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Nematicidal Activity of Methanolic Extracts of Leaf, Stem Bark, and Root of *Azadirachta Indica* (Neem) Against *Haemonchus Contortus*

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ABSTRACT

Livestock, especially small ruminants, represent a significant asset among resource-poor smallholder farmers, particularly the pastoralist communities. The main aim of this study is to evaluate the potency of methanolic extracts of *Azadirachta indica* (neem) leaves, stems, barks, and roots against *Haemonchus contortus*. Different parts (leaf, stem bark, and root) of *A. indica* (neem) were collected in Gwagwalada. The plant was identified in the Herbarium Section of the Department of Biological Sciences, University of Abuja. Abuja. The different parts of *A. indica* (neem) were air-dried at ambient temperature, after which each was pounded separately using a pestle and mortar and later sieved using a sieve mesh. 500g of each plant part was stored concealed at room temperature until required for use. 500g of each plant part collected was defatted in 2.5 litres of absolute methanol at room temperature for 8 hours to obtain the methanolic extract. The extract was filtered, and the filtrate evaporated to dryness at 50 °C under reduced pressure. The extracts were collected and concealed in aluminium foil paper and stored in a refrigerator at 4 °C until required. The goat's intestinal waste was obtained from the Gwagwalada abattoir and conveyed in clean polythene bags to the Biology Laboratory, Department of Biological Science, University of Abuja. The waste content was placed on a fluorescent picking board; the parasites were picked with a picker and transferred to Petri dishes containing water. 120 larvae in 0.1 ml in wells of microtitre plate (10 larvae in each microtitre) and concentrations of each of the extracts (leaf, stem, bark, and root) at 0.1 mg/ml, 1.0mg/ml, 10.0mg/ml, 100mg/ml, negative control (water) and positive control (Levamisole) was added to each of the wells. The phytochemical screening showed the presence of carbohydrates, cardiac glycosides, saponins, flavonoids, tannins, and alkaloids and absence of anthraquinones derivatives in stem bark, root, and leaf of *A. indica* (neem), presence of steroids in leaf and absence in stembark and root, presence of triterpenes in both stembark and root and its absence in leaf. Mortalities recorded were high in the positive control wells, increasing mortality as the time of exposure increased. At 24 hours of parasites exposure to the positive control, there was almost complete mortality of the parasites; none of the parasites survived in the positive control wells from the 30" hour of exposure. Only one mortality was observed in the negative control wells; even in wells with the highest exposure time, mortalities were not recorded. Mortalities of the parasites increased with an increase in concentration and with the time of exposure.

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INTRODUCTION

1.1 Background of the Study

Livestock, especially small ruminants, represent a significant asset among resource-poor smallholder farmers, particularly the pastoralist communities (Mini, 2012; Alemu *et al*., 2014). Diseases caused by helminth parasites in small ruminants continue to be a significant productivity constraint in the tropics and subtropics (Verissimo *et al*., 2012; Mini *et al*., 2013). In developed countries, the most significant impact is on the control costs of helminth parasites. In contrast, in developing countries such as Nigeria, the effect is direct and potential productivity losses (Ombasa *et al*., 2012). Among different types of helminths, nematodes are the most important as far as their prevalence and adverse effects on

animal health and productivity are concerned. They cause retarded growth (Mini, 2012), lowered productivity (Ombasa *et al*., 2012), mortality (Mohammed *et al*., 2013), and high economic losses (Alemu *et al*., 2014), which adversely affect the livelihood of small farmers.

Haemonchus is the highly pathogenic nematode parasite of livestock, especially small ruminants capable of causing acute disease and high mortality (Soulsby, 2006; Ombasa *et al*., 2012). Haemonchosis is characterised by hemorrhagic anaemia due to the blood-sucking activities of the worms in the abomasum (Chartier *et al*., 2001; Soulsby, 2006; Mohammed *et al*., 2013); this is one of the most critical parasites causing reduced production in livestock throughout the tropics and most countries (Alemu *et al*., 2014). Over the past five decades, the control of this parasite has been achieved mainly through intensive chemoprophylaxis based on the repeated use of anthelmintic drugs (Mohammed *et al*., 2013). The emergence of a nematode population resistant to one or more of the currently available broad spectra anthelmintics is a worldwide phenomenon and is particularly severe in small ruminants (Ombasa *et al*., 2012).

The control of nematode infections has traditionally been done using anthelminthic drugs (chemotherapy), with the best results obtained when this approach is initiated with proper grazing management and resistant animals. However, in the last two to three decades, misuse of synthetic antiparasitic products has led to the development of Anthelmintic Resistance (AR) (Arunachalam, 2008; Mohammed *et al*., 2013). In developing countries such as Nigeria, the production and availability of these drugs are highly variable and often too expensive. Moreover, this approach has become a source of public concern in terms of both the use of proprietary medicines in farm production and the risk of chemical residues in food products (Muhammad *et al*., 2004; Mohammed *et al*., 2013). Hence, novel approaches to nematode parasite control are needed for small ruminants in the tropics and sub-tropics to counteract the problem of AR (Ombasa *et al*., 2012), creating a trend towards organic farming and sustainable animal husbandry. (Mohammed *et al*., 2013). The development of livestock breeds resistant to parasites and plants with anti-parasitic properties and traditional herbal remedies, or Ethnoveterinary Medicine (EVM), are becoming more relevant (Pereira *et al*., 2013). Plant-based anthelmintics have been known and used in many parts of the world for a long time, but little research has been done to validate their use, especially in veterinary medicine.

Three approaches to the control of nematodiasis are; chemotherapy, chemoprophylaxis, and vector control. Attempts to develop a vaccine against nematodes have been dwarfed by the parasite's ability to avoid complete detection by the host's immune system. In some cases, there are drug resistance problems. Deficiencies such as the lack of efficacy of anthelminthic drugs against all stages of the diseases, the need for parenteral administration, and the inability to eliminate all strains of species of nematodes make treatment costly and complex. Furthermore, the methods used to control insects/vectors are expensive or tedious.

Ethnoveterinary medicine refers to people's beliefs, knowledge, skills, and practices relating to the care of their animals (McCorkle *et al*., 1996). In human healthcare, 30-90% of the planet's inhabitants still rely mainly on traditional treatment and practitioners (Ferreira *et al*., 2013). Similar figures appear for animal health care (Mini, 2012). These local knowledge systems are reported as effective and cheaper than their orthodox equivalents (Sibanda and Okoh, 2008). Natural products from plant materials are increasingly being explored to extract bioactive agents to develop drugs against diseases. Different regions of Nigeria are affected by nematode vectors hence endemic for nematodiasis. In this light, there is the need to create and enrich the pool of information on plants with a medicinal value which will serve as a referral in the future for the development of drugs against several pathogens causing diseases.

MATERIALS AND METHODS

Study Area

The study was carried out in the Biology Laboratory University of Abuja Main Campus, Federal Capital Territory (FCT), Nigeria, located between latitude 8° and longitude 7°, and has an area of 1,043 km^2. The area's temperature ranges from 30-37°C yearly, with the highest temperature experienced in March and with mean total rainfall of approximately 1,650 mm per annum. About 60% of this rain falls between July to September.

Collection of Plant Materials

Different parts (leaf, stem bark, and root) of A. indica (neem) were collected in Gwagwalada. The plant was identified in the Herbarium Section of the Department of Biological Sciences, University of Abuja, Abuja.

Preparation of the Plant Parts

Different parts (leaf, stem bark, and root) of A. indica (neem) were air-dried at ambient temperature, after which each was pounded separately using a pestle and mortar and later sieved using a sieve mesh. 500g of each plant part was stored concealed at room temperature until required for use (Nwude *et al*., 1983).

Methanolic Extraction

the solvent extraction technique described by Nwude *et al*. (1983) and Sofowora (1982) was performed. Five hundred grams (500g) of each plant part collected was defatted in 2.5 litres of absolute methanol at room temperature for 8 hours to obtain the methanolic extract. The extract was filtered, and the filtrate evaporated to dryness at 50^0C under reduced pressure. The extracts were collected and concealed in aluminium foil paper and stored in a refrigerator at 4 °C until required.

Phytochemical Analysis of the Neem Plant

The qualitative analysis of the Neem plant (Leaves, stems, barks, and root) methanol extracts was carried out for

flavonoids, anthraquinone, alkaloids, saponins, steroids, terpenoids, cardiac glycoside, anthocyanin, tannins, and carotenoids use the standard procedures described by Edeoga et al. (2005).

Test for Alkaloids

5.0 cm^3 of the extracts was added to 5 % HCI (2.0 cm^3), followed by Dragendorff's reagent (1.0 cm^3). If an orange precipitate is produced, that indicates the presence of alkaloids.

Test for Glycosides

5.0 cm^3 of the extracts were dissolved in 5.0 cm^3 of distilled water, and 2.0 cm^3 of glacial acetic acid containing 1.0 cm^3 of ferric chloride solution was added and followed by the addition of 1.0 cm^3 of concentrated sulphuric acid. A brown ring at the interface of the two solutions indicates the presence of glycoside.

Test for Anthraquinones

5.0 cm^3 of the extract was boiled with 5 % H_25O_4 (10.0 cm^3) and filtered while hot. The filtrate was shaken with 5 cm^3 of chloroform. The chloroform layer was pipette out into another test tube, and 1.0 cm^3 of dilute ammonia was added, and the resulting solution was observed for colour change. No colour change indicates the absence of anthraquinones.

Test for Saponins

5.0 cm^3 of the crude extracts were boiled with distilled water (5.0 cm^3) and filtered; 2 drops of olive oil were added to the filtrate. The formation of emulsion shows the presence of saponins.

Test for Tannins

5.0 cm^3 of the extract each, a few drops of 1 % lead acetate were added, and yellow precipitate formation indicates the presence of tannins.

Test for Flavonoids

1.0 cm^3 of the extract each, a few drops of dilute sodium hydroxide were added. An intense yellow colour formed after adding a few drops of dilute HCI shows the presence of flavonoids.

LCS0/EC Values Analysis

The lethal concentration LCSO/EC values for the leaf, stem bark, and root of A. indica were computed using the SPSS Analysis Programme after 48 hours of exposure to the various plant extracts (Lorke, 1983).

Procedure for Collection of Eggs

The collection of *H. contortus* eggs for the in vitro studies was done using the modified McMaster method described by Sloss et al. (1994). The goat intestinal waste was obtained from Gwagwalada Abattoir and conveyed in clean polythene bags to the Biology Laboratory, Department of Biological Science, Faculty of Science, University of Abuja. The waste content was placed on a fluorescent picking board; the parasites were picked with a picker and transferred to Petri dishes containing water. Female adult *H. contortus* identity was confirmed using standard taxonomic features. The parasites were crushed in a mortar with a pestle. 60 ml of water was added to the crushed worms and filtered in a 100-mesh sieve (150um).

Cultivation and Recovery of *Haemonchus contortus* Larvae

Eggs of *H. contortus* were cultured in culture plates and maintained a 27°C in an incubator. The infective third-stage larvae (L3) were recovered from 7–9-day old sterile faecal cultures using a modified Baermann Apparatus according to the method of Suleiman (2002). Cultured plates were filled with tap water and inverted onto clean Petri dishes. 10 ml of water was then placed around the bottle in the Petri dishes. It was then allowed to stand for at least 5 hours to allow the larvae to migrate out of the culture materials in the inverted sample bottles. The water in the Petri dishes was then transferred into clean 250 ml beakers and allowed to stand for an hour to allow the parasites to settle at the bottom of the beaker. After that, the water in the beaker was decanted to concentrate the parasites. The larvae harvested were concentrated at 1000 rpm for 15 minutes. About 0.1 ml of the concentrated larvae was pipetted and spread onto a glass slide, and a drop of Lugol iodine was applied to immobilise the L3 larvae. The slide was mounted on a microscope, and the L3 was observed and counted at ×40 magnification. The process was repeated twice, and the mean average numbers of larvae were recorded. Water was added where necessary, and the volume was adjusted such that 0.1 ml of the solution contained about 120 infective larvae (L3).

In-vitro Larval Mortality Test

120 larvae in 0.1 ml in wells of microtitre plate (10 larvae in each microtitre) and concentrations of each of the extracts (leaf, stem, bark, and root) at 0.1mg/ml, 1.0mg/ml, 10.0mg/ml, 100mg/ml, negative control (water) and positive control (Levamisole) was added to each of the wells, with two replication each.

After adding the treatments, it was then viewed under the microscope once in 6 hours for 48 hours, and the number of mortalities was recorded. Data will be expressed as percentage mortality using the formula of Cavier (1973);

N-n/N x 100

Where N=Total number of larvae in control wells.

n= number of mortalities.

Statistical Analysis

Statistical Package for the Social Science (SPSS) was used for the data analysis. The mortality rate of *Haemonchus contortus* was expressed in percentage at the different plant parts, the various concentrations, and the time of exposure; Data was also presented in tabulating and chart forms.

RESULTS

1. **Phytochemical Constituents**

The Phytochemical screening of the methanolic extracts of leaf, stem bark, and root of *A. indica* (neem) showed the presence of the following secondary metabolites: carbohydrate, cardiac glycosides, saponins, flavonoids,

tannins, and alkaloid; steroids were absent in stem-bark and root while leaf showed the presence of steroids, whereas anthraquinones derivatives were absent in all three plant part extracts (Table 1).

Table 1: Qualitive Analysis of the Phytochemicals of Leaf, Stembark and Root of *Azadirachta indica*

Constituents	Leaf	Stem-bark	Root
Saponin	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Alkaloids	+	+	+
Anthraquinones	+	+	+
Steroid	+	+	+
Cardiac glycosides	+	+	+
Carbohydrates	+	+	+

Keys: + = Present
- = Absent

4.2 In-vitro Larval Mortality Test

The in vitro nematocidal activity of methanolic extracts of leaf, stem bark, and root of A. indica (neem) showed that the three extracts affected the mortality of the parasite, the negative control was not effective as there were no mortalities or only one mortality recorded, but the positive was the most effective. Mortalities increased with an increase in concentration and with the time of exposure.

Table 2: percentage mortality of *Haemonchus contortus* after incubation in Methanolic Extract of Leaf, for 6 – 48 Hours

Conc. (%)	Number of Sample	Mortality in % 6	12	18	24	30	36	42	48
0.1	10	3	3	3	5	8	10	15	18
1	10	3	8	10	13	16	18	20	25
10	10	5	10	15	28	30	32	35	40
100	10	15	25	25	30	40	45	60	70
Positive (Control)	10	65	80	85	100	100	100	100	100
Negative (Control)	10	0	0	0	0	0	0	0	0

Table 3: Percentage Mortality of *Haemonchus contortus* after incubation in Methanolic Extract of Step-bark, for 6 – 48 Hours

Conc. (%)	Number of Sample	Mortality in % 6	12	18	24	30	36	42	48
0.1	10	0	3	5	5	8	10	13	15
1	10	3	5	5	15	17	19	22	25
10	10	5	10	18	30	33	35	40	42
100	10	18	25	27	35	45	50	60	75
Positive (Control)	10	65	80	85	100	100	100	100	100
Negative (Control)	10	0	0	0	0	0	0	0	0

Table 4: Percentage Mortality of *Haemonchus contortus* after incubation in Methanolic Extract of Root, for 6 – 48 Hours

Conc. (%)	Number of Sample	Mortality in % 6	12	18	24	30	36	42	48
0.1	10	3	3	3	5	8	10	15	18
1	10	3	8	8	15	18	19	20	24
10	10	5	10	15	28	30	32	38	42
100	10	18	24	24	32	45	48	60	70
Positive (Control)	10	65	80	85	100	100	100	100	100
Negative (Control)	10	0	0	0	0	0	0	0	0

DISCUSSION, CONCLUSION, AND RECOMMENDATION

Discussion

The phytochemical screening showed the presence of carbohydrates, cardiac glycosides, saponins, flavonoids, tannins, and alkaloids and absence of anthraquinones derivatives in stem bark, root, and leaf of *A. indica* (neem), presence of steroids in leaf and absence in stembark and root, presence of triterpenes in both stembark and root and its absence in leaf. Perhaps the in vitro effects of the neem plant are due to the presence of the enumerated secondary metabolites. In-vitro studies by Kahiya *et al*. (2003) revealed that condensed tannins from the leaf extract of *Acacia nilotica* inhibited the development of *H. contortus* larvae obtained from goats. Also, Athanasiadou *et al*. (2001), in in-vitro and in-vivo studies, reported the anthelminthic activity of condensed tannins extracted from Quebracho on the larvae of *H. contortus*, *Teladorsagia circumcinta*, and *Trichostrongylus vitrinus*. Mortalities recorded were high in the positive control wells, increasing mortality as the time of exposure increased. At 24 hours of parasites exposure to the positive control, there was almost complete mortality of the parasites; none of the parasites survived in the positive control wells from the 30'hoursr of exposure. Only one mortality was observed in the negative control wells; even in wells with the highest exposure time, mortalities were not recorded. This explains that mortalities recorded were due to the effects of the extracts on the parasites since the parasites thrive well in the negative control wells and could not survive in the positive control wells and the extracts. The negative control did not affect the parasite; mortality observed may be due to stress. Mortalities of the parasites increased with an increase in the concentration and with the time of exposure. At the peak time of exposure which is 48 hours, and at the highest concentration of 100mgml-l, mortalities were higher than those of the least time of exposure which is 6 hours, and with the lowest concentration of 0.1 mg ml-1. Similar to 1000 ug concentration of azadirachtin, mortality of *Haemonchus contortus* larvae was observed by Assis *et al.* (2003) at higher concentrations of plant extracts of hexane and methanol, indicating the relevance of this screening test in judging anthelminthic properties of *Afzelia africana* and Combretum mole extracts demonstrated significant nematocidal activity against *H. contortus* in in-vitro egg hatch assay (Simon, 1997).

Conclusion

Mortalities of the parasites increased with an increase in concentration and with the time of exposure; mortalities recorded were high in the positive control wells, with an increase in mortality as the time of exposure increased. In the negative control wells, only one (1) mortality was observed; even in wells with the highest time of exposure, mortalities were not recorded.

Recommendations

i. Although the extracts displayed a good nematocidal activity, further investigation/ research should be conducted to understand the fundamental mechanism of action by which the extracts eliminate the parasites.

ii. Further studies should be carried out to isolate, identify, characterise and elucidate the structure of the bioactive compounds.

iii. The antimicrobial activities of the plant for the treatments of diseases as claimed by traditional healers should also be investigated.

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