Bayero Journal of Pure and Applied Sciences, 11(2): 274 - 278
Received: May, 2018
Accepted: October, 2018
ISSN 2006 – 6996

IN VITRO STUDY OF THE ANTHELMINTIC ACTIVITY OF AQUEOUS AND METHANOL EXTRACT OF *Guiera senegalensis* AGAINST EGG HATCH AND LARVAL DEVELOPMENTAL OF *Caenorhabditis elegans*

[1]Haladu Ali Gagman, [2]Nik Ahmad Irwan and [2]Hamdan Bin Ahmad
[*1]Department of Biological Sciences, Faculty of Science, Bauchi State University Gadau, 751 Itas Gadau, Nigeria
[2]School of Biological Sciences, Universiti Sains Malaysia

ABSTRACT
The anthelmintic potential of methanol and aqueous extracts of G. senegalensis in vitro against the egg hatch and larval development of C. elegans Bristol N2 (susceptible to ivermectin) and C. elegans DA1316 (ivermectin resistant strain) was evaluated.. The eggs and larvae were incubated in aqueous and methanol extracts of the stem bark of G. senegalensis with the serial concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/ml. Methanol extracts of G. senegalensis which inhibited above 80% egg hatch in both strains of C. elegans were moderately effective whereas the aqueous extracts were ineffective. Both aqueous and methanol extracts which recorded up to 90% inhibition of larval development against C. elegans DA1316, as well as C. elegans Bristol N2, were considered effective. However, methanol extract proved more efficient than the aqueous extract against larval development as well as egg hatch in both strains of C. elegans (P < 0.05). The effects of the extracts on the egg hatch and larval development of C. elegans Bristol N2 was similar compared to that of C. elegans DA1316 (P > 0.05). Therefore, extract from G. senegalensis can be use against ivermectin resistant parasitic nematodes. Guiera senegalensis may be used as a natural source of lead compounds to produce anthelmintic drugs. Cytotoxicity test should be conducted before the extracts are tested in vivo on the infected ruminant animals.
Key words: Activity; Anthelmintic; Caenorhabditis elegans; Egg hatch; G. senegalensis; Larval development.

INTRODUCTION

Caenorhabditis elegans is a free-living non- parasitic soil nematode which naturally exists in the soil within the temperate climate region (Brenner, 1974). The organism is easily maintained in the laboratory on nematode growth agar with a lawn of *E. coli* OP50. The single self-fertilizing hermaphrodite can produce up to 300 offspring throughout it life time. The adult organism grows to the size of 1.3 mm with an average diameter of 80 µm (Brenner, 1974; Kumarasingha *et al.,* 2014). *Caenorhabditis elegans* has been used over a decade as an invaluable model for drugs discovery and development as well as a major tool for biomedical research by several researchers (Katiki *et al.,* 2011).

The difficulties experienced when screening plants as an alternative to synthetic anthelmintic drugs has been overcome through the application of *C. elegans* as a model organism in the screening of plant and its products. For example, difficulty in obtaining adult parasitic nematode organism that can remain alive outside the host organism for a long time to be used in the evaluation of the efficacy of anthelmintics was overcome using adult *C. elegans.*The application of *C. elegans* as a model in the screening of plants and plant products for anthelmintic have generated the advantages of cheap *in vitro* activities, easy maintenance, short generation time coupled with the transparent body of *C. elegans* which may permit easy observation of the drug's effects against the adult organism.

Caenorhabditis elegans is often used to replace adult parasitic nematodes organisms especially those of the family trichostrongylidae such as *Haemonchus contortus, Ostertagia ostertagi* among others for drug screening. This is because of the anatomical and physiological similarities of *C. elegans* to the members of trichostrongylidae. Therefore, it is reasonably assumed that drug effects on *C. elegans* might be reproduced on any member trichostrongylidae (Katiki et al., 2011, Kumarasingha *et al.,* 2014). For instance, Moxidectin confirmed as a potent drug against the wild type and ivermectin resistant strain of *C. elegans* was equally confirmed to be effective against ivermectin resistant *H. contortus* (Ménez *et al.,* 2016).

Guiera senegalensis is among other traditional herbal medicines been used in the treatment of diverse ailment, with no proper scientific validation and documentation. *Guiera senegalensis* is a small tropical shrub which belongs to the family Combretaceae. The stem is grayish with several branches arising from knots on the stem. *Guiera senegalensis* has been in use exclusively in several West African countries for both human and veterinary medicine (Ademola, 2016). This work was aimed at investigating the anthelmintic potential of methanol and aqueous extracts of *G. senegalensis in vitro* against the egg hatch and larval development of *C. elegans* Bristol N2 (susceptible to ivermectin) and *C. elegans* DA1316 (ivermectin resistant strain).

MATERIALS AND METHODS

Collection of Plant Materials

Collection of the stem bark of *G. senegalensis* shrub was carried out during the month of December 2016, in the wild bush of Azare, in Katagum Local Government area of Bauchi State, Nigeria.

This was authenticated at the Department of Biological Sciences, Bauchi State University Gadau and the Voucher No. 900103 was assigned to the specimen.

Phytochemical extraction

The Stem bark was transported and extracted at the Universiti Sains Malaysia using Soxhlet method of extraction as described by Foo *et al.* (2015). Water and 80% methanol was used as the extraction solvents. Twenty (20) g of the dried powdered plant sample was placed in a thimble. Exactly 150 ml of the solvent used for the extraction was placed in the round bottom flask portion of the soxhlet apparatus. The process of extraction was run for 24 hours at 150^{o}C. The liquid extract was concentrated using rotary evaporator at 40^{o}C under reduced pressure and was lyophilized into the dried powdered extract. The powdered extract was weighed, transferred into label specimen vial and stored at 4 ^{o}C ready for use.

Phytochemical Screening of the Plant Extracts

Secondary metabolites in the extracts were detected based on the methods of Maoebe *et al.* (2013) and Gaziano *et al*. (2015) as follows: Saponins (froth formation on shaking with water), Alkaloids (Dragendorff's reagents and 2M of H_2SO_4), Salkowski's test for steroids (acetic anhydride + 2% + H_2SO_4), Flavonoids (2% ammonia solution + 2 % NaOH + 2% HCl), Terpenoids (Chloroform + H_2SO_4), Phenols (using 2% $FeCl_3$), Tannins (2% $FeCl_3$).

Phytochemical analysis

Phytochemical analysis was carried out to determine the total phenolics and total tannins content in the crude aqueous and methanol extracts of *G. senegalensis* using Folin-Denis spectrometric method described by Oliveira *et al.* (2009). A standard curve for quantification of tannins was established using tannic acid solution with varying concentrations of 0, 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 mg/ml and optical density was obtained at 765 nm. The tannic content was expressed in Tannic Acid Equivalent (TAE/mg). The same procedure was applied for total phenolic content; however, the standard used was a gallic acid with the standard concentrations of 0, 0.5, 1, 1.5, 2, 2.5, 3.3.5 mg/ml instead of tannic acid. Phenolic content was calculated in mg Gallic Acid Equivalence (GAE/mg).

Egg Hatch Bioassay

A solution of the required extract of *G. senegalensis* of serial concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml was prepared using an M9 buffer. Ivermectin stock solution was prepared by dissolving 10 mg of the dry sample in 10 ml of (DMSO) and subsequently 1 ml of the solution was diluted with 100 ml of M9 buffer to yield 0.02 µg/ml. Fifty (50) µml of the suspension containing approximately 100 eggs obtained from gravid adults *C. elegans* of the required strain was pipetted into each of the 24 macro-wells. Exactly 1 ml of each of the desired plant extract concentration was added to the eggs in the macro-wells in 3 replications. Up to 1 ml of 0.02 µg/ml ivermectin solution was also applied to the eggs in the macro wells in 3 replications and served as positive control. The M9 buffer which was applied to eggs in another 3 wells served as negative control. The set up was incubated for 24 hours at 20^{o}C by placing in an incubator shaker. The experiment was repeated three times and the number of L1 larvae was recorded at the end of each test. The percentage egg hatch was calculated according to the formula adapted from Molan, (2014) as follows:

EHA % = $\frac{Number\ of\ larvae\ dead\ or\ alive}{Number\ of\ eggs\ incubated}\ x\ 100$

Where EHA% = percentage egg hatched.

Larval development bioassay

Evaluation of the efficacy of the extracts was based on the standard set by the World Association for the advancement of veterinary Parasitology (WAAVP) which consider the ovicidal or larvicidal efficacy of the anthelmintic agent to be effective when it is up to 90% but moderately effective when it is lower than 90% but up to 80% (Ferreira *et al.*, 2013). The method of Brenner, (1974) and Al-Roofai *et al.* (2012) was adapted for this assay. Exactly 50 µl of the suspension containing approximately 100 L1 larvae was pipetted into each well of the 24 well macro plate containing 1 ml of nematode growth medium(NGM) (a mixture of 3 g NaCl, 17 g agar and 2.5 g of Bacto-peptone in 1000 ml of distilled water) seeded with *E. coli* OP50. One (1) ml each of the concentration of 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 mg/ml of the plant extract to be tested was added into separate well of the 24 wells plate in 3 replications. One (1) ml of 0.02 µg/ml of ivermectin was added to three of the wells and served as positive control. M9 buffer was added to three of the wells and served as negative control. In each of the well, 8 µl of 5 µg/ml of amphotericin B was added to prevent fungal growth. The set up was incubated for 30 hours at 20^{o}C at the end of which all the larvae were killed and the L4 larvae were counted and recorded. The percentage larval migration was calculated based on the formula of Ademola *et al.* (2011) as follows:

LD% = $\frac{\text{Number of L4 larvae in the treatment well}}{Total\ number\ of\ L1\ larvae\ incubated}$ x 100

Where LD% = percentage larval development.

RESULTS AND DISCUSSION

The result of preliminary phytochemical screening of methanol and aqueous extracts of *G. senegalensis* revealed more varieties of secondary metabolites in the methanol extract than in the aqueous extract. The secondary metabolites confirmed in the methanol extract include saponins, tannins, terpenoids, anthocyanins, flavonoids, and phenols glycoside, carbohydrate. Screening of the aqueous extract revealed the presence of saponins, tannin, steroids, glycoside, and phenols. Higher quantities of both phenolic and tannins compounds were recorded in methanol extract than aqueous extract as shown in Table 1. The total phenolic content of 288.67 GAE/mg and 326.37 GAE/mg were recorded for aqueous and methanol extracts of *G. senegalensis* respectively.

Similarly, the quantity of tannin recorded in the aqueous extract of *G. senegalensis* was 2.78 TAE/mg whereas 3.85 TAE/mg was recorded in methanol extract (Table 2).

More varieties and higher quantities of metabolites recorded in the methanol extract than aqueous extract might be due to the exhibition of both polar and non-polar characteristics by the methanol. This enables the methanol to extract both polar and non-polar compounds, unlike water which exhibits only polar characteristic that enable it to extract only polar compounds (Bowyer *et al*. 2015). Also, the non-polar characteristic of methanol might have facilitated its ability to dissolve the polar cell wall to release greater quantity and varieties of secondary metabolites (Tiwari *et al*. 2011).

The efficacy of aqueous and methanol extract of *G. senegalensis* against the egg hatch of *C. elegans* Bristol N2 and *C. elegans* DA 1316 is presented in Table 3. The percentage egg hatch was observed to decrease as the concentration of the extract increased from 0.2 mg/ml to 2.0.mg/ml and the highest performance of the extracts was recorded at 2.0 mg/ml similar to the findings of Kanojiya *et al.* (2015) who reported the ovicidal activity of extract of *Eucalyptus globulus* increased with increase in concentration of the extracts against the egg hatch of *H. contortus*. For instance, at the lowest concentration of 0.2 mg/ml up to 91.3% and 81.2% eggs of *C. elegans* Bristol N2 incubated in aqueous and methanol extracts respectively were able to hatch. Similarly, up to 94.3% and 84.82% egg hatch was recorded in the aqueous and methanol extract respectively against the eggs of *C. elegans* DA1316. The lowest percentage egg hatch which indicated increase in the efficacy of the extracts was recorded at the highest concentration of 2.0 mg/ml where aqueous extract recorded only 27% eggs hatched (73% inhibition). The aqueous extract was, therefore, considered ineffective as less than 80% of the eggs were inhibited from hatching. However, the methanol extract which was moderately effective recorded the lowest percentage egg hatch of 13.3% (87% inhibition) against the eggs of *C. elegans* Bristol N2. On the other hand, the percentage egg hatch of 29.2% and 15.4% was recorded in aqueous and methanol extracts respectively against the eggs of *C. elegans* DA1316.

The percentage inhibition ± standard error of aqueous and methanol extract of *G. senegalensis* against larval development is shown in Table 4. Up to 65.2% of the larvae of *C. elegans* Bristol N2 developed to adult in 0.2 mg/ml of aqueous extract whereas 60.1% developed in methanol extract at the same concentration. The percentage larval development of 67.2% and 62.2% were recorded in aqueous and methanol extract respectively at the concentration of 0.2 mg/ml against *C. elegans* DA1316. The extracts demonstrated the highest efficacy at the highest concentration of 2.0 mg/ml where both aqueous and methanol extracts were effective. This was evidence as the lowest percentage larval development of 9.2% was recorded in aqueous extract and 5.3% in methanol extract against *C. elegans* Bristol N2. On the other hand, *C. elegans* DA1316 recorded the percentage development of 10.0% in the aqueous extract whereas 6.20% was recorded in the methanol extract. The extract was more effective against larval development than egg hatch. This was consistent with the work of Al- Roofai *et al.* (2012) who suggested that the effect of the extract against the L1 larvae might be due to lack of egg shell or sheath on the body of L1 larvae for protection against the activities of metabolites from the plant extracts.

The methanol extract was more efficient than aqueous extract against the egg hatch and larval development of both strains of the *C. elegans* at $P < 0.05$. Kanojiya *et al.* (2014) reported high performance of methanol of *E. globulus* than aqueous extracts against egg hatch of *H. contortus* which belongs to the same Clade V with *C. elegans*. The performance of methanol extract above that of the aqueous extracts might be attributed to more varieties and more quantities of metabolites in the methanol extract than aqueous extract as revealed in the phytochemical analysis. This is in conformity with the findings of Metrouh-Amir *et al.* (2015) who reported that methanol extracts of *Matricaria pubescent* which contained more varieties of metabolites and higher phenolic and tannins content than pure water extract exhibited the highest antibacterial activities. Another reason could be attributed to the presence of the enzyme polyphenol oxidase which is very active in aqueous extracts as it can degrade the polyphenols in the aqueous extracts and render them inactive against the nematodes. On the other hand, polyphenol oxidase is inactive in methanol extract. Therefore, it has no effect on the performance of polyphenol as anthelmintics active principle in the methanol extract (Tiwari *et al.,* 2011).

There was no significant difference in the efficacy of the plant extracts against the egg hatch as well as the larval development of *C. elegans* Bristol N2 and *C. elegans* DA1316 ($P > 0.05$). However, ivermectin was more effective than the plant extracts against the egg hatch and larval development of *C. elegans* Bristol N2 but completely inactive against the egg hatch and larval development of *C. elegans* DA1316. This could be attributed to the fact that ivermectin which contained a pure compound has a narrow spectrum of action against *C. elegans* DA1316 whereas plant extracts have several varieties of secondary metabolites which interact to produce a synergistic effect with multiple mechanisms of action that could compromise resistance by the worm (Ademola *et al.,* 2011).

Generally, the effectiveness of *G. senegalensis* against egg hatch and larval development of *C. elegans* in this work could be due to the presence of tannins and other phenolic compounds in both the aqueous and methanol extracts of *G. senegalensis.* This is because tannins and phenolic compounds formed the major anthelmintic properties of the plant. For instance, tannin was reported to have binding affinity to the free protein on the nematodes as well as its binding ability to the worm's digestive and reproductive tracts (Debiage *et al.,* 2016).

In addition, tannin interferes with energy phosphorylation of the nematodes thereby leading to energy starvation. All the above roles played by tannins may result in the paralysis of the nematodes and might eventually lead to their death. In addition, this might also lead to inhibition of egg hatch as well as larval development (Debiage et al., 2016; Williams et al., 2014). Saponin increases the permeability of nematodes cell membrane by the formation of more pores that might interferes with the development of the organism within the egg or dead of the organism within the egg (Wang *et al*., 2010).

Table 1: Secondary Metabolites Present in Aqueous and Methanol Extracts of *G. senegalensis*

	alk	sap	tan	ter	ste	fla	phe	anth	glyc	chy
Aqueous extract	-	+	+	-	+	-	+	-	-	+
Methanol extract	+	+	+	+	+	+	+	+	+	+

Key: alk = Alkaloids, sap = saponins, tan = tannins, ter = terpenoids, ste = steroids, fla = flavonoids, phe; phenols, anth = anthocyanide, glyc = glycoside, chy = carbohydrate, + = presence of metabolite, - = absence of metabolite.

Table 2: Total phenolic and tannins contents of aqueous and methanol extracts of *G. senegalensis*

Extract	Phenolic contents (GAE/mg ± SD)	Tannins content (TAE/mg± SD)
Aqueous extract	326.37	2.78
Methanol extract	288.67	3.85

Table 3: The percentage inhibition/ ± standard error of aqueous and methanol extract of *G. senegalensis* against the egg hatch of *C. elegans* Bristol N2 and *C. elegans* DA 1316

	***C. elegans* Bristol N2**		***C. elegans* DA1316**	
Conc. mg/ml	**Aqueous extract**	**Methanol extract**	**Aqueous extract**	**Methanol extract**
0.2	91.3 ± 0.55	81.2 ± 0.61	94.3 ± 0.58	84 .8 ± 0.72
0.4	86.6 ± 0.67	75.2 ± 0.61	90.1 ± 0.66	75.0 ± 0.45
0.6	82.2 ± 0.58	68.3 ± 0.58	84.2 ± 0.61	67.0 ± 0.77
0.8	76.3 ± 0.52	56.2 ± 0.55	78.3 ± 0.58	53.6 ± 0.36
1.0	56.3 ± 0.58	36.2 ± 0.55	52.7 ± 0.74	43.7 ± 0.74
2.0	27.0 ± 0.49	13.3 ± 0.58	29.2 ± 0.64	15.4 ± 0.50
Ivermectin	5.60 ± 0.57	6.40 ± 0.44	96.0 ± 0.77	95.6 ± 0.85
Neg. control	96.9 ± 0.38	97.0 ± 0.38	97.4 ± 0.34	97.1 ± 0.55

Data was based on percentage ± standard error of 3 independent experiments.

Table 4: The Percentage Inhibition/ ± Standard Error of Aqueous and Methanol Extract of *G. senegalensis* against the Larval Development of *C. elegans* Bristol N2 and *C. elegans* DA1316

	***C. elegans* Bristol N2**		***C. elegans* DA1316**	
Conc. mg/ml	**Aqueous extract**	**Methanol extract**	**Aqueous extract**	**Methanol extract**
0.2	65.2 ± 0.55	60.1 ± 0.52	67.2 ± 0.55	62.2 ± 0.58
0.4	60.2 ± 0.55	55.2 ± 0.58	62.8 ± 0.64	57.2 ± 0.52
0.6	55.2 ± 0.58	46.9 ± 0.63	57.2 ± 0.58	48.9 ± 0.72
0.8	45.2 ± 0.61	35.8 ± 0.68	47.3 ± 0.49	37.9 ± 0.67
1.0	23.3 ± 0.58	15.5 ± 0.67	24.6 ± 0.77	16.2 ± 0.55
2.0	9.20 ± 0.55	5.30 ± 0.37	10.0 ± 0.50	6.20 ± 0.57
Ivermectin	1.30 ± 0.39	1.00 ± 0.20	94.4 ± 0.97	94.4 ± 0.58
Neg. control	96.0 ± 0.34	96.8 ± 0.35	95.9 ± 0.65	96.1 ± 0.95

Data was based on percentage ± standard error of 3 independent experiments.

CONCLUSION

The methanol extract at the concentration of 2.0 mg/ml was moderately effective against the egg hatch of *C. elegans* Bristol N2 as well as *C. elegans* DA1316. The aqueous extract was ineffective against the egg hatch of *C. elegans* Bristol N2 as well as *C. elegans* DA1316 even at the highest concentration of 2.0 mg/ml. For larval development, both aqueous and methanol extracts of *G. senegalensis* were confirmed to be effective against the development of both *C. elegans* Bristol N2 as well as *C. elegans* DA1316 for inhibiting up to 90% larval development. Generally, methanol extract was more potent than the aqueous extract against both the egg hatch and larval development of *C. elegans.* Ivermectin was ineffective against the egg hatch and larval development of *C. elegans* DA1316. The effectiveness of the extracts against the larvae of ivermectin resistant strain of *C. elegans* is a clear indication that the extract from *G. senegalensis* maybe used against ivermectin resistant parasitic nematode such as *H. contortus*, *T. colubriformis* and *Ostertagia ostertagi* which belongs to the same Clade V with *C. elegans*. *Guiera senegalensis* may be used as a natural source of lead compounds to produce anthelmintic drugs.

Recommendation

The pure compounds in the extract should be identified isolated and further tested against the eggs and larvae *in vitro.* Cytotoxicity test should be conducted before the extracts are tested *in vivo* on infected ruminant animals.

Acknowledgements

The author is grateful to Universiti Sains Malaysia for their laboratorial and instrumental supports, and all laboratory technicians for technical aids in this research.

REFERENCES

Ademola, I. O. and Eloff, J. (2011). The anthelmintic activity of acetone extract and fractions of *Vernonia amygdalina* against *Haemonchus contortus* eggs and larvae. *Tropical Animal Health and production, 43*(2), 521-527.

Al-Rofaai, A., Rahman, W., Sulaiman, S., & Yahaya, Z. (2012). *In vitro* activity of neem (*Azadirachta indica*) and cassava (*Manihot esculenta)* on three pre-parasitic stages of susceptible and resistant strains of *Teladorsagia (Ostertagia)circumcincta.Veterinary Parasitology, 188*(1), 85-92.

Bowyer, M. C., Van Vuong, Q., Van Altena, I. A., & Scarlett, C. J. (2015). Phytochemicals and antioxidant capacity of Xao tam phan (*Paramignya trimera*) root as affected by various solvents and extraction methods. *Industrial Crops and Products, 67*, 192-200.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics, 77*(1), 71-94.

Debiage, R. R., Gonçalves, F. F., Pereira, A. R., da Silva, R. G., Yoshihara, E., & de Mello Peixoto, E. T. (2016). Anthelmintic potential of *Psidium guajava* in sheep. *Planta Medica, 81*(S 01), P997.

Ferreira, L., Castro, P., Chagas, A., França, S., & Beleboni, R. (2013). *In vitro* anthelmintic activity of aqueous leaf extract of *Annona muricata* L.(Annonaceae) against *Haemonchus contortus* from sheep. *Experimental parasitology, 134*(3), 327-332

Foo, L. W., Salleh, E., & Mamat, S. N. H. (2015). Extraction and qualitative analysis of *piper betle* leaves for antimicrobial Activities. *International Journal of Engineering Technology Science and Research 2,* 1-7.

Gaziano, T. A., Abrahams-Gessel, S., Denman, C. A., Montano, C. M., Khanam, M., Puoane, T., & Levitt, N. S. (2015). An assessment of community health workers' ability to screen for cardiovascular disease risk with a simple, non-invasive risk assessment instrument in Bangladesh, Guatemala, Mexico, and South Africa: an observational study. *The Lancet Global Health, 3*(9), e556-e563.

Kanojiya, D., Shanker, D., Sudan, V., Jaiswal, A. K., & Parashar, R. (2015). *In vitro* and in vivo efficacy of extracts of leaves of *Eucalyptus globulus* on ovine gastrointestinal nematodes. *Parasitology research, 114*(1), 141-148.

Katiki, L. M., Ferreira, J. F., Zajac, A. M., Masler, C., Lindsay, D. S., Chagas, A. C. S., & Amarante, A. F. (2011). *Caenorhabditis elegans* as a model to screen plant extracts and compounds as natural anthelmintics for veterinary use. *Veterinary Parasitology, 182*(2), 264-268.

Kumarasingha, R., Palombo, E., Bhave, M., Yeo, T., Lim, D., Tu, C., Boag, P. (2014). Enhancing a search for traditional medicinal plants with anthelmintic action by using wild type and stress reporter *Caenorhabditis elegans* strains as screening tools. *International Journal for Parasitology, 44*(5), 291-298.

Maobe, M. A., Gatebe, E., Gitu, L., & Rotich, H. (2013). Preliminary phytochemical screening of eight selected medicinal herbs used for the treatment of diabetes, malaria and pneumonia in Kisii region, southwest Kenya. *European journal of applied sciences, 5*(10), 01-06.

Molan, A.-L. (2014). Effect of purified condensed tannins from pine bark on larval motility, egg hatching and larval development of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Nematoda: Trichostrongylidae). *Folia Parasitologica, 61*(4), 371.

Ménez, C., Alberich, M., Kansoh, D., Blanchard, A., & Lespine, A. (2016). Acquired tolerance to ivermectin and moxidectin after drug selection pressure in the nematode *Caenorhabditis elegans. Antimicrobial agents and Chemotherapy, 60*(8), 4809-4819.

Metrouh-Amir, H., Duarte, C. M., & Maiza, F. (2015). Solvent effect on total phenolic contents, antioxidant, and antibacterial activities of *Matricaria pubescens*. *Industrial Crops and Products, 67*, 249-256.

Oliveira, L., Bevilaqua, C., Costa, C., Macedo, I., Barros, R., Rodrigues, A., Vieira, L. (2009). Anthelmintic activity of *Cocos nucifera* L. against sheep gastrointestinal nematodes. *Veterinary Parasitology, 159*(1), 55-59.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., & Kaur, H. (2011). Phytochemical screening and extraction: a review. *Internationale pharmaceutica sciencia, 1*(1), 98-106.

Wang, G.-X., Han, J., Zhao, L.-W., Jiang, D.-X., Liu, Y.-T., & Liu, X.-L. (2010). Anthelmintic activity of steroidal saponins from *Paris polyphylla*. *Phytomedicine, 17*(14), 1102-1105.

Williams, A. R., Fryganas, C., Ramsay, A., Mueller-Harvey, I., and Thamsborg, S. M. (2014). Direct anthelmintic effects of condensed tannins from diverse plant sources against *Ascaris suum*. *PLoS One, 9*(5), e97053.