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Research Article

Studies on Anthelmintic Activity of *Tithonia Diversifolia* in Mbinga District, Tanzania

Lutfrid Samwel Komba[1], Frida Richard Mgonja[1], Amina Ramadhani Issae[2] and Abdul Seleman Katakweba[2]*

[1]*Department of Veterinary Physiology, Biochemistry and Pharmacology, College of Veterinary and Biomedical Sciences (CVMBS) Sokoine University of Agriculture (SUA), Morogoro, Tanzania*

[2]*Institute of Pest Management, Sokoine University of Agriculture, Morogoro, Tanzania*

Abstract

Gastrointestinal nematode parasitism is a global problem in both sub-tropical and tropical countries. Due to frequent administration of chemical anthelmintics the gastrointestinal nematodes have developed resistance hence giving rise to the search of alternative anthelmintics. This study was carried out to evaluate anthelmintic effects of *Tithonia diversifolia* in Mbinga district, Ruvuma region, Tanzania. The study specifically dealt with evaluation of the efficacy of *T. diversifolia* extracts against adult Haemonchus contortus worms in a controlled critical test. Further, the toxicity of the plant was evaluated using the brine shrimp lethality test. A total of fifteen goats which were free from helminthosis were purchased and quarantined for 60 days. They were then administered 1250 larvae of Haemonchus contortus. On day 29 after infection the egg per gram of faeces (epg) count was done. The goats were randomly divided into three groups of five goats each. The groups were negative control, treated and positive control. The treatment group was administered 50 mg/kg of *T. diversifolia* orally and the positive control group was administered 8 mg/kg of albendazole orally. The epg count was then carried out on day 4, 7, 10 and 14, after which animals were sacrificed for total worm count. The results show that *Tithonia diversifolia* is not effective against adult Haemonchus contortus worms based on epg count and post-mortem worm counts reduction tests. From the study, it is recommended that more studies should be carried out so as to validate the anthelmintic effects of *T. diversifolia* by investigating its activity on other specific species of the nematodes which parasitize animals.

Keywords: Haemonchus; *Tithonia diversifolia*; Worm; Anthelmintic; Albendazole

Introduction

For centuries, medicinal plants have been used in different parts of the world as source of both preventive and curative traditional medicine preparations for both human and livestock. The traditional medicine also creates income to the indigenous people by exporting the dried form of the medicinal preparations [1]. The plants were used by people without the knowledge of their active ingredients. The plant materials used include seeds, berries, roots, leaves, barks or flowers [2]. Those people took the crude extract orally, the practice which was extremely hazardous since the extracts may contain some toxic constituents [3]. They acquired knowledge of medicinal plants by methods of trial and error [4,5]. The indigenous people used the medicinal plants for medicine [6]. Currently it is estimated that more than 80% of the people living in developing countries rely on traditional medicine [7-10]. Most of the people in developing countries continue to rely on traditional medicines due to its accessibility and affordability. For example, the research which was conducted in Uganda showed that the ratio of traditional practitioner to population was between 1:200 and 1:400 compared to ratio of the allopathic practitioner to population which was 1:20,000 or less. From this difference most of the traditional practitioners are easily available compared to the allopathic ones [7]. On the other hand the cost of the modern drugs are extremely high compared to the traditional ones as it was revealed in a research carried out in Ghana, Kenya and Mali whereby it was observed that the course of sulfadoxine/pyrimethamine was estimated to be about several dollars although in the actual fact the per-capita-out-pocket health expenditure was US$ 6 per year while the traditional medicines used in malaria treatment were cheaper and sometimes it was just charged according to the wealth of the patients [7]. Some people in developing countries do not go straight to the public health centres as it has been shown that in Dar es Salaam (Tanzania) more than 21% of the patients who attended public health services had consulted a traditional healer before going to the hospitals [6].

The traditional medicines practices are also evident in developed countries, where the reports show that different countries have been documented with percentage in brackets; China (40%), Australia (48%), Canada (70%), USA (42%), Belgium (38%) and France (75%) [8]. In some African countries people have been using herbal medicine as primary treatment for Human immunodeficiency virus (HIV) related problems. There are also evidences of some people taking anti-retroviral drugs (ARVs) and traditional medicinal plants simultaneously [11]. Research has shown that some traditional medicines have powerful immune-stimulant effects which have raised hope to the HIV victims [7]. Some people believe that there are some diseases which cannot be cured in the hospitals so they use to go and consult the traditional healers [12]. Many drugs used in conventional medicine are derived from plants [2,6,13]. The medicinal plants contribute about 90% of the newly discovered pharmaceuticals [14]. For example, many years ago a plant chemical was discovered in a

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***Corresponding author:** Frida Richard Mgonja, Department of Veterinary Physiology, Biochemistry and Pharmacology, College of Veterinary and Biomedical Sciences (CVMBS), Sokoine University of Agriculture (SUA), Morogoro, Tanzania

tropical plant, Cephaelis ipecacuanha, and the chemical was named emetine. A drug was developed from this plant chemical called Ipecac which was used for many years to induce vomiting mostly if someone accidently swallowed a poisonous or harmful substance [15].

So, there is a need to make strong collaboration between traditional medicine and conventional medicine so as to fight diseases of priorities such as tuberculosis, malaria, hypertension, diabetes mellitus and HIV/AIDS [8]. The use of traditional medicine in developing countries such as Tanzania is empowered due to its accessibility and affordability in the majority of resource-poor communities. Sometimes ethnoveterinary medicine can be obtained free of charge among the traditional healers found in African villages [16,17]. Due to non-regulated use of several herbal medicines the health of the users may be at risk due to toxicity. This is due to the fact there is limited scientific evidence in evaluating the safety and effectiveness of traditional medicine products and practices [2]. The uses of plants as medicine differ from one place to another. Therefore, there is the need to study the plants so as its traditional knowledge can be validated by taking small locality and a number of plants, in each study. Based on above facts researches have been carried out for many plants and their preparations, to investigate the pharmacological activities. It is therefore important to validate traditional medicine knowledge so as to conserve the plants as well as documenting them for future generations. The present study focused on *Tithonia diversifolia* as an anthelmintic used by people living in Mbinga district, and the aim of the study is to provide scientific validation on the alleged activity of the plant.

Materials and Methodology

Study area

The field work which included gathering of the information on plant parts used and preparations of the plant was done at Mbinga district, Ruvuma Region. The laboratory work was done at the College of Veterinary Medicine and Biomedical Science, and in the Department of Animal Science and Production (DASP), Sokoine University of Agriculture (SUA).

Collection of plant samples

Informal interviews were conducted in three wards namely Litembo, Myangayanga and Mbaha at Mbinga District, Ruvuma Region. The interviews were carried out among farmers based on the uses of the parts of the plant, and how the plant parts were prepared for administration to animals (Appendix 1**).** The specimens were photographed, collected and carried to the Department of Crop Science and Production, SUA for further identification and confirmation by a botanist.

Preparation and Extraction of The Plant Materials

The leaves of *Tithonia diversifolia* were cleaned with water, chopped into small pieces and air-dried for 14 days under the shade. The air-dried leaves were ground into fine powder using laboratory mill (Christy Hunt Engineering Ltd, England) at the DASP, SUA.

Preparation of aqueous extracts

The aqueous extracts of the leaves were prepared by mixing 100 g of the ground powder of the leaves with 1000 ml of distilled water. The mixture was left to soak over-night at room temperature. The mixture was then filtered using a filter funnel fitted with Whatmann® filter paper No. 1 (Whatman International Ltd, England).

Preparation of ethanolic extracts

This was done by dissolving 138 g of *T. diversifolia* leaves in 250 ml of 80% ethanol overnight at room temperature. After 24 hours the mixture was filtered using a filter funnel fitted with Whatmann® filter paper No. 1 (Whatmann International Ltd, England). The filtrates were concentrated on water bath at 50°C using Rotavapor (BUCHI Labortechnik AG, Switzerland).

Experimental Animals and their management

Fifteen young goats were purchased from Kingolwira and Mkundi villages in Morogoro municipality, Morogoro region, and transferred to the Animal Research Unit (ARU) at the Faculty of Veterinary Medicine, SUA. The animals were divided into three groups of five goats each and kept in separate pens with slatted floor (Appendix 2). The animals were treated with albendazole (Albandazole®, Hebei Yuanzheng Pharmaceutical Company Ltd, China) at a dose of 8 mg/kg body weight; sulfadimidine (S-Dime®, Cosmos company, Kenya) at a dose of 99 mg/kg body weight, ivermectin (Kelamectin®, Kela company, Belgium) at a dose of 0.2 mg/kg body weight. The drugs were given for the control of endoparasites and ectoparasites. The animals were left to acclimatize for 2 months while being provided with worm free hay and grasses (fetched from areas known to be free of nematode larvae infestation). The animals were also supplemented with maize bran and minerals (Superlick®, Farmers centres, Tanzania). The meal was prepared by mixing 2 kg of Superlick® with 38 kg of maize bran and 10 kg seed cake. Each animal was given 0.25 kg per day.

The ingredients of the Superlick are given below:

- Vitamin A 12 000 000 IU
- Vitamin D_3 4 000 000 IU
- Vitamin E 10 000 mg
- Iron 250 000 mg
- Manganese 200 000 mg
- Copper 40 000 mg
- Zinc 80 000 mg
- Cobalt 6 000 mg
- Iodine 10 000 mg
- Selenium 200 mg
- Calcium 22.5%
- Chloride 13.2%
- Sodium 8.8%
- Phosphorus 9.0%

Analysis of faeces for eggs per gram (epg) count

The egg count per gram (epg) of faecal sample from each goat was done by using the McMaster method with slight modification. Four grams (4 g) of faeces were weighed and placed in container 1 and 56 ml of flotation fluid was added. The contents were mixed thoroughly with a stirring device. The faecal suspension was filtered through a tea strainer or a double-layer of cheese cloth into container 2. While stirring the filtrate in container 2, sub-samples were taken using a

Pasteur pipette. Both sides of the McMaster counting chamber were filled with sub-sample. The counting chamber was allowed to stand for 5 minutes. The sub-sample of the filtrate was examined under a microscope at 10x10 magnification. Eggs and coccidian oocysts within the engraved area of both chambers were counted. The epg was calculated as follows: Add the egg count of the two chambers together. Multiply the total by 50.

Solvents

Distilled water and ethanol were used for aqueous and ethanolic extracts preparation respectively. Ethanol (99.9%, Harris reagent, Philip Harris limited, Sheristone, England) was purchased from a local dealer in Morogoro. Distilled water was obtained from the Department of Veterinary Microbiology and Parasitology, SUA.

The test organisms

Artemia salina Leach (Artemia salina sanders™ Great Salt Lake, Brine Shrimp Company L.C., USA) was used for brine shrimp lethality bioassay. The eggs were donated by Dr. Joseph Jangu Magadula, Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences (MUHAS).

Larvae

Adult Haemonchus contortus worms were isolated from the abomasa of goats purchased from Melela slaughter slabs in Mvomero District in Morogoro region from December 2011 to February 2012. The worms were isolated by incising the greater curvature of the abomasa. The female worms were then ground using mortar and pestle to liberate the eggs. The suspension was centrifuged (Kubota 5100, Japan) for 5 minutes at 1500 rpm and the supernatant was discarded [18]. The sediment which contained eggs was then incubated in a conical flask for 7 days (Appendix 3) and 3rd stage larvae were then harvested, and stored at 4°C. The larvae were used to infect goats in Groups 1-3 as described in section 3.10.

Brine Shrimp lethality assay for *T. diversifolia*

Hatching of the brine shrimp: Brine shrimp (Artemia salina Leach) eggs (Artemia salina sanders™ Great Salt Lake, Brine Shrimp Company L.C., USA) were hatched in simulated seawater prepared from sea salt and acted as culture medium. (Sea salt is usually prepared by boiling seawater to evaporation). The simulated seawater was prepared by dissolving 3.8 g sea salt in 1 litre of distilled water. Rectangular glass chamber divided into two unequal compartments with holes on the divider was used for hatching. The eggs were sprinkled into the large compartment that is darkened, while small chamber was illuminated. After 24 hours incubation at room temperature, nauplii (larvae) were collected by Pasteur pipette from the lighted chamber, while their shells were left in the darkened chamber.

The bioassay: The brine shrimp lethality test was carried out using the standard procedure as described by [19,20] with slight modifications. The stock solution of the study plants extracts was prepared by dissolving 160 mg of the dry extract in 4 ml of DMSO to get a concentration of 40 $mgml^{-1}$. Taking 30, 15, 10, 5, 3 and 1µl of the stock solutions the final concentrations of 240, 120, 80, 40, 24 and 8 μgml^{-1} were obtained by dilution with 5 ml of the sea salt solution in vials. Each concentration was tested in duplicate making a total of 12 vials using DMSO as a negative control as previously described by [21,22]. Ten larvae of brine shrimps suspended in a small amount of the culture medium (sea salt solution) were transferred, using Pasteur pipettes, into each of the vials containing test extract, followed immediately with adjusting the volume of the sea salt solution to 5 ml mark. The vials were left on the laboratory bench, at room temperature (25°C), for 24 hours in order to determine survival rate of the larvae at the different concentrations of *T. diversifolia* extract. Survivors were counted after 24 h and from these the percentage death at each concentration was determined according to [19,20] (Appendix 4).

Experimental design

The parasite free goats described in section 3.4 were divided into three groups, Group 1, 2 and 3, consisting of five goats each. All animals were then administered 1250 Haemonchus contortus larvae orally. On Day 21 after administration of larvae, faecal samples were collected from each goat and analysed for epg count to establish presence and magnitude of worm burden. Goats in Group 2 were treated with extracts of *Tithonia diversifolia* leaf extract at an oral dose of 50 mg/kg body weight, while goats in Group 3 were treated with albendazole (Albandazole®) at a dose of 8 mg/kg body weight orally. Goats in Group 1 were left untreated and acted as negative control. Following treatment, faecal samples were collected from each goat on Day 0, Day 4, Day 7, Day 10 and Day 14 and analysed for epg. On day 14 all animals were sacrificed and *H. contortus* worms isolated from the abomasa and transferred into a petri-dish where the total worm counts were carried out and recorded for each goat.

The epgs were used to calculate faecal egg count reduction (FECR) according to Coles et al. (1992). FECR%=100 (1- $\bar{X}_t/\bar{X}_c$) where $\bar{X}_c$ and $\bar{X}_t$ represent the arithmetic means of the control and treated groups respectively. From the same data the lower confidence limit (95% CI) was determined using the formula: 100(1- $\bar{X}_t/\bar{X}_c$ exp (+2.048√Y^2) where $\bar{X}_c$ and $\bar{X}_t$ represent the arithmetic means of the control and treated groups respectively and Y^2 represents variance of reduction.

- The percentage efficacy of the drug against the worms was calculated by using the formula:

$$\% \text{ efficacy} = \frac{C-T}{C}$$

Where C is the arithmetic mean of the worms in the control group and T is the arithmetic mean of worms in the treated group.

- Group mean for the treated group was calculated by using the formula:

$$\overline{X} = \frac{\sum fX}{\sum f}$$

$\overline{X}$ is the mean

f is the number of occurrences

$\sum fX$ sum of products fX

$\sum f$ is the total number of occurrences

Procedures for total worm count

This was done according to [23]. The abomasum was opened along its greater curvature and the contents were spilled into a black flat tray. The abomasum was then spread on another flat tray. Worms attached to mucosal folds of the abomasum were removed manually

and placed on the tray. The abomasal contents were transferred into a 50 µm sieve and then washed using gentle jet of tap water to leave *H. contortus* worms on the sieve, with dirt and fine particles removed. The washed contents were then taken and poured into another illuminated black flat tray. The worms were collected using forceps and then counted manually. The procedure was done to all abomasa from the experimental goats.

Data analysis

Data were entered in Microsoft Excel and the Means±SD of total worm count in each group was calculated. For the determination of LC_{50}, data were analyzed by using single-factor ANOVA and the regression line was plotted using the Microsoft Excel Program. The faecal egg count reduction percentage was obtained by using the formula described by [24].

Results

Informal interviews which were conducted in five villages (located in three wards in Mbinga District) revealed that 69% of the villagers were aware that *T. diversifolia* is used as anthelmintic in goats. They said that the plant parts which are commonly used are the leaves and it is given to the animals as decoction preparation. Seventy-six (76%) of the respondents revealed that the plant is used as anthelmintic in human beings while 11% knew nothing about the uses of the plant. The plant was identified by a botanist at SUA as *Tithonia diversifolia* (Figure 1).

Figure 1: *Tithonia diversifolia* plant showing branches, leaves and flowers.

Twenty-nine days after experimental infection of 1250 larvae to the goats the epg was determined. It was found that the epg in all animals was high. This shows that all animals were successfully experimentally infected. On the same day of counting the animals were treated with either albendazole or *T. diversifolia* extracts (day 0). The epg on day 7 (post-treatment) were higher in the negative control group as compared to the *Tithonia diversifolia* treated group. On the other hand, the epg in albendazole treated group (positive control group) was 0 on Days 7 and 14 (post-treatment), showing that albendazole was 100% effective. The epg in the negative control group on Day 14 was higher compared to the treated group (Table 1). On Day 14 all animals were sacrificed (Figure 2) and epg analysis was carried out in all groups of animals. The percentage reduction in epg in the *Tithonia diversifolia* treated group was 29%, while the percentage reduction in the albendazole treated (positive control) group was 100% (Table 2).

With respect to the total worm count, results show that on Day 14 the mean of the total worm count in the untreated group (negative control) was 155 worms while that of the treated group was 158 worms (Figure 3). The percentage efficacy in the *T. diversifolia* group was 0 while that in albendazole treated group was 100% (Table 3).

When the brine shrimp lethality test was carried out, results show that the LC_{50} was 58.5 µg/ml (Figure 4). Therefore, the extract from the study plant is considered to be safe when used as medicinal drug. In addition, the drug is considered to be cytotoxic if the value of LC_{50} is less than 20 µg/ml and it is bioactive if it is less than 100 µg/ml.

Figure 2: Sacrificed goats.

Table 1: Mean±SD egg counts in faeces after infecting goats with 1250 larvae of Haemonchus contortus

	Faecal egg counts per gram of faeces (epg)		
	Control Group (Untreated)	T. diversifolia treated Group	Albendazole treated Group
Day 0	2260±2145	1140± 680	2680 ±1530
Day 7	3420±3282	1140 ±764	0
Day 14	4780±2926	3420 ±1432	0

Table 2: Post-treatment faecal egg-count on Day 14

Faecal egg counts per gram of faeces (epg)			
	Control Group (Untreated)	T. diversifolia treated group	Albendazole treated Group
Number in group	5	5	5
Arithmetic mean	4780	3420	0
variance of counts	8567000	2052000	0
Percentage reduction		29	100
Variance of reduction (log		0.11	Undefined
scale)			
Approximate 95% confidence	-	-	-
Limits			
Lower confidence limit		80	Undefined

Table 3: Percentage efficacy on Day 14

Groups	Percentage efficacy
T. diversifolia treated group	0
Albendazole treated group	100

Discussion

Informal interviews conducted in five villages in Mbinga District revealed that 69% of the villagers were aware that *T. diversifolia* is used as anthelmintic in goats. Most of these respondents had been using the plant for the regular deworming of their goats. The commonly used plant parts were leaves. Upon the interviews most of the people

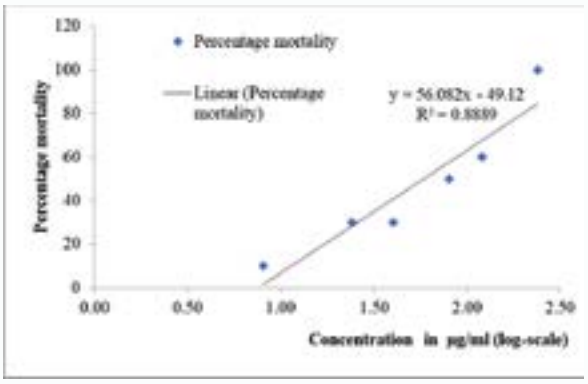

Figure 3: Percentage of larvae mortality against *T. diversifolia* concentration in µg/ml (log-scale).

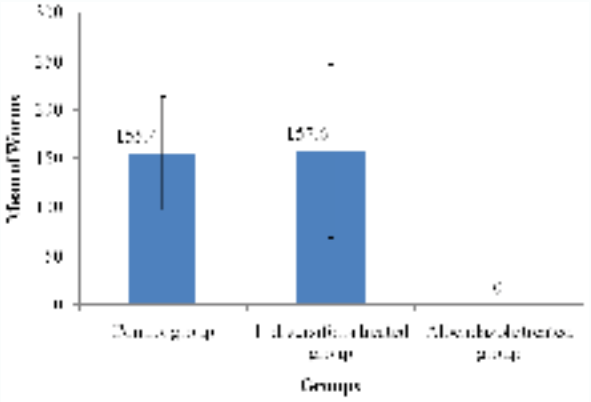

Figure 4: Means and standard deviations of the total worm count on Day 14.

said that they use the leaves simply because they are easy to boil and are given to the animals as decoction preparation. The same plant was observed to be used as anthelmintic in human. The villagers said that they use it due to its availability and affordability. These reasons also agree with [25] who reported the uses of plants as anthelmintics. The goats were dewormed before administration of the larvae. This was done so as to clear gastro-intestinal helminths. After the deworming the goats were analysed for epg and all animals had zero epg. After 22 days all goats were infected with infective larvae of Haemonchus contortus. On Day 21 epg was done before the administration of the drugs in the two groups (*T. diversifolia* and albendazole treated groups) and it was found that all animals had significant levels of epgs. This shows that the animals in all groups had acquired infection successfully. The percentage reduction in epg after deworming with albendazole shows that the drug is 100% effective against Haemonchus contortus. Similar results were obtained by [26] when they used albendazole as anthelmintic against Haemonchus contortus. Seven days after treatment there was a significant difference between the *T. diversifolia* treated group and albendazole treated group. The albendazole treated group had 0 epg showing that the drug was more effective than the extracts of *T. diversifolia* plant. The difference which was observed on Day 14 was not due to pharmacological activity of the drug since the differences were not statistically significant (P=0.964). The results of the total worm count done on Day 14 after treatment of the experimentally infected goats show that the difference between the means from untreated and *T. diversifolia* treated groups was not statistically significant (P=0.987). This shows that the plant had no pharmacological activity against adult worms. The mean worm count in the albendazole treated group (positive control) was 0 showing that albendazole is very effective in controlling the Haemonchus contortus worms as compared to *T. diversifolia*. The results shown by albendazole agree with those reported by [27]. The results from this study show that the aqueous extract of *T. diversifolia* plant was not effective against *H. contortus*. Although the people in Mbinga district have been using this plant for deworming animals, it is for certain that it is not effective against *H. Contortus* based on the results of this study. According to WAAVP an anthelmintic is considered to be effective if it has percentage faecal egg count reduction of 95% and above, with lower confidence limit of 90% and above [28]. Basing on this fact, the plant studied did not meet the criteria of being considered effective against *H. contortus*. In previous studies, it has been noted that not all cases of alleged anthelmintic activity of certain plant extracts reported was confirmed by controlled experimentation. For example, the administration of Myrsine africana and Rapanea melanophloeos extracts to parasitized sheep in Kenya did not result in reduction of the level of parasitism in controlled experimental studies [25].

There are a number of factors which could have led to the observed ineffectiveness of the anthelmintic plants. These factors include the process of collection/ harvesting and storage. The dose of the drug given to the animals, may be was small thus failing to kill the worms in the group treated with *Tithonia diversifolia*. The physical and chemical properties of anthelmintic may be affected by season at which the plant is harvested. This is due to the fact that the concentration of the active ingredients may vary with the season. If the plant is stored for a long time, there are changes in plant availability in nutrients and metabolites which affect the reproducibility of anthelmintic activity. In the study conducted by [29] on the neem tree, the fresh leaves were collected and given to the animals on daily basis while in another study done by [25] the animals were fed with conserved leaves. The results were inconsistent. The inconsistency could be attributed to the method of preservation which may have affected the plant properties. In most of the medicinal plants the doses and duration of the treatment are not well known and established. For example, some plants become effective anthelmintics only if they are consumed regularly by animals [25,30].

On the other hand, the livestock keepers may claim anthelmintic effect of the different plants if they observe parts of the worms expelled with faeces, but it becomes difficult to appreciate the effect when the species of worms are nematodes whose identification needs specialized techniques and equipment's. In this study we observed FECR percentage of 29% which is very low for *T. diversifolia*. Similar (low FECR percentage) was documented by [31] who demonstrated that the FECR percentage of 62% of abomasal nematode *H. contortus* egg counts after the animals were fed with whole plant preparation. The observed low FECR percentage could also have been contributed by small sample size per test group since the recommended sample size is 6 animals per group.

Although this plant did not show significant anthelmintic activity against *H. contortus*, other plants have been reported recently to have anthelmintic activity against nematodes. These include *Artemisia herba-alba* [32], *Anarcadium occidentale* [33], *Nauclea latifora* [34], *Musa paradisiaca, Anogeissus leiocarpus*, and *Daniellia oliveri* [35]. The brine shrimp toxicity results show that, the plant is not acutely

toxic due to its high LC_{50} value of 58.5µg/ml. A substance is regarded as acutely toxic to biological systems, if it has an LC_{50} value of not more than 20 μgml^{-1} (8,20,36). Furthermore, it has been established that, for a compound to be considered completely safe, it should have LC_{50} value greater than 100 μgml^{-1} [36,37,38,]. Therefore, more elaborate toxicity studies are needed to establish the safety of the extract from *T. diversifolia*.

Conclusion

From this study it can be concluded that, based on the dosage and formulation used it appears that *Tithonia diversifolia* did not show effectiveness on both faecal egg count and post-mortem worm count reduction tests carried out in this study as compared to conventional drugs.

Recommendations

From the study it can be recommended that more studies should be carried out so as to validate the anthelmintic effects of the plant by investigating other specific species of the nematodes. Since the understanding of most livestock keepers of the word 'worms' refers to macroscopic gastrointestinal parasites (tapeworm segments); it would be worthwhile to test the effects of this plant against cestodes and trematodes.

The studies using laboratory animals should be carried out so as to demonstrate the efficacy of the plant.

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