*., 2005). Moreover, for in vitro studies, *H. contortus* proved to be a good experimental worm due to its longer survival in Phosphate buffered saline (PBS). The present result is comparable to those obtained utilizing different kinds of parasites reported by some researchers. Mumed *et al*. (2022) reported that the methanolic extract of leaves of Croton macrostachyus showed paralysis and death of *H. contortus* worm than the reference drug albendazole. Abon (2021) reported the ability of *C. tiglium* seeds in native chickens (*Gallus domesticus*) particularly against *Ascaridia galli* and *Heterakis gallinarum*as alternative anti-worms. Bodas *et al*. (2014) reported that the *C. tiglium* extracts showed paralysis and death of Indian earthworms than the reference drug albendazole. Liu (2014) reported the ability of *C. tiglium* extract caused 100% mortalities of the root-knot nematode at 1000 µg/ml for 72 h. Dohutia *et al*. (2015) reported that the extract of *C. tiglium* seeds had remarkable mosquito larvicidal activity *Anopheles stephensi*. This may be attributed to the fact that the *C. tiglium* seeds extracts are considered poisonous plants and can eliminate many kinds of parasitic worms. And, it was observed that Croton tiglium seeds extract has acaricidal activity and larval efficacy at different concentrations and periods (Mares *et al*., 2022).

Histological preparations showed changes in *H. contortus* after in vitro exposure to *C. tiglium* seed extract at a concentration of 150 mg/ml, RPMI-1640 medium and ivermectin 0.1%. Histological observations of *H. contortus* at RPMI-1640 medium, the cuticle was seen covering the outer surface of the body of the worm that the cuticle layer was intact and thick (Fig 2). The hypodermal cord below the cuticle layer is still attached to the cuticle and the muscular layer looks elongated. The struts present beneath the cuticular ridges. The nucleus and most of the cytoplasm (or sarcoplasm) reside in a massive, bulging cell body. Meanwhile, in histological observations of *H. contortus* at a concentration of 150 mg/ml (Fig 3), there was a similarity with ivermectin 0.1% (Fig 4). The cuticle layer was eroded so that it looked thinner than the cuticle layer of *H. contortus* at RPMI-1640 medium and the muscular layer look stringy and wrinkled. These situations made the muscular layer of *H. contortus* at a concentration of 150 mg/ml looked shorter than the negative control. Histological observations of *H. contortus* at a concentration of 150 mg/ml and ivermectin 0.1% caused damage to the integumentary structure of *H. contortus*, especially in the cuticle and muscular layer. The increase in the level of concentration is directly

Table 1: *In vitro* worm lethal effect of *C. tiglium* seeds extract on *H. contortus.*

Extract concentration	Average number of worm dead (average of mortality±SD) after exposure		
(mg/ml)	2 h	4 h	6 h
150	7.00±0.00	8.66±0.57	10.00±0.00
125	6.33±1.15	8.00±0.57	10.00±0.00
100	6.00±1.00	8.00±1.00	10.00±0.00
75	4.66±1.52	6.66±1.00	7.66±0.00
50	3.00±1.00	4.66±0.57	6.00±0.57
25	0.66±0.57	2.66±0.00	4.66±0.00
Ivermectin 0.1%	10.00±0.00	10.00±0.00	10.00±0.00
RPMI-1640 medium	0.00±0.00	0.00±0.00	0.00±0.00

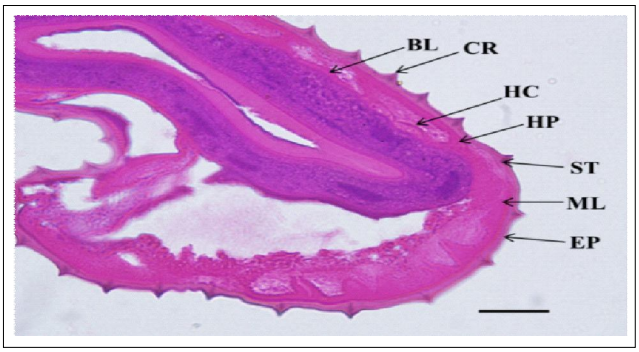

Fig 2: Transverse section of *H. contortus* at normal RPMI-1640 medium showing the epicuticle (EP), cuticular ridges (CR), struts (ST), hypodermal pore (HP), hypodermal cord (HC), muscular layer (ML) and basal layer (BL) of the body wall.Scale bar = 20 µm.

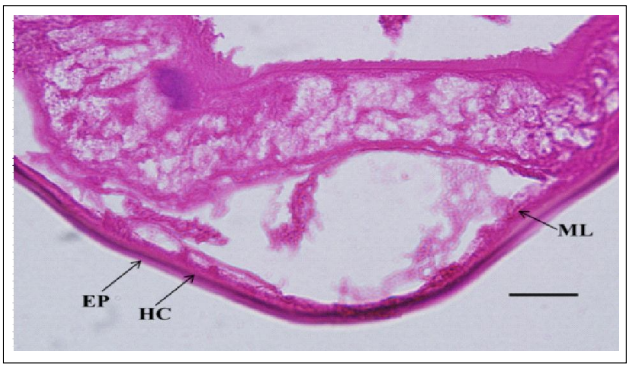

Fig 3: Transverse section of *H. contortus* at 150 mg/ml of *C. tiglium* seed extract showing the epicuticle (EP), hypodermal cord (HC) and muscular layer (ML). Scale bar = 20 µm.

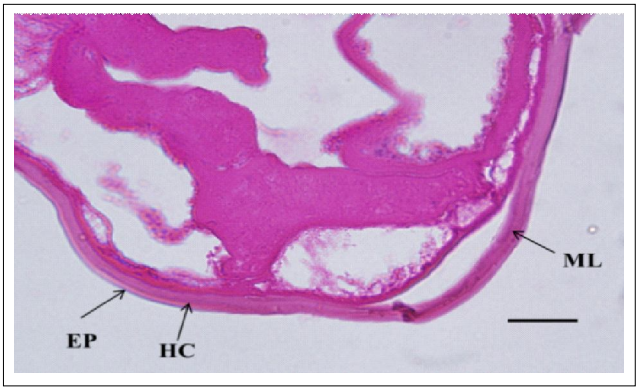

Fig 4: Transverse section of *H. contortus* at ivermection 0.1% showing the epicuticle (EP), hypodermal cord (HC) and muscular layer (ML). Scale bar = 20 µm.

proportional to the increase in damage to the cuticle and muscular layer compared to RPMI-1640 medium. Abbas *et al*. (2013) reported that all the *Croton* seed extracts have *in vitro* anthelmintic potential on *H. contortus*. The bioactive compounds responsible for these activities could be secondary metabolites that are present in the extract such as alkaloids, flavonoids, terpenoids, tannins and saponins. While the *in vitro* activities are comparable to those of a commercial anthelmintic.

CONCLUSION

The current study concluded that the medicinal plant tested showed a promising lethal effect against *H. contortus* worm that could be utilized as a possible alternative to replace commercially available drugs. More in vivo and in vitro studies are needed to better evaluate the possibility of these extracts.

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Authors' contributions

MMM (orcid.org/0000-0002-0662-2113) Conceptualization, Data curation and Methodology, RA (orcid.org/0000-0002-0422-8286) and SA (orcid.org/ 0000-0003-4204-3124) Formal analysis and Writing - review and editing, MMM and RA Writing original draft.

Conflict of interest declaration

The authors declare that they have no conflict of interest.

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