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In vitro anthelmintic activities of aqueous crude extract of *Azadirachta indica* on *Paramphistomum cervi* and *Fasciola hepatica*

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Abstract
For centuries, medicinal plants have been used to combat parasitism, and in many parts of the world they are still being used for this purpose. The *in vitro* anthelmintic properties of *Azadirachta indica* on *Paramphistomum cervi* and Fasciola hepactica was investigated in this study. Five different concentrations of *Azadirachta indica* crude leaf extract (2, 4, 6, 8 and 10mg/ml) were prepared and administered *in vitro* in sample petridishes containing *P. cervi* and *F. hepatica* collected from cattle slaughtered at Apiapum abattoir. A total of 10 of each of the parasites were kept inside each sample petridish. Mortality of the parasites was monitored for 1, 2, 3, 4 and 5 hour respectively. The mean cumulative mortality and probit analysis were performed to determine the lethal concentration (LC_{50}) of the plant crude leaf extract on the parasite. The anthelmintic activities of Azadirachta *indica* plant was found to be time and concentration dependent. Cumulative mortality (%) range from 10.10 – 23.33% at the end of 5 hour at normal control (0mg/ml) and 0.00 -23.33% for reference control in all the parasites. At the highest concentration (10mg/ml) of *A. indica*, 100% mortality was attained in just 2 hours of exposure of *P. cervi* whereas 3 hours was required to achieve the same result for F. hepatica. The LC_{50} values decreased with increase in time of exposure for both species of parasites. The 5h LC_{50} of *A. indica* (1.65mg/ml) recorded against *P. cervi* was higher than the corresponding values 1.44 for *Fasciola hepatica*. *A. indica* therefore exhibited anthelmentic activities against both trematodes *in vitro* with higher lethality to *F. hepatica* relative to *P. cervi* which is both concentration and time dependent.

Keywords: Anthelmintic, *Azadirachta indica*, *Paramphistomum cervi*, *Fasciola hepatica*

Introduction
In ethno veterinary medicine, which draws inspiration from traditional practice, there seems to be a range of plants or plant extract suitable for treating almost every parasitic disease of livestock. Reports from around the world include exhaustive lists of plants that have been reported to have medicinal properties [1, 2, 3]. Although a number of medicinal plants have been evaluated through these methodologies and have been found to be active against parasites, the purported antiparasitic properties of a large variety of plants have not been reproduced under controlled experimentation. Commercial anthelmintics have been used for some decades throughout the world to minimize the losses caused by helminth infections. The threats of anthelmintic resistance, risk of residue, availability and high cost, especially to farmers of low income in developing countries, have led to the notion that sustainable helminth control cannot be achieved with commercial anthelmintics alone. The emergence of resistance to anthelmintic drugs, which is now a worldwide phenomenon [4], and the increased awareness of consumers about drug residues that potentially enter the food chain have stimulated investigation into alternatives to commercially available anthelmintics, such as medicinal plants. Their persistence in various sources in many parts of the world has resulted in medicinal plants attracting attention from the scientific community.
In an attempt to utilise as effectively as possible the information available from ethno veterinary and medicinal reports on the anthelmintic activity of plants, there is a current trend to validate such plants under controlled experimental conditions. For example, the consumption of leaves of wormwood in the form of powder (*Artemisia brevifolia*), one of the bitterest of plants, has been tested in a controlled study for its anthelmintic activity [5].

Demonstrated that the consumption of the whole plant resulted in a 62% reduction of the abomasal nematode *Haemonchus contortus* egg counts. The consumption of fagara leaves (*Zanthoxylum zanthoxyloides*), a native tree from Africa, believed to have antiparasitic activity, resulted in reduced egg excretion by the same nematode in sheep, when consumed regularly in small amounts. Similarly, lespedeza (*Sericea lespedeza*) a grazing perennial legume native of Eastern Asia showed promising anthelmintic activity when offered to goats either fresh [6] or as hay [7]. Ethno veterinary sources from south-east Asia report that cassava forage (*Manihot esculenta*) has been used by traditional healers with success for the control of internal parasitism [8]. Rich literature is available on ethno veterinary use of medicinal plants as anthelmintics and other diseases.

Traditionally, leaves root and bark of *Carissa spinarum* are used against Gastro Intestinal Track parasites and ring worm [9, 10]. The leaves and fruits of *Phytolacca dodecandra* are used against endoparasites [11], and the leaves as antiseptic [12]. The root, bark and inner bark of *Acacia tortilis* are used to treat diarrhea [9] and the bark is used topically to treat ring worm [10]. The consumption of cassava hay resulted in lower faecal egg counts and worm burdens in sheep parasitised with abomasal and intestinal nematodes compared with unsupplemented controls [8].

The neem tree (*Azadirachta indica*) is known for its medicinal properties and has been recommended for use against gastro-intestinal nematodes and related problems in many parts of the world [13]. *Melia azederach*, another plant that belongs to the same family as the neem tree, has also been reported to have anthelmintic activity. However, under controlled experiments, no anthelmintic activity has been demonstrated [14]. In addition, there is a large number of grazing forages, including *Lotus pedunculatus* and *Hedysarum coronarium*, whose anthelmintic activity has been shown to be rather inconsistent across the various studies around the world [15, 16]. Earlier studies showed that diamondback moth larvae had mortality of 14.8% when treated with 1% aqueous extract of *Azadirachta indica* (Neem leaves) and 37% mortality on 5% treatment [17, 18] reported that 5% and 10% aqueous solutions of neem leaf extracts inhibited normal development of the tobacco cutworm, *Spodoptera litura* l., whereas a 2% solution distinctly decreased fecundity and egg fertility. When [19] applied 2.5, 5.0, and 10% solutions to bean leaves, the growth of 1st-instar of the Mexican bean Beetle *Epilachna varivestis* muls., was inhibited and 100% larval mortality occurred. The author also found that 4th-instar larvae of Diamondback moth *Putella xylostella L*., treated with 2.5 and 5.0% methanol-water extract (1:1) solutions of neem leaf extract could not pupate. In Togo (West Africa), the application of a 4% aqueous extract was not efficient enough to protect corn and cabbage against destruction by insects [20]. Helminthosis play a crucial role in small ruminant production leading to enormous economic losses particularly in areas where extensive grazing is practised [21] *Fasciola hepatica* is distributed worldwide and has been known to be an important trematode of cattle and sheep for hundreds of years and it causes great economic losses in sheep and cattle. The present study is designed to investigate the *in vitro* anthelmintic activities of aqueous extract of *Azadirachta indica*, on *Fasciola hepatica* and *Paraphistomum cervi* trematodes.

Materials and methods

Collection of Plant Material

The *Azadirachta indica* (neem leaves), were collected from Ovonum village in Obubra Local Government council of Cross River State of Nigeria. The leaves were identified by a taxonomist in the Department of Forestry and Wildlife management CRUTECH Obubra Campus and voucher specimen deposited in the same Department. The leaves were chopped into pieces shade dried at room temperature for two week according to [22]. The dried sample was ground into fine powder using mortar and pestle and stored in an airtight container prior to the test.

Preparation of *Azadirachta indica* (*A. indica*) leaf extract

Five hundred (500g) grams of shade dried leaves of *A. indica* were pounded in a mortar with pestle and ground to powder. The powdered form was poured into a conical flask containing 100ml of sachet water and stirred every six hours. This was done for 48 hours to allow water penetrate the powder. The suspension was filtered with wire guaz sieve and the filtrate evaporated to dryness in DHG-9101ISA oven. The un-evaporated dried filtrate (extract) was kept in clean 50ml conical flask for further action.

Preparations of different concentrations of crude extract of *A. indica* shade dried leaves

Five test tubes numbered 1-5 containing 20ml of water each were set in a test tube racket. Two to ten milligrams (40-200mg/20ml representing 2-10mg/ml) of the crude extract of *A. indica* were added to the test tubes. Forty/40mg of the crude extract of *A. indica* was added to the first test tube. The second received 80mg/2oml, the third 120mg/20mlm, the fourth 160mg/20ml and the fifth 200mg/20ml representing the different concentrations used in this study. The dried crude extract was measured out using weighing balance model KD-CN number 110726042 having a capacity to measure 0.01g or mg. The solutions were poured out into five/5 labeled petridishes (A-E) for the dipping of the parasites. This was replicated 5times representing the hourly (immersion of the parasites) duration of the experiment. The control experiment contains only the distilled water with rumen content for *P. cervi* and distilled water with liver tissues for *F. hepatica*.

Dipping of parasites in prepared concentration of *A. indica* crude extract

The labeled petridishes containing different concentrations of the crude extract were set in laboratory bench table. Ten/10 *P. cervi* were immersed in the first petridish for one hour, then for 2hours, 3, 4 and 5 hour duration. This same process was repeated for the rest four concentrations of the crude extract. (ie 4mg, 6mg, 8mg and 10mg/ml). Mortality of the parasites in different concentrations and time duration were recorded. The same procedure was carried out using ten/10 *F. hepatica* in equal concentration of crude extract and same time duration and their mortalities recorded also.

Collection of parasites

Adult specimens of *Fasciola hepatica* and *Paramphistomum cervi* were collected from the liver and the rumen of the cattle into different containers from freshly slaughters cattle in Apiapum slaughter slab, Obubra in Cross River State. Identification was done based on their morphological characteristic. The parasites were immediately placed in a normal saline (Goodwin's physiological) solution and taken to parasitology laboratory for the *in vitro* bioassay.

Identification of live helminths

The living (live) helminth of *F. hepatica* wriggles as soon as it's outside the host animal. The wriggling movement continues for some time as long as the parasites are alive. If

the helminth is resting, it retracts its head close to the body. If the helminth is dead, every bodily movement comes to a halt. For the *P. cervi*, the helminth appears in cluster of 5 to 10 *P. cervi*. If they are alive they elongates or lengthen their head but at death they retract and assume roundish shape. If the cluster is separated they try to re-cluster themselves again. When the helminths are finally dead, the separated *P. cervi* remains permanently separated without re- clustering together.

Plate 1: Fresh *P. cervi*

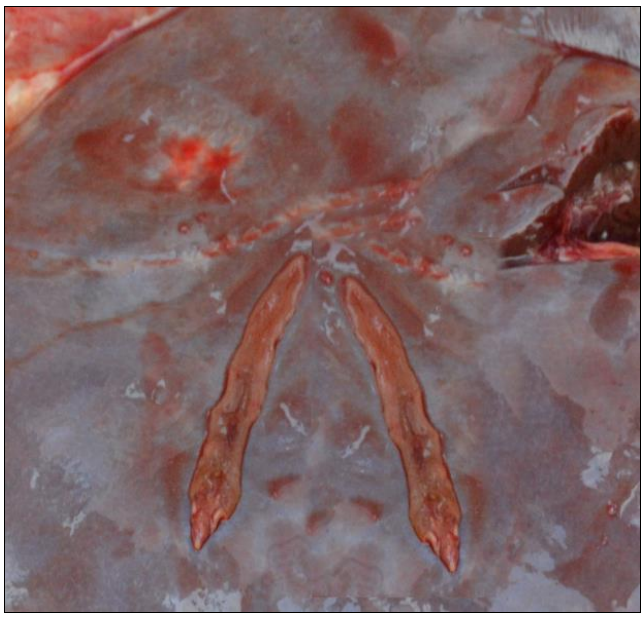

Plate 2: Fresh *F. hepatica*

Experimental procedure

The collected worms were kept in distilled water with collected rumen ingesters for *P. cervi* and distilled water with liver tissues for *F. hepatica* just to mimic the natural predilection sites which serves as the reference control. The test was performed in 5cm diameter plastic Petri dishes. Ten/10 worms each of *P. cervi* and *F. hepatica* was put in 5 different concentrations in milligram per millilitre (mg/ml) of the plant extract in triplicates. The *in vitro* lethality of aqueous extract of *A. indica* was performed in petri dishes. Ten *P. cervi* and ten of *F. hepatica* were dipped in different concentrations of *A. indica*. Mortality of the parasites were monitored at five/5 different (2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml) concentrations at different time intervals of 1, 2, 3, 4, 5 hours. Death was confirmed by cessation of movement by the parasite when touched with sharp instrument or vigorously shaken. The set up was allowed for 12 hours and mortality figures recorded and percentage mortality calculated.

Data collection and statistical analysis

Simple descriptive statistics of percentages were used to calculate the mortality rates and 5x5 factorial analysis of variance used to test for significant differences among and within groups of treatment. Probit analysis was used to estimate dose quantal response of the parasites and the median lethal concentration LC50 of the extract determined.

Result

Cumulative Mortality (%) of Adult *Paramphistomum cervi* and *Fasciola hepatica* exposed to Concentrations of *Azadirachta indica* for 5 hours

The results of the mean cumulative [%] mortality of *P. cervi* and *F. hepatica* exposed to various concentration of *A. indica* are shown in table 1.1 and 1.2 respectively. The result revealed that mortality was both time and concentration dependant. Mortality increase with both time of exposure and concentration of *A. indica*. In both *P. cervi* and *F. hepatica* there were no mortality recorded in reference control treatment. The normal control treatment for *P. cervi*, recorded no mortality between the first and fourth hour and only 10% at the end of five hours of exposure. At 2.00mg/ml, more than 50% mortality was recorded at the second hour and about 86.67% was recorded at the fifth hour of exposure. At 6.00mg/ml, more than 70% mortality of the flukes was recorded at the first hour while 100% mortality of the parasites was recorded from 3rd to 5th hours of exposure. At the highest concentration of 10mg/ml, 90% mortality of the parasite was recorded at the first hour and 100% from the second to the fifth hour. For *F. hepatica*, about 24.42% mortality of the worms were recorded at the end of five hours. At the least concentration (2.00mg/ml), 53.33% mortality of the warms was recorded at the first and 96.67% at the fifth hours of exposure. Similar trends of mortality were also observed as in *P. cervi* except that only 86.61and 96.67% mortality was recorded for first and second hour at 10.00mg/ml for *F. hepatica*. Significant ($P<0.05$) differences exist in mortality of parasite between two concentrations exposed to the same length of time and similar significant pattern of mortality occurred in parasites exposed to same concentration but different times exposure. This was true for both parasites. There was a strong deviation from this pattern as was observed in 8mg/ml for 1 hour exposure time which may be attributed to experimental error. The probit data showed a strong inverse association between lethality of extract and its duration of exposure as depicted in table 1.3. The longer the length of exposure time, the lower the LC_{50} figure but the more lethal the extract is to the parasite.

Table 1: Cumulative Mortality (%) of *Paramphistomum cervi* exposed to Concentrations of *Azadirachta indica* for 5 hours.

Conc. (mg/ml)	Time (hr)				
	1	2	3	4	5
Ref. Contr.	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0	0.00±0.00	0.00±0.00	0.00±0.00	3.33±1.22	10.16±5.77
2	36.67 ± 5.77^{a}	53.33 ± 5.77^{b}	70.00 ± 10.00^{c}	80.00 ± 0.00^{d}	86.67 ± 5.77^{d}
4	50.00 ± 10.00^{e}	73.33 ± 5.77^{f}	86.67 ± 5.77^{g}	96.67 ± 5.77^{h}	100.0 ± 00.00^{h}
6	73.33 ± 5.77^{i}	83.33 ± 5.77^{j}	100.00 ± 0.00^{k}	100.00 ± 0.00^{k}	100.00 ± 0.00^{k}
8	63.33 ± 5.77^{l}	86.67 ± 5.77^{m}	96.67 ± 5.77^{n}	100.00 ± 0.00^{n}	100.00 ± 0.00^{n}
10	90.00 ± 10.00^{o}	100.00 ± 0.00^{p}	100.00 ± 0.00^{p}	100.00 ± 0.00^{p}	100.00 ± 0.00^{p}

Mean is ± SD. Mean values on the same row with different superscripts are significantly (P<0.05) different.

Table 2: Cumulative Mortality (%) of *Fasciola hepatica* exposed to Concentrations of *Azadirachta indica* for 5 hours.

Conc. (mg/ml)	Time (hr)				
	1	2	3	4	5
Ref. Contr.	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0	0.00±0.00	0.00±0.00	0.00±0.00	10.00±10.55	24.54±2.47
2	53.33 ± 15.28^{a}	70.00 ± 10.00^{b}	83.33 ± 5.77^{c}	93.33 ± 5.77^{d}	96.67 ± 5.77^{d}
4	56.67 ± 5.77^{e}	76.67 ± 5.77^{f}	86.67 ± 5.77^{g}	100.00 ± 0.00^{h}	100.00 ± 0.00^{h}
6	73.33 ± 5.77^{i}	80.00 ± 0.00^{j}	90.00 ± 0.00^{k}	96.67 ± 5.77^{k}	100.00 ± 0.00^{k}
8	63.33 ± 5.77^{l}	83.33 ± 5.77^{m}	90.00 ± 10.00^{n}	100.00 ± 0.00^{n}	100.00 ± 0.00^{n}
10	86.67 ± 5.77^{o}	96.67 ± 5.77^{p}	100.00 ± 0.00^{p}	100.00 ± 0.00^{p}	100.00 ± 0.00^{p}

Mean is ± SD. Mean values on the same row with different superscripts are significantly (P<0.05) different

Median Lethal Concentration (LC_{50}) of Azadirachta indica

Table 3: Lethal concentration (LC_{50}) and associated 95% confidence limit of *A. indica* exposed to *P. cervi* and *F. hepatica* for 5 hours

Time (h)	LC_{50} (95% Confident Limit)	
	P. cervi	*F. hepatica*
1	4.51 (2.56 – 6.31)	4.51 (2.56 – 6.31)
2	2.95 (1.28 – 4.17)	2.95 (1.28 – 4.17)
3	1.70 (0.72 – 2.50)	1.86 (0.00 – 0.00)
4	1.62 (0.00 – 0.00)	1.44 (0.00 – 0.00)
5	1.62 (0.00 – 0.00)	1.44 (0.00 – 0.00)

The lower the LC_{50}, the more the lethality of plant extracts to the parasite and the more susceptible the parasite is to the extract.

Discussion

The results of the present study clearly demonstrate that *Azadirachta indica* is potent source of helminthicide. This finding agrees with the previous report that indigenous plants are useful in the treatment of helminthiasis [1]. Preserved in the natural host environment, both parasites could live longer than the five hour treatment duration as is observed in reference control experiment. The anthelmintic property of plants is dependent on numerous substances that are found in them. These could be alkaloids, sugars, saponins, aromatic oils, resins and other medicinally useful chemicals. [23] Reported that substances like steroids, coumarins, tannins, and triterpenoids and other chemical constituents of plants like alkaloids, glycosides, enzymes, anthraquinones, tannins, gums, fixed oils, fats, waxes, volatile oils, proteins and carbohydrates all have medicinal or pharmaceutical value of anthelmintic agent for the treatment of Ascariasis, Trichuriasis, and ancylostomiasis [23]. *Azadirachta indica* contains the secondary metabolite azadirachtin which is capable of suppressing feeding by many insects and parasites while maintaining very low mammalian toxicity. Although this metabolite is resident in the seed, and methanol leaf extract is effective against Haemonchus contortus [24]. The findings are in consonance with the report of [25] on aqueous and alcoholic extracts showing anthelminthic activity against Setaria cervi and [26] on aqueous leaf extract exhibiting anthelminthic activities in dose-dependent manner showing maximum efficacy at 40mg/ml against *Ascaridia galli* and *Raillietina* species. The presence of alkaloids, flavonoids and saponins in leaf extract of *Azadirachta indica* has been reported [27]. Alkaloid may act on the central nervous system to cause paralysis of the parasite and death [28] while saponin affects the permeability of the cell membrane of the parasite and cause vacuolization, disintegration of teguments and eventual death [29]. Flavonoid (isoflavones) inhibits the enzyme of glycolysis and disturbs the Calcium homeostasis and Nitrous Oxide activity and eventual death of the parasite [30]. while maintain low toxicity in mammalian animal host The above reports give credence to the current study which revealed anthelminthic activities of *Azadirachta indica* aqueous leaf extract *in vitro* on both *Paramphistomum cervi* and *Fasciola hepatica* with higher susceptibility exhibited by *F. hepatica*. The non-significant mortality rate observed at higher times of exposure and higher concentrations of *A. indica* extract may be due to the attainment of maximum mortality rate by the parasite or it may be due to the uptake of the active moiety which progressively increases in the parasites' body with increase in exposure period. *A. indica* therefore may be a good alternative to tackle the stiff resistance developed by the parasites against orthodox anthemintics.

Conclusion

The *in vitro* anthelmintic characteristics of *Azadirachta indica* aqueous leaf extract was investigated on *P. cervi* and *F. hepatica* in this study and the result showed a greater than 50% mortality of both parasites occurring across the 5 hour duration from 4mg/ml concentration of the extract and above. The result attests to the *in vitro* anthelmintic efficacy of the extract and toxicity in both parasites reaching 100% at higher concentration and time of exposure. As this study is aimed at finding a suitable alternative to orthodox anthelmintics, *Azadirachta indica* has sufficiently proved the herbal leave of choice.

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