# THE EFFICACY OF AQUEOUS AND METHANOL EXTRACTS OF *Detarium microcarpum* AGAINST MIGRATION OF *Ostertagia ostertagi* AND *Trichostrongylus colubriformis*

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## ABSTRACT

The use of synthetic drugs to control gastrointestinal parasite of ruminant animals has resulted to several complications which call for exploring the use of plant material as an alternative anthelmintics. This research compared the sensitivity of *O. Ostertagia* and *T. colubriformis*, against methanol and aqueous extracts of *D. Microcarpum* using larval migration assay. The L3 larvae of *O. Ostertagia* and *T. colubriformis* were treated with aqueous and methanol extracts of *D. microcarpum* with the serial concentrations of 0.2, 0.4, 0.6, 0.8 1.0 and 2.0 mg/ml for two hours and were later transferred to migratory plates. Some of the larvae were treated with 0.02 µg/ml of ivermectin which served as positive control, whereas those treated with only M9 buffer solution served as negative control. Observation and counting of the migrated larvae were carried out after 24 hrs. Good larvicidal activity against the migration of both species was demonstrated by both the aqueous and methanol extract of *D. microcarpum*. Methanol extract demonstrated higher anthelmintic potential than aqueous extract against both larval species ($P < 0.05$). There was no significant diffrerence in the sensitivity between the two larval species to both the aqueous and the methanol extract as *O. ostertagi* recorded the $IC^{50}$ values of 0.56 mg/ml and 0.351 mg/ml for aqueous and methanol extracts, whereas, *T. colubriformis* recorded0.59 mg/ml and 0.38 mg/ml aqueous and methanol extracts respectively. Even though ivermectin demonstrated a higher efficacy compared to the plant extracts ($P < 0.05$), *D. microcarpum* may serve as a source of lead compounds for the development of natural anthelmintic. There is need for isolation of active compounds in the extracts to be tested against the larvae, as well. There is need for the extracts to be tested *in vivo*.

**Keywords**: anthelmintics; concentrations; migration; larvae; extracts.

## INTRODUCTION

Intestinal helminthes parasites infections are serious Global challenges to live stocks industries (Mechineni *et al.*, 2014). This is more pronounce where grazing of animals on the pastureland is been practice extensively (Tembely*et al.*, 1994). The effect could leads to ailments such as diarrhoea, weight loss, reduction in milk production, loss of wool, depletion of mineral level, anaemia, loss of plasma

protein and mortality may result due to excesses of some of the effects mention above (André *et al.,* 2017; Mohammed*et al.,*2013). Economic lost may result from the cost for control of the parasites, reduction in the quality of carcass and wool, increase in the cost for better feeding aim at regaining the lost weight (Mohammed *et al.,* 2013).

*Ostertagia ostertagi* and *T. colubriformis* belongs to the family Trichostrongylidae which are the most prevalent parasitic nematodes that cause severe and acute gastroenteritis, anemia among other ailments in sheep and goats with considerable morbidity and increase in mortality rate in the developing countries (Faria *et al.,* 2016). The sensitivity of the parasites to drugs sometimes varied from one species has to another (Roeber *et al.,* 2013b; Mackie, 2016). The variation in the sensitivity of the nematodes parasites to drugs based on the species could be due to genetic diversity among the different species. Therefore, if the worms whose genotype renders them susceptible are eliminated, automatically the susceptible genes are not transferred to the offspring (Roger *et al.,* 2001).

Mixed infections often demonstrate greater impact on the host's wellbeing than mono specie infections. The severity of the infection depends on the composition of the species, number, and the burden of the nematode's parasites (Roeber*et al.,* 2013; Wimmer*et al.,* 2004). Rare cases of monospecie infection of gastrointestinal parasitic nematodes in the naturally infected host occur. However some species are found to be dominant in number than the others in the mixture, for instance in Australia, *Haemonchus contortus* formed 80 % of the mixture of 3 species of trichostrongylid nematode tested using larval migration assay against Macrocyclic lactones (Kotze, Le Jambre, & O'Grady, 2006).

Plants and plant products have been widely applied in the control of gastrointestinal parasites of ruminant animals to boost livestock production by local animal breeders (Abdu *et al.,* 2000). Medicinal plants are easily accessible, cheap, because of their abundance in nature and ease of application. Alternative anthelmintic from the plant is sustainable and environmentally friendly(McCorkle, 1995). They are comparatively less harmful with a minimal side effect on the animals and play important roles for future control of helminth infection in tropical and subtropical countries (Tariq, 2017). *Detarium microcarpum* is one of the herbal plants used in Africa for the treatment of several infections such as venereal diseases, dysentery, diarrhoea as well as dewormer (Akah *et al.,* 2012). It is a shrub which belongs to the family of Fabiacea and grows up to the height of 10 m tall under favourable climatic condition. It is widely distributed within the dry savannah region of Central and West Africa (Akah *et al.,* 2012). This research was aimed at comparing the sensitivity of *O. ostertagi* and *T. colubriformis* against the aqueous and methanol extracts of *D. Microcarpum* using larval migration assay.

## MATERIALS AND METHODS

### Collection and Extractions of Plant Materials

The collection of stem bark of *D. microcarpum* was done in the Sahel savannah region of Azare in Katagum

Local Government Area, of Bauchi State, Nigeria within the month of December 2016. The plant was authenticated in the Biological Science Department of Biological Sciences Bauchi State University Gadau where the voucher specimen with the registration **No. 900103** was preserved. The stem bark was crushed into semi powdered form after washing thoroughly with distilled water. The specimen was shade dried for 3 weeks before finally pulverized into powdered form. The phytochemicals extraction of the specimen was carried in the School of Biological Universiti Sains Malaysia. Maceration in water (aqueous) and methanol as solvents was adopted as the extraction methods according to Cesar *et al*. (2015) and Lienou *et al*. (2015).

**Aqueous Extraction**

Up to 50 g of a dry powdered sample of *D. microcarpum* was macerated in distilled water for five days at room temperature in 250 ml (1:5 w/v) of distilled water. Filtration of the liquid extract after maceration was performed with a Whatman filter paper No. 1. The filtrate was concentrated at 45$^{o}$C in an oven and the dry extract was obtained. The dry extract was preserved in a labeled sterile specimen bottle at the temperature of 4$^{o}$C before use (Cesar *et al.*, 2015; Lienou *et al.*, 2015).

**Methanol Extraction**

Exactly 50 g of the powdered stem bark of *D. microcarpum* was soaked in 250 ml of 80 % (1:5 w/v) methanol for 5 days at room temperature. The liquid extract was filtered through a Whatman filter paper No. 1. The dry extract was obtained after concentration of the liquid extract at 45$^{o}$C in an oven. The dried extract was placed in a labeled steriled specimen bottle and stored at 4$^{o}$C till further use (Cesar *et al.*, 2015; Lienou *et al.*, 2015).

**Phytochemical Test of Plant Extracts**

The preliminary phytochemical screening was carried out by mixing appropriate quantity of reagents and the extracts solution which reacted to produce certain colour changes that was used for the identification of secondary metabolites in the extracts of *D. microcarpum* according to the methods of Maoebe *et al.* (2013)and *Gaziano et al.(*2015) as follows:
Saponins (froth formation on shaking with water), Alkaloids (Dragendroff's reagents and 2ml of $H_2SO_4$), Flavonoids (2% ammonia solution + 2 % NaOH + 2% HCl), Phenols (using 2% $FeCl_3$), Tannins (2% $FeCl_3$), Terpenoids (Chloroform + $H_2SO_4$), Salkowski's test for steroids (acetic anhydrite + 2% + $H_2SO_4$).

**Analysis of Total Phenolic and Total Tannin content**

The total phenolic content of *D. microcarpum* extracts was determined according to the method of Orak. (2007). Gallic acid of serial concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3.3.5 mg/ml) was used as tandard. Each of the concentration was mixed with 100 µl of 25% (v/v) Folin-Ciocalteau reagent kept for 3-5 minutes. 80 µl of 1% solution of sodium carbonate ($Na_2CO_3$) was added to the mixture in 96 well plates and incubated for 1 hour in darkness. The absorbance values against the various concentrations of Gallic acids were used to draw the calibration curve from which the regression equation (y = ax + b) was obtained which was used to compute the phenolic content. The phenolic

content is expressed in mg Gallic Acid Equivalence (GAE/mg). Same procedure was applied for the determination of total tannins content but Folin- Denis reagent was used instead of Folin-Ciocalteau reagent. Also, tannic acid was used as the standard and the tannins content was expressed as Tannic Acid Equivalent (TAE/ mg).

**Collection of Faecal Sample and Coproculture of L3 larvae of *O. ostertagi* and *T. colubriformis***

Faecal samples were collected from sheep and goats naturally infested with mixed species of trichostrongylid nematode son a private farm located on Penang Island, Malaysia according to the guidelines of World Association for the Advancement of Veterinary Parasitological (WAAAVP) described by Coles *et al,* (1992). Middle and index fingers were directly inserted into the rectum of the animal and approximately 4 g of faecal sample was collected at a time. Harada *et al.*(1955) method was adapted for the Coproculture of the L3 larvae of the nematodes. Five grams of faecal pellets were crushed to form a mash and was incubated in a widely open-mouthed sterile specimen bottle. The sample was cover with Petri dish and the setup was incubated for 2 weeks at the temperature between 25-28$^{o}$C. The larvae were harvested after 14 days during which the incubated faecal mash was smeared on the upper part of a strip of filter paper. The lower unsmeared portion of the filter paper was dipped into the distilled water in the test tube. Water was soaked up the filter paper through capillary action and the larvae migrated on the wet surface of the filter paper and were collected in the water. Exactly 10 µl of 5 µg/ml of amphotericin B was added to the larval suspension which inhibited fungal growth in the suspension that preserved at 4$^{o}$C (Al-Rofaai *et al.*, 2012).

**Bioassay of Aqueous and Methanol Extracts *D. microcarpum* Against the Migration of the L3 Larvae**

Evaluation of the efficacy of the extracts was based on the WAAVP standard which considers anthelmintic agent as effective when the ovicidal or larvicidal efficacy is up to 90% and moderately effective when it is less than 90% but up to 80%. The stock solution of 2.0 mg/ml of an aqueous and methanolic extract of *D. microcarpum* was prepared by dissolving 200 mg of a dry sample of the extract type in 5 ml of 1% Tween 80. Exactly 95 ml of M9 buffer was added to the 5 ml of the dissolved extract and 200 mg/ml of the stock solution was obtained. Portions of the2.0 ml of were further diluted with various volumes of M9 solution to give serial concentrations of 0.2, 0.4, 0.6, and 1.0 mg/ml and 2.0 mg/ml as described by Kumarasingha *et al*. (2014). 1 mg of ivermectin was dissolved in 1 ml of 1% DMSO and subsequently diluted with M9 solution to obtained 0.02 µg/ml solution.

Larval migratory inhibition bioassay conducted as described by Almeida *et al.* (2013). Exactly 2 ml of 0.08% of sodium hypochlorite was mixed with 5 ml of the larval suspensions and kept for 4 minutes until the larvae were unsheathed. Distilled water was added to stop the action of sodium hypochlorite and the content was subjected to centrifugation at 1200 rpm for 2 minutes3x according to the method of Almeida *et al.* (2013). About100 unsheathed larvae in 0.3 ml of the

suspension were added to each of the 24 macro wells. One ml of each serial concentration of the required plant's extract (0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/ml) was added to each of the well in 3 replications. The 0.02 µg/ml ivermectin solution which served as positive control was added to the larvae in 3 wells. One ml of M9 solution was applied on the larvae in 3 of the wells and served as negative control. The setup was incubated at 27$^{o}$C for 2 hours.

The larvae in each well were transferred on to a migratory test sieve with the mesh of 20 µm placed across the mouth of Petri dish. Each migratory plate was half filled with M9 solution and 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 at 27$^{o}$C for 24 hours after which each test sieve was removed and the liquid content containing the migrated larvae was drained into the migratory plate. Non-migrated larvae which remained on the surface of the sieve were washed into another Petri dish. The migrated larvae in the migratory plates were killed by the adding 50 µml of Lugol's iodine solution. The experiments were repeated 3x and at the end of each experiment, the larvae were identified based on the keys of Van Wyk *et al.* (2013). The larvae were separated according to species. The non-migrated larvae were also identified after been killed with Lugol's iodine solution and separated according to species. Both the migrated and none migrated larvae were counted using a stereo inverted microscope at x 100 magnification. At the end of each experiment, the total number of each of the species of larvae used in the experiment was computed by adding the number of migrated larvae and non-migrated larvae of the same species. The percentage larval migration for each species was calculated at the end of each experiment according to the formula used by (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**

The percentage means and standard error of each species migration against the various extracts' concentrations were calculated using Microsoft® Excel version 2016 software. IBM SPSS® statistic version 24 was used to carry out the statistical analysis. The comparison between mean percentage migrations for each species at different concentration against control was performed using one - way ANOVA. The post hoc statistical significance used was least square difference (LSD). The difference between the means was considered significant at $P < 0.05$. The efficacy of aqueous and methanol extract was compared using paired sample T-test. The concentration required to inhibit 50 % ($IC_{50}$) migration for each nematode species was calculated using probit analysis.

## RESULTS

### Phytochemical Test of Plant Extracts

**Table 1:** Results of Phytochemical screening

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

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

More varieties of secondary metabolites were recorded in methanol extracts than aqueous extract of *D. microcarpum* during the phytochemical screening as shown in Table 1 above.

### Analysis of Total Phenolic and Total Tannin content

Higher quantities of phenolic and tannin compounds where detected in methanol extract than in aqueous extract ($P < 0.0\ 5$) as shown in Table 2.

**Table 2:** Results for Total Phenolic and Total Tannin content

| | **Total tannins** (TAE/mg) | **Total Phenolic** (GAE/mg) |
|---|---|---|
| **Aqueous Extracts** | 4.79 | 376.74 |
| **Methanol Extracts** | 6.23 | 484.91 |

### Efficacy of *D. microcarpum* Extracts Against the Migration of the Larvae

Both aqueous and methanol extracts of *D. microcarpum* were effective against the both species. The efficacy increased with increase in the concentration of the extracts. Up to 64.7% of *O. ostertagi* larvae incubated in 0.2 mg/ml aqueous extract migrated whereas 58.5% migration was recorded by the larvae incubated in the methanol extracts at 0.2 mg/ml (Table 3). At the highest concentration of 2.0 mg/ml the percentage migration of *O. ostertagi* decreased to 7.20% (92.80% inhibition) whereas 2.90% (97.1% inhibition) were recorded by the larvae incubated in the aqueous and methanol extracts respectively. For *T. colubriformis*, 65.5% (34.5% inhibition) migration was recording for the larvae treated in aqueous extract at 0.2 mg/ml whereas 58.9% (41.1% inhibition) migration was recorded for those treated in methanol extract at the same 0.2 mg/ml. At the highest concentration of 2.0mg/ml, only 8.10 % (90.9 % inhibition) of *T. colubriformis* treated in aqueous extract were able to migrate and 3.30 % (96.7 % inhibition) was recorded by the larvae treated in methanol extract. There was no significant difference in the response of *O. ostertagi* compared to those of *T. colubriformis* at $P > 0.05$. However significant difference was recorded between the efficacies of aqueous and methanol extract at $P < 0.05$ as methanol extract proved to be more potent than aqueous extract. Ivermectin (the commercial drug) was more potent than the plant extract. This is because the sensitivity of each of the worm species treated with ivermectin was above 95% (Table 3).

**Table 3:** Efficacy of *D. microcarpum* extracts against the migration of the four species of trichostrongylid nematodes L3 larvae

| Concentrations (mg/ml) | *O. ostertagi* | | *T. colubriformis* | |
|---|---|---|---|---|
| | Aqueous | Methanol | Aqueous | Methanol |
| **0.2** | 64.7 ± 0.74 | 58.5 ± 0.67 | 65.5 ± 0.59 | 58.9 ± 0.64 |
| **0.4** | 57.7 ± 0.75 | 46.8 ± 0.81 | 58.3 ± 0.52 | 47.7 ± 0.43 |
| **0.6** | 50.2 ± 0.77 | 38.7 ± 0.68 | 51.1 ± 0.87 | 40.3 ± 0.47 |
| **0.8** | 40.3 ± 0.64 | 32.4 ± 0.61 | 41.6 ± 0.46 | 33.1 ± 0.57 |
| **1.0** | 28.7 ± 0.70 | 18.6 ± 0.72 | 29.7 ± 0.68 | 20.6 ± 0.79 |
| **2.0** | 7.20 ± 0.44 | 2.90 ± 0.61 | 8.10 ± 0.57 | 3.30 ± 0.55 |
| **IVM** | 0.70 ± 0.23 | 0.70 ± 0.23 | 0.60 ± 0.19 | 0.80 ± 0.47 |
| **Negative control** | 95.2 ± 0.58 | 96.2 ± 0.58 | 65.5 ± 0.59 | 58.9 ± 0.64 |

A similar trend was observed in the inhibitory concentartion ($IC_{50}$) of *D. microcarpum.* The $IC_{50}$ of 0.56 mg/ml and 0.59 mg/ml were recorded against *O. ostertagi* and *T. colubriformis* and respectively treated in aqueous extract whereas those treated in methanol extract recorded the $IC_{50}$of 0.35mg/ml and 0.38mg/ml for *O. ostertagi* and *T. colubriformis* and respectively.

**Table 4:** The $IC_{50}$ for the efficacy of aqueous and methanol extracts of *D. microcarpum* against the migration of L3 larvae *O. ostertagi and T. colubriformis*

| Plant extract | Aqueous $IC_{50}$ (mg/ml) | Methanol $IC_{50}$ (mg/ml) |
|---|---|---|
| *O. ostertagi* | 0.56 | 0.35 |
| *T. colubriformis* | 0.59 | 0.38 |

## DISCUSSION

The diluents used in the preparation of the various extracts concentrations did not interfere with the anthelmintic activity of the extracts because the percentage larval migration remained high in the negative control during the assay and also there were differences in the percentage larval migration among the different concentrations of the extracts. Based on the WAAVP standard, aqueous extract of *D. microcarpum* was effective against the migration of *O. ostertagi* and *T. colubriformis.* Currently, there is scarce scientific report on the anthelmintic activity of *D. microcarpum* but previous studies comfirmed the seed coat of *D. microcarpum* as a broad spectrum antimicrobial (Ebi *et al.,* 2011). Ethnoveterinary application of stem bark of *D. microcarpum* for treatment of the gastrointestinal disorder as well as a dewormer in many African countries was also reported by Djoueche *et al.* (2011).

That appart, previous researches reported the anthelmintic potentials of aqueous and methanolic extracts of some plants against some members of trichostrongylid which belongs to the same family with *O. ostertagi* and *T. colubriformis.*For instance, Acharya *et al.* (2014) reported high inhibitory activity of *Lotus corniculatus* against the migration of L3 larvae of *H. contortus*. Extracts from Tillia flowers and willow bark were reported to inhibit the migration of L3 larvae of *Oesophagostomum spp* (Williams *et al.*, 2014). The sensitivity of *O. ostertagi* and *T. colubriformis* was statistically the same ($P > 0.05$). This is similar to findings

of Athanasiadou *et al*,(2001) who recorded similarities in the *in vitro* larvicidal activity of condense tannin extract from quebracho against *H. contortus, T. colubriformis* and *O. ostertagi*. The similarity in the sensitivity of these species to the extracts might result from sharing of common genetic mechanisms; as genetic diversity among different larval species could leads to variation in their sensitivity to anthelmintics (Prichard 2001).

Throughout the assay, the methanol extract of *D. microcarpum* was observed to be more efficient against all the larval migration of all the species than the aqueous extracts ($P < 0.05$). This is similar to the findings of Attindéhou *et al.* (2016) who reported that methanol extract of *Khaya senegalensis* exhibited the highest inhibition against larval migration of *H. contortus.* The high efficacy of methanol extract above aqueous extract could be as a result of more varieties of secondary metabolites as well as thehigher quantity of tannin and phenolic compounds in the methanol extracts than aqueous extract as revealed in the results of the preliminary phytochemical test and phytochemical analysis respectively. Ina related finding, Neffati *et al*. (2017)recorded a higher quantity of tannins compounds in the methanol extracts than the aqueous extract of several plants investigated in Tunisia and further suggested that the higher quantity of tannins and phenolics content was responsible for the antimicrobial activity of the methanol extract than aqueous extract.

The migration inhibitory activity of the extracts was dependent on concentration similar to the findings of Kanojiya *et al.*, (2015). Ivermectin was more efficient than the plant extracts in the inhibition of larval migration. This could be attributed to the fact that crude plant extracts contained mixtures of impurities that might have interferred with its potency whereas ivermectin contained pure compounds (Al-Rofaai *et al.*, 2012). In addition, plant extracts might exhibit a mechanism or mode of action against the larvae different from that of ivermectin as suggested by Kumarasingha *et al.* (2014).

## CONCLUSION

The extracts of *Detarium microcarpum* was effective against *O. ostertagi* and *T. colubriformis.*The methanol extract was more efficient than aqueous extract. There was no significant difference in the sensitivity of *O. ostertagi* and *T. colubriformis*to the extracts of *D. microcarpum.* Methanol extract contained a higher quantity of phenolics and tannins compounds than the aqueous extract. Isolation of pure compounds from the extract should be carried out before further *in vitro* test on the larvae. The efficacy of the extracts should be tested *in vivo* on the infected ruminant animls.

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