IN VITRO ANTHELMINTIC ACTIVITY OF AQUEOUS AND METHANOLIC EXTRACTS OF *Guiera senegalensis* AGAINST FOUR SPECIES OF TRICHOSTRONGYLID NEMATODES

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ABSTRACT

Gastrointestinal nematodes increasing resistance to synthetic drugs and the side effects of the drugs as it interferes with the quality of animal products call for a search of alternative anthelmintic from plants. This research evaluates the anthelmintic potential of methanol and aqueous extracts of *G. senegalensis* against the migration of *H. contortus, T. colubriformis, O. circumcincta* and *Oesophagostomum spp*. Each of the species was incubated in 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 mg/ml of aqueous and methanol extracts of *G. senegalensis* and later transferred to migratory plates. Larvae in the positive control were treated with 0.02 µg/ml of ivermectin while those in the negative control were treated with M9. Both methanol and aqueous extracts exhibited good larvicidal activities against the migration of all the species of the nematodes. However, the efficacy of methanol extract was higher than aqueous extract in every tested species ($P < 0.05$). The most sensitive of all the species to the extracts was *H. contortus* which recorded the lowest IC_{50} values of 0.631 and 0.350 mg/ml for aqueous and methanol extract respectively. The less sensitive of all the species was *Oesophagostomum spp* as it recorded the highest IC_{50} values of 0.840 and 0.535 mg/ml for aqueous and methanol extracts respectively. Ivermectin was more efficient that the plant extracts.

Keywords: *Guiera senegalensis*, Trichostrongylid Nematodes, Methanol

INTRODUCTION

Gastrointestinal parasitic nematode infection in small ruminants is normally caused by trichostrongylid nematodes of the family Trichostrongylidae. The most important economically influential species responsible which cause serious infection include *Haemonchus contortus, Trichostrongylus colubriformis, Ostertagia circumcincta, Cooperia species, Oesophagostomum species* among others (Zarrin *et al.*, 2015). These parasites cause one or several pathogenic effects that may sometimes lead to the death of the infected host animal (Pestechian *et al*., 2014).Each of these parasites species possesses a variable degree of sensitivity and resistance to treatment with drugs as reported in the previous research work (Roeber *et al.*, 2013b; Mackie, 2016). The variation in the drugs sensitivity among the different species of nematodes parasites could be due to genetic diversity among the different species.

The plant *G. senegalensis* is among the small tropical shrubs widely distributed within the Sudan and Sahel Savannah countries of Africa such as Nigeria, Mali, Niger, Cameroun and Senegal. *Guiera senegalensis* belongs to the family Combretaceae. The plant can grow up to a height of 3-5 meter and the stem is grayish with several branches arising from knots on the stem. *Guiera senegalensis* has been in use exclusively across West African countries for both human and veterinary medicine. It is reported to be used for the treatment of diarrhoea, dysentery, respiratory tract infections as antimalarial among others (Alshafei*et al.,*2016). It is also used as dewormer in Nigeria ethno veterinary industry (Ademola, 2016).

There is not any scientific evidence on the anthelmintic efficacy of *G. senegalensis* irrespective of it traditional application in the treatment of human and animal gastrointestinal infection as well as a dewormer. Therefore, the need for validation of the ethno-veterinary use of *G. senegalensis* becomes necessary. Recently, secondary metabolites such as tannins, alkaloids, flavonoids among others were observed in the ethanolic and aqueous extracts of *G. senegalensis* and were hoping to confer on its anthelmintic potentials of *G. senegalensis* (Besier *et al.,* 2016).This research was therefore aimed at evaluating the anthelmintic potential of methanol and aqueous extracts of *G. senegalensis* against the migration of four species of trichostrongylid nematodes (*H. contortus, T. colubriformis, O. circumcincta* and *Oesophagostomum spp*).

MATERIALS AND METHODS

Collection of plant materials

Collection of stem bark of *G. senegalensis* was carried out in Azare wild forest, Katagum Local Government Area, of Bauchi State during the month of December 2015. The plant was authenticated in the department of Biological Science Bauchi State University Gadau, Nigeria. *Guiera senegalensis* with the voucher specimen No. 900103 was deposited in the herbarium of the Biological science Department of the same university. The stem bark of *G. senegalensis* was collected washed and shade dried for 3 weeks before finally pulverized into powder form using pistil and mortar

Phytochemical extractions

The extraction of phytochemicals was carried in the Biological science department of Universiti Sains Malaysia. Maceration method using distilled water (aqueous) and 80% methanol as solvents was applied for the extraction, described by Cesar *et al.,* (2015) as follows: Up to 50 g of powdered sample of *G. senegalensis* was macerated in 250 ml (1:5 w/v) of distilled water for five days at room temperature. The infusion was filtered with a Whatman filter paper No. 1. The filtrate was dried in an oven at the temperature of 45^{o}C. The dry extract was transferred into labeled sterile specimen vials and stored in a refrigerator at 4^{o}C awaiting further use. The same procedure was applied for 80% methanol as solvent.

Phytochemical test of plant extracts

Identification of secondary metabolites in the aqueous and methanol extracts of *G. senegalensis* was carried out based on the observation of colour changes that occurred due to reactions after mixing appropriate quantity of extract's solution and reagents according to the methods of Gaziano *et al.*, (2015) as follows: Alkaloids (Dragendroff's reagents and 2m H_2SO_4), Saponins (froth formation shaking with water), Phenols (using 2% $FeCl_3$), Flavonoids (2% ammonia solution + 2 % NaOH + 2% HCl), Tannins (2% $FeCl_3$), (Salkowski's test for steroids (acetic anhydrite + 2% + H_2SO_4, Terpenoids (Chloroform + H_2SO_4),

Phytochemical analysis

The method of Orak, (2007),was used for the determination of the total phenolic content of both aqueous and methanol extracts of *G. senegalensis.* The standard used was Gallic acid serial concentration of (0, 0.5, 1, 1.5, 2, 2.5, 3.3.5 mg/ml). Folin-Ciocalteau reagent was separately mixed with 100 µl of 25% (v/v) of each standard and plant extract concentration and allowed to stay for 3-5 minutes. 1% of 80 µl solution of sodium carbonate (Na_2CO_3) was added to the mixture in 96 wells plate and incubated for 1 hour in darkness. The regression equation (y = ax + b) was obtained from the calibration curve drawn from the absorbance values against various concentrations of the gallic acids. Total phenolic content expressed in mg Gallic Acid Equivalence (GAE/mg) was computed using the regression equation. The same procedure was applied for the determination of total tannic content but Folin- Denis reagent instead of Folin-Ciocalteau reagent was used and tannic acid was the standard used. The tannic content of the samples was expressed as Tannic Acid Equivalent (TAE/ mg).

Collection of faecal sample and Coproculture of L3 larvae of trichostrongylid nematodes

Faecal samples were collected from goats and sheep that are naturally infected with mixed species of trichostrongylid nematodes in private farm, located on Penang Island Malaysia. The faecal samples were collected according to the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAAVP) described by Coles *et al.* (1996). The faecal sample was collected directly from the animal's rectum using the middle and index fingers after wearing hand glove. About 4 g of faeces was collected at a time. The collected sample on the glove was turned inside out to encase the faecal sample as the glove was removed.

The method of Harada *et al.*, (1955) was adapted for the Coproculture of the nematode's larvae. Five grams of pellets faecalsamplewere crushed to form a mash. The faecal mash was placed into a sterile, widely open-mouthed specimen vial. The specimen vial was covered with a glass Petri dish. The setup was incubated for 14days at the room temperature of about 25-28^{o}C. The culture was kept constantly damped by routine sprayed with water once daily.

After 14 days, larvae were harvested by smearing the incubated faecal mash on the upper portion of filter paper about half its

total length. The remaining portion of the filter paper free of the faecal mash was dipped in the pool of distilled water inside the test tube. The water absorb through the paper via capillary action. Larvae were collected after 24 hours as they migrated from the faecal mash through the moist surface of the filter paper into the distilled water (Harada *et al.*, 1955).

The larval suspension was transferred into asterile glass vial and distilled water was added until the concentration was adjusted to approximately100 larvae/ml of the distilled water. A total of 10 µl of 5 µg/ml of amphotericin B was added to the larval suspension to avoid the growth of fungal organisms. Suspension of the larvae was stored at 4^{o}C prior to use.

Bioassay of *G. senegalensis* aqueous and methanol extracts against migration L3 larvae of the four species of trichostrongylid nematodes

The 2.0 mg/ml stock solution of an aqueous and methanolic extract of *G. senegalensis* was prepared by dissolving 200 mg of the solid sample of the required extract type in 5 ml of 1% Tween 80 and was diluted with 95 ml of M9 buffer. Further dilution of the stock solution with various volumes of M9 based on dilution ratio was carried out to obtain the solution of serial concentrations of 0.2, 0.4, 0.6, and 1.0 mg/ml and 2.0 mg/ml according to the method of Kumarasingha *et al.* (2014). A solution of ivermectin was prepared by first dissolving1 mg of the solid sample in 1 ml of 1% DMSO and subsequently diluted to 0.0 2 µg/ml usingM9 buffer.

Larval migratory inhibition experimental procedure described by Almeida *et al.*, (2013) was adapted for this bioassay. The L3 larvae were first exsheathed by adding 2 ml of 0.08% of sodium hypochlorite to about 5 ml suspensions of the larvae and allowed to stand for about 4 minutes. The solution of hypochlorite was removed by addition of distilled water and centrifuged at 1200 rpm for 2 minutes three times as described by Almeida *et al.*, (2013). The suspension of 0.3 ml containing approximately 100 exsheathed larvae was dispensed into each of the 24 wells of the macro well plate. Up to 1 ml of each of the required extract solution of the given concentrations 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/ml was added to each of the well in triplicate. Exactly 0.02 µg/ml ivermectin was added to the larvae in 3 wells and served as positive experiment whereas larvae in 3 wells treated with anM9 solution only served as negative control. The setup was incubated for 2 hours at 27^{o}C.

The samples were transferred on to the migratory test sieves with the mesh of 20 µm placed across the openings of Petri dishes (migratory plates) each containing M9 solution. The sieve was submerged in the M9 solution but not touching the bottom of the migratory plate (Wagland *et al.*, 1992). The setup was incubated for 24 hours at the temperature of 27^{o}C after which each test sieve was removed and the liquid content was drained into the migratory plate. Non-migrated larvae on the sieve were washed into another Petri dish. About 50 µml of aqueous Lugol's iodine was added to the content in the migratory plate and all the migrated larvae were killed. The experiment was repeated 3 times. At the end of each experiment, the larvae were separated according to species after identification using the identification keys of Van Wyk *et al.*, (2013). The non-

migrated larvae were also treated with Lugol's iodine solution. The larvae were identified, separated according to species and counted using stereo inverted microscope at x 100 magnification. The total number of each larval species used at the end of each experiment was obtained by adding the number of migrated larvae and non-migrated larvae of the same species. The percentage migration for each species at the end of each experiment was calculated according to the formula adapted from Jackson *et al.*,(2010) as follows:

$$\text{LMI\%} = \frac{A-B}{A} \text{ x } 100$$

Where A = the total number of larvae of the species used during the experiment,
B = the number of larvae of that species which migrated through the sieves

Statistical analysis

Computation of the percentage means and standard error of the migratory inhibition for each species for the various extracts concentrations was carried out using Microsoft® Excel 2016 software. Statistical analysis was carried out using IBM SPSS® statistic version 24. The comparison between mean percentage migratory inhibitions for each species at different concentration against control was done using one - way ANOVA. The post hoc statistical significance used was least square difference (LSD) and the difference between the means was considered significant at $P < 0.05$. Comparison between the efficacy of aqueous and methanol extract was carried out using paired sample T-test. Probit analysis was used to compute the extract concentration required to inhibit 50% (IC_{50}) migration for each nematode species.

RESULTS

Phytochemical screening

Table 1: Results of Phytochemical screening

Extracts	**Secondary Metabolites**						
Aqueous	sap	tan	terp	**x**	**x**	**x**	phen
Methanolic	Sap	tan	terp	antho	anthra	flav	phen

Key: Sap = Saponins, tan = tannins, terp = terpenoids, antho = anthocyanins, anthra = anthraquinones, flav = flavonoids, phen = phenols, x **=** absence

The preliminary phytochemical screening revealed more types of secondary metabolites in methanol extract than in aqueous extract of *G. senegalensis* as shown in Table 1 above.

Phytochemical analysis

Table 2: Results for Phytochemical analysis

	Total tannins (TAE/mg)	**Total Phenolic (**GAE/mg)
Aqueous Extracts	2.78	288.67
Methanol Extracts	3.85	326.37

The result of phytochemical analysis revealed higher quantities of both phenolic and tannins compound in methanol extract than in aqueous extract. The total phenolic content of 288.67 GAE/mg and 326.37 GAE/mg were recorded in aqueous and methanol extracts of *G. senegalensis* respectively. Similarly, the total of 2.78 TAE/mg was recorded for aqueous extract

whereas 3.85 TAE/mg was recorded in methanol extract (Table 2)

Efficacy of *G. senegalensis* extracts against the migration of the various species of trichostrongylid nematodes

The extracts performance increased as the concentration of the extracts increased. The highest performance of the extracts was recorded at the concentration of 2.0 mg/ml. At 2.0 mg/ml, the sensitivity of all the worms was statistically significant ($P < 0.001$) compared to negative control (Table 1). For instance, at 2.0 mg/ml, only 10.5% of the *H. contortus* incubated in aqueous extract were able to migrate. On the other hand, only 4.8%of the larvae incubated in methanol extract were able to migrate. *Haemonchus contortus* proved more sensitive than any of the trichostrongylid species to both aqueous and methanol extracts of *G. senegalensis*. *Ostertagia circumcincta* was the next most sensitive species with percentage migration of 11.1% for those treated in 2.0 mg/ml aqueous extracts whereas those treated in methanol extract recorded the percentage migration of 5.60% at the same concentration of 2.0 mg/ml. On the other hand, *T. colubriformis* sensitivity was indicated by the percentage migration of 13.2% (aqueous extract) whereas the percentage migration of 6.10% was recorded against those incubated in 2.0 mg/ml of methanol extract. *Oesophagostomum spp* was the less sensitive of all the species tested as the percentage migration of 18.2% was recorded for aqueous extract and 11.3% for methanol extract at 2.0 mg/ml. These are higher compared to those of the rest of the species at $P < 0.05$ (Table 1).

The sensitivity of *T. colubriformis, O. circumcincta* and *H. contortus* were statistically the same ($P > 0.05$). Ivermectin was more efficient than the plant extract in the inhibition of larval migration in all the species. This is evidenced by the percentage larval migration inhibition above 90% for every species. The sensitivity of all the species to ivermectin was the same among all the different species of the parasite ($P > 0.05$).

Table 4 shows the inhibitory concentration (IC_{50}) (concentration at which 50% of the larvae failed to migrate through the sieve mesh) for the four species of the parasitic nematodes after treated in aqueous and methanol extracts. *Haemonchus contortus, T. colubriformis*and *O. circumcincta* were more sensitive to the extracts of *G. senegalensis* than *Oesophagostomum species*. The responses of *H. contortus, T. colubriformis* and *O. circumcincta* to both aqueous and methanol extract of *G. senegalensis* were statistically the same ($P >0.05$). This is justified by the closeness of the IC_{50} values of aqueous extract of 0.631 mg/ml 0.695 mg/ml and 0.647 mg/ml against the migration of *H. contortus, T. colubriformis* and *O. circumcincta* respectively. Similarly, the IC_{50} of 0.474 mg/ml 0.492 mg/ml and 0.482 mg/ml were recorded against the migration of *H. contortus, T. colubriformis* and *O. circumcincta* respectively treated in methanol extract. *Oesophagostomum spp* was the lowest sensitive species with the highest IC_{50} value of 0.840 mg/ml (aqueous) and 0.628 mg/ml (methanol) compared to the rest of the species ($P < 0.05$).

Table 3: Efficacy of *G. senegalensis* extracts against the migration of various species of trichostrongylid nematodes

Conc.	Aqueous extract				Methanol extract			
(mg/ml)	H. C	T. C	O. C.	O. S	H. C	T. S	O. C.	O. S
0.2	65.7 ± 0.48	68.3 ± 0.67	67.7 ± 0.74	74.3 ± 0.58	60.9 ± 0.80	61.2 ± 0.52	60.8 ± 0.32	68.3 ± 0.55
0.4	59.4 ± 0.61	62.0 ± 0.84	61.4 ± 0.52	67.2 ± 0.53	54.2 ± 0.55	56.2 ± 0.58	55.9 ± 0.40	61.2 ± 0.61
0.6	55.2 ± 0.58	56.8 ± 0.52	53.2 ± 0.77	59.2 ± 0.55	48.2 ± 0.61	47.2 ± 0.54	46.8 ± 0.37	52.2 ± 0.58
0.8	48.2 ± 0.55	49.2 ± 0.58	47.2 ± 0.55	55.2 ± 0.64	40.3 ± 0.58	40.2 ± 0.56	40.0 ± 0.64	47.2 ± 0.61
1.0	25.2 ± 0.55	28.2 ± 0.61	27.0 ± 0.35	34.3 ± 0.61	17.2 ± 0.55	21.2 ± 0.61	20.2 ± 0.58	23.8 ± 0.88
2.0	10.5 ± 0.86	13.2 ± 0.61	11.1 ± 0.44	16.3 ± 0.58	4.80 ± 0.68	6.10 ± 0.47	5.60 ± 0.37	10.0 ± 0.56
IVM	0.80 ± 0.32	0.90 ± 0.31	0.80 ± 0.30	0.80 ± 0.32	0.90 ± 0.40	0.80 ± 0.30	1.70 ± 0.57	0.90 ± 0.40
Neg. con.	95.5 ± 0.68	96.3 ± 0.33	96.9 ± 0.38	95.5 ± 0.60	95.5 ± 0.44	96.7 ± 0.33	97.0 ± 0.38	95.5 ± 0.44

The data are reported as mean percentage ± standard error of 3 independent experiments. H. C = *Haemonchus contortus*, T.C = *Trichostrongylus colubriformis*, O.C = *Ostertagia circumcincta*, O.S = *Oesophagostomum spp.*

Table 4: Inhibitory concentration of *G. senegalensis* against the larval migration of the various species of trichostrongylid nematodes

Parasite	Aqueous extract	Methanol extract
	IC50	IC50
H. contortus	0.631	0.350
T. colubriformis	0.695	0.376
O. circumcincta	0.647	0.351
Oesophagostomum spp	0.840	0.523

DISCUSSION

The anthelmintic potential of *G. senegalensis* was clearly revealed as the percentage larval migration in the negative control remained very high at the end of the assay. This is further proven by the variation in the percentage larval migration in accordance with the variation in the concentrations of the aqueous and methanol extracts of *G. senegalensis.*

There has been scarce scientific record on the *in vitro* study of the anthelmintic potential of *G. senegalensis* against trichostrongylid nematodes. However, there are previous reports on ethno-medicinal use of *G. senegalensis* in the treatment of diarrhoea, microbial infections, dysentery and gastrointestinal tract infection in many African countries as well as its application as dewormer in the ethno veterinary practice in Nigeria (Alshafei *et al.,* 2016; Ademola *et al.,* 2016). Also, similar records on the the sensitivity of some of these species of trichostrongylid nematodes to some plants were documented. For instance, the high inhibitory activity of *Lotus corniculatus* wasreported against the migration of L3 larvae of *H. contortus* by Acharya *et al.*

(2014). It was also reported that extracts from two plants (Tillia flowers and willow bark) inhibited the migration of L3 larvae of *Oesophagostomum dentatum* Williams *et al.*(2014).

There was no significant difference in the sensitivity of *H. contortus, T. colubriformis* and *O. circumcincta*. However,*H. contortus*proved to be the most sensitive to both aqueous and the methanol extracts of *G. senegalensis* compared to the rest of species. *Oesophagostomum spp* exhibited the lowest sensitivity to both methanol and aqueous extracts of *G. senegalensis*. The reason for the variation in the sensitivity to extracts among the species, could be due to genetic diversity among the different species, based on the assumption of Prichard *et al.* (2005).

All the species were more sensitive to methanol extracts than aqueous extracts. This is similar to the findings of Attindéhou *et al.*(2016) where they reported that methanol extract of *Khaya senegalensis* exhibited the highest inhibition of larval migration among the extractsof different solvents tested against the migration of *H. contortus*. High efficacy of methanol extract in this present work could be as a result of a higher quantity of tannin and phenolic compounds in the methanol extracts than aqueous extract as revealed in the results of the phytochemical analysis as well as more varieties of secodary metabolites in the methanol extract than aqueous extract. This is similar to the findings of Badar *et al.* (2011) who reported that crude aqueous methanolic extracts of stembark of *Acacia nilotica* were more potent than the aqueous extracts in the inhibition of motility of adult *H. contortus.* The disparity could be due to more varieties/quantity of active principle/compounds in the extract obtained usingmethanol as solvent compared to aqueous extract. Neffati *et al.*, (2017) also recorded higher quantity of tannic compounds in the methanolic extracts than the aqueous extract of several plants investigated in Tunisia.They further suggested that the higher quantity of tannins and phenolics content was responsible for better antioxidant and antimicrobial activity of the methanol extract than aqueous extract. The relationship between the phenolics and tannins content of the extracts and the larvicidal efficacy was also reported by Alanso Diaz *et al*., (2008) who confirmed that plant extracts with the highest level of total phenolics and total tannins inhibited the exsheathment and migration of *H. contortus*.

Tannins and other phenolic compounds are responsible for anthelmintic properties of plants. Despite lack of complete understanding of the exact mechanism of anthelmintics of tannin, saponin, and flavonoids among other phenolic compounds against the worms, several hypotheses have been formulated to explain the anthelmintics properties of some of these metabolites against gastrointestinal nematodes. For instance, tannins, flavonoids and saponins were reported to interfere with the protective function of the cuticles of the nematodes by increasing the permeability of the membranes and sometimes destroying the membranes (Williams *et al.,* 2014). Failure of the worms to passed through the sieves might have resulted from the paralysis and interference of neuromuscular coordination of the larvae by condensed tannin (Molan, 2014).

The variation in the phytochemical contents of aqueous and methanol extracts might be attributed to the difference in the polarity of water and methanol as solvents. Water is a polar solvent which extracts only polar compounds. On the other hand, methanol behaves as a polar solvent and as nonpolar solvent. The exhibition of both polar and nonpolar characteristic of methanol enables it to extract both polar none polar compound hence, the reason for more varieties and quantities of plant's secondary metabolites in methanol extract than aqueous extract (Dailey *et al.,* 2015). Furthermore, the non-polar characteristic of methanol enables it to be more effective in the degradation of plant cell walls which are also non-polar to release more secondary metabolites (Tiwari *et al.*, 2011).

The larvicidal efficacy of the extracts was concentration dependent, as reported in a similar finding by Kanojiya *et al.*(2015). The larvae were more sensitive to ivermectin than the plant extracts this could be due to the fact that plant extracts are in crude forms with mixtures of impurities that interfere with its potency whereas ivermectin contained pure compounds (Al-Rofaai *et al.,* 2012).

CONLUSSION

The extracts of *G. senegalensis* was found to be effective in the inhibition of all the four species of trichostrongylid nematodes although methanol extract was more efficient than aqueous extract. *Haemonchus contortus* was the most sensitive of all the trichostrongylid species tested whereas *Oesophagostomum spp* was the less sensitive of all the species. *Guiera senegalensis*, therefore,could serve as a source of lead compounds for natural anthelmintics.Active compounds in the extracts of *G. senegalensis* should be isolated and tested. The extracts should also be tested *in vivo* on the infected animals.

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