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In - Vitro And *In - Vivo* Evaluation Of Anthelmintic Activity Of Methanolic And Aqueous *Moringa pterygosperma* Leaf Extracts On *Haemonchus Contortus*.

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ABSTRACT

Haemonchus contortus (*H. contortus*) is one of important helminths that causes significant losses in the small ruminant industry in many countries. Good management practice to control the infestation of the worm must be attempted to break its life cycle through anthelmintics, animal management and/or pasture management. Anthelmintic resistance resulted from the extensive use of the drugs is an important emerging threat in small ruminant industry. The development of anthelmintic resistance against chemical anthelmintic drugs has resulted in an increased level of *H. contortus* infestation in small ruminants in Malaysia. Previous studies have reported *Moringa pterygosperma* (*M. pterygosperm*) leaf extract possess antifungal, antibacterial, anti-inflammatory and antioxidant activities. Its anthelmintic activity is evaluated in this study. Four different concentrations (1.25, 2.5, 5 and 10 mg/mL) of *M. pterygosperma* leaf extracts methanol (MPME) and water (MPAE) were tested for their effects on larval motility. For adult worm motility test, 25 mg/mL of each extract was tested to evaluate adult worm paralysis. For both tests, levamisole (1.5 mg/mL) was used as a positive control. Further *in vivo* evaluation on the effectiveness of the extracts as anthelmintic was performed on Boer does that were experimentally infected with 9000 L3 *H. contortus*. A total of 25 Boer does were divided equally into 5 groups consisted of control (A), negative control (B), positive control-treated with Ivermectin® (C), MPME-treated group (D) and MPAE-treated group (E). Fifteen weeks prior to the experiment, groups D and E were fed with fresh *M. pterygosperma* leaves daily. Results revealed both MPME and MPAE induced L3 motility within 24 hours and paralysis of adult *H. contortus* within 6 hours after treated with the extracts. Mortality of the L3 and motility of the adult worms increased with increase in the concentrations and with the times of exposure. The percentage (mean ± SE) of dead L3 *H. contortus* in every 2 hours for 24 hours were 38.16 ± 8.26 and 22.25 ± 5.51 at concentrations of 10 mg/mL and 5 mg/mL of MPME, respectively and 28.83 ± 6.964 MPAE at concentration of 10 mg/mL. The mortality index (MI) of the highest concentration of MPME was almost 100% effective as levamisole. For adult motility test, at 25 mg/mL, both MPME and MPAE 100% reduced adult *H. contortus* motility. In *in vivo* study, both MPME and MPAE were effective as anthelmintic against *H. contortus* infection in Boer does. Supplementation of fresh *M. pterygosperma* leaves prior to L3 *H. contortus* infection significantly reduced FEC compared to negative and positive control groups.

Keywords: *M. pterygosperma, in vitro* anthelmintic activity, infective larvae, adult *Haemonchus contortus.*

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INTRODUCTION

Conventional chemical anthelmintic agents can be used to control *H. contortus*, but development of anthelmintic resistance by *H. contortus* populations may occur [1, 2]. Hence, the need for alternative anthelminthic drugs for curbing this emerging health hazard is imperative. Recently, researchers have discovered natural anthelmintic substance from local and available medicinal plants, which have been used in ancient times to treat diseases of man and animals [3,4]. These plants are generally consumed as food, but have been reported to possess ethnoveterinary medicinal (EVM) value as well [5, 6, 7]. Many researchers are also focusing on secondary plant metabolites with promising anthelmintic activity [8, 9]. The efficacy of some plants such as *M. pterygosperma* against some parasites will pave way for cheaper means to control helminthic diseases compared to chemical therapy [10, 11, 12, 13, 14].

The leaf of *M. pterygosperma* is considered a miracle tree in most countries of the world [15, 16, 17]. The leaf is rich in ascorbic acid, flavonoids, phenolic and carotenoids [12, 18, 19, 20]. In the Philippines, it is known as 'mother's best friend' because of its utilization to increase woman's milk yield and is sometimes prescribed for anaemia [19, 20, 21, 22]. *M. pterygosperma* leaf is high in protein, lipid, amino acid, iron and sulphur in addition to calcium, potassium, waxes, alkaloids, kaempferol and flavonoids [17, 23, 24].

Therapeutic uses of leaves of *M. pterygosperma* include anti-inflammatory, anodyne, immunomodulation and enhancing immunity, improve growth and [25, 26, 27, 28, 29, 30, 31]. In humans, it has also been used as anthelmintics in India, Malaysia and Nigeria. In addition, *M. pterygosperma* is also used to treat scurvy, wounds and tumours [15, 30, 31, 32].

Synthetic anthelmintics residues in meat could pose potential public health hazards [33]. However, there are limited studies and paucity of data on the effect of *M. pterygosperma* leaves as anthelmintic regimen. Screening and proper *in vitro* and *in vivo* evaluation of ethnoveterinary medicinal plants could offer alternatives that may both be sustainable, environmentally friendly and socially acceptable. Hence, the aim of this study is to determine the anthelmintic activity of *M. pterygosperma* leaves and its extracts against *H. contortus* parasite. The specific objectives of the study are: (i) To evaluate *in vitro* anthelmintic activity of *M. pterygosperma* leaves and its extracts on adults *H. contortus* and its larval. (ii) To evaluate and compare the methanolic extract and aqueous extract (MPME and MPAE) in Boer does as an alternative treatment against third stage larvae (L3) and adult stage *H. contortus*.

MATERIALS AND METHODS

Identification and preparation of *M. pterygosperma* leaves

Leaves of *M. pterygosperma* were purchased from the Unit Biodiversity, Institute of Bioscience, UPM. The purchased leaves were identified and authenticated by a botanist in the Laboratory of Botany, Institute of Bioscience, Universiti Putra Malaysia. The leaves were then dried in shade for four days. The dried leaves were ground into fine powder using an electric blender. The leaf powder was packaged in an airtight cellophane bag and stored at 4°C before extraction.

Preparations of methanolic extract (MPME)

Moringa pterygosperma powder (100 g) was weighed into a glass beaker containing 1000 mL of methanol. The mixture was sonicated in a water bath for 30 min and then filtered using Whatman No 1 filter paper. This process was repeated three times. The entire solution was evaporated in a vacuum rotary evaporator (under reduced pressure) at 40°C to obtain crude *M. pterygosperma* extract (24.9 gm). The MPME was packaged in an airtight container and stored at 4 °C until further use.

Preparation of aqueous extracts (MPAE)

Moringa pterygosperma powder (100 g) was added to 1000 mL of distilled water in a glass container. The mixture was allowed to dissolve for 24 h at room temperature (21–23°C), after which it was sonicated for 30 min and filtered using Whatman No 1 filter paper. The process of percolation was repeated three times,

followed by evaporation at 40°C in a vacuum rotary evaporator to obtain crude *M. pterygosperma* aqueous extract (MPAE). Final aqueous extract was packaged in an airtight vial and stored in a refrigerator (4°C) until further use.

Preparation of infective larvae from *H. contortus* eggs

Goats' abomasum were obtained after necropsy of animals in a private small ruminant farm in Selangor state, for recovering of adult female *H. contortus*. Adult female *H. contortus* worms were crushed to liberate eggs. The eggs were then cultured *in vitro* at room temperature. At the end of seven days of the egg culture, infective larvae L3 *H. contortus* were harvested.

Preparation of donor does infected with *Haemonchus contortus*

A Boer doe was initially infected with 3000 L3 of *H. contortus*. Another nematode free Boer doe was inoculated with 9000 of L3 [34]. Both does were kept in a separate pen at the Small Ruminant Experimental House, Malaysian Agricultural Research and Development Institute (MARDI). Both does served as *H. contortus* eggs donors [35].

Preparation of infective larvae of *H. contortus* from the donor does

Faecal samples were manually collected from the donor goats and processed in the Parasitology Laboratory, Faculty of Veterinary Medicine, UPM. The samples were crushed and then placed in a glass container, moisturized by a few drops of distilled water for seven days as described by [36]. After that, L3 was harvested by filling a glass container with warm distilled water and then inverting the container on a petri dish. L3 harvested after more than 30 min were observed under a Stereo Microscope. The L3 collected in glass container were kept in a refrigerator.

***In vitro* evaluation of anthelmintic activity of *M. pterygosperma* extracts against *H. contortus* L3and adult**
***In vitro* L3 mortality test**

Approximately 100 active L3 were collected in to each petri dish containing distilled water and 1.5% dimethyl sulfoxide (DMSO) as negative controls. For positive control, 1.5 mg/mL of levamisole (Nilverm Orench, India) was used in another petri dish containing 100 active L3. *M. pterygosperma* extracts (MPAE and MPME) were diluted with distilled water and 1.5% DMSO, respectively, in quadruplicate, each containing 100 active L3 and 1.25, 2.5, 5 and 10 mg/mL of the diluted extract. The petri dishes were shaken for 2 min and incubated at room temperature, and checked for L3 motility every 2 h for 24 h. The test was replicated four times for each treatment. In each treatment, the number of motile or live L3 was checked and recorded and the non-motile or dead L3 was also recorded. After 24 hours, L3 that remained alive in both plant extracts were counted. The formula for mortality index (MI) of L3 is shown below:

$$\text{Mortality Index (\%)} = \frac{\text{number of dead larvae}}{\text{total number of L3 in a petri dish}} \times 100$$

***In-vitro* assay for adult worm motility test**

Motility assay was performed on live adult mature *H. contortus* as described by [37]. Mature adult *H. contortus*, obtained from freshly slaughtered goats in a private small ruminant farm in Selangor state, were washed in phosphate buffered saline (PBS). Ten worms were exposed to the treatment as tabulated in Table 1 in separate petri dishes at room temperature in triplicate.

Experimental design for *in vivo* anthelmintic activity of the extracts on *H. contortus* in does

Fifteen adult Boer does, 2 to 2.5 years of age, weighing 30 to 36 kg (32.69 ± 0.4 kg) with no history of gastrointestinal parasite infestation was used in this experiment. Prior to the onset of the experiment, all goats were screened for gastrointestinal parasite eggs using McMaster modified technique. The animals were confirmed to be free of infection of any species of gastrointestinal nematodes, by faecal examination, using McMaster procedure every week for 2 months. All goats were kept in the Small Ruminant Experimental House

at the Research Unit, MARDI, Malaysia and fed with fresh cut grass and commercial goat pellet. Before starting of the experiment, the goats were administered with single dose of 1% Ivermectin® 1mL per 50 kg body weight subcutaneously. Tap water was provided for drinking and the experiment was conducted in an intensive farm condition.

The goats were divided randomly into five groups (A, B, C, D and E) (Table 2) where three goats were allocated in each group and kept in a separate pen. Group A was a control group. Groups B, C, D and E were administered with 9000 L3. At week 3 post L3 infection, group C was treated with ivermectin, while groups D and E were treated with MPME and MPAE extracts orally for 6 days, group B served as a negative control group. Faecal samples of each group were collected from rectum in the morning every week for 6 weeks. They were evaluated for the presence of eggs, counted using the modified McMaster method [38, 39].

Statistical analysis

Data obtained were analysed using JMP statistical software (version 9.0.1 SAS Institute Inc., Cary, NC, USA). All values obtained were reported as mean ± standard error. Significant differences were assessed by comparing mean with control using Dunnett's test and considered significant at $p<0.0001$. Through log–probit analyses, LC_{50} and LC_{90} values were done by computer software Excel 2013, regression equation (Y= mortality, X = concentration).

RESULTS AND DISCUSSION

***In vitro* evaluation on anthelmintic activity of *M. pterygosperma* extracts**

***In vitro* mortality test for L3**

Results obtained showed that MPME at 10 mg/mL and 5 mg/mL, MPAE at 10 mg/mL showed significant ($p<0.0001$) nematocidal effect against L3 (Table 3). The nematocidal effect of both extracts concentration and MPME at 10 mg/mL showed the most comparable nematocidal effect to levamisole (positive control). The LC_{50} was 1.5 mg /ml for MPME and 2.92 mg/ml for MPAE, while LC_{90} was 10.02 mg/ml for MPME and 14.42 mg/ml for MPAE. The mortality effect of MPME and MPAE on L3 increased with increasing exposure times and doses (Figure 1). This indicates that the tested extracts are moderately effective [40].

The anthelmintic effect of MPME and MPAE on L3 mortality was increased in a time and dose-dependent manner, as the number of larvae mortality increased with increasing dose and time. This is mainly related to increase in the concentration of anthelmintic compounds in the extract, as the concentration increased from 1.25 to 10 mg/mL. There was no significant difference ($P < 0.0001$) between the effects of levamisole at 1.5 mg/mL and MPME (5 and 10 mg/mL) and MPAE (10 mg/mL). Levamisole killed worms by depolarizing nicotinic acetylcholine receptors in muscular junction and cause paralysis and death of worms [41]. MPME and MPAE might exhibit similar action like levamisole, which might bedue to precences of saponin, flavonoids and alkaloids in *M. pterygosperma* leaf [28].

Alcoholic extracts of plants contain some non-polar organic chemicals with lower polarity than the aqueous extracts [32; 42], rendering compound more lipid soluble than the aqueous extracts and hence better anthelmintic activity [42]. Lipophilic anthelmintics have a greater capability to cross the external surface of the helminths than the hydrophilic compounds (10, 23, 43, 44, 45]. According to [46], methanol extract of *Artemisia brevifolia* at the concentration of 25 mg/mL exhibited significant *in vitro* anthelmintic activity on adult *H. contortus*. The finding is in agreement with the finding in this study.

In vitro* motility test for adult *H. contortus

The MPME and MPAE exhibited anthelmintic potency by inhibiting motility of adult *H. contortus*. There were three replicates were performed for each treatment, after 6 h, all 10 adults worms tested if revived, when placing in PBS for 30 min. Meanwhile, all worms exposed to levamisole died at 6 hours of exposure. Thus, adult stage *H. contortus* was proved to be a good *in vitro* test for the evaluation of anthelmintic activity of herbal extracts.

The findings in this study showed anthelmintic activity of MPME and MPAE against *H. contortus* was similar to levamisole (Table 4 and Figure 2). Exposure of adult worms to MPME and MPAE at 25 mg/mL for 6 houtrs significantly reduced the worm motility compared to negative control.

In vitro test is very important to evaluate the inhibition and/or mortality of and adult worm at the concentration of 25 mg/mL [47, 48, 49, 50, 51, 52] and widely used in veterinary parasitology researches to develop novel anthelmintic agents in place of chemical anthelmintic which are expensive, besides, development of resistance by the parasite against these drugs [53, 54].

In vivo* evaluation on anthelmintic activity of *M. pterygosperma* extracts against adult *H. contortus

Faecal egg count

Faecal samples of 15 Boer does (inoculated with 9000 of L3) were collected every week starting from week 0. At week 3 post infection prior to anthelmintic chemical, MPMA and MPAE treatment, FEC were calculated to be more than 2500/2 g.

Effect on faecal egg counts

Table 5 shows FEC results throughout the experiment. The *H. contortus* eggs was started to observed at week 2 post L3 infection in all group (except control group). The FEC for the negative group control group was increased from 20 epg at week 2 to above 1000 eggs from weeks 3 to 6. At week 3 post exposure, MPME and MPAE groups caused significant ($p<0.0001$) lower FEC compared to positive and negative control groups, and the values was only slightly above 500 epg. The FEC for MPME group was below 100 epg starting from week 4 until the end of the experimental period. While the FEC of MPAE group was between 150 to 350 epg starting from week 4 until the end of the experimental. For positive control group, the EPG was started to observe below 500 epg starting from week 5. The results indicate that both MPME and MPAE exhibit effective anthelmintic activity against *H. contortus*.

In the present study, *H. contortus* eggs in faecal samples of infected Boer does was first detected after 2^{nd} weeks of infection with the EPG value ranging from 15-70, but all the infected goats were highly positive on week 3. Only infected goats in group B recorded significant persistent EPG levels until experiment end. Similar result of activity of herbs as anthelmintics got by [48], but Synthetic anthelmintics residues in meat could pose potential public health hazards [33]. However [55] reported that infected sheep with 5000 L3 showed parasitic eggs in their faeces as early as 9 day post infection, and on 2 week was 701±74.

Effect of *Moringa pterygosperma* extracts on body weight of does experimentally infected with L3 *Haemonchus contortus*

Table 6 shows body weight of all does in all groups. Does treated orally with either MPME or MPAE at week 3 post L3 infection for 6 days showed significant ($p<0.0001$) increase in body weight throughout the last 3 weeks of the experimental period.

Significant increases in body weight were observed in groups D and E. This observation is in agreement with a previous study conducted by [55] who reported that infected goats showed weight gain after 10 weeks of treatment with Fumatiaceae plant extract. It can be concluded that *M. pterygosperma* extracts, besides deworming can increase animal's body weight.

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Table 1: Experimental design for exposure of adult *H. contortus* to MPME and MPAE

Group	Treatment composition and concentration
Positive control group	Levamisole (0.55 mg/mL)
Treatment groups	MPME (25 mg/mL) MPAE (25 mg/mL)
Negative control groups	Distilled water DMSO (1.5%)

The inhibition of motility and mortality of the worms due to anthelmintic activity were recorded. The motility was recorded at 0, 1, 2, 3, 5 and 6 hrs. After 6 hrs of exposure, the worms were kept for 30 min in lukewarm fresh PBS to observe the revival of motility.

Table 3: *In vitro* Anthelmintic potency of MPME and MPAE at various concentrations against *H. contortus* L3 for 24h

Concentration (mg/mL)	Mean of dead L3 ± SE	Mortility Index (MI)
MPME 10	**38.16±8.26**	**89**
MPME 5	**22.25±5.51**	**60**
MPME 2.5	20.33±5.51*	58
MPME 1.25	16.83±4.07*	45
MPAE 10	**28.83±6.96**	**72**
MPAE 5	14.41±3.69*	43
MPAE 2.5	12.16±3.69	41
MPAE 1.25	11.40±2.70*	30
Levamisole 1.5	42.83±9.04	100
Distilled water	0.00±0.00*	0
DMSO 1.5%	0.00±0.00*	0

*** Significantly different at P< 0.0001 to levamisole 1.5. ** Denotes mean of dead L3 every 2 hrs in 24 hrs (mean± SE). Bold values denote the results are not significantly difference from levamisole. MPME and MPAE (Methanolic and Aqueous *Moringa pterygosperma* Leaf Extracts).**

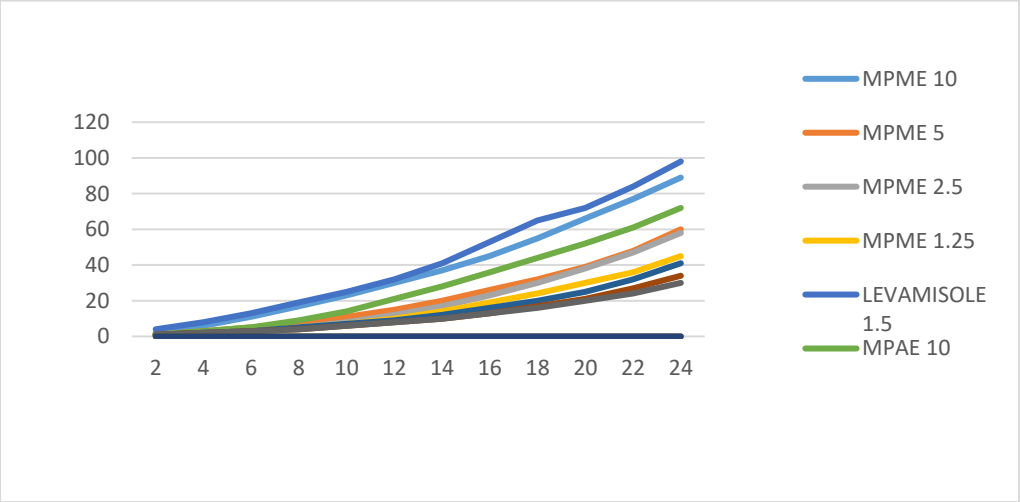

Figure 1 : Larval motility (%) of different concentrations of Methanolic (MPME) and Aqueous (MPAE) *Moringa pterygosperma* Leaf Extracts show larvacidal activity within 24 h compare with levamisole.

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MPME 10 mg/mL, MPME 5 mg/mL, MPME 2.5 mg/mL, MPME 1.25 mg/mL, Positive control= levamisole 1.5 mg/mL, MPAE 10 mg/mL, MPAE 5 mg/mL MPAE 2.5 mg/mL, MPAE 1.25 mg/mL, Negative control 1= distilled water, Negative control 2= 1.5% DMSO.

Table 4: Anthelmintic potency of MPME and MPAE on *H. contortus* motility

Group	Mean ± SE**	Motility index
Negative control 1	10.0 ± 00.0 *	0
Negative control 2	10.0 ± 00.0 *	0
Levamisole (0.5 mg/mL)	3.83 ± 1.49	100 ± 1.94
MPME (25 mg/mL)	4.66 ± 1.45	100 ± 1.45
MPAE (25 mg/mL)	4.00 ± 1.43	100 ± 1.43

*** Denotes the values are significantly different at P< 0.0001 from levamisole.**

**** Denotes the mean of immotile adult worms (*Haemonchus contortus*) for every 2 hours in 6 hours**.

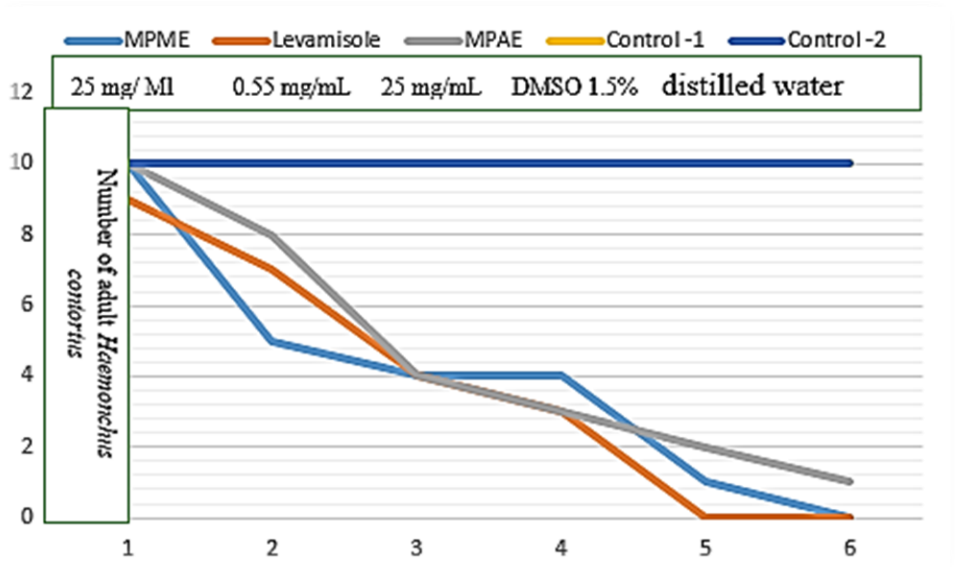

Figure 2 : *In vitro* assay for the evaluation of anthelmintic potency of MPME and MPAE on adult worm motility adult *H. contortus* in all groups within 6 hours.

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Table 2: Experimental design for the *in vivo* anthelmintic activity evaluation of MPME and MPAE on *H. contortus* in Boer does

Groups		WK 0	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6
A	Control	0.00±00.00	0.00±0.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00
B	Control -	00.00±00.00	00.00±00.00	20.0±15.2	1250.0±292.9	1483.3±130.2	1416.7±187.8	1300.0±76.40
C	Ivermectin+	00.00±00.00	00.00±00.00	33.3±16.67	1533.3±317.9	600.00±150.00*	266.67±72.56*	166.67±44.10*
D	MPME	00.00±00.00	00.00±00.00	16.67±1.67	1016.67±196.50	516.67±120.19*	200.00±28.87*	233.33±60.09*
E	MPAE	00.00±00.00	00.00±00.00	50.00±28.87	1116.67±248.89	316.67±33.33	233.33±60.09	183.33±83.33*

*** Denotes the does were infected with 9000 L3 *H. contortus'* larvae.**

Note: Group A (control), group B (negative control), group C (positive control - chemical anthelmintic treatment), groups D and E (treated with MPME and MPAE, respectively).

Table 5: Mean faecal egg counts (FEC) of Boer does challenged with L3 *H. contortus* and treated with MPME and MPAE

Group		Description	Treatment
A	Control	Control (not infected)	-
B*	Control +	Negative control (infected)	No treatment
C*	Control + (Ivermectin)	Positive control	Treated with ivermectin at week 3 post L3 infection
D*	MPME		Treated with MPME X mg/kg for six days starting at week 3 post L3 infection
E*	MPAE		Treated with MPAE Y mg/kg for six days starting at week post L3 infection

*** Denotes significantly different at P< 0.0001; positive control (treated/not treated) and negative control.**

Note: Group A (control group). Group B (negative control group). Group C (positive control - chemical anthelmintic treatment). Groups D and E (treat with Methanolic and Aqueous *Moringa pterygosperma* leaf extracts, respectively.

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Table 6: Effects of MPME and MPAE on body weight (Kg) of Boer does infected with *H. contortus* L3 larvae

	Groups	Wk 0	Wk 4	Wk 6
A	Control	33.83±0.60	34.26±0.72	34.33±0.72
B	Control+ Chemical	35.33±0.72	33.66±0.72	32.66±0.16
C	anthelmintic	34.67±0.60	33.27±0.16	32.28±0.72
D	MPME	34.50±0.28	**36.67±0.6***	**38.5±1.25[a]**
E	MPAE	35.50±0.50	**36.67±0.33***	**38.26±0.33[a]**

*** Denotes significantly different at (P< 0.0009). [a] Denotes significantly different at p<0.0026 between groups. MPME, MPAE denotes Methanolic and Aqueous *Moringa pterygosperma* leaf extracts. Note: Group A (control group), group B (negative control group), group C (positive control - chemical anthelmintic treatment), groups D and E (treated with and, treat with Methanolic MPME and Aqueous MPAE *Moringa pterygosperma* leaf extracts, respectively respectively).**

CONCLUSION

The *M. pterygosperma* leaf extracts had the anthelmintic activity (nematocidal effect) as was demonstrated against L3 and adult stage of *H. contortus* for the first time. Results in this study also demonstrate potential anthelminthic properties of *M. pterygosperm*a extracts as well as increase body weight of does.

REFERENCES

[1] Sangster NC 1999; 29:115–124.
[2] Papadopoulos E 2008; 76 (1-2):99–103.
[3] Li TS. *Chinese & Related North American Herbs: Phytopharmacology & Therapeutic Values.* CRC press. 2016.
[4] Váradyová Z, Kišidayová S, Čobanová K, Grešáková Ľ, Babják M, Königová A, Urda Dolinská M, M Várady 2017; 151, 124-132.
[5] Kahiya C, Mukaratirwa S, Thamsborg SM 2003; 115: 265–274.
[6] Nchu F, Githiori JB, McGaw LJ, Eloff JN 2011183: 184-188.
[7] Vatta AF, Kandu-Lelo C, Ademola IO, JN Eloff 2011; 180: 279-286.
[8] Getachew T, Alemu B. Sölkner J, Gizaw S, Haile A, Gosheme S, DR Notter, 2015; 47(5), 961-968.
[9] Omar AI, Alam MBB, Faruque MO, Mondal MM, Raihan MS. Adetula AA Wu Z, Bhuiyan AA, Soudy F Cao, J, S Zhao 2017; 27(4), 473-481.
[10] Tadesse D, Eguale T, Giday M, A Mussa 2009; 122(2), 240-244.
[11] Amaglo NK, Bennett RN, Lo Curto RB, Rosa EAS, Lo Turco V, Giuffrida A, Lo Curto A, Crea F, GM Timpo 2010; 122:1047–1054.
[12] Moyo B, Masika PJ, Hugo A, V Muchenje 2011; 10(60), 12925-12933.
[13] Rahuman AA Efficacies of medicinal plant extracts against blood-sucking parasites. In *Nature Helps.*2011, (pp. 19-53). Springer Berlin Heidelberg. Mehlhorn: http://www.springer.com/978-3-642-19381-1, Accessed 29/10/15.
[14] Piluzza G, Virdis SF, Serralutzu F, S Bullitta 2015; 168: 87–99.
[15] Morton JF 1991; 45: 318- 333.
[16] Arora DS, Onsare JG, H Kaur 2013; 1(6).
[17] Mawouma S, Ponka R, CM Mbofung 2017; 5(2), 344-348.
[18] Dillard CJ, JB German 2000; 80(12), 1744-1756.
[19] Siddhuraju P, K Becker 2003; 51(8), 2144-2155.
[20] Popoola JO, OO Olawole 2013; 150, 2: 682-691.
[21] Njan AA, Amali MO, Olatunji LO, OE Olorundare 2014; 2, 135-45.
[22] Kumssa DB, Joy EJ, Young SD, Odee DW, Ander EL, MR Broadley 2017; 12(4), e0175503.
[23] Jose TA, Oliveira SB A Silveira A 1999;*I* 79: 815-20.
[24] Yaméogo CW, Bengaly MD, Savadogo A, Nikiema PA, S Traore 2011; 10 (3), 264-268.
[25] Mughal MH, Ali G, Srivastava PS, M Iqbal 1999; 42: 37 – 42.

[26] Anwar F, Latif S, Ashraf M AH Gilani 2007; 21(1), 17-25.
[27] Coppin JP, Xu Y, Chen H, Pan MH, Ho CT, Juliani R, Simon JE, Q Wu 2013; *5*(4),:1892–1899. doi: 10.1016/j.jff.2013.09.010.
[28] Witt KA 2013; 1-6.
[29] Zaki SS MM Rady 2015; 8(11): 120-134.
[30] Abdel-Latif M, El-Shahawi,G, Aboelhadid SM, H Abdel-Tawab 2017;1-12
[31] Fahey JW 2005; 1(5).
[32] Maroyi A 2013; 9 (1), 31.
[33] Rodrigues AB, Athayde AC, Rodrigues OG, Silva WW, EB Faria 2007; 27. 162–166.
[34] Paolini V, Bergeaud JP, Grisez C, Prevot F, Dorchies P, H Hoste 2003; 113 (3), 253-261.
[35] Barrau E, Fabre N, Fouraste I, H Hoste 2005; 131(4), 531-538.
[36] Chandrawathani P, Adnan M, PJ Waller 1999; 82, 305–310.
[37] Iqbal Z, Lateef M, Jabbar A, Muhammad G, MN Khan 2005; 102(2), 256-261.
[38] Ministry of Agriculture, Fisheries and Food Manual of Veterinary parasitological laboratory techniques, 3rd ed. Her Majesty's Stationery Office, London, U.K, 1986, 38.
[39] Zajac AM, GA Conboy Veterinary clinical parasitology. John Wiley and Sons, 2012.
[40] Molan, AL, AM Faraj 2010; 57(1), 62.
[41] Rehni AK, TG Singh 2010; 382(3), 279-285.
[42] El Sohaimy SA, Hamad GM, Mohamed SE, Amar MH, RR Al-Hindi 2015; 4(4), 188-199.
[43] Alvarez L, Mottier M, Sánchez S, C Lanusse 2001; 87(11), 929-934.
[44] Mottier L, Alvarez L, Ceballos L, C Lanusse 2006; 113(1), 49-57.
[45] Jabbar A, Zaman MA, Iqbal Z, Yaseen M, A Shamim 2007; 114(1), 86-91.
[46] Iqbal Z, Lateef M, Ashraf M, A Jabbar 2004; 93 (2-3), 265-268.
[47] Hounzangbé-Adote MS, Paolini V, Fourasté I, Moutairou K, Hoste H 2005a; 78:155–160**.**
[48] Hounzangbe-Adote S, Zinsou FE, Hounpke V, Moutairou, K H Hoste 2005b; 37: 205–214.
[49] Kotze AC, Le Jambre LF, J O'Grady 2006; 137(3), 294-305.
[50] Ademola IO, JN Eloff 2011; 43: 521-527
[51] Ademola IO, JN Eloff 2010; 169 (1-2): 198 – 203.
[52] Adamu M, Oshadu OD, CI Ogbaje 2010; 7: 279-285.
[53] Maciel MV, Morais SM, Bevilaqua CML, Camurça-Vasconcelos ALF, Costa CTC, CMS Castro 2006; 140 (1), 98-104.
[54] Camurça-Vasconcelos ALF, Bevilaqua CML, Morais SM, Maciel MV, Costa CTC, Macedo ITF, Oliveira LMB, Braga RR, Silva RA, LS Vieira 2007; 148(3), 288-294.
[55] Bahrami A, Ahmady-Asbchin S, Araash B, LM Ali 2011); 15 (9): 1267-1273.