Evaluation of anthelmintic activity of Nigerian ethnoveterinary plants;
Cassia occidentalis and *Guiera senegalensis*

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Abstract

Aim: This study was designed to evaluate the anthelmintic activity of the stem-barks of *Cassia occidentalis* and *Guiera senegalensis* which are used traditionally to treat gastrointestinal helminth infections in goat.

Materials and Methods: Phytochemical analysis using standard techniques was used to detect secondary metabolites contained in the plants. *In vitro* anthelmintic activity of the crude methanol extracts of the plants was determined using egg hatch inhibition test (EHIT) and larval development inhibition assay (LDIA).

Results: The extracts of *C. occidentalis* and *G. senegalensis* inhibited hatching of eggs and larval development of *Haemonchus contortus* in a concentration-dependent manner. At concentrations of 0.1, 1, 10 and 100 mg/ml, the extract of *C. occidentalis* produced significant ($p < 0.05$) inhibition of egg hatching of *H. contortus* eggs when compared with the untreated (distilled water) control group. *C. occidentalis* inhibited, by up to 86%, the larval development of *H. contortus*. Similarly, the extract of *G. senegalensis* at concentrations of 10 and 100 mg/ml produced significant ($p < 0.05$) inhibition of egg hatching of *H. contortus* eggs and inhibited larval development by up to 85% of the parasite. *C. occidentalis* ($EC_{50} = 4.23$ mg/ml) was found to be more efficacious than *G. senegalensis* ($EC_{50} = 88.24$ mg/ml) against *H. contortus* eggs. However, *G. senegalensis* ($EC_{50} = 0.0012$ mg/ml) was more effective than *C. occidentalis* ($EC_{50} = 0.11$ mg/ml) against the larvae of *H. contortus*.

Conclusion: The crude methanol extracts of *C. occidentalis* and *G. senegalensis* possess *in vitro* anthelmintic activity against *H. contortus* that requires detailed *in vivo* pharmacological and toxicological trials to justify their use in clinical veterinary practice.

Keywords: anthelmintic, *Cassia occidentalis*, *Guiera senegalensis*, *Haemonchus contortus,* medicinal plants.

Introduction

Gastrointestinal nematode infections pose a major threat to small ruminant production. *Haemonchus contortus* is a helminth parasite that feeds on blood of small ruminant animals and causes anaemia, anorexia, loss of condition, and eventual death of the host animal. The parasite is particularly found in tropical and sub-tropical regions worldwide [1]. In Nigeria and most developing countries, parasitic helminths cause large scale loss in animal productivity and pose a great threat to livestock development. The climate in Nigeria is humid and favours the development of helminth parasites [2].

Control of gastrointestinal nematodes, particularly in ruminants over the past 3 decades has been carried out almost entirely using conventional anthelmintic drugs. Most of these anthelmintics are derived from synthetic or semi-synthetic sources. These compounds are mainly considered to be the only effective way of controlling helminthosis in livestock. [3]. Some of the important drawbacks associated with the use of modern anthelmintics include resistance developed by the parasites to the drugs, which is a serious problem for livestock and a potentially growing problem for human helminth infections [4]. A good number of helminth parasites of domestic animals have the capacity to develop resistance to anthelmintics. Resistance to anthelmintics in sheep and goats is rapidly increasing, especially in warm and humid climatic regions, this could be as a result of increased rate of dosing with anthelmintics and also the adoption of common management, nutritional, and therapeutic strategies [5]. In addition, anthelmintic substances have been reported to cause considerable toxicity to humans and livestock, posing serious threat to human health [6]. In Africa, the rising cost of veterinary drugs and services has made it difficult for resource-poor farmers to access modern veterinary care including treatment of gastrointestinal helminth parasites in livestock [7]. Other issues of concern, such as drug residues in animal products and environment have limited the use of modern anthelmintic in veterinary practice [8, 9]. *Cassia occidentalis* (*Linn*) is a flowering plant belonging to the family Caesalpinaceae [10]. The entire parts of

the plant have medicinal values. In studies conducted by different workers, the plant showed antibacterial [11], antimalarial [12], hepatoprotective and antioxidant [13,14] activities. *Guiera senegalensis* (Combretaceae) is a shrub that grows to about 3 m high. The Hausa and Fulani tribes of northern Nigeria call the plant 'sabara' and 'geloki', respectively. All the plant parts are used for medicinal purposes. The leaf has a bitter taste and is widely acknowledged as a 'cure-all' medicine. The leaf part has been shown to have gastroprotective effect when tested experimetally against ethanol, water immersion stress and aspirin-induced gastric ulcer in rats [15].

Due to the problems arising from the use of conventional anthelmintics highlighted above, there is the need to find alternative therapeutic agents for the treatment and control of helminth infections. One practical way of developing cheaper and effective anthelmintics is to study indigenous herbal remedies that are used as anthelmintics [16]. There have been many reports indicating the effectiveness of plant products against helminth infections in animals mainly from Africa and Asia [17,18,19]. This will suggest a high chance of discovering compounds with anthelmintic effect in some of such traditional medicinal plants.

The aim of this study was to evaluate the effect of the stem-barks of *C. occidentalis* and *G. senegalensis* against gastrointestinal nematode *H. contortus* under *in vitro* condition.

Materials and Methods

Plant collection, identification and processing: Fresh stem-barks of *C. occidentalis* and *G. senegalensis* were collected around Zaria, Nigeria in the month of July, 2012. The plants were identified and authenticated in the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria and voucher specimen numbers of 4751 and 1823 for *C. occidentalis* and *G. senegalensis*, respectively, were deposited. The stem-barks of the plants were individually chopped into smaller pieces, air-dried and pulverized into powder by pounding in a mortar using a pestle. Similarly, the powder of *C. occidentalis* (230 g) and *G. senegalensis* (150 g) were individually cold-extracted exhaustively in a separating funnel by using methanol and , subsequently concentrated *in vacuo* at 55 °C to solid dark-brown and brown extracts for *C. occidentalis* and *G. senegalensis*, respectively [20]. The extracts were kept in a refrigerator at 4 °C until required.

Two grams of each extract were dissolved in 20 ml of distilled water to produce a stock solution of 100 mg/ml. Thereafter, 10-fold serial dilutions were made. The solutions were used for the anthelmintic tests.

Phytochemical screening: The extracts of *C. occidentalis* and *G. senegalensis* were screened for the presence of carbohydrate (Molisch's test and Fehling's test), glycosides (modified Borntrager's test), anthraquinones (Borntrager's test), cardiac glycosides (Kedde test and Kella-killian test), saponins (froth test), steroids and triterpenes (Libermann Burchard's and Salkowski's

test), flavonoids (lead acetate test), tannins (gelatine test), and alkaloids (Mayer's and Wagner's tests) by the method described by Tiwari *et al.* [20].

Recovery and preparation of *Haemonchus contortus* eggs: The abomasums of 5 goats naturally infected with *H. contortus* were obtained from Zaria abattoir in Nigeria. Both ends of the collected abomasums were ligated and immediately taken to the Veterinary Helminthology laboratory of the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria for the recovery, quantification and identification of *H. contortus* using the procedure described by Hansen and Perry [21]. The abomasums were opened along their greater curvature and their contents washed into a bucket, a total volume of 2 litres was obtained. Mature female adult *H. contortus* were picked, crushed and sieved to obtain the eggs. About 40 ml of the filtrate was collected and the concentration of eggs was estimated in 200 µl of the solution. By dilution and concentration, about 100 eggs in 200 µl of the filtrate were obtained. The *H. contortus* eggs were viewed under a light microscope at $\times 40$ magnification.

Egg hatch inhibition assay (EHIA): The *in vitro* egg hatch assay was done as described by Coles *et al.* [22]. Approximately, 100 eggs in 200 µl of distilled water were pipetted into each well of a 48-well microtitre plate. To each of the test wells, 200 µl of each plant extract was added to a final volume of 400 µl per well. The plant extracts were tested at concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 mg/ml. Similarly, albendazole (200µl) (standard anthelmintic drug) at concentration of 0.25 mg/ml was used as a positive control, while distilled water (200µl) was used as a non-treated control. Each test was done in three replicates. The plate was incubated in a humidified incubator at 37 ºC for 48 h. Thereafter, a drop of Lugol's iodine solution was added to each well to stop further hatching. All unhatched eggs and L_1 larvae in each well were counted. The percentage inhibition of eggs hatching was calculated.

Larval development inhibition assay: The larval development inhibition assay (LDIA) as described by Hubert and Kerboeuf [23] with minor modification was used to determine the effect of the extract on the viability of *H. contortus* larvae. About 150 µl of the filtrate which contained approximately 100 eggs were distributed to each of the 48 wells in a microtitre plate. This suspension was supplemented with 30 µl of nutritive medium of Earle's balanced salt solution (1 g of yeast in 90ml of saline solution). The plate was incubated in a humidified incubator at 37 ºC for 6 days. Thereafter, 200 µl of the extracts were individually added to each well. The plant extracts were tested at concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 mg/ml. Similarly, albendazole (200 µl) (standard drug) at concentration of 0.25 mg/ml was used as a positive control, while distilled water (200 µl) was used as a negative control for each plant extract. Each test was
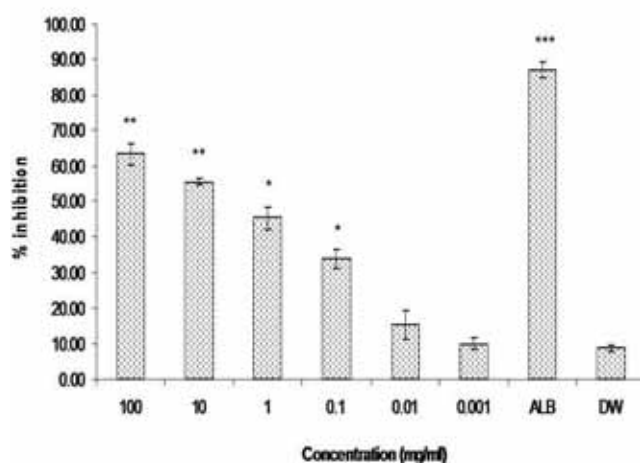
Figure-1: The effect of *Cassia occidentalis* crude methanol extract (0.001-100 mg/ml) and albendazole (ALB) (0.25 mg/ml) on hatching of *Haemonchus contortus* eggs. Control group was dosed with distilled water (DW) at 5 mL/kg. Values are mean ± S.E.M. ***p < 0.001, **p < 0.01 and ***p < 0.05 showed significant difference when compared with distilled water-treated control group.
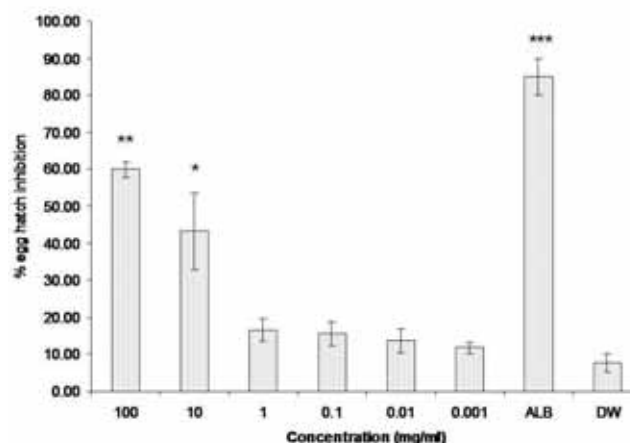

Figure-2: The effect of *Guiera senegalensis* crude methanol extract (0.001-100 mg/ml) and albendazole (ALB) (0.25 mg/ml) on hatching of *Haemonchus contortus* eggs. Control group was dosed with distilled water (DW) at 5 ml/kg. Values are mean ± S.E.M. ***p < 0.001, **p < 0.01 and *p < 0.05 showed significant difference when compared with distilled water-treated control group.

done in three replicates. At the end of the sixth day after incubation, the different larval stages of the parasites (L_1, L_2 and L_3) from each well were recovered and counted under a light microscope at × 40 magnifi-cation.

Statistical analysis: The percent (%) inhibition of egg hatching and larval development was calculated by using the formula described by Coles *et al.* [16].

Percent inhibition = $100 (1-P_{test}/P_{control})$

where P_{test} = number of eggs hatched (or larval forms) in the case of egg hatch test (EHT) assay, or the number of hatched larvae that developed into infective (L_3) in the larval development inhibition assay (LDIA). $P_{control}$ = number of hatched eggs or developed larvae in the distilled water treated control wells.

All values were transformed into means ± S.E.M. Comparisons between groups of data were conducted using Student's *t*-test. *p* values of < 0.05 were considered significant. The concentrations of the plant extract and the controls were transformed into natural logarithm and plotted against the percentage inhibition using Microsoft Excel, 2003 software. The straight line graph obtained from the best line of fit was used to calculate the ED_{50} as a measure of the effectiveness of the plant extracts.

Results

Extract yield: The percentage yields of the extracts after *in vacuo* concentration were 18.5% and 23.3% for *C. occidentalis* and *G. senegalensis*, respectively.

Phytochemical test: The methanol extracts of *C. occidentalis* and *G. senegalensis* were both positive for carbohydrate, glycosides, tannins, saponins, flavonoids, cardiac glycosides, steroids and triterpenes. Moreover, the extract of *G. senegalensis* contained alkaloids.

EHIA: The extracts of *C. occidentalis* and *G. senegalensis* inhibited hatching of eggs of *H. contortus* in a

concentration-dependent manner (Figure-1 and 2). At concentrations of 0.1, 1, 10 and 100 mg/ml, the extract of *C. occidentalis* produced significant (p<0.05) inhibition of egg hatching of *H. contortus* eggs when compared with the group treated with distilled water. However, at the concentrations of 0.01 and 0.001 mg/ml the extract showed no significant anthelmintic effect when compared with distilled water (untreated control). Similarly, the extract of *G. senegalensis* at concentrations of 10 and 100 mg/ml produced significant (p < 0.05) inhibition of egg hatching of *H. contortus* eggs when compared with the group treated with distilled water. However, at the concentrations of 0.001 and 0.01 mg/ml the extract showed no significant difference when compared with the untreated control group. Equation of the dose-response curves of the extracts of *C. occidentalis* and *G. senegalensis* were y = 4.94x + 42.88 and y = 4.11x + 31.58, respectively. The correlations of the dose-response curves were 98% and 78 % for *C. occidentalis* and *G. senegalensis*, respectively. The concentration that is effective in producing 50% anthelmintic effect also know as effective concentration 50 (ED_{50}) of the extracts of *C. occidentalis* and *G. senegalensis* against hatching of *H. contortus* eggs deduced from the dose-response curves were, therefore, 4.23 mg/kg and 88.24 mg/kg, respectively.

Larval development Inhibition assay: The extracts of *C. occidentalis* and *G. senegalensis* inhibited the development of L_1 to L_3 of *H. contortus* larvae in a concentration-dependent fashion. At concentrations of 0.001, 0.01, 0.1, 1, 10 and 100 mg/ml, the extract of *C. occidentalis* inhibited development of *H. contortus* larvae by 18, 36, 56, 63, 76 and 86 %, respectively (Figure-3), while that of *G. senegalensis* inhibited the development of *H. contortus* larvae by 55, 59, 68, 75, 82 and 85 %, respectively (Figure-4). The dose-response curves of the extracts of *C. occidentalis* and *G. senegalensis* against larvae of *H. contortus* were
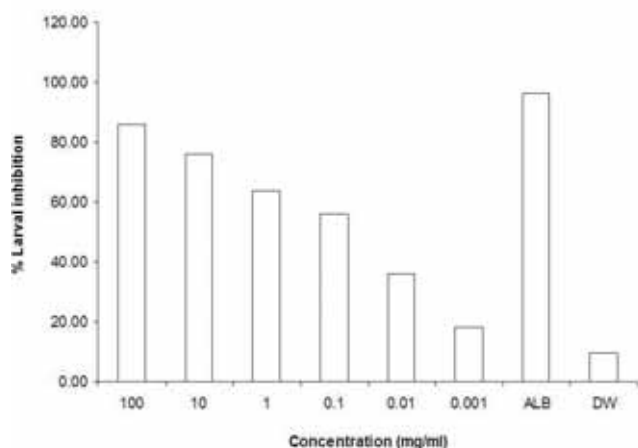
Figure-3: Effect of different concentrations of crude methanol extract of *Cassia occidentalis* on the *in vitro* development of *H. contortus* larvae.

Figure-4: Effect of different concentrations of crude methanol extract of *Guiera senegalensis* on the *in vitro* development of *H. contortus* larvae.

defined by y = 5.79x + 62.54 and y = 3.43x + 72.94, respectively. The correlations of the dose-response curves were 96% and 97 % for *C. occidentalis* and *G. senegalensis*, respectively. The efficacies (ED$_{50}$) of the extracts of *C. occidentalis* and *G. senegalensis* against larval development of *H. contortus* larvae were therefore 0.11 and 0.0012 mg/ml, respectively.

Discussion

The problem of anthelmintic resistance, toxicity and the increasing concern over the presence of drug residues in animal products has led to a renewal of interest in the use of plant-developed drugs, in the form of extracts containing mixture of different plant secondary compounds [8]. The utilization of plants for the treatment of diseases of human and animal origin continues to rise although with few studies demonstrating proof of these effects [24]. Some plant extracts that were found to exhibit *in vitro* anthelmintic activity are *Thespesia lampas* [25], *Khaya senegalensis* [2], *Anona senegalensis* [18], *Luffa cylindrica* [26], *Combretum molle* [27] and *Vernonia amygdalina* and *Alstonia boonei* [28]. The extracts of *Cassia occidentalis* and *Guiera senegalensis* were evaluated for anthelmintic activity in this study because reports had shown their traditional use as anthelmintic agents [10, 29].

The safety and efficacy of *C. occidentalis* and *G. senegalensis* as traditional medicinal herbs have been demonstrated [30]. Scientific evaluation is, however, required for the demonstration of the safety and efficacy potentials of medicinal plants [31]. The present study evaluated the possible inhibition of *Haemonchus contortus* eggs and larvae by the methanol extracts of *C. occidentalis* and *Guiera senegalensis*. *H. contortus is a* good test screen for *in vitro* studies because of its longer survival rate in distilled water. Different stages of the parasite and that of other similar *Strongyloides* had been used for *in vitro* anthelmintic screening by other workers [32-35].

Both the extracts of *C. occidentalis* and *G. senegalensis* inhibited *in vitro* hatching and development of *H. contortus* eggs and larvae in concen-
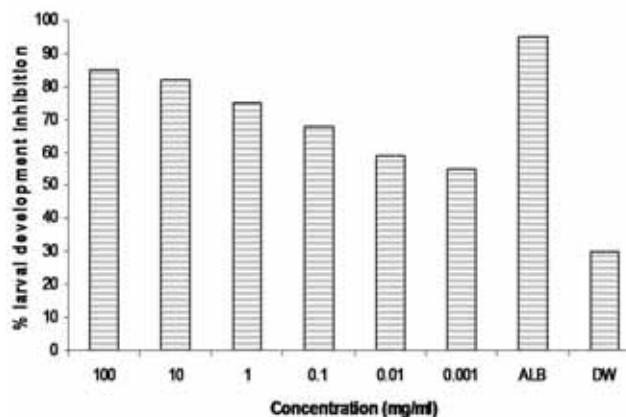
tration-dependent fashions. The main advantages of using *in vitro* assays to test for the anti-parasitic properties of plant and plant extracts are the low costs and rapid turnover which allow large scale screening of plants [36]. Also due to high cost of *in vivo* tests, *in vitro* tests have been used for initial screening of plant extracts for their anthelmintic activity [37]. Albendazole showed higher anthelmintic action than any of the extracts. The superior anthelmintic action of albendazole could perhaps be attributed to its purity when compared with the extracts that have many compounds contained in it. Perhaps if the active compound(s) is isolated from the extract it may have comparable anthelmintic action when compared with albendazole.

The extracts appeared to be having more effect against the larvae of *H. contortus* than the eggs. This could be attributed to egg shell resistance. The egg shell comprises 3 layers; an external lipoprotein layer, a middle chitinous protein layer and an inner lipid layer. The middle and inner layers are resistant to salts and chemicals and also protect the eggs from desiccation, strong acids and bases, oxidants, reductive agents, detergents and proteolytic compounds [38].

Secondary chemical metabolites like saponins, tannins, phenolic glycones, flavonoids, triterpenes and steroids were demonstrated in the extracts of *C. occidentalis* and *G. senegalensis*. In addition, alkaloids were also present in the extract of *G. senegalensis*. The observed anthelmintic effect of the extracts could be attributed to one or more of these secondary metabolites contained in the extract. Monodesmoside saponins destabilize membranes and increase cell permeability in helminth parasites by combining with membrane-associated sterols [39,40]. Monomers of condensed tannins are reported to have the capacity to bind to protein and prevent the exsheathment of nematode third stage larvae [41]. Another possible anthelmintic effect of tannins is binding to free proteins in the gastrointestinal tract of host animals [8] or glycoproteins on the cuticle of the parasite [42] resulting in the death of the parasite. The direct anthelmintic effect

of condensed tannins from an extract of *Schinopsis quebracho* has been reported [8]. Substituted phenols such as disophenol, niclofolan and nitroxynil are established anthelmintics which act by uncoupling the mitochondrial reaction involved in electron transport-associated event of ATP generation by helminths. This causes exhaustion and death of helminths [3]. These compounds may act singly or in synergy to produce the observed anthelmintic effect. The extract of *G. senegalensis* was shown to have strong antioxidant effect attributed to the tannins and alkaloids [43]. This could be responsible for enhancing the anthelmintic action of the extract. The antioxidant effect of *G. senegalensis* against DPPH radical has been demonstrated [44]. The various proposed mechanisms of action of antioxidants are: direct radical scavenging, inhibition of enzymes (such as NO-synthase, xanthine oxidase, cyclooxygenase and lipoxygenase), iron chelating, and direct inhibition of lipid peroxidation [45].

The difference of *in vitro* and *in vivo* studies with regards to drug action is greatly due to differences in the physiology of the host animals and of the *in vivo* mechanisms that inactivate exogenous substances and the toxic effects of plant preparations within the animal host [7]. Hence an appropriate *in vivo* study that would involve toxicological and residual effect is required before a conclusion is made on the usefulness of *C. occidentalis* and *G. senegalensis* as an effective anthelmintic.

Conclusion

The findings from this study suggest that the crude methanol extracts of the stem-bark of *C. occidentalis* and *G. senegalensis* contain constituents with anthelmintic properties. The results obtained, therefore, suggest that the extracts of *C. occidentalis* and *G. senegalensis* are good candidates for developing potentially useful and effective drugs for the control of *H. contortus* in livestock. However, further studies on the active principles and the development of quality assurance protocol involving the use of reference substance of plant origin are required.

Authors' contributions

MMS, AS and EJI designed and performed the experiment. MMS and AMT analysed the data. MM, MMS and MT had given technical guidance during the experiment, drafted and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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