



RESEARCH ARTICLE

Evaluation of *in vitro* anthelmintic activity of Indian acalypha (*Acalypha indica*) root and Indian borage (*Plectranthus amboinicus*) extract against L3 stage strongyle larvae in sheep

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ABSTRACT

Gastrointestinal nematode (GIN) infection has been identified as the second most important cause of small ruminant mortality and morbidity in Malaysia. To control GIN infection, commercial anthelmintic drugs (AHD) are used. However, continuous unscrupulous usage of AHD has inevitably resulted in rampant anthelmintic resistance in numerous small ruminant farms in Malaysia. Hence, ethnoveterinary medicine is actively explored to find an alternative to commercial AHD. This study aims to evaluate the anthelmintic activity of Indian acalypha (*Acalypha indica*) and Indian borage (*Plectranthus amboinicus*) against third stage strongyle larvae (L3 larvae) in sheep. A total of 300 L3 larvae was assigned to 8 experimental groups (40, 50 and 60 mg/ml Indian Acalypha Root Methanolic Extract (IARME), 10, 30 and 50 mg/ml Indian borage extract (IBE), ivermectin and distilled water). The average percentage of L3 larvae mortality was recorded at time 0, 10 and 30 minutes, 1, 2, 3, 4 and 24 hours. The results revealed that 50 mg/ml IBE is the most efficient treatment, followed by 60 mg/ml IARME. Probit analysis revealed that both IARME and IBE are effective anthelmintic agents with time-dependent increases in efficacy. Therefore, this study showed that IARME might be optimal for rapid treatment, while IBE could be suitable for sustained anthelmintic treatment.

Keywords: *Acalypha indica*; anthelmintic drugs; ethnoveterinary medicine; *Plectranthus amboinicus*; strongyle.

INTRODUCTION

Gastrointestinal nematode (GIN) infection remains as one of the major issues affecting the small ruminant industries worldwide, particularly in tropical regions with suitable climate that favour parasite survival and transmission (Chengaiyah *et al.*, 2009; Várady *et al.*, 2011). These infections do not cause the death of livestock but can lead to growth disorders, impaired productivity, affected reproduction and low quality of meat that can significantly impose a substantial economic burden on livestock producers.

In Malaysia, helminthiasis has been recognized as the second most important cause of small ruminant mortality and morbidity (Chandrawathani *et al.*, 2013). A previous study by Department of Veterinary Services reported high prevalence rates of strongyle infections, with 64.9% of goats and 49.4% of sheep testing positive on farms in Perak, Malaysia (Azima *et al.*, 2020). A similar study in

Negeri Sembilan reported that 16.7% of goats were infected with strongyles (Paul *et al.*, 2020). These prevalence rates highlight an urgent need for effective parasite control measures across Malaysian small ruminant farms.

Currently, the control of helminths in Malaysia heavily relies on commercial anthelmintic drug (AHD) like benzimidazole (Bz), levamisole (Lv), closantel (Clo) and ivermectin (IVM). However, extensive use of these drugs has led to widespread anthelmintic resistance in Malaysian farms (Nor-Azlina *et al.*, 2011a, 2011b; Sabariah *et al.*, 2017; Wong *et al.*, 2018; Khadijah *et al.*, 2018; Abd Majid *et al.*, 2022). This resistance is a growing concern, as it limits the effectiveness of anthelmintic drugs and alternative approaches to control GIN sustainably is needed.

In recent years, the search for natural anthelmintics has gained recognition among researchers and farmers since a large biodiversity of tropical plants with potential larvicidal activities. Several studies

have demonstrated the anthelmintic properties of plants such as Indian acalypha (*Acalypha indica*) and Indian borage (*Plectranthus amboinicus*), particularly against ascarids and *Haemonchus contortus*, one of the most pathogenic GINs affecting small ruminants (Tadesse *et al.*, 2009; Wadekar *et al.*, 2010; Mutiarawati, 2020; Laut *et al.*, 2022). These findings present promising prospects for incorporating plant-based anthelmintics into integrated parasite management strategies, potentially reducing dependence on synthetic AHDs. Therefore, this study aimed to investigate the *in vitro* anthelmintic efficacy of *A. indica* and *P. amboinicus* extract on third stage strongyle larvae (L3 larvae) from sheep, comparing their effectiveness against commercially used drug IVM.

MATERIAL AND METHODS

Ethical approval

The animal protocol and sample collection procedures in this study was completed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (AUP no: UPM/IACUC/AUP-U035/2019).

Plant extract preparation

One kilogram (kg) of *P. amboinicus* leaves was collected from naturally growing plants. The leaves were washed and air-dried overnight. Once dried, the leaves were separated from branches. The leaves were placed in an oven at 60°C for 2 days to further dry the leaves. The dried leaves were then ground into powder using a coffee grinder. For *A. indica*, 1 kg of roots was harvested. The roots were washed and cut into 1-inch pieces and air-dried in root temperature for 12 hours. Both powdered leaves and dried roots were kept in a clean, dry, air-tight container at room temperature before being transported for further processing.

Crude methanolic extraction preparation

Methanolic extraction technique using Soxhlet apparatus was adapted from Ahmad *et al.* (2017). The extraction was performed for a minimum of 8 hours to ensure optimal recovery of bioactive compounds from the leaves and roots. The solvent was removed by using a rotary evaporator. The yield was collected in the form of a dark brown semi-solid and stored in a Petri dish. The leaf and root extracts were oven-dried at 60°C for 24 hours, weighed, labeled and stored at 4°C until further use.

Faecal collection and egg count

A sheep farm in Selangor with a history of high faecal egg per gram (e.p.g) was identified for this study. Faecal samples were collected per rectum using gloved hands, bagged separately into individual gloves and transported to the lab. A total of 21 samples were labeled and stored at 4°C overnight before culturing.

The next day, faeces were taken out from the fridge, and the McMaster technique was performed at room temperature to determine the fecal egg count (FEC) (Gordon & Whitlock, 1939). For each sample, 1 g of faeces, composed of small amounts taken from multiple pellets, was placed into a plastic beaker and weighed using an electronic balance. Then, 14 ml of saturated sodium chloride (NaCl) solution was added and the faeces were mashed up and mixed homogeneously with the solution. The mixture was filtered into another plastic beaker using a tea sieve. The filtrate was mixed well using a disposable pipette before being pipetted into two chambers of the McMaster slide. The residue was discarded.

The slide was examined under a light microscope at 100x magnification. The FEC of strongyle eggs in both chambers were counted and the e.p.g was calculated using the following formula:

e.p.g = Total number of strongyle oocysts counted in both chambers x 50

Faecal culture and L3 harvesting

Faecal culture and L3 harvesting techniques were adapted from Hansen & Perry (1990) with slight modification. Briefly, faecal were combined as needed to reach a total FEC of 1500 e.p.g and were emptied into a mortar and crushed using a pestle. Non-infected faeces were added to increase the sample volume. The compacted faeces were transferred into glass jar and ensuring they occupied one-third of each jar. Distilled water was carefully added to moisten the faeces without creating a water film on the surface. A square piece of gauze was cut was placed over the mouth of each jar and secured with a rubber band. The gauze was moistened with distilled water. The faecal cultures were kept at room temperature for seven days with daily gauze moistening using distilled water.

After seven days, L3 larvae were harvested using a modified Baermann's technique (Rugai *et al.*, 1954). The faeces in each jar were transferred onto 10 x 15cm pieces of gauze. The gauze was tied with a rubber band and attached to a small stick. These bundles were placed in sedimentation cylinders filled with warm water (~37°C) until submerged. The ensemble was left overnight. The larvae were harvested the next morning by carefully removing the water from each sedimentation cylinder, leaving about 5ml at the bottom. The residual water was transferred to a petri dish and examined under stereomicroscope for L3 larvae. The water was then transferred into a 50 ml Falcon tube and diluted with distilled water to a total volume of 30 ml. The tubes were centrifuged at 4000 rpm for 10 minutes and 25 ml of the supernatant was discarded. A 1 ml aliquot of larvae stock was transferred to a clean petri dish and enumerated. The remaining contents were diluted with distilled water, labeled and stored at 4°C until further use.

Extraction and drug preparation

The Indian acalypha root methanolic extract (IARME) concentrations of 40 mg/ml, 50 mg/ml, 60 mg/ml and Indian borage extract (IBE) concentrations of 10 mg/ml, 30 mg/ml, 50 mg/ml were prepared by dissolved 0.2 g, 0.25 g and 0.3 g of crude IARME and 0.05 g, 0.15 g and 0.25 g of crude IBE with 5 ml of distilled water at room temperature. These treatment solutions were filtered using a 0.22 µm filter to remove any debris and placed in 10 ml syringes that labeled accordingly.

Ivermectin (Vetoquinol Biowet) 1mg/ml served as the positive control, while distilled water was used as negative control. A 10 ml solution of 0.1% IVM was prepared by diluting 1 ml of 10 mg/ml IVM with 9 ml of distilled water. Both control solutions were labeled appropriately.

Mortality assessment of L3 strongyles

The L3 strongyle solution was removed from the fridge and placed in a water bath at 37.5°C for 5 to 10 minutes to activate dormant larvae. A six well plate was labelled for each control and treatment. All tests conducted in duplicate. A total of 300 L3 larvae was assigned to 8 experimental groups (40, 50 and 60 mg/ml Indian Acalypha Root Methanolic Extract (IARME), 10, 30 and 50 mg/ml Indian borage extract (IBE), ivermectin and distilled water). The well plates were left at room temperature for up to 24 hours to access final larvae viability. The average percentage of L3 larvae mortality was recorded at time 0, 10 and 30 minutes, 1, 2, 3, 4 and 24 hours. Larvae were considered alive if they exhibited any movement while dead larvae appeared translucent and straight.

Statistical analysis

All data were corrected for control mortality using Abbot's formula (Busvine, 1972):

$$P = \frac{P_1 - C}{100 - C} \times 100$$

Where P = corrected percentage inactivation;

P1 = observed percentage inactivation;

C = control percentage inactivation.

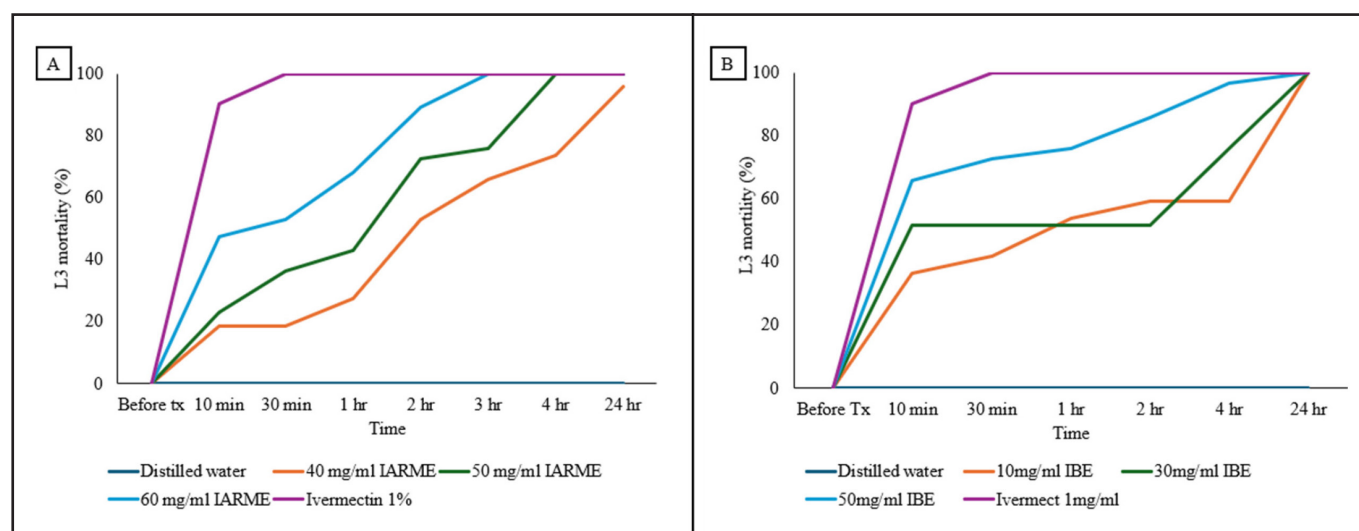


Figure 1. Percentage mortality of L3 strongyle against all groups over 24 hours post treatment for (A) IARME and (B) IBE.

Statistical analysis was performed using Microsoft Excel 2013 and SPSS version 25.0.

Since the data was not normally distributed according to the Shapiro-Wilk test, Kruskal-Wallis and Mann-Whitney U test were used to determine the significant difference of L3 mortality at all concentrations of IBE (10 mg/ml, 30 mg/ml and 50 mg/ml) and IARME (40 mg/ml, 50 mg/ml and 60 mg/ml) and to determine the significant difference of L3 stage strongyle mortality at all concentrations of IBE and IARME compared to control drug, ivermectin (1 mg/ml) respectively. Probit analysis (Finney, 1952) and lethal time (LT) values were calculated in this study.

RESULTS

Both IARME and IBE treated groups showed an increasing trend in the percentage of L3 strongyle larvae mortality over 24 hours (Figure 1). IARME at 60 mg/ml was highly effective and reached 100% mortality within just 3 hours of post-treatment. In contrast, 10 mg/ml, 30 mg/ml and 50 mg/ml IBE reached 100% mortality only by 24 hours post-treatment.

For both plant extracts, Kruskal-Wallis H test revealed significant difference ($p < 0.05$) in the percentage of L3 mortality between the control groups and all treatment groups. Mann Whitney test expressed that the average percentage of L3 mortality recorded by the negative control and all concentration of IARME and IBE were significantly different ($p < 0.05$) when compared to positive control group (Ivermectin 0.1%) and negative control (distilled water). For

IBE, there was no significant difference ($p > 0.05$) in the average percentage of L3 strongyle mortality between 30 mg/ml and 10 mg/ml as well as 30 mg/ml and 50 mg/ml. On the other hand, for IARME, there is no significant difference ($p > 0.05$) in the average percentage of L3 strongyle mortality between 40 mg/ml and 50 mg/ml as well as 50 mg/ml and 60 mg/ml. However, there was significant difference ($p < 0.05$) between 40 mg/ml and 60 mg/ml. Based on the results, the highest average percentage of L3 mortality is 0.1% IVM, followed by 60 mg/ml IARME and 50 mg/ml IBE (Table 1).

Both IARME and IBE exhibited decreasing LC_{50} values over time which indicated an increased efficacy with extended exposure (Table 2). The LC_{50} for IBE was lower than that of IARME. All concentration of IARME had steeper slopes compared to

Table 1. The average percentage of L3 mortality across all treatment groups

Group	Treatment	L3 Mortality (%) Mean $\pm$ SEM
1	Positive control (Ivermectin 0.1%)	86.84 $\pm$ 8.502
2	40 mg/ml IARME	48.56 $\pm$ 7.842
3	50 mg/ml IARME	59.63 $\pm$ 8.579
4	60 mg/ml IARME	71.14 $\pm$ 8.724
5	10 mg/ml IBE	52.04 $\pm$ 29.66
6	30 mg/ml IBE	60.44 $\pm$ 26.32
7	50 mg/ml IBE	70.90 $\pm$ 32.50
8	Negative control (DW)	8.55 $\pm$ 2.224

Table 2. *In vitro* sensitivity of IARME and IBE with determination of its effective lethal concentration (mg/ml) against L3 strongyle

Plant	Time	LC50(mg/ml)	Fiducial limits		Regression equation	Chi-square value (χ^2)
			Lower	Upper		
IARME	10 min	69.491	53.477	90.301	$y = 4.3105x - 2.9436$	0.238
	30 min	61.195	49.404	75.801	$y = 5.1401x - 4.184$	0.945
	1 hr	53.981	44.450	65.555	$y = 5.5104x - 4.5455$	0.972
	2 hrs	41.149	33.432	50.647	$y = 5.252x - 3.4785$	0.917
	3hrs	37.873	31.269	45.871	$y = 6.04x - 4.5295$	0.567
	4hrs	31.346	24.613	39.920	$y = 5.2696x - 2.8814$	0.728
	24 hrs	—	—	—	—	—
IBE	10 min	30.439	10.160	91.197	$y = 0.9556x - 3.0863$	0.735
	30 min	24.072	7.908	73.272	$y = 0.9405x + 3.7014$	0.455
	1 hr	15.056	2.728	83.096	$y = 0.6099x + 4.2828$	0.280
	2 hrs	10.957	2.682	44.765	$y = 0.7495x + 4.2224$	0.144
	4hrs	9.518	4.305	21.044	$y = 1.4246x + 3.6074$	0.617
	24 hrs	—	—	—	—	—

Table 3. *In vitro* sensitivity of IARME, IBE and 0.1% IVM with determination of its effective lethal time (minute) against L3 strongyle

Treatment	0.1% IVM	IARME			IBE		
		40	50	60	10	30	50
Concentration (mg/ml)							
LT ₅₀	0.000	102.053	46.920	10.745	48.344	25.113	1.275
LT ₉₀	0.367	951.474	434.040	232.078	1208.821	1218.039	246.117
LT ₉₅	9.847	1257.746	573.194	340.753	1807.650	1978.707	475.156
LT ₉₉	136.875	1572.352	716.009	463.323	2494.113	2917.143	804.250
Slope	1.520	17.917	17.980	13.018	12.426	10.305	7.601

IBE, suggesting that IARME has a more rapid effect at higher concentration than IBE.

Based on Table 3, 0.1% IVM showed high efficacy against L3 strongyle as evidenced by its exceptionally low LT₅₀ value (1.36 x 10⁻¹² minutes). In contrast, both plant extracts exhibited slower lethality compared to 0.1% IVM. Among the plant extracts, 50 mg/ml IBE has the shortest LT₅₀ (1.275 minutes), followed by 60 mg/ml IARME (10.745 minutes). However, the lower slope value of 50 mg/ml IBE compared to IARME indicates a less steep increase in mortality over extended periods. In other words, while IBE at 50 mg/ml is potent at early exposure, it might not sustain the same level of mortality over time.

DISCUSSION

The emergence of anthelmintic resistance in Malaysian farm has raised a concern in the search of natural anthelmintics. Plant extracts have therefore gained attention as potential alternatives. The *in vitro* testing of L3s is considered among the best methods to screen the anthelmintic activity of plant compounds (Hernández-Villegas et al., 2011). Previous studies have demonstrated the potential of *A. indica* root (50 mg/ml) and *P. amboinicus* (100% concentration) as anthelmintic plants, with alcoholic extraction of both plant producing the shortest time to worm death of 30 and 29 minutes respectively (Chengaiyah et al., 2009; Wadekar et al., 2010). Therefore, this study aimed to investigate the anthelmintic activity of methanolic extract from *A. indica* root and *P. amboinicus* leaf against L3 strongyle larvae.

Based on the results, both IARME and IBE demonstrated a decreasing trend of LC₅₀ value over time. This indicates an increasing efficacy with extended exposure. A notable difference was that the LC₅₀ of IBE is consistently lower compared to IARME. This difference might be attributed to variations in the phytochemical composition of the two plant extracts. Previous phytochemical screening of *A. indica* roots has identified compounds such as alkaloids, flavonoids, saponins, tannins, terpenoids, and coumarins, with anthelmintic properties (Nazri et al., 2016; Tasmim et al., 2021; Sahukari et al., 2021). Flavonoids and tannins have also been reported in *P. amboinicus* leaves (Paramasivam et al., 2020). Future studies are recommended to confirm the association between the presence of bioactive compounds with its anthelmintic strength.

In addition, slope analysis of the data revealed that all concentrations of IBE had lower slope values as compared to IARME, suggesting that although IBE potent at lower concentrations, it acts more gradually against the L3 strongyle which might be better for lower-dose, sustained treatments in anthelmintic management. On the other hand, high slope values of IARME indicating its faster onset action which might be beneficial in situations requires a quick and strong response where rapid elimination of larvae is a priority. However, this requires further confirmation in future study.

Both IARME and IBE showed slower lethality when compared to 1% IVM. This is because IVM is a highly effective synthetic anthelmintic which indicates a rapid and efficient action against L3 strongyle as this study showed a very low LT₅₀ (1.36 x 10⁻¹² minutes). In contrast, both plant extracts exhibited longer LT₅₀, with

50 mg/ml IBE being the most effective plant extract (1.275 minutes), followed by 60 mg/ml IARME (10.745 minutes). The rapid action of IVM is due to its mechanism of action that interferes with the GABA receptors and glutamate-gated chloride channels in parasites (Campbell et al., 1983; Cheeseman et al., 2001). This will lead to immediate paralysis and death. Although the plant extracts show slower lethality compared to IVM, they might offer a safer, natural alternative with potential additional benefit for the small ruminant industry.

A limitation of the present study is that phytochemical screening of the plant extracts was not performed. Another limitation is that the L3 strongyle larvae were not identified to the genus or species level. Therefore, the observed anthelmintic activity cannot be attributed to a specific strongyle species. Further studies with phytochemical profiling and L3 species identification is necessary to identify the active compounds and determine the species-specific sensitivity to plant extracts.

CONCLUSION

This study evaluated the anthelmintic efficacy of *A. indica* root and *P. amboinicus* leaf methanol extract against L3 strongyle and comparing their effects with the synthetic drug IVM. Among the plant extracts tested 50 mg/ml IBE demonstrated the highest anthelmintic activity against L3, followed by 60 mg/ml IARME. Probit analysis revealed that both IARME and IBE are effective anthelmintic agents with time-dependent increases in efficacy where IARME might be optimal for rapid treatment, while IBE could be suitable for sustained anthelmintic treatment. Future research is needed to investigate their *in vivo* efficacy and the potential synergistic effects of their available bioactive compounds in integrated parasite management for small ruminants.

Competing of Interest Statement

The authors declare that they have no conflicts of interest.

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