

Research Article

***In vitro* anthelmintic activity of *Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolor*, *Teclea trichocarpa* and *Zanthoxylum usambarense* using sheep nematodes**

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Background: *Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolor*, *Teclea trichocarpa* and *Zanthoxylum usambarense* are used to treat different ailments in many parts of the world. For instance, *A. gummifera* is used to treat stomach pains, malaria, diarrhoea and sleeping sickness while *C. axillaris* treats ophthalmic disorders and kidney problems. *Manilkara discolor* stem bark infusion is used to treat stomach disorders and as an astringent while *T. trichocarpa* has been used to treat malaria, helminth infections and fever. *Zanthoxylum usambarense* is used to treat rheumatism, backache, painful joints, fever, sore throat, tonsillitis, chest pains, malaria, abscesses and wounds.

Objective: The aim was to determine whether the crude plant extracts have *in vitro* anthelmintic activity.

Materials and methods: Different parts of the plants were acquired from Ngong Hills forest, Kajiado County, Kenya in May 2012, dried and macerated to exhaustion with dichloromethane: methanol (1:1, v/v) solution. Nematode eggs (*Haemonchus Spp*, *Trichostrongylus Spp* and *Oesophagostomum Spp*) were obtained from infected sheep rectums at Department of Vet Farm, University of Nairobi based at Kabete. Varying concentrations of water solutions of the dry crude extracts were prepared. Egg hatch (EHA) and larvae development assays (LDA) were used to test the extracts' effects on nematode egg hatching and larvae development, respectively.

Results: *Albizia gummifera* (root bark) and *Zanthoxylum usambarense* (stem bark) showed high activity (IC₅₀ below 300 µg / mL) in both tests. *Albizia gummifera* (root, stem bark and pods), *Zanthoxylum usambarense* root bark, *Crotalaria axillaris* twigs and *Teclea trichocarpa* root and stem bark showed high activity in LDA but moderate activity (300 µg / mL < IC₅₀ < 500 µg / mL) in EHA. *Teclea trichocarpa* twigs showed moderate activity in LDA but low (IC₅₀ > 500 µg / mL) activity in EHA. *Manilkara discolor* extracts showed low activity in both tests.

Conclusion: Different extracts of the plants tested may inhibit nematode growth and development and hence warrant *in vivo* tests which would support their ethnomedicinal application.

Key words: Anthelmintic activity, egg hatch, larval development, *Haemonchus*, *Trichostrongylus*, *Oesophagostomum*

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1. Introduction

Helminthiasis is a common human and animal infection in the world and though usually non-fatal can cause serious health, social and economic deprivation. It is considered as a neglected infection and drug resistance by the causative agents has been reported (Maingi, 1991; Kaplan, 1994; WHO, 2008; WHO, 2009; Easwaran et al, 2009).

Majority of the people in low-income countries where helminth infections abound continue to rely on alternative medicines for their health requirements (Raaman, 2006; WHO, 2008; Priya, 2012). Although past research has resulted in eighty pure chemical substances currently used as medicines against various illnesses, there is a dearth of research into the plants used to treat helminthiasis providing an opportunity to discover efficacious anthelmintic agents or lead molecules from such plants (Farnsworth et al, 1985).

Albizia gummifera, *Crotalaria axillaris*, *Manilkara discolor*, *Teclea trichocarpa* and *Zanthoxylum usambarensis* are some of the plants that have been used to treat different ailments in Kenya and other parts of the world. Different parts of *A. gummifera* have been used to treat stomach pains, malaria, diarrhoea and sleeping sickness while *C. axillaris* has been used to treat ophthalmic disorders and kidney problems. *Manilkara discolor* stem bark infusion is used to treat stomach disorders and as an astringent (Gachathi, 1989; Kokwaro, 1993; Freiburgauss, 1996; Kareru et al, 2007; Maroyi, 2007). *Teclea trichocarpa* has been used by the Kamba community in Kenya to treat malaria, helminth infections and fever while *Z. usambarensis* is used to treat rheumatism, backache, painful joints, fever, sore throat, tonsillitis, chest pains, malaria, abscesses and wounds (Bussmann et al, 2006; Mwangi et al, 2010; Matu, 2011).

Although the above plants have been used to treat helminth infections and/or stomach problems among other diseases, no studies have been carried out to confirm their anthelmintic activity. The aim of the current work was to evaluate the *in vitro* anthelmintic activity of extracts from the five plants.

2. Materials and Methods

2.1 Plant material collection and extraction

Plant materials (*Albizia gummifera* root, stem bark and pods; *Crotalaria axillaris* twigs, *Manilkara discolor* root bark and stem bark, *Teclea trichocarpa* root, stem bark and twigs and *Zanthoxylum usambarensis* stem and root barks) were collected from Ngong Hills forest, Kajiado County, Kenya in May 2012. Voucher specimens were identified and deposited at Department of Botany Herbarium, University of Nairobi under voucher numbers **BMDK01**, **BMDK02**, **BMDK03**, **BMDK04** and **BMDK05**.

The clean plant materials were air-dried and ground into a coarse powder. About 250 g of the plant material were macerated with about 500 ml of dichloromethane:methanol (1:1, v/v) solution for 48 h with regular agitation. The solution was filtered off using Whatmann's no.1 filter paper and fresh solvent added

to the plant material to exhaustion. The filtrates were mixed and dried with the rotary evaporator (Heidoph). The extracts were stored at 4 °C until the time of use.

2.2 Harvesting and identification of nematode species

Nematode eggs were obtained from rectums of naturally infected sheep at Department of Vet Farm, University of Nairobi based at Kabete. Dichloromethane and methanol for extraction and Tween-80 for preparation of extract solutions and the negative controls were from Lobachemie (Mumbai, India).

The fecal material collected was moistened using distilled water and ground into a fine paste followed by incubation in a loosely covered container at 27 °C for 7 days to allow the eggs to hatch and develop into stage-3 larvae. At this stage, the larvae had acquired different morphological features used to identify the genera when observed under the microscope (GBMAFF, 1986).

2.3 Preparation of stock solutions of plant extracts

Approximately 50 mg/ml stock solutions of plant extracts were prepared using 0.3 % tween-80 v/v in distilled water and mixed thoroughly with a vortex mixer and stored at 4 °C. The negative control was prepared by thoroughly mixing 30 µl of tween-80 with 970 µl of distilled water. It was stored at 4 °C until the time of use.

2.4 Preparation of egg suspension

Strongyle eggs were separated from crushed fecal material by floatation on brine, picked up by placing a perspex Petri-dish on the fluid whereupon they stuck to the bottom of the dish. The dish was rinsed with fresh salt solution. Floatation process was repeated but washing done using distilled water making the egg suspension. The concentration of the eggs in the egg suspension was estimated by thoroughly mixing and pipetting 20 µl of the solution onto a microscope slide followed by counting the number of nematode eggs under the microscope. The egg concentration was adjusted to about 40-50 eggs per 80 µl.

2.5 Egg hatch assay

A 96-well plate was marked to hold a serial dilution of plant extract assigned randomly along a column or row. Twenty µl of plant extracts prepared on the plates contained 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.157 mg/ml and each well was topped with 80 µl of the egg suspension. The plate was then covered with a foil paper and incubated for 48 h at 27 °C after which a drop of Lugol's iodine was added to each well to stop further development of the nematode eggs. The number of eggs and larvae in each well were counted under the inverted light microscope and recorded in a table. Six to eight replicates for each plant extract were carried out.

2.6 Larval development assay

Serial dilutions of plant extract solutions on the 96-well plate were prepared as was done for the egg hatch assay. However, the egg suspension was first incubated at 27 °C for 48 h allowing the nematode eggs to hatch

into larvae stage-I (L1). Eighty micro-litres of this suspension containing about 40 - 50 larvae were then pipetted into each well of the prepared plate and incubated for 5 days at 27 °C with regular mixing of well contents for aeration after which a drop of Lugol's iodine was added to each well to stop further development of larvae. The fully developed (up to stage-L3) and the underdeveloped were counted and recorded. Six to nine replicates for each plant extract were done.

2.7 Statistical analysis

Data was processed using SPSS version 17.0 to determine the concentrations of extracts that prevented hatching of 50% (IC₅₀) of the eggs for egg hatch assay or prevented complete development of 50% (IC₅₀) of L1 larvae into L3 larvae for larval development assay by probit analysis.

3. Results

The sheep were co-infected with *Haemonchus contortus* which comprised 60% of the larvae while *Oesophagostomum* spp. and *Trichostrongylus* spp. comprised 30% and 10%, respectively. *Strongyloides* spp. was present in negligible numbers.

In the egg hatch assay, nine extracts had moderate activity against sheep nematode eggs with IC₅₀ values between 200 µg/ml and 500 µg/ml as shown in **Table 1**. These were *Albizia gummifera* (root, root and stem barks; and pods), *Zanthoxylum usambarensis* (root and stem barks), *Crotalaria axillaris* twigs and *Teclea trichocarpa* root and stem bark. Three of the twelve extracts had low activity with IC₅₀ values above 500 µg/ml. These were *T. trichocarpa*-twigs (820 ± 192 µg/ml), *M. discolor* (RB) (950 ± 224 µg/ml) and *M. discolor* (SB) (1036 ± 251 µg/ml).

The differences between activities of the extracts as determined by Welch ANOVA were significant, [F(11,62) = 16.021, *p* = 0.000]. Games-Howell post hoc test showed that *M. discolor* stem bark had significantly lower activity than all the *Albizia gummifera* extracts (pods, root, root bark and stem bark) as well as *Z. usambarensis* root bark (*p*-value = 0.010) and stem bark (*p*-value = 0.007); and *Crotalaria axillaris* twigs (*p*-value = 0.012). In addition, its activity was lower than *T. trichocarpa* root (*p*-value = 0.042) and stem bark (*p*-value = 0.041). The activity of *M. discolor* stem bark against hatching of sheep nematode eggs was comparable to that of *M. discolor* root bark (*p*-value = 1) and *T. trichocarpa* twigs (*p*-value = 0.843).

In the larval development assay, *A. gummifera* (R), *Z. usambarensis* (RB), *A. gummifera* (RB), *Z. usambarensis* (SB) and *A. gummifera* (SB) had high activity against sheep nematode larval development with IC₅₀ values below 200 µg / ml as shown in **Table 2**. Five extracts exhibited medium activity against larval development with IC₅₀ values between 200 µg / ml and 500 µg / ml. These were *C. axillaris* (T), *A. gummifera* (P), *T. trichocarpa* (R), *T. trichocarpa* (T) and SB. *Manilkara discolor* extracts had low inhibitory activity: RB had IC₅₀ of 587 ± 227 µg / ml and SB had 1265 ± 322 µg / ml.

In addition, Welch ANOVA showed the differences in larval development inhibitory activity between different extracts was significant [F (11, 25.462) = 10.739, *p* = 0.000]. Post hoc tests showed that potency of *M. discolor* (SB) was significantly lower than all the other extracts (*p*-values between 0.005 and 0.045) except *M. discolor* (RB) (*p* = 0.051). *Albizia gummifera* (R), *Z. usambarensis* (RB), *A. gummifera* (RB), *Z. usambarensis* (SB), *A. gummifera* (SB), *C. axillaris* twigs and *A. gummifera* pods were the most active extracts in larval development inhibition using sheep nematodes, respectively.

Table 1: Mean IC₅₀ values and confidence limits for egg hatch assay values

Plant extract	IC ₅₀ (µg/ml) ± SD	95 % CI (µg/ml)	Replicates
Ag-RB	219 ± 94	140 - 344	6
Zu-SB	297 ± 122	210 - 422	6
Zu-RB	320 ± 214	247 - 406	6
Ca-Twigs	325 ± 226	213 - 583	6
Ag-R	336 ± 118	226 - 484	6
Ag-SB	345 ± 145	253 - 451	6
Ag-Pods	377 ± 58	247 - 579	6
Tt-R	476 ± 196	345 - 643	6
Tt-SB	492 ± 72	353 - 688	6
Tt-Twigs	820 ± 192	570 - 1305	8
Md-RB	950 ± 224	652 - 2017	6
Md-SB	1036 ± 251	548 - 1752	6

IC₅₀: Inhibitory concentration, CI: 95% Confidence interval, SD: Standard Deviation

Ag: *A. gummifera*, Ca: *C. axillaris*, Md: *M. discolor*, Tt: *T. trichocarpa*, Zu: *Z. usambarensis*, SB: stem bark, RB: root bark, R: root.

Table 2: Mean IC₅₀ values and confidence limits for larval development assay values

Plant extract	IC ₅₀ (µg/ml) ± SD	95 % CI (µg/ml)	Replicates
Ag-R	154 ± 41	101 - 235	6
Zu-RB	160 ± 41	125 - 196	6
Ag-RB	187 ± 83	128 - 277	9
Zu-SB	196 ± 51	145 - 257	6
Ag-SB	197 ± 59	153 - 251	6
Ca-Twigs	243 ± 61	185 - 324	6
Ag-Pods	277 ± 98	203 - 392	6
Tt-R	295 ± 73	202 - 451	7
Tt-Twigs	346 ± 141	208 - 654	7
Tt-SB	443 ± 124	356 - 551	7
Md-RB	587 ± 227	436 - 816	6
Md-SB	1265 ± 322	960 - 1732	6

IC₅₀: Inhibitory concentration, CI: 95% Confidence interval, SD: Standard Deviation

Ag: *A. gummifera*, Ca: *C. axillaris*, Md: *M. discolor*, Tt: *T. trichocarpa*, Zu: *Z. usambarensis*, SB: stem bark, RB: root bark, R: root.

4.0 Discussion

Some phytochemical and pharmacological tests have been carried out on some of the plants investigated in this work or closely related species. In a thesis, Ayoo (2001) reported that dichloromethane extract of *Z. usambarensis* root bark contains alkaloids (enzophenanthridine and anthranilic acid derived), triterpenes and lignans. In addition, pharmacological investigations showed the plant extracts to possess larvicidal activity against second and fourth instar larvae of *Aedes aegypti*. *Crotalaria axillaris* seeds contain pyrrolizidine alkaloids including axillarine and axillaridine (Crout, 1969; Asres, 2004). The activity observed may be due to the secondary metabolites identified in previous work, which are present in these plants. Review of literature shows no anthelmintic activity tests have been reported before for these two plants.

Manilkara zapota is reported to yield antioxidant polyphenols in the methanolic extracts of the fruits (Ma, 2003). Acetone extract of *M. zapota* seed is reported to possess antibacterial activity while the leaf and fruit pulp have antidiabetic and antilipidemic activity (Kothari, 2010; Barbalho, 2015). Kumar (2012) reports the plant to have anthelmintic activity when the chloroform and ethanolic extracts were tested against the common earthworm, *Pheretima posthuma*.

Teclea trichocarpa is reported to yield alkaloids and tannins. Two acridone alkaloids were shown to possess nematode egg hatching inhibitory activity albeit at higher concentrations than the crude extract. These were melicopicine and 6-methoxytecleanthine (Mule et al, 2014).

Albizia gummifera yields a variety of chemical classes of phytochemicals including alkaloids, saponins, terpenes and flavonoids. Condensed tannins produced by the plant were shown to have anthelmintic activity in egg hatch and larval development assays (Bekele et al, 2011). The plant also possesses other pharmacological activities including antiviral, molluscicidal, antimalarial,

insecticidal, antitumor and antiplatelet aggregation activity (Egualé et al, 2006; Kokila et al, 2013). According to Egualé (2006), the crude extract of the plant also showed a 52.2 % fecal egg load reduction when administered in vivo. In addition, the authors reported that the hydroalcoholic extract of the stem bark showed an ED₅₀ in egg hatch assay of 0.48 mg/ml while the aqueous extract showed an ED₅₀ of 0.67 mg/mL. This compares favourably with the dichloromethane: methanol (1:1, v/v) extract which showed an ED₅₀ of 0.345 mg/mL in this work. Higher anthelmintic activity may be attributed to extraction of more lipophilic phytochemicals by the solvent used in this work. Water- alcoholic and aqueous extracts of *Albizia gummifera* bark are reported to have shown IC₅₀ values of 0.48 mg/ml and 0.67 mg/ml, respectively, in an egg hatch assay using *Haemonchus contortus* eggs (Thoithi et al, 2002; Tadesse et al, 2006) similar to what was observed in this work.

5.0 Conclusion

Extracts derived from *Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolor*, *Teclea trichocarpa* and *Zanthoxylum usambarensis* exhibited varying potencies against sheep nematodes in the *in vitro* egg hatch and larval development assays. Further *in vivo* investigations are required to warrant the plants' ethnomedicinal application as anthelmintic agents.

Conflict of Interest declaration

The authors declare no conflict of interest.

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