Efficacy of *Azadirachta indica* in the Treatment of Gastrointestinal Helminthiasis

Abstract:

The study was carried out to assess the anti-helminthic efficacy of *Azadirachta indica* commonly known as Neem (Dogoyaro). The phytochemical components of the leaf extract was accessed using Gas Chromatography Mass Spectrometry and the efficacy was carried out *invitro* and *invivo* using *Toxocara canis* (as worm model) of dogs. Twenty peaks of compounds were revealed in the GS-MS chromatogram, with Hexadecanoic acid which is saddled with antihelminthic effect as one of its major constituents. In the *invitro* study, the lethal concentration was 92.48ml/mg at 6hrs. Percentage egg reduction of 76.2% and 83.9% from the F~~F~~aecal Egg Count (FEC) at LC25 and LC50 respectively was recorded for the *invivo* study. The overall blood count of all the puppies used for the experiment were 4.46 X 10^6µL for RBC, 31.32% for PCV and 11.23 X $10^3$for WBC. Puppies treated with *Azadirachta indica* leaf extract showed minimal alteration of the three heamatological parameters (WBC, PCV and RBC) analyzed compared to the uninfected control group and group treated with Mbendazole. This ~~justifies~~indicates that *Azadirachta indica* leaf extract is effective in the treatment of gastrointestinal helminthiasis and also further investigations~~studies~~are~~is~~ recommended as it may be useful in the treatment of other diseases.

Key words: Efficacy, Gastrointestinal helminthiasis, phytochemical, antihelminthic

Introduction

Gastrointestinal helminthiasis refers to helminth infections that primarily affect the gastrointestinal tract in humans andother animalsref. These infections are caused by various types of parasitic worms, including roundworms, hookworms, whipworms, and tapewormsref. Infections can lead to symptoms like abdominal pain, diarrhea, nausea, vomiting, anemia, digestive issues, weight loss, vitamin deficiencies and malnutrition and growth retardation in children (Bethony *et al.,* 2006; CDC, 2013; Hotez *et al.,* 2012), and inother animals, loss of production through mortality, weight loss, reduced milk, meat and wool production (Ketzis et al., 2002; Githiori et al., 2006; Eguale et al., 2007). In severe cases, a large number of worms can cause intestinal blockage both in animals? and man (Otubajo, 2013).

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Treatment for gastrointestinal helminthiasis involves administration of specific antiparasitic medications prescribed by healthcare professionals or veterinarians, depending on the species of

thehelminth involved, and are referred to as aAntihelminthic medications or antihelminthicsref. These medications are specifically designed to target and eliminate the worms from the bodyref. There are different types of antihelminthic drugs available, depending on the specific type of helminth infection being treated. Some common classes of antihelminthics include: Albendazole, Mebendazole, Ivermectin, Praziquantel and Pyrantel Pamoate (CDC, 2013).The drug commonly used by control programmes targeting helminth infections in man and animals have different species efficacy, but the fact remains that a large proportion of the world's population affected by these parasites do not have access or cannot afford it, particularly in the rural communities of developing countries (WHO, 2012). Also, there is a growing concern about potential reduction in the effectiveness or even emergence of drug resistance through wide spread and frequent use of this anti-helminthic chemotherapy (Sangaster, 2008; Jackson and Coop, 2000). These have created an urgent need for newer, inexpensive and accessible drugs, and traditional herbal medicine based largely on medicinal plants.Theseplants offers a major and accessible source of health care to the people in remote parts of most developing countries (Tandon *et al.,* 2011). Therefore, there is need for proper scientific documentation of the efficacy of some of the plants extracts used as anthelminthic.

The use of plant extracts as antihelminthic agents has been explored in traditional medicine systems for centuries due to various factors ranging from drug resistance to inability to accessess/purchase the prescribed drugs (Al-shaibani et al., 2009; Fajmi and Taiwo,2005)). Various plants contain bioactive compounds that have shown potential in combating helminth infections (Eguale and Giday, 2009). However, itis's important to note that while traditional knowledge and anecdotal evidence exist, the efficacy and safety of plant extracts as anti-helminthics have not been extensively studied or confirmed through rigorous scientific research, which is the case with Neem and Bitter leaf plants.

Neem (*Azadirachta indica*) leaf extract hasve been traditionally used in various cultures for its potential anti-helminthic properties (Athanasiadou *et al.*, 2007). Neem, commonly known as

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Dogoyaro in Nigeria, is a tree native to the Indian sub-continent although in recent times it has naturalized and grown throughout the tropics and subtropical regions and is known for its medicinal properties which have been explored widely (Porter, 2006). Although, its leaf extracts have shown some activity against certain helminths, it is's important to note that the scientific evidence supporting its effectiveness as an anti-helminthic, is limited and this work is aimed at validating its use.

Materials and Methods

The study was carried out in Akwa Ibom Sstate, Nigeria which lies between latitude 4^0 32' and 5^0 53' North and longitude 7^0 25' and 8^0 25' East with total land area of 7.249 km^2. The plant leaves of *Azadirachta indica* were collected from matured plants and the ethanolic extraction was carried out using maceration method (Sujon *et al*., 2008). Dogs (puppies) agedof about 7 months weighing about 7 – 8 kg were bought from vendors and used for the studies. The crude leaf extract of *Azadirachta indica* commonly known in the area as neem (Ddogoyaro) was screened for antI-helminthic effects *invitro* using adult worms (*Toxocara canis*) and also *invivo* using experimentally infected dogs (puppies infected with *Toxocara canis*) according to the method of Sujon *et al*., (2008) modified.Detail the modificationif not referenced

GC-MS analysis of the plant extract was carried out on an Agilent technology 7890 GC system equipped with a mass spectrometric detector (MSD). Ms model was agilent technology 5975 ms, the column used was HP-5MS agilent technology, length of the column 30 m, internal diameter 0.320 mm, thickness of 0.25 μm. Volume of sample injected was 1 μL. Oven temperature program with initial temperature of 80°C to hold for 2 minutes at 10°C/min to final temperature of 240°C to hold for 6 minutes with injector temperature of 250°C. The mobile phase was helium gas while the stationary phase was the column.

Detection of Components Analysis of mass spectrum GC-MS was conducted by the database of National Institute Standard and Technique (NIST) having more than 62,000 patterns. The spectrum of the unidentified components were compared with the spectrum of the identified

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components stored in the NIST library. The name, molecular weight, structure of the components in the test material were ascertained (Oshiobugie *et al*., 2017)

Invitro analysis of the extract was carried out using the method of Sujon *et al*. (2008). All the dogs (except the reservoir host) were treated with anthelminthic drug administered orally and faecal analysis carried out to ascertain a worm free colony prior to the experiment. Faeces containing *Toxocara canis* eggs was obtained from a naturally infected puppy (reservoir host). Dogs (worm free colony of puppies) (These puppies werepurchased from vendors. Howwould you know that they wereworm free?)were infected with *Toxocara canis* (round worm species associated with dog) by contaminating their food and water with the infected faecal sample that was collected from the reservio~~u~~r host and kept moist for 7 days at room temperature, and infection was confirmed through faecal analysis after three weeks. Some of the dogs that were passing eggs in faeces were slaughtered using appropriate humane method and the small and large intestinecarefully extracted from the body of the dogs ~~carefully~~. The intestines were opened with a scissor and the content washed with tap water into a clean plastic container, then the adult worms were collected carefully with the aid of a needle into petri dishes containing physiological saline (buffered saline).Two sets each of 5 petri dishes (groups) containing 10 adult worms each (total of 100 worms) was prepared and the plant extract was diluted at various concentrations 10%, 20%, 50% and 100% and a control with only physiological saline. 0.1ml of each diluted plant extracts (extract of *Azadirachata indica*) at various concentrations were pipetted into the different petri dishes (groups) leaving a control and then allowed for 12hours at room temperature. The groupings were as follows:

Group 1: adult worms treated with 10% extract

Group 2: adult worms treated with 20% extract

Group 3: adult worms treated with 50% extract

Group 4: adult worms treated with 100% extract (crude extract)

Group 5: adult worms which received only physiological saline

The non-motile worms were counted from the various plates (groups) after 30mins, 1hour, 6hours and 12hours respectively and then percentage mortality calculated.

$$\text{Percentage death} = \frac{\text{Number of dead parasites}}{\text{Total number of parasites}} \times 100$$

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Lethal concentration at 25 and 50% was calculated using probit analysis (AAT Bioquest Inc., 2022). The GC-MS Chromotogram of Ethanolic Extract of *A. indica* Leaf is presented in figure 1 and the identified phyto-component are presented in table 1.

The *invivo* screening was carried out according to the modified method of Sujon *et al*. (2008), the plant ethanolic extract was tested using 4 groups of experimentally infected puppies and one group of worm free colony of puppies which were made up of two males and two females respectively i.e. in duplicate, (making a total of 40 puppies).

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Three of the experimentally infected groups were treated orally with LC50 and LC25 of the extract and Mmebendazole, all at the dosage of 50 mg/kg body weight divided into three doses for 3 consecutive days (as it is the recommended dosage of Mmebendazole for therapeutic use in Nigerian dogs) and one group was left untreated (positive control). The groupings were:

Group 1: Infected puppies treated with LC25obtained from the *invitro* studies
Group 2: Infected Puppies treated with LC50obtained from the *invitro* studies
Group 3: Infected Puppies treated with Mebendazole
Group 4: Infected puppies without treatment
Group 5: Non-infected puppies
The efficacy of the extract on the experimental animals was determined by faecal egg count from direct smear faecal analysis using the formula:

$$\text{Faecal Egg Count Reduction (\%)} = \frac{\text{Pre- treatment egg count} - \text{post treatment egg count}}{\text{Pre-treatment egg count}} \times 100$$

(Salien *et al.*, 2019).

Heamatological test which included white bloodcell count (WBC), packed cell volume (PCV) and red blood cellcount (RBC) was carried out on the puppies before and after the experiment.

Packed cell volume (PCV) was assessed by the method of Nweze *et al.* (2013). Blood was collected from the tail veins of the dogs into heparinized capillary tubes. One end of the tube was sealed using sealer. The tubes were placed on a capillary tube rack and centrifuged with a micro-hematocrit centrifuge at 5000 revolutions/min for 5 min. The PCV was then read using a hematocrit reader. White and red blood count was carried out using the Neubauer haemocytometer counting chamber. BehaviourBehavioural changes observed from the experimental animals was noted. The *invivo* screening results are presented in table 2 and figure 2.

All data obtained were subjected to analysis using Microsoft Excel and SPSS Version 21 data packages. Chi-square test was used to compare differences based on the level of statistical significance of P≥0.05 with 95% confidence interval and results are presented in table 3.

Twenty peaks of compounds were revealed in the GS-MS chromatogram of *Azadirchta indica*as shownin(Fig 1), and the major constituents were Oleic acid (26.04%), Nonadecanoic acid (21.07%), Methyl-beta–D-Arabinopyranosidde (12.82%), 11-Octadecenoic acid (8.04%), 9,12-Octadecadienoic (5.17%), n-Hexadecanoic acid (5.00%), while the minor compounds were Hexadecanoic acid (4.93%), Eicosanoic acid (4.85%), Pentadecanoic acid (2.04%), 3-n-Hexyltioslane S, S-dioxidee (1.96%), 1-Hexacosanol (1.21%), 2H-Pyran, 2-(7-Heptadecynyloxy) tetrahydro (1.19%), Octanoic acid (1.10%), Decanoic acid, methyl ester (1.04%), Docosanoic acid methyl ester (0.57%), Acetic acid (0.72%), Heneicosanoic acid methyl ester (0.57%), 1-Nonanol (0.55%) and Divalonic acid (0.72%) as shown in Table 3. The plant contained Hexadecanoic acid which is saddled with antihelminthic effect as one of their major constituent.

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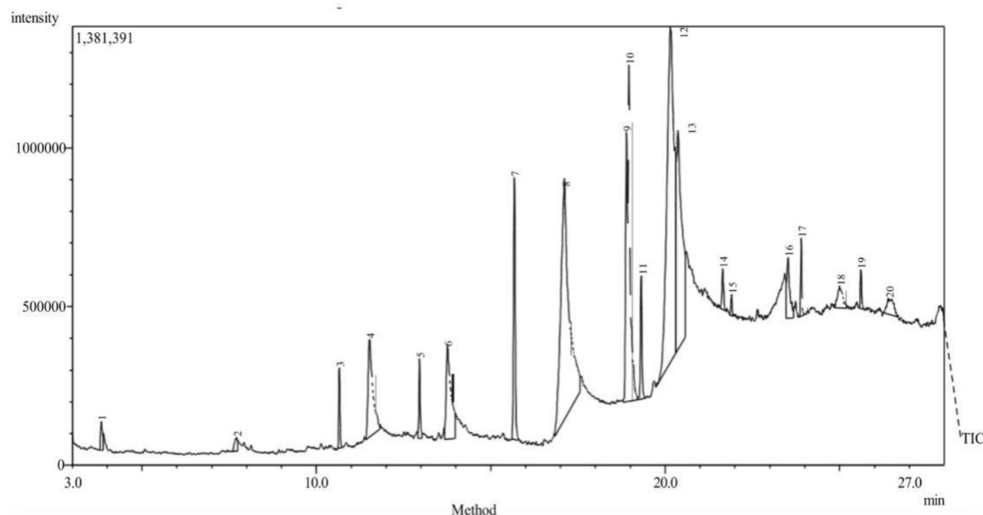

Figure 1: GC-MS Chromotogram of Ethanolic Extract of *A. indica* Leaf

Table 1: Phyto- Components Identified in the *Azadirachta Indica* Leaf Extract

Peak	Retention time	% Peak Area	Compound Analyzed	Molecular Weight	Molecular formular
1.	3.835	0.55	1-Nonanol	144	$C_9H_{20}O$
2.	7.713	0.45	Divalonic acid	130	$C_6H_{10}O_3$
3.	10.656	1.04	Decanoic acid, methyl ester	186	$C_{11}H_{22}O_2$
4.	11.524	5.00	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$
5.	12.957	1.10	Octanoic acid	158	$C_9H_{18}O_2$
6.	13.760	4.85	Eicosanoic acid	312	$C_{20}H_{40}O_2$
7.	15.677	4.93	Hexadecanoic acid	270	$C_{17}H_{34}O_2$
8.	17.111	21.07	Nonadecanoic acid	298	$C_{19}H_{38}O_2$
9.	18.893	5.17	9,12-Octadecadienoic acid	294	$C_{19}H_{34}O_2$
10.	18.932	8.04	11-Octadecenoic acid	296	$C_{19}H_{36}O_2$
11.	19.314	2.04	Pentadecanoic acid	270	$C_{17}H_{34}O_2$
12.	20.147	26.04	Oleic Acid	282	$C_{18}H_{34}O_2$
13.	20.365	12.82	Methyl-.beta.-D-arabinopyranoside	164	$C_6H_{12}O_5$
14.	21.650	0.72	Acetic acid	200	$C_{12}H_{24}O_2$
15.	21.902	0.28	Pentanoic acid, 4-methyl-, methyl	130	$C_7H_{14}O_2$
16.	23.521	1.96	3-n-Hexylthiolane, S,S-dioxide	204	$C_{10}H_{20}O_2S$
17.	23.900	0.95	Docosanoic acid, methyl ester	354	$C_{23}H_{46}O_2$
18.	25.001	1.21	1-Hexacosanol	382	$C_{26}H_{54}O$

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19.	25.611	0.57	Heneicosanoic acid, methyl ester	340	$C_{22}H_{44}O_2$
20.	26.384	1.19	2H-Pyran, 2-(7-heptadecynyloxy)tetrahydro-	336	$C_{22}H_{40}O_2$
Total		**100.0**			

Table 2: *Invitro* screening of the *Azadirachta indica* extracts on adult worms.

	Percentage (%) of Dead Adult Worms N=100			
Concentration (ml/mg)	**30mins**	**1hr**	**6hrs**	**12hrs**
10ml/mg (10%)	_	_	_	60
20ml/mg (20%)	_	_	20	50
50ml/mg (50%)	_	_	40	100
100ml/mg (100%)	_	_	50	100
Control	_	_	_	_

LC25= 25.85ml/mg, LC50=92.48ml/mg

Azadirachta indica plant extract screening on adult *Toxocara canis* (shown in Table 4) revealed that the extract was observed to be effective within 6-12 hrs. The percentage death at 6hrs was 20, 40 and 50% for 20ml/mg, 50ml/mg and 100ml/mg respectively, while no death was recorded for the control. At 12 hours the percentage death was 60, 50, 100 and 100 at the dose of 10ml/mg, 20ml/mg, 50ml/mg and100ml/mg respectively. The lethal concentration was observed at the 6hrs and was calculated to be 92.48ml/mg.

Invivo screening of *Azadirachta indica* leafextract on puppies in Figure 2 shows a high percentage egg reduction of 76.2% and 83.9% from the Faecal Egg Count (FEC) at LC25 and LC50 respectively, while Mebendazole showed a 92.5% reduction.

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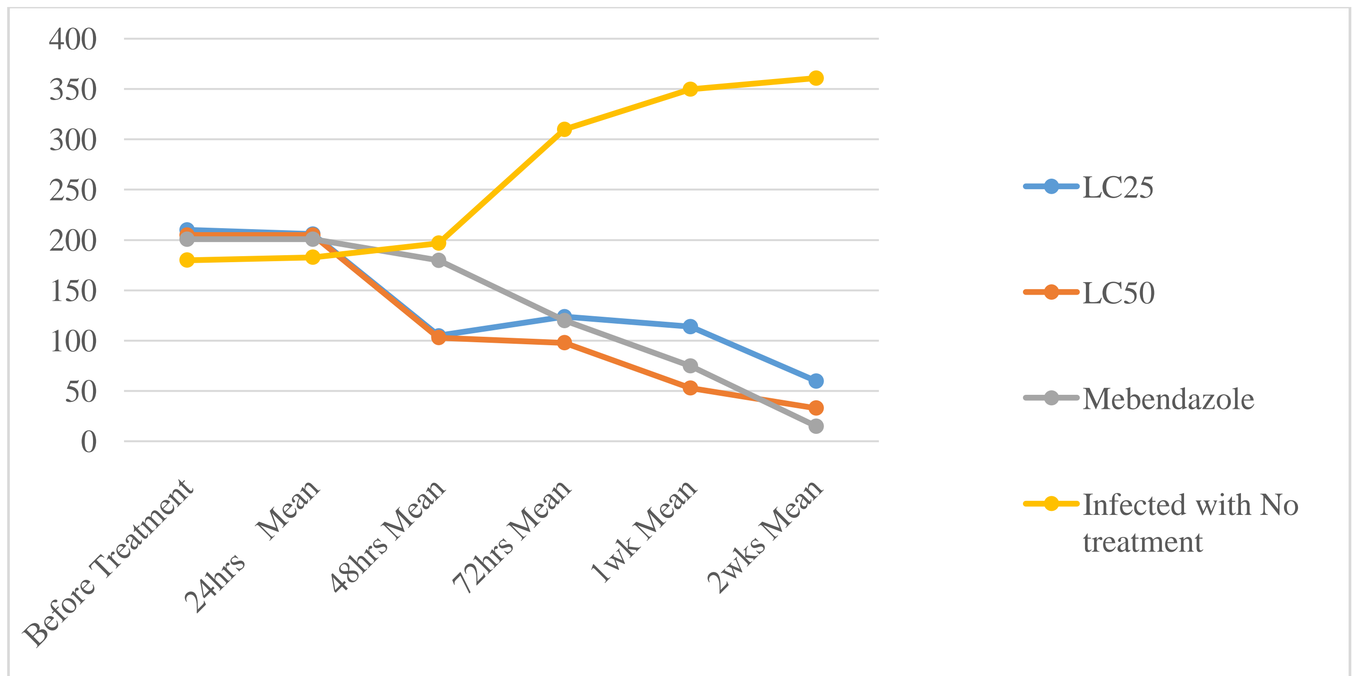

Figure 2: *Invivo* screening of the *Azadirachta indica* extracts on puppies faecal samples

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The overall blood count of all the puppies used for the experiment as shown in Table 5 were 4.46 X 10^6µL for RBC, 31.32% for PCV and 11.23 X $10^3$for WBC. A marked reduction was observed in the pack cell volume (PCV) and Red blood count (RBC) of the infected untreated puppies (23%, 3.70 X10^6µL) and also those that were treated with Mebendazole (25.83%, 4.40 X10^6µL) while those treated with LC25 and LC50 extracts of *Azadirachta indica* recorded 33, 4.80 X10^6µL and 32%, 4.50 X10^6µL respectively. The difference was statistically significant.

The WBC revealed an increase in the infected untreated (positive control)(14 x $10^3\mu$L) and also those treated wth Mebendazole (11.63 x $10^3\mu$L) while those treated with LC_{25} and LC_{50} extracts concentrationss of *Azadirachta indica* recorded 10 and 11x$10^3\mu$L respectively. The difference was statistically significant(quote thep value). LC50 of *Azadirachta indica* revealed a minimal alteration of the three heamatological parameters (WBC, PCV and RBC) analyzed compared to the uninfected control group and group treated with Mbendazole.

Table 3: Heamatological analysis of the puppies used for the *Invivo* screening

Treatments	**RBCs(μL) Mean±SD**	**PCV (%) Mean±SD**	**WBCs(μL) Mean±SD**
Non-infected	5.30±0.12a	42.00±1.73a	9.33±0.17cd
Infected untreated	3.70±0.12f	23.77±0.62c	14.17±0.88a
Infected treated with Mbendazole	4.40±0.06de	25.83±0.83c	11.63±0.52b
Infected treated with LC25 OF *Azadirachta indica*	4.80±0.15b	33.00±1.53b	10.00±0.29bcd
Infected treated with LC50 of *Azadirachta indica*	4.10±0.06e	32.00±1.15b	11.00±0.50bc
Total	**4.46±0.11**	**31.32±1.17**	**11.23±0.47**
p Value	<0.001*	<0.001*	<0.001*

Means along the same column with different Alphabet(s) are statistically significant at p≤0.05, ns – Not significant at p>0.05, * - significant at p≤0.05.

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Discussion

The leaves of *Azadirachta indica* (Neem) in the study showed an anthelmintic efficacy of 76.2% and 83.9% egg reduction in faecal egg count (FEC) among the litters used for the experiment which agrees with the report of Sujon *et al*. (2008) who also reported a high percentage efficacy of 85% on the gastrointestinal nematodes of goats, but disagrees with the report of Nambiar *et al*. (2016) who reported that neem leaves had no anti-helmintic effect on the test organisms (goats). The differences in the reports on neem leaves anti-helmintic efficacy could be attributed to the solvent used for extraction which was ethanol and water respectively and also time in which the leaves were harvested for the experiment (Dele *et al.*, 2020). Although, phytochemicals present in the plant such as phenols, alkaloids and trepenoids are said to be antioxidants and inhibitors of carcinogenic cell, microorganisms, parasites and other foreign bodies in the body thus revealing a high potential of the plant having an anti-helmnthic property but its use was justified by the presence of n-Hexadecanonic acid and decanonic acid which are nematicidal compounds in the leaf extract.

The reduction of egg count by the leave extracts of *Azadirachta indica* in this study is a positive and welcomed development in our local helminths struggle because the plants are available year-round in Nigeria. The easy access to this plant and its availability might mean that the cost of medication would be drastically reduced.

The activities shown by the plant extract is of considerable importance and justified its use as anthelmintic in folklore traditional medicine as the use of medicinal herbs in treatment of infectious diseases has attracted the attention of scientist worldwide (Aswathi and Dhivya, 2017). Studies show that the plant contain alkaloids, saponins, tannin, glycosides, flavonoid, terpeniod and phenol which indicates that the plant could be useful in treatment of other diseases for example Alkaloid has antimalarial, analgesic value and can equally be used as stimulant, flavonoids for gastric infections (Arona and Tamrakar, 2017). GC-MS analysis revealed that the plant contained n-Hexadecanioc acid as one of its major compound which accounts for their anthelmintic effect and also anti-inflammatory compounds such as Octadecanoic acid and Decanoic acid. This agrees with the findings of Abirami and Rajendran, (2012) who also reported the presence of n-Hexadecanioc in *Azadirachta indica*. Other compounds with bioactivities such as antibacterial, antifungal, antioxidant were encountered in the study which could be useful in the treatment of other ailments.

Conclusion

Aside the use of medically prescribed drugs, enema/ drinking of various plant extracts (both water and ethanolic extract) have been used by traditional/herbal medicine in the treatment of gastrointestinal helminthiasis in both man and animals. The leaves of Neem (*Azadirachta indica*) which is one of the most commonly used plants were collected, identified and anthelmintic efficacy on *Toxocara canis* as helminth model was tested *invivo* and *invitro*. LC50 of 92.48ml/mg at 6hours with percentage egg reduction of 83.9% was reported for *Azadirachta indica* respectively. This revealed that the plant leaves had anthelmintic potentials with minimal alteration of heamatological parameters and could be attributed to the presence of flavonoids which is associated with treatment of gastric infections and also n-Hexadecanioc acid which accounts for their anthelmintic effect, alongside Octadecanoic acid and Decanoic acid which are anti-inflammatory compounds.

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More recentcitations recommendedfor introduction and discussion.

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