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ANTHELMINTIC ACTIVITY OF *HYMENODICTYON PACHYANTA* STEM BARK EXTRACTS AGAINST HAEMONCHUS CONTORTUS

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ABSTRACT

The development of host resistance to anthelmintics and the increasing cost of commercial anthelmintics have encouraged the need for the *in vitro* anthelmintic evaluation of crude extract and fractions of *Hymenodictyon pachyanta* plant as alternative drugs against *Haemonchus contortus*. *H. contortus* is one of the most prevalent and highly pathogenic parasitic nematodes in small ruminant farming globally. *H. pachyanta* stem bark is a prospective plant used by the local and indigenous farmers of Nsukka, Enugu state, Nigeria. The stem bark of *H. pachyanta* were collected, dried, pulverized and extracted with 80 % methanol. The purpose of this study was to investigate the *in vitro* anthelmintic effects of these crude extract and fractions against *H. contortus* in sheep and goats. The two extracts (crude and fractions) of *H. pachyanta* were tested by the egg hatch assay (EHA) and the larval development inhibition assays (LDIA) and to compared the results with albendazole (as the positive control). The concentrations for the crude extract and albendazole used for this study were 0.78, 1.56, 3.125, 6.25 and 12.5 mg.ml^{-1}. The results demonstrated that the crude extracts, fractions and albendazole all at the concentration doses of 12.5 mg.ml^{-1} produced 100 % inhibition of egg hatching and larval development. Statistically, there was no significant difference ($P > 0.05$) in the mean percentage inhibition of egg hatching and larval development inhibition of the crude extracts and fractions when compared with albendazole. However, a significant difference ($P < 0.05$) was observed with n-butanol fraction which inhibited 96.17 % of egg hatchability. All of the extracts and albendazole showed ovicidal and larvicidal effects and were able to induce over 50 % of the egg hatching and mortality of larvae at the concentration ranges of 0.78—12.5 mg.ml^{-1}. The results obtained from our study suggest that *H. pachyanta* had ovicidal and larvicidal activity against *H. contortus* and that the bioactive plants compounds responsible for this effect could be attributed to the presence of tannins, alkaloids and the saponins contained in the crude extracts.

Key words: anthelmintic; extract; fractions; *H. pachyanta*; *H. contortus*; larvicidal; ovicidal; resistance

INTRODUCTION

Sheep and goat parasitism in the past has been a continuous problem experienced by the small livestock producers. Parasitic gastro-enteritis is a clinical and sub-clinical condition resulting from one or in combinations of two or more parasitic infections of the gastrointestinal (GI) tracts. This poses a serious health challenge to the ruminant animals by limiting their productivity due to morbidity and mortality of the animals [16, 24]. Diseases due to gastro-intestinal nematodes are major economic constraints to grazing sheep production globally [19]. *Haemonchus contortus* is one of the GI parasites that causes an alarming threat to the development and production of ruminant livestock. They limit the production by causing high mortality rates in herds during the rainy season [26]. Among helminths types that infect livestock, *H. contortus* ranks highest in importance globally. They are considered to be the most prevalent and devastating species, thriving mostly in warm and humid areas. The death rate due to haemonchosis is very high and may be up to 50 % in some small ruminant communities [10].

The continuous and indiscriminate use of the synthetic anthelmintics has increased the resistance of parasitic helminths to anthelmintics [15]. Anthelmintic resistance is considered a major challenge in most sheep-rearing countries [17]. The current alternative and approach to reduce anthelmintic resistance is: phototherapy, use of medicinal and herbal plants (leaves, stem, barks, roots and seeds) to formulate good alternative herbal preparations with high anthelmintic effects that will complement the commercial anthelmintics [14, 25, 34].

The *Hymenodictyon pachyantha* (*Rubiaceae*) genus comprises twenty two species of which eleven are mostly found in the Madagascar region, four in Asia and seven in Tropical Africa [6, 29]. It is a tree found in the tropical forest of Africa, Cameroon, Nigeria (oke igbo), Niger and Benin [12]. They are medium-sized trees and grow up to 35 meters tall, with simple, opposite, decussate, subcoriaceous or coriaceous and membranaceous leaves. The West African *H. pachyantha* has the largest leaf blades, 8—31 by 5—11 cm. A previous study revealed that the *H. floribundum* trunk bark has been used in Angola folk medicine to treat fever, while the *H. excelsum* bark was used to kill tapeworms. The leaves and bark of *H. excelsum* possess pharmacological activities: such as antimicrobial, anticoagulant, anti-inflammatory, antioxidant, analgesic, and antipyretic activities [2, 7, 8, 23, 32].

This study evaluates the *in vitro* anthelmintic properties of the crude extracts and fractions of the *H. pachyanta* stem-bark extracts against *H. contortus* eggs and larval inhibition assay and validate its anthelmintic potentials.

MATERIALS AND METHODS

Plant collection and extraction

Fresh leaves of *H. pachyanta* were collected from Orba, in Udenu Local Government Area, Enugu state, Nigeria. The plants were identified by a Taxonomist, Mr. A. Ozioko of the Department of Biological Science University of Nigeria, Nsukka and a voucher specimen was deposited at the Department of Parasitology and Entomology, University of Abuja, Nigeria. The leaves were air-dried, pulverized and sieved. Three hundred grams of the pulverized plant material was extracted using 80 % methanol in a Soxhlet apparatus. The crude extract was concentrated in vacuo using a rotary evaporator coupled to a thermo-regulator.

Table 1. Qualitative phytochemical analysis

Phytochemical analysis	Plant constituents	*H. pachyanta* extract
Froting test	Saponin	+
Dragendoff test	Alkaloid	+
Molish test	Carbohydrate	+
Leiberman Bucchard test	Steroid	–
Leiberman Bucchard test	Triterpine	+
Keller kiliani test	Cardiac glycoside	–
Ferric chloride test	Tannin	+
Sodium hydroxide	Flavonoid	+
Bontrager's test	Anthraquinone	+
Ferric chloride test	Phenol	+

(+) indicates the presence of the component, while (–) indicates the absence of the phytochemical compound in the extract. The plant extract had all the listed secondary metabolites except for steroid and cardiac glycoside.

Solvent partitioning

The crude extract was suspended in water and partitioned subsequently using petroleum ether, ethyl acetate and n-butanol, using 150 ml of each solvent according to their polarity. The whole process was repeated three times for each solvent [30].

Collection of eggs and parasites

The collection of the parasites was done immediately after evisceration. The abomasum of a sheep naturally infected with *H. contortus* was incised and the contents washed in a clean plastic bucket and then taken to the laboratory. Parasites were recovered by passing the abomasal contents through a sieve of 100-µm-diameter mesh. Adult female *H. contortus* obtained from the abomasal washings were individually picked up with a wire loop under an illuminator (Picker X-ray). The female *H. contortus* were identified and separated from other parasites and were crushed in a mortar, using pestle, to obtain the eggs. The eggs were further mixed with autoclaved horse faeces and were incubated at 27 °C for 8 days after which the larvae (L1) were harvested using modified Baerman's apparatus [31].

***In vitro* egg hatch assay**

The Egg Hatch Assay (EHA) was performed using the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines [9]. Adult female *H. contortus* were obtained from the abomasum of naturally infected sheep slaughtered at the dogarawa abattoir, Zaria. The abomasa were removed soon after the evisceration and parasites were recovered by passing the abomasal contents through a sieve of 100-µm-diameter mesh. The parasites were individually picked out with a wire loop under an illuminator (Picker X-ray). The female *H. contortus* were identified and separated from other parasites [33]. The female worms were separated and suspended in distilled water and later crushed with mortar and pestle to liberate the eggs as described by Simon et al. [31].

Approximately 200 eggs contained in 0.08 ml were pipetted into a 96-flat-bottomed micro titre plate in addition with 0.5 ml at different concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 $mg.ml^{-1}$) of the crude extract and the fractions. Similarly, the same process was repeated with albendazole in addition with 0.5 ml at different concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 $mg.ml^{-1}$) and distilled water (0.5 ml). The eggs were incubated for 48 hours at 27 °C and 70 % relative humidity. Each concentration was done in triplicate. After 48 hours of incubation, a drop of Lugol's iodine was added to stop further hatching. Thereafter, using a pipette, the content of each well of the microtitre plate was placed on a glass slide and examined microscopically at ×40 magnification. All the unhatched eggs as well as the larvae (L1) in each well were counted and recorded.

Evaluation of the larvicidal activity of the extract

The evaluations of the larvicidal activities of the different portions of the extracts and fractions were done according to the methods described by Wabo et al. [34]. Albendazole, at different concentration and distilled water, were used as treated and untreated controls, respectively. One hundred (100) larvae of *H. contortus* contained in 0.1 ml of a suspension were added into each of a labelled 96-well flat-bottom microtitre plate. Thereafter, 0.5 ml of the different concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 $mg.ml^{-1}$) of the extracts and fractions were added. Each test was done in triplicate. The plates were covered with foil and left at room temperature (25 °C) for 24 hours. Thereafter, the content of each well was stirred and pipetted onto a clean glass slide and then examined under a microscope at ×4 magnification to count the number of larvae that were dead or alive. The movements or migration of the larva from one point to the other was used to consider if the parasite was still alive or dead. The larva was considered dead if it showed no observable motion after 5—10 seconds.

Statistical analysis

The mean (± SD) percentages inhibition of eggs hatched and larval development at different concentrations with the controls were compared and performed by one-way ANOVA. The statistical analysis was also determined by using the SPSS version 20 to aid easy analysis. The Post Hoc statistical significance test employed was the least square difference (LSD), the difference between the means was considered significant at $P < 0.05$.

RESULTS

The crude extracts of *H. pachyanta* stem bark and fractions significantly ($P < 0.05$) inhibited the hatching of eggs and larval development of *H. contortus* in a concentration-dose response. There was a positive correlation between the

Table 2. Mean (± SD) percentage inhibition of the egg hatch of *H. contortus* at different concentrations of *H. pachyanta* extract [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Treatments	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
H. pachyanta	96.3 ± 1.53^{b}	97.67 ± 1.04^{b}	98.67 ± 0.58^{b}	99.6 ± 0.289^{b}	100 ± 0.00^{b}
Albendazole	96.8 ± 1.04^{b}	98 ± 0.50^{b}	99 ± 0.00^{b}	99.8 ± 0.289^{b}	100 ± 0.00^{b}
DW	6.2 ± 2.33				

DW—distilled water; means with different superscript letters ($^{a, b, c}$) differed significantly ($P < 0.05$) from the positive control group

Table 3. Mean (± SD) percentage inhibition of larval development of *H. contortusat* different concentrations of H. *pachyanta* extract [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Treatments	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
H. pachyanta	70.6 ± 4.70^{b}	75.67 ± 3.50^{b}	82.3 ± 5.85^{a}	92 ± 3.00^{a}	100 ± 0.00^{b}
Albendazole	73.47 ± 2.25^{b}	77 ± 1.73^{b}	91.3 ± 3.20^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}
DW	13 ± 1.45				

DW—distilled water; means with different superscript letters ($^{a, b, c}$) differed significantly ($P < 0.05$) from the positive control group

Table 4. Mean (± SD) percentage inhibition of the egg hatch of *H. contortusat* different concentrations of *H. pachyanta* fraction [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Fractions	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
n-butanol	87.7 ± 6.89^{a}	90.17 ± 5.96^{a}	92.5 ± 4.48^{a}	94.3 ± 4.48^{a}	96.17 ± 3.55^{a}
Ethylacetate	90 ± 6.50^{c}	92.17 ± 5.90^{c}	94.5 ± 4.82^{c}	96.77 ± 3.3^{c}	100 ± 0.00^{b}
Petroleum ether	93.07 ± 2.72^{d}	95.03 ± 2.25^{d}	96.7 ± 2.60^{d}	98.3 ± 2.47^{e}	100 ± 0.00^{b}
Aqueous	89.17 ± 1.44^{e}	92.5 ± 1.81^{e}	93.6 ± 1.65^{e}	95.83 ± 1.89^{b}	99.5 ± 0.87^{b}
Albendazole	96.83 ± 1.04^{b}	98 ± 0.50^{b}	99 ± 0.00^{b}	99.83 ± 0.28^{b}	100 ± 0.00^{b}
DW	6.2 ± 2.33				

DW—distilled water; means with different superscript letters ($^{a, b, c, d, e}$) differed significantly ($P < 0.05$) from the positive control group

Table 5. Mean (± SD) percentage inhibition of larval development of *H. contortusat* different concentrations of *H. pachyanta* fraction [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Fractions	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
n-butanol	55.6 ± 3.00	60.5 ± 2.00^{a}	60.5± 2.00^{a}	80 ± 2.00^{a}	99 ± 1.32^{b}
Ethylacetate	62 ±2.00^{c}	72.0 ± 4.00^{c}	75.3 ± 5.03^{c}	90.17 ±1.61^{c}	99 ± 1.00^{b}
Petroleum ether	60 ± 2.00^{d}	67.3 ± 1.85^{d}	78.17 ± 2.05^{d}	92 ± 2.00^{d}	100 ± 0.00^{b}
Aqueous	65 ± 5.00^{e}	74.3 ± 2.00^{b}	82.0 ± 4.0^{e}	90.0 ± 4.00^{e}	100 ± 0.00^{b}
Albendazole	73.47 ± 2.25^{b}	77 ± 1.73^{b}	91.3 ± 3.2^{b}	100 ±0.00^{b}	100 ± 0.00^{b}
DW	13 ± 1.45				

DW—distilled water; means with different superscript letters ($^{a, b, c, d, e}$) differed significantly ($P < 0.05$) from the positive control group

concentrations of the crude extract, fraction, albendazole and the rates of egg hatch inhibition such that, as the drug concentration increased, the egg hatch and larval development inhibition rate increased (Tables 2, 3, 4 and 5). Although there were variations in the concentrations ($mg.ml^{-1}$) required for each of the crude extracts and fractions to show individual anthelmintic activity and efficacy. At the concentration of $12.5\,mg.ml^{-1}$ the crude extracts and albendazole inhibited 100 % of the hatching of eggs and larval development of the *H. contortus* showing no significant difference ($P > 0.05$) in their anthelmintic activity, while a significant difference ($P < 0.05$) was observed with 96.17 % inhibition of egg hatching by n-butanol when compared with albendazole.

DISCUSSION

All of the extracts of *H. pachyanta* showed significant inhibitory effects on the egg hatching of *H. contortus*. Even though there were differences in activity between the extracts of the plant, they were statistically insignificant ($P > 0.05$). However, a significant difference ($P < 0.05$) existed between n-butanol and albendazole which inhibited the hatching of eggs at 96.17 %. The inhibitory effect of the crude extract and fractions on the egg hatching of *H. contortus* eggs were not significantly different ($P > 0.05$) from the effects produced by albendazole (standard drug). Previous studies revealed that plant extracts usually produce graded dose response when tested on helminth eggs [2, 3, 27]. This study also showed an increase in the mean larval mortality rates with an increase in concentration of all the extracts tested. The extracts caused significant ($P < 0.05$) larva mortality rates from concentration ranges of 0.78–12.5 $mg.ml^{-1}$. The larvicidal activity of the crude and fractions of *H. pachyanta* on *H. contortus* larvae were not significantly different ($P > 0.05$) from the effects produced by albendazole. The larvicidal inhibitory effects observed in this study was due to the penetration of the active chemical constituents of the extracts across the cuticle of the larvae into their circulatory system when the larvae were brought in contact with the extracts [27].

This study confirms the anthelmintic effects of *H. pachyanta* against *H. contortus*. At the concentration of $12.5\,mg.ml^{-1}$, all of the crude extracts and albendazole produced 100 % inhibition of the egg hatch and larval development on *H. contortus* except for n-butanol fraction which had 96.17 %. However, there was no significant difference ($P > 0.05$) between the extracts and albendazole. An increase in the concentrations of the crude extract and fractions increased the percentage of inhibition of the anthelmintic activities of the extracts on the parasites in a dose-dependent manner (Tables 2, 3, 4 and 5). Our study compared with the previous studies done by Wabo et al. [34] and Gatachew et al. [13]. Our study also agreed with Passo et al. [28] who demonstrated that the extracts of the essential oil of *ocimum gratissimum* inhibited 96.94 % of the egg hatching of *H. contortus* at the lowest concentration of $2.5\,mg.ml^{-1}$, which was compared with $0.78\,mg.ml^{-1}$ of the crude extract that inhibited 96.3 % of the egg hatching of *H. contortus* in our study. With the ovicidal and larvicidal effects of *H. pachyanta* on *H. contortus* eggs and larvae, our study agreed with Maitreya [20], who reported the use of *H. excelsum* bark to kill tapeworms.

In our study, the anthelmintic effects of *H. pachyanta* could be attributed to one or more of the phytochemicals present in the stem bark such as tannins, triterpenes, saponin, polyphenols anthraquinones and contained toxic alkaloids (hymenodictyonim and hymenodictine) a bitter substance, which could also may be responsible for the activity observed in our study [7, 11, 18]. The tannins and saponins contained in medicinal plants have been reported to possess anthelmintics compounds [1, 4, 22, 31]. The effect of tannins is similar to some synthetic phenolic anthelmintics like niclosamide and nitroxynil, which interferes with the generation of energy by uncoupling oxidative phosphorylation in the helminth parasites [21]. Tannins also have the ability to bind free protein available for larval nutrition and reduce the nutrients available for the parasites chemical metabolism or directly through inhibition of oxidative phosphorylation which results in larval starvation and finally larval death [5].

CONCLUSIONS

Our study established the anthelmintic effects of *H. pachyanta* crude extract and fractions which offers a potential inhibitory ovicidal and larvicidal effects against the eggs hatching and larval development of *H. contortus*. Therefore, *in vivo* studies are required to investigate the safety, toxicity profile and to authenticate the therapeutic poten-

tials of *H. pachyanta* extracts for use as an anthelmintic compound in the treatment *H. contortus* parasites. Further isolation and screening of the plant bioactive compounds of *H. pachyanta* responsible for this ovicidal and larvicidal activity are needed.

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