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Anti-helminthic properties evaluation of extracts from *Heliotropium indicum* plant against free-living and parasitic stage of *Trichuris* spp

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Abstract
The Continued reliance on the limited number of synthetic anthelmintics has the potential to augment widespread anthelmintic drug resistance, a serious problem already observed in many livestock production systems. The use of natural dietary compounds has the potential to be a complementary control option which may reduce this reliance on drug treatment, and slow the development of resistance. Various crude extracts from *Heliotropium indicum* (basic, neutral and acidic compounds) at 10 mg/mL, 50 mg/mL, 100 mg/mL and 150 mg/mL were used to evaluate the anthelmintic effect on *Trichuris* spp. Phytochemical analysis on the crude extract revealed the presence of plant metabolites such as Alkaloids, saponins, tannins, Reducing sugar, flavonoids and Anthraquinones. Brown crystals were obtained for all crude extracts. HPLC revealed the presence of 5, 7, and 2 different isolated in the basic, acidic, and neutral crude extract from *H. indicum*. All extracts had effect on the motility of *Trichuris* spp. basic crude extracts had the lowest motility score at all concentration levels followed by Albendazole, Acid crude extract and basic crude extract within the 150 minutes of test. ANOVA performed at 0.05 significant level reveals that, there was significant reliable difference in motility above 30 minutes at all test levels both within and between groups at all the concentration level. The basic crude extract exhibited the lowest paralysis and death time at all concentrations levels with (158.4±0.59) min and (190.4±0.32) min respectively for paralysis and death at the 150 mg/mL. The acidic crude basic at the highest concentration caused paralysis and death at (406.7±0.56) min and (430.2 ±0.34) min respectively. The Neutral crude extract exhibited the highest paralysis and death time at all concentrations levels with (412.6±0.43) min and (425.3 ± 0.43) min respectively at 150 mg/mL. The extracts exhibited anthelmintic activity and thus could be an inexpensive and readily available source of *Trichuris* spp treatment.

Keywords: *Heliotropium indicum*, *Trichuris* spp, Crude, Motility, Time of paralysis and death.

1. Introduction
Phytomedicine refers to the using of plant's seed, berries, roots, leaves, bark, or flowers for medicinal purposes. It is becoming more acceptable for using herbal medicine for the treatment and prevention of diseases. This is as a result of advance improvement in quality control analysis along with clinical research (Stephen and Ehrlich, 2011) [11, 12]. The importance is further enhanced due to the cost and inaccessibility of orthodox drugs that are used for the treatment of diseases. This therefore makes the search for alternative ones with lower cost and easily accessible inevitable (Marcus, 2009) [5].
According to World Health Organization Technical Report Series 749 on the Prevention and Control of Intestinal Parasitic Infections (2007), one major health problem in West Africa today is worm infection among farming communities especially in children. The study showed that, the period under review from 2001 to 2011 saw a total number of 47147 patients reporting to the Laboratory for intestinal parasitic investigations (Walana *et al.,* 2011). Scholars estimate that over a quarter of the world's population is infected with an intestinal worm of some sort, with roundworm, hookworm and whipworm infecting 1.47 billion people, 1.05 billion people and 1.30 billion people respectively. Furthermore, the World Bank estimates that 100 million people may experience stunting or wasting as a result of infection (John and Petri, 2006) [4].

Heliotropium indicum is a common plant in Ghana and other parts of Africa. It is commonly known as 'Indian heliotrope', a traditional medicinal plant with many uses. The plant possesses antibacterial, antitumor, uterine stimulant effect, antifertility, wound healing, anti-inflammatory, anti-nociceptive and diuretic activities, anti-tumor and rheumatism (Singh *et al.,* 2012 and Bero *et al.,* 2009) [3]. It is also periodically used to treat intestinal worm infestation. In vitro anthelmintic properties of medicinal plants including *Heliotropium indicum* are assessed on earth worm *spp* due to its anatomical and physiological resemblance with the intestinal roundworm parasites in human and easy availability (Vidyasarathi, 1977. Thorn *et al.*, 1977. Oluwakemi *et al*., 2015) [14, 13, 7].

Although some work has been done on the anthelmintics activity of *Heliotropium indicum,* most of them were performed on common earthworm spp with no partial purification of the extract.

The aim of the study therefore is to investigate the anthelminthic activity of various crude compounds of *Heliotropium indicum* using isolated *Trichuris spp* from ruminants.

2. Methods

2.1 Extraction of plant material

The *Heliotropium indicum* plant was collected in Adoagyiri, located in the Eastern region of Ghana. The plant was air dried at room temperature (28 °C) for eight (8) days. The whole plant was pulverized prior to extraction. Extract was prepared by soaking 500g of the dry powdered plant materials in 1.5 L of water: ethanol (50:50) at room temperature for 48 hours (Phrompittayarat *et al.,* 2007) [8]. The extracts was filtered, first through a Whatmann filter paper No.42 (125mm) and then through cotton wool. The extracts was concentrated to approximated volume of 200 mL using a rotary evaporator at 40 °C. Phytochemical screening was then performed.

The crude basic, crude neutral and crude acidic components of the plant were separated as follows: 5 mL of 1.0 M ammonia was added to make the medium alkaline. 3 separate portion of 50 mL of diethyl ether were used to extract the basic and neutral compound from the aqueous phase.

To the aqueous phase, 5 mL of 2.0 M acetic acid was added to acidify the medium. 30 mL, 10 mL and 10 mL of diethyl ether was used successively to extract the acidic compound. The organic solvent was evaporated and the crude acidic residue was weighed and stored.

The organic phase containing the basic and neutral compound was then evaporated on a water bath at a temperature of 40 °C. The residue was reconstituted in 50 mL of distilled water. The medium was acidified using 5 mL of 1.0 Molar acetic acid. The neutral compounds present were extracted with 3 successive 30 mL Chloroform, evaporated to dryness and stored for the test.

Finally, the aqueous phase was made alkaline with 5 mL of 2.0 M ammonia to make the medium alkaline. It was then extracted with 3 successive 50 mL of diethyl ether successively to obtain the basic compound. The resultant solution which is the organic phase were evaporated to dryness and stored for further analysis. All the residues extracted were stored in an amber airtight glass.

2.2 Phytochemical screening of crude extract.

Phytochemical screening were performed using standard procedures (Sofowora, 1993) [9].

Test for Alkaloid: Approximately 5 mL of extract was diluted to 10 mL with acid alcohol, boiled and filtered. To 5 mL of the filtrate, 2 mL of dilute ammonia and 5 mL of chloroform was added. Sample was shaken gently to extract the alkaloid base. The chloroform layer was extracted with 10 mL of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

Test for saponins: Approximately 5 mL of extract was pipetted into a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for flavonoids: Approximately 2 mL of extract was pipetted in to a test tube. 5 mL of dilute ammonia was added and agitated to mix. Concentrated sulphuric acid (1 mL) was added. A yellow colouration that disappear on standing indicates the presence of flavonoids.

Also, a portion of the extract was heated with 10 mL of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

Test for tannins: Approximately 5 mL of the extract was boiled in a test tube after which the sample was filtered. A few drops of 0.1% ferric chloride was added. Brownish green or a blue-black colouration indicated the presence of tannins.

Test for reducing sugars (Fehling's test): Approximately 5 mL was added to boiling Fehling's solution in a test tube. The solution was observed for a colour reaction.

Test for Anthraquinones: Approximately 5 mL of the extract was boiled with 10 mL of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer was pipette into another test tube and 1 mL of dilute ammonia was added. The resulting solution was observed for colour changes.

HPLC finger printing of crude extract: For crude basic extract: Mobile phase include acetonitrile (60) and potassium phosphate buffer (40) adjusted to pH 8.2, used as diluent and mobile phase.

For Crude neutral extract: Mobile phase include acetonitrile and de ionized water (60:40).

For Crude Acid extract: Mobile phase included acetonitrile, ionized water and acetic acid (60:39:1)

The analyte were detected at 254 nm at a flow rate of 1 mL/ min. An ODS column (C18) with column dimension 250mm x 4.6mm (5 microns) was used. Column temperature also was set at 30 °C and Injection volume was 50 µL.

2.3 Culture media preparation.

The media consist of nutrient broth, 100 μg/mL of Amoxicillin and 75 μg/mL of clotrimazole. The combination was to provide nutrient and to prevent the growth of other microorganisms. HEPES was used as buffering agent. After sterilization 4 mL media were poured into each well of the 12 well plates and kept for cooling.

2.4 Preparation of Plant Extracts and standard Solutions.
Test extract (crude base, crude acid and crude neutral extract) and Albendazole were prepared separately as 10 mg/mL, 50mg/mL, 100 mg/mL and 150 mg/mL concentrations using distilled water. The standard drug Albendazole was used as positive control and distilled water was used as negative control.

2.5 Collection of *Trichuris spp*
Helminthes (*Trichuris spp)* were collected from the Gut of matured sheep under the guidance of qualified veterinary personnel. Adult worms were manually plucked from the gut contents with forceps and washed well with warm saline. The worms were then taken to the laboratory and washed repeatedly in the same sterile culture medium prepared. The anthelmintic activity was carried out as per the method described by Ajaiyeoba *et al.,* 2001, [1]. With little modification.

2.6 Evaluation of Anthelmintic Activity *Trichuris spp*.
In each of the 12 well plates containing 4 mL of the prepared medium, 2 mL of the various extract concentrations, 10 mg/mL, 50mg/mL, 100 mg/mL and 150 mg/mL were added and swilled to mix. Albendazole solution was used as reference standard drug and distilled water as control (Murugamani *et al.,* 2012) [6]. In each well, one *Trichuris spp* averagely 40 mm were added.
All the well plates were incubated at 37 °C. The worms were observed for motility. This was done after tapping the edges of the well plates and allowing the worms to move freely towards the well, the worms that were alive would be seen moving. The motility of the worm were scored at 30 minutes interval for 150 minutes. Motility was scored on a 0–5 scale where 0 is no movement and 5 is vigorous movement, as described by Stepek *et al*., 2005. [10]. Time of paralysis was noted when no sort of any movement was observed except when the worms were vigorously shaken. Time of death were noted ascertaining that the worms did not move neither when shaken vigorously nor when dipped in warm water (50 °C).

2.7 Statistical Analysis.
Worm counts were expressed as mean ± SEM. The significance of difference between the means were determined by ANOVA Using software package (SPSS for Windows) and considered as significant when $P < 0.05$.

3. Results and Discussion
3.1 The phytochemical screening
The phytochemical screening of whole plant extract of *Heliotropium indicum* using ethanol and water mixture (50:50) revealed the presence of alkaloid, saponins, flavonoid, tannins, reducing sugars and Anthraquinones. The detection of saponins and tannins in *Heliotropium indicum* plant supports the earlier report of Akinlolu *et al*., (2008).
The percentage crude extract yielded 4.2%, 3.4% and 1.3% respectively for Crude basic extract, Acidic crude extract and neutral crude extract. HPLC analysis of the various extracts revealed that approximately 14 different phytochemicals or more could be present in *Heliotropium indicum.* 7 compounds were detected in the crude acid isolates whilst the crude basic isolate revealed 5 phytochemicals and the least composition was the neutral crude isolates with two peaks. Refer to Figure 1 to Figure 3.

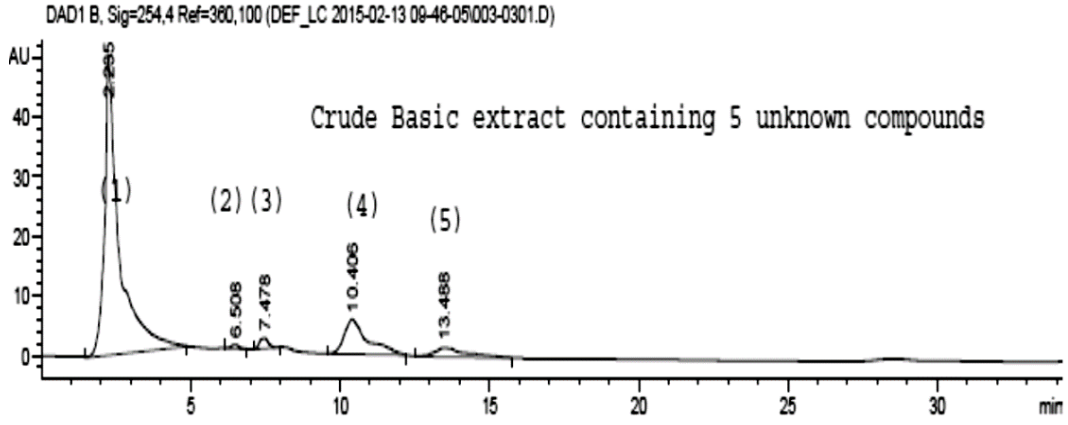

Fig 1: Chromatogram of basic crude extract

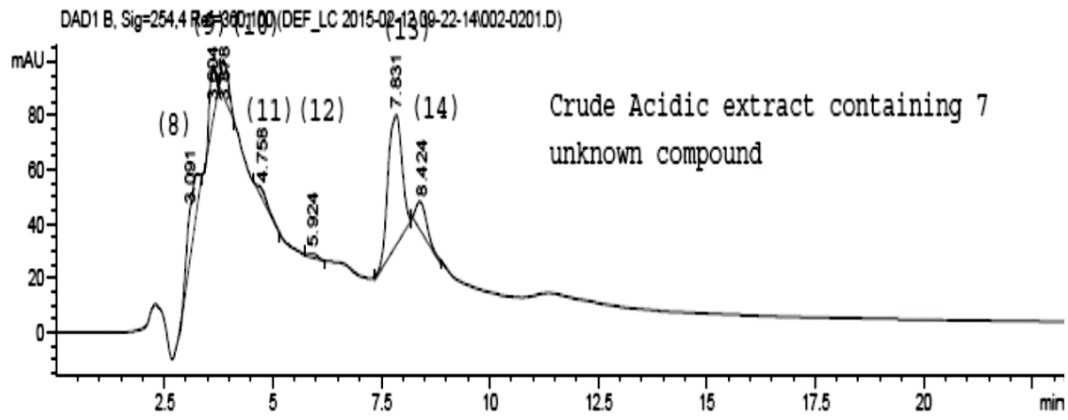

Fig 2: Chromatogram of Acidic crude extract

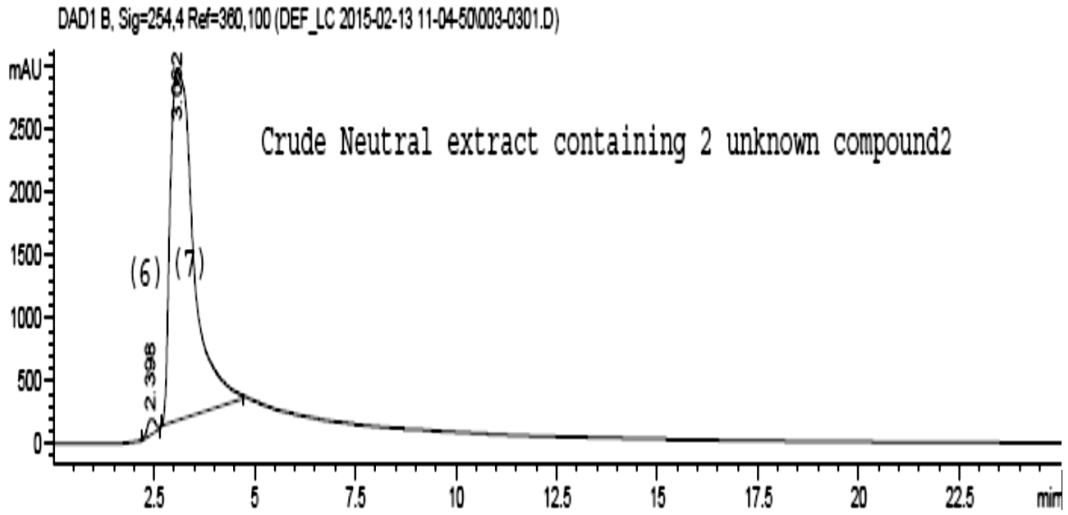

Fig 3: Chromatogram of Neutral crude extract

3.3 Motility test

Anthelmintic effects of the plant extracts fractions, standard Albendazole and control were assessed in vitro against the *Trichuris spp*. After incubation in each of the three plant extracts and control at concentrations range of 10 mg/mL to 150 mg/mL added at 2 mL to 4 mL nutrient broth, a reduction in motility was noted across all concentrations within 150 minutes of test (Figure 5). ANOVA performed at 0.05 significant level reveals that, there is significant reliable difference in motility above 30 minutes at all test levels both within and between groups at all the concentration level.

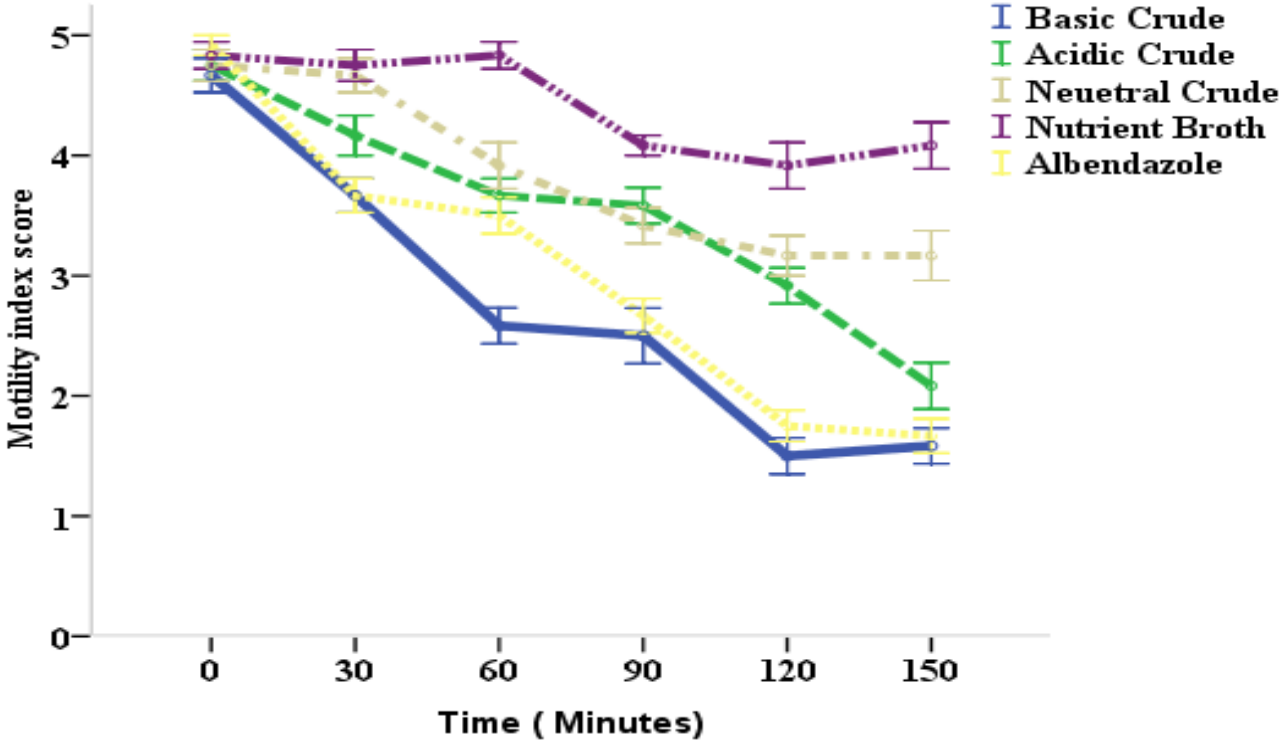

Fig 5: Effects of Crude extract, standard and control on the motility of *Trichuris spp.*

3.3 Time of Death and Paralysis

From the observation in Table 1, it was found that the higher the concentration of the extract, the faster was the paralytic effect and the shorter was the death time for all helminthes. From table one, crude base extract with concentration 150 mg/mL gave shorter paralysis and death time to be 158.4 min ± 0.59 min and 190.4 min ± 0.32 min respectively. (170.3 ± 0.51) min and (202.3 ± 0.23) min all at the highest concentration levels were the respective paralysis and death time for Albendazole. The acidic and crude extract at 150 mg/mL affected the *Trichuris* spp by paralyzing them at (406.7± 0.56) min and (412.6 ± 0.43) min respectively. All the investigational extract exhibited anthelmintic activity from a minimum dose of 10 mg/mL. Crude basic extract exhibited the shortest paralysis and death time followed by Albendazole, acidic crude extract and the least neutral crude extract. There were statistical reliable difference at 0.05 significant level for paralysis and death for all concentration levels when compared to standard drug Albendazole.

Table 1: *In-vitro* anthelmintic activity of crude extract of *Heliotropium indicum*

Drug treatment	Concentration (mg/mL)	Time taken for paralysis (min)	Time taken for Death (min)
Control	----------	----------	----------
Albendazole	10	247.8 ± 0.53	360.4 ± 0.35
Albendazole	50	209.5 ± 0.32	320.4 ± 0.43
Albendazole	100	190.5 ± 0.21	250.5 ± 0.25
Albendazole	150	170.3 ± 0. 51	202.3 ± 0.23
Basic Crude	10	247.8 ± 0.98	357.4 ± 0.43
Basic Crude	50	200.3± 0.78	312.3± 0.45
Basic Crude	100	180.4 ± 0.91	241.3 ± 0.32
Basic Crude	150	158.4 ± 0.59	190.4 ± 0.32
Acidic Crude	10	484.2 ± 0.64	521.3 ± 0.04
Acidic Crude	50	441.4 ± 0.39	500.3 ± 0.23
Acidic Crude	100	401.6 ± 0.34	451.6 ± 0.23
Acidic Crude	150	406.7 ± 0.56	430.2 ± 0.34
Neutral Crude	10	484.2 ± 0.68	524.4 ± 0.68
Neutral Crude	50	454.8 ± 0.67	491.2 ± 0.32
Neutral Crude	100	440.4 ± 0.34	464.4 ± 0.25
Neutral Crude	150	412.6 ± 0.43	425.3 ± 0.43

4. Conclusions

There crude extracts of *Heliotropium indicum* (Basic compounds, acidic compounds and neural compound) exhibited anthelmintic properties against the *Trichuris spp.* The basic crude exhibited the highest effect whilst the Neutral compounds exhibited the least. These findings may partly explain some of the folklore use of these plants in the treatment of worm infestations.

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