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Original article

COMPARATIVE SENSITIVITY OF HAEMONCHUS CONTORTUS AND OESOPHAGOSTOMUM VENULOSUM TO AQUEOUS AND METHANOL EXTRACT OF DETARIUM MICROCARPUM

[*1, 2]Gagman, H. A., [1]Hamdan, A. and [1]Nik Ahmad, I.I.N

[1]School of Biological Science, Universiti Sains Malaysia, 11800 Penang, Malaysia
[2]Department of Biological Sciences, Faculty of Science, Bauchi State University Gadau, 751 Itas Gadau, Nigeria

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ABSTRACT

Complications associated with the use of synthetic drugs draw the attention of researchers to explore plant as alternative anthelmintics. This research compared the sensitivity of *Haemonchus contortus* and *O. venulosum* to aqueous and methanol extracts of *D. Microcarpum* using larval migration assay. During the bioassay, all the species were treated in aqueous and methanol extracts of *D. Microcarpum* with the serial concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 mg/ml and later transferred to migratory plates. Larvae in the positive control were treated with 0.02 µg/ml of ivermectin whereas larvae treated with M9 solution served as a negative control. Larvicidal activities against the migration of all the species of the nematodes were demonstrated by both the aqueous and methanol extracts of *D. microcarpum*. However, methanol extract demonstrated higher efficacy than aqueous extract against each of the species tested ($P < 0.05$). *H. contortus* recorded the lowest IC_{50} (concentration at which 50% of the larvae are inhibited) values of 0.631 and 0.350 mg/ml for aqueous and methanol extract respectively and was the most sensitive species compared to *Oesophagostomum venulosum,* which recorded the IC_{50} of 0.74 and 0.52 for aqueous and methanol extracts respectively. Therefore, *D. microcarpum* may serve as a source of lead compounds for the development of natural anthelmintic.

Key words: anthelmintics, concentrations, concentrations, *D. microcarpum;* migration, larvae, bioassay

***Corresponding author**: degagman@gmail.com

INTRODUCTION

Gastrointestinal helminthes infections are serious challenges to livestock's industries worldwide [1]. The effect could be direct when it leads to ailments such as diarrhoea, weight loss, reduction in milk production, loss of wool and mortality may result due to excesses of some of the effects [2]. Indirect effects are associated with the economic aspect, which could result from the cost for control of the parasites, poor sales due to the low quality of carcass and wool as well as increase in the cost for better feeding aimed at regaining the lost weight [2].

Haemonchus contortus and *Oesophagostomum venulosum* belong to members of the family Trichostrongylidae. They are the most prevalent parasitic nematodes, which cause severe and acute gastroenteritis, anemia among other ailments in sheep and goats with considerable morbidity and increase in mortality rate in most of the third world countries [3]. Each of these parasites species has a variable degree of sensitivity and resistance to drugs [4]. The variation in the drugs sensitivity among the different species of nematodes parasites could be due to genetic diversity among the different species [5].

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 [6]. Medicinal plants are easily accessible and cheap, because of their abundance in nature and ease of application. Alternative anthelmintics from plants are sustainable and environmentally friendly [7]. They are comparatively less harmful with a minimal side effect on the animals and play important roles for future control of helminth infections in tropical and subtropical countries [8].

Detarium microcarpum is one of the herbal plants used in Africa for the treatment of several infections such as venereal diseases, dysentery, diarrhoea and also use as dewormer [9]. 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 [9]. This research was aimed at comparing the sensitivity of *H. contortus* and *O. venulosum* to aqueous and methanol extracts of *D. Microcarpum.*

MATERIALS AND METHODS

Collection and extractions of plant materials

The stem bark of *D. microcarpum* was collected in the Sahel savannah vegetation of Azare in Katagum Local Government Area, of Bauchi State, Nigeria. The plant was authenticated in the Department of Biological Science Bauchi State University Gadau, Nigeria and was given the voucher specimen No. 900103. The specimen was crushed into semi powdered form with a set of pistil and mortar before finally pulverized into powdered form after shed drying. The phytochemicals extraction of the specimen was carried out in the School of Biological Sciences Universiti Sains Malaysia.

Preparation of aqueous extracts

Total of 50 g of a dry powdered sample of *D. microcarpum* was macerated in 250 ml (1:5 w/v) of distilled water for five days at

28°C. Filtration of the infusion was carried out using Whatman filter paper No. 1. The filtrate was concentrated at 45°C in an oven and the dry extract was obtained. The dry extract was preserved in a labeled sterile specimen bottle at 4°C until further use [10]. The same procedure was applied for extraction using 80% methanol. The dry extract's percentage yields were calculated according to the formula used by [11] as follows:

$$EY = \frac{weight\ of\ dryextract}{weight\ of\ original\ sample} X\ 100$$

EY = Extract yield

Phytochemical screening of plant extracts

The phytochemical test of the plant extracts was carried out as described by [12] for detection of the various such as saponins, flavonoids, terpenoids, phenols, tannins and Steroids.

Phytochemical analysis

The determination of the total phenolic content of both aqueous and methanol extracts of *D. microcarpum* was carried out according to the methods described by [13]. Gallic acid of serial concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3.3.5 mg/ml) was used as a standard. Total of 20 µl of each of the concentration and the extract were mixed with 100 µl of 25% (v/v) Folin-Ciocalteau reagent and allowed to stay for 3-5 minutes. This was followed by the addition of 80 µl of 1 % solution of sodium carbonate (Na_2CO_3) in 96 well plates and incubated in the darkness for 1 hour. The absorbance values against the various concentrations of Gallic acids were used to draw the calibration curve from which the regression equation ($y = ax + b$) which was used to calculate the phenolic content of each extract (aqueous and methanol). The phenolic content is expressed in mg Gallic Acid Equivalence (GAE/mg). Determination of total tannins content was carried out using the same procedure. However, 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

The faecal samples were collected from sheep and goats naturally infected with mixed species of trichostrongylid nematodes on a private farm located on Penang Island, Malaysia. The guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAAVP) described by [14] were adopted for the collection of the faecal sample. Approximately 4 g of faecal sample was collected directly from the animal's rectum.

Coproculture of L3 larvae of trichostrongylid nematodes

The method of [15] was adapted for the Coproculture of the L3 larvae of trichostrongylid nematodes. 5 g of faecal pellets was incubated in a specimen bottle cover with Petri dish at 25-28°C for 2 weeks. Harvesting of the larvae was carried out by smearing the incubated faecal mash on the upper part of 8 cm x 12 cm strip of filter paper. The lower portion of the filter paper free of faecal matter was dipped into the distilled water inside a test tube. Water move up the filter paper through capillary action and the larvae migrated on the wet surface of the filter

paper into the water and were collected after 24 hours. Approximately 10 µl of 5 µg/ml of amphotericin B was added to the larval suspension, which inhibited fungal growth in the suspension. The larval suspension was preserved at 4°C before use [16].

Bioassay of aqueous and methanol extracts *D. microcarpum* against the migration of the *H. contortus* and *O. venulosum* of trichostrongylid nematodes L3 larvae

Evaluation of the efficacy of the extracts was based on the WAAVP standard, which considers anthelmintics 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 methanol extract of *D. microcarpum* was prepared by dissolving 200 mg of a dry sample of each 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 to obtained 200 mg/ml of the stock solution. The stock solution was further diluted to give serial concentrations of 0.2, 0.4, 0.6, and 1.0 mg/ml and 2.0 mg/ml according to Kumarasingha *et al.* (2014). Total of 1 mg of the solid sample of ivermectin was dissolved in 1 ml of 1% DMSO and subsequently diluted with M9 solution to obtained 0.02 µg/ml solution.

The procedure for larval migratory inhibition bioassay was carried out according to the method described by [17]. Total of 2 ml of 0.08% of sodium hypochlorite was added to approximately 5 ml of the larval suspensions and allowed to stand for 4 minutes until the larvae were unsheathed. The action of the hypochlorite was stopped by the addition of distilled water and the content was centrifuged at 1200 rpm for 2 minutes three times. Approximately 100 exsheathed larvae in 0.3 ml of the suspension were dispensed into each well of the 24 macro well plate. Total of 1 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 a positive control was added to the larvae in 3 wells. Three of the wells containing larvae treated with only M9 solution served as negative control. The setup was incubated at 27°C for 2 hours.

The larvae in each well were transferred on to a migratory test sievewith 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 [17]. The setup was incubated at 27°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. 3 independent experiments were performed. At the end of each experiment, the larvae were identified using the identification key of [18]. The identified larvae were separated according to species. The non-migrated larvae were also identified and separated according to species. Both the migrated and none migrated larvae were counted using a stereo inverted microscope Model SM – 6 at x 100 magnification. At the end

of each experiment, the total number of each larval species used in the experiment was obtained by adding the number of migrated larvae and non-migrated larvae of the same species as follows: A + B

Where A= number migrated larvae

B= none migrated larvae [17].

Percentage migration for each species at the end of each experiment was calculated according to the formula adapted from [17] as follows:

$$LMI\% = \frac{A}{A+B} \times 100$$

Where A = number migrated larvae

A + B = total number of larvae that migrated.

Data 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

Extract yields

The percentage yields of the dry extracts were 9.90% for aqueous extract whereas the 10.80% yield was recorded for methanol extract.

Phytochemical components of plant extracts

The secondary metabolites revealed during the preliminary phytochemical screening of the aqueous extract of *D. microcarpum* were saponins, flavonoid tannin, terpenoids and phenols. Terpenoids, anthocyanins, anthraquinones, saponins, tannins, flavonoids and phenols were confirmed in methanol extract.

Phytochemical analysis

Phytochemical analysis of the extracts showed higher quantities of phenolics and tannins compounds in methanol extract than in aqueous extract ($P < 0.0 5$). The aqueous extract of *D. microcarpum* has the total phenolic content of 376.74 GAE/mg whereas the methanol extract recorded the total phenolic content of 484.91 GAE/mg. Similarly, the total tannins content of 4.79 TAE/mg was computed in the aqueous extract of *D. microcarpum* compared to 6.23 TAE/mg for methanol extract.

Efficacy of *D. microcarpum* extracts against the migration of *H. contortus* and *O. venulosum*.

The methanol extracts of *D. microcarpum* were effective against both *H. contortus* and *O. venulosum*. However, the aqueous extract was moderately effective against *O. venulosum*. The efficacy of the extracts increased with increase in the concentration. At the highest concentration of 2.0 mg/ml, only 6.40% (93.6% inhibited) and 1.70% (98.3% inhibited) of *H. contortus* treated in aqueous and methanol extracts

respectively migrated through the mesh. Based on the percentage larval migration, *H. contortus* was considered more sensitive than *O. venulosum* at 2.0 mg/ml as up to 15.2% and 9.70% migration was recorded by *O. venulosum* treated in aqueous and methanol extract respectively (Table 1). Both *H. contortus* and *O. venulosum* were more sensitive to 0.02 μg/ml of ivermectin compared to the plant extracts ($P < 0.05$). This is because the migration of each of the worm species treated in ivermectin was above 95% (Table 1).

Similar trend was observed in the inhibitory concentartion (IC_{50}) of *D. microcarpum* against the two species. *Haemonchus contortus* was the most sensitive species as it recorded the lowest IC_{50} values of 0.350 mg/ml and 0.537 mg/ml for methanol and aqueous extracts respectively compared to the IC_{50} values of 0.744 and 0.523 mg/ml for aqueous and methanol extracts respectively recorded by *O. venulosum.* Significant difference was recorded in the sensitivity between *H. contortus* and *O. venulosum* at $P < 0.05$.

Table 1: Efficacy of *D. microcarpum* extracts against the migration *Haemonchus contortus* and *Oesophagostomum venulosum*

Conc.	*H. contortus*		*O. venulosum*	
(mg/ml)	Aqueous	Methanol	Aqueous	Methanol
0.2	63.9 ± 0.58	57.4 ± 0.59	70.2 ± 0.58	65.2 ± 0.61
0.4	56.7 ± 0.69	45.7 ± 0.68	64.2 ± 0.61	55.2 ± 0.58
0.6	47.8 ± 0.74	38.0 ± 0.37	57.2 ± 0.58	49.3 ± 0.55
0.8	39.6 ± 0.88	31.8 ± 0.75	47.2 ± 0.60	40.2 ± 0.52
1.0	28.4 ± 0.61	19.5 ± 0.64	33.2 ± 0.55	22.2 ± 0.58
2.0	6.40 ± 0.67	1.70 ± 0.42	15.2 ± 0.58	9.70 ± 0.32
IVM	1.00 ± 0.38	0.70 ± 0.23	0.70 ± 0.23	0.80 ± 0.47
Neg. con.	96.0 ± 0.38	95.4 ± 0.68	95.2 ± 0.49	96.3 ± 0.67

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

Table 2: IC_{50} for the efficacy of *D. microcarpum* extracts against the migration of *H. contortus* and *O. venulosum*

Parasite	Aqueous extract IC_{50} mg//ml	Methanol extract IC_{50} mg//ml
H. contortus	0.63	0.35
O. venulosum	0.74	0.52

DISCUSSION

This research was aimed at comparing the sensitivity of *H. contortus* and *O. venulosum* against the methanol and aqueous extracts of *D. Microcarpum* using larval migration bioassay. The diluents used in the preparation of the various extracts contractions did not interfere

with the anthelmintic activity of the extracts. This may be due to the high percentage larval migration in the negative control during the bioassay coupled with the difference in the percentage larval migration among the different concentrations of the extracts.

Based on the WAAVP standard, aqueous extract of *D. microcarpum* was therefore effective against the migration of *H. contortus.* However, only methanol extract was effective against the migration of *O. venulosum* whereas aqueous extract was moderately effective against *O. venulosum*. Currently, there is scarce scientific record on the anthelmintic activity of *D. microcarpum* but it has been observed previously that the seed coat of *D. microcarpum* has a broad spectrum antimicrobial [19]. Stem bark of *D. microcarpum* has been used for treatment of the gastrointestinal disorder as well as a dewormer in many African countries as reported by [20].

Previous studies reported the anthelmintic potentials of aqueous and methanolic extracts of some plants against *H. contortus* and *Oesophagostomum* spp. For instance, [21] 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 [22].

H. contortus was more sensitive to both aqueous and methanol extracts compared to *O. venulosum.* The variation in the drugs sensitivity among the different species of nematodes parasites could be due to genetic diversity among the different species [5]. Furthermore, if the worms whose genotype renders them susceptible are eliminated, automatically the susceptible genes are not transferred to the offspring [23]. In a similar development, [24] recorded a significant difference in the *in vitro* larvicidal activity of condense tannin extract from quebracho against *H. contortus* and *Trichostrongylus colubriformis.*

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 [25] 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 the higher 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. In a similar investigation, [26] observed crude methanol extracts from stembark of *Acacia nilotica* to be more potent than the extracts from the leaves against the motility of adult *H. contortus*. They suggested that the variation in the anthelmintic activity of the extract could be attributed to the disparity in the types/quantity of active principle/compounds in the extract from bark compared to leaves. In related findings, [27] 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 [28]. 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 [29]. In addition, plant extracts might exhibit a mechanism or mode of action against the larvae different from that of ivermectin as suggested by [16].

CONCLUSION

The extracts of *Detarium microcarpum* was effective against *H. contortus* and *O. venulosum*. The methanol extract was more efficient than aqueous extract. *Haemonchus contortus* was more sensitive than *O. venulosum*. 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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Conflict of interest

Authors declare that there were no any conflicts of interest in doing this research.

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