UNIVERSITI PUTRA MALAYSIA

IN VITRO ANTHELMINTIC ACTIVITY OF NEEM LEAVES (*AZADIRACHTA INDICA*) CHLOROFORM EXTRACT AGAINST THE THIRD-STAGE LARVAE OF STRONGYLES FROM SHEEP

NURUL HAIRUNNISA BINTI SUHAIMI

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NURUL HAIRUNNISA BINTI SUHAIMI

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It is hereby certified that we have read this project paper entitled "*In vitro* anthelmintic activity of neem leaves (*Azadirachta indica*) chloroform extract against the third-stage larvae of strongyles from sheep", by Nurul Hairunnisa Binti Suhaimi and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project.

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DR SITI ZUBAIDAH RAMANOON

DVM (UPM), MSc (Guelph)

Senior Lecturer

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

_____

DR WAN MASTURA SHAIK MOHAMED MOSSADEQ

DVM (UPM), MSc (Guelph), PhD (UPM)

Senior Lecturer

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

DEDICATION

I dedicate this thesis with love and appreciation to:

My parents and siblings

SUHAIMI BIN MOHD MUKHTAR and ZAIDAH BINTI ZAKARIA

NOR FARAHIYAH, NUJAIMI, BAZILAH, NASUHA, IZZAH and ZIA

My Friends and FYP mates

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LIST OF ABBREVATIONS

ACE	Neem Leaves (*A. indica*) Chloroform Extract
A. indica	*Azadirachta Indica*
df	Degree of freedom
DMSO	Dimethyl sulfoxide
EPG	Egg per gram
GI	Gastrointestinal
H. contortus	*Haemonchus contortus*
IBM SPSS	IBM Statistical Software for Social Sciences
KW	Kruskall-Wallis
L3	Third-stage larvae
p	p-value

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Tahun Akhir

AKTIVITI ANTELMINTIK *IN VITRO* EKSTRAK KLOROFORM DAUN SEMAMBU (*AZADIRACHTA INDICA*) PADA LARVA STRONGIL PERINGKAT KETIGA DARIPADA BEBIRI

Oleh:

Nurul Hairunnisa Binti Suhaimi

2016

Penyelia: Dr. Siti Zubaidah Ramanoon

Penyelia bersama: Dr. Wan Mastura Shaik Mohamed Mossadeq

Penyakit berparasit menyebabkan kematian dan morbiditi pada bebiri di Malaysia. Semambu (*Azadirachta indica*) telah dibuktikan mempunyai nilai perubatan seperti anti-kulat, anti-bakteria, anti-radang dan antelmintik. Kajian ini dijalankan untuk menentukan kesan antelmintik *in vitro* daun semambu pada larva peringkat ketiga (L3)

strongil dari bebiri. Sampel najis terkumpul daripada 22 bebiri yang mempunyai sejarah gastrousus berparasit dikulturkan untuk menuai L3. Tiga ribu L3 telah dibahagikan kepada lima kumpulan mengandungi enam ceper petri setiap satu, yang mana dalam setiap ceper petri terkandung seratus L3. Tiga kumpulan telah diuji dengan ekstrak kloroform daun semambu *Azadirachta indica* (ACE) berkepekatan 5, 10 dan 15mg/ml, satu kumpulan kawalan positif (levamisol, 10 mg/ml), dan satu negatif (0.01% DMSO + air ternyahion). Mortaliti L3 diperhatikan dalam masa 2, 4, 6, dan 24 jam. Keputusan menunjukan bahawa peratus mortaliti L3 adalah sebanyak 93% pada kumpulan rawatan ACE 5 mg/ml selepas 24 jam; dan 83% ACE 10 mg/ml, selepas 2 jam. Kumpulan ujian ACE menunjukkan keberkesanan antelmintik ketara pada L3 berbanding dengan kumpulan kawalan negatif (KW = 93,55, df = 4, $p < 0.05$). Kajian lanjut berkaitan dengan kesan ACE berkepekatan lebih rendah terhadap mortaliti L3 strongil pada bebiri dicadangkan.

Kata kunci: daun semambu, *Azadirachta indica*, ekstrak kloroform, antelmintik, strongil, larva, bebiri

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, UPM in partial requirement of the course VPD 4999- Final Year Project

IN VITRO ANTHELMINTIC ACTIVITY OF THE NEEM LEAVES (_AZADIRACHTA INDICA_) CHLOROFORM EXTRACT AGAINST THE THIRD–STAGE LARVAE OF STRONGYLES FROM SHEEP

By

Nurul Hairunnisa Binti Suhaimi

2016

Supervisor: Dr. Siti Zubaidah Ramanoon

Co-supervisor: Dr. Wan Mastura Shaik Mohamed Mossadeq

Parasitic disease causes mortality and morbidity of sheep in Malaysia. Neem plant (*Azadirachta indica*) has been shown to possess medicinal values such as anti-fungal, antibacterial, anti-inflammatory and anthelmintic activity. This study was conducted to determine the *in vitro* anthelmintic effect of neem leaves on the third-stage larvae (L3) of strongyles from sheep. Pooled faecal samples from 22 sheep with the history of gastrointestinal parasitism were cultured to harvest the L3. Three thousands L3 were

divided into five groups of six petri dishes each containing one hundred L3 per dish. Three groups were tested with the neem leaves (*Azadirachta indica*) chloroform extract (ACE) at 5, 10 and 15mg/ml concentrations, one positive control group (levamisole, 10 mg/ml) and one negative (0.01% DMSO + deionized water). The L3 mortality was observed for 2, 4, 6, and 24 hours. Results showed that the percentage L3 mortality were 93% (ACE 5 mg/ml of 24 hours post-treatment) and 83% (ACE 10 mg/ml 2 hours post-treatment). All ACE treated groups showed significant anthelmintic efficacy against L3 compared to the negative control group ($KW=93.55$, $df=4$, $p<0.05$). Further studies related to the effect of using lower ACE concentrations on L3 mortality of strongyles from sheep are suggested.

Keywords: neem, *Azadirachta indica*, chloroform extract, anthelmintic strongyles, larvae, sheep

Chapter 1

INTRODUCTION

1.1 Background

Helminthiasis is considered the major cause of mortality and morbidity in goats and sheep in Malaysia (Sani *et al.*, 2004) and elsewhere throughout the humid tropical/subtropical countries of the world (Perry *et al.*, 2002). Neem (*Azadirachta indica*, *A. indica*), a tree in the mahogany family Meliaceae, is native to India, Pakistan and Burma, growing in tropical and semi-tropical regions (Neem Foundation, 2001). Some parts of neem have been used in traditional medicine to treat diseases such as gastrointestinal (GI) nematodes infection (Arunachal *et al*., 2002). Literature on the usage of neem leaves as anthelmintics for sheep especially in reducing the worm load of *Haemonchus contortus* (*H. contortus*) is very limited. Though Costa *et al*. (2006) reported that there was no significant anthelmintic activity observed after feeding sheep with neem leaves. Furthermore, studies on anthelmintic properties of neem through feeding the leaves to sheep are limited. Therefore, there is a need to study the anthelmintic effect of neem leaves in sheep.

1.2 Justification

Drug resistance from prolong use at high levels and increasing frequency and inappropriate doses of chemical anthelmintics, to control gastrointestinal parasitism in sheep is considered an important problem in small ruminant husbandry. Additionally, anthelmintic drugs are costly and may cause drug residues in food products that will cause public health concern for food safety. Neem leaves as an alternative anthelmintic treatment for GI parasitic infections in sheep need to be investigated.

1.3 Study Objectives

a) to determine the *in vitro* anthelmintic efficacy of ACE on the L3 of strongyles from sheep

b) to determine whether or not ACE has the same anthelmintic efficacy as levamisole

1.4 Study Hypothesis

i. ACE at different concentrations show anthelmintic activity against L3 of strongyles from sheep.

ii. ACE has same efficacy as levamisole

Chapter 2

LITERATURE REVIEW

2.1 Strongyles in Small Ruminants

Helminthiasis in small ruminants in Malaysia has been recognised as the second most important cause of mortality in sheep after pneumonic pasteurellosis (Sani and Rajamanickam, 1990). Faecal culture studies in Peninsular Malaysia indicated *H. contortus* (85–90%) was the most common trichostrongylid of sheep and goats on all farms studied (Chandrawathani *et al.,* 1999). Other strongyles species recorded were *Trichostrongylus colubriformis* (5–10%), and *Oesophagostomum* sp. and *Cooperia* sp. (1–5%) (Chandrawathani *et al.,* 1999). Young animals and females in periparturient period or in lactation are most susceptible, causing weight loss of 6–12 kg/animal/year (Githigia *et al*., 2001). Mortality can be more than 40% which *H. contortus* and *Trichostrongylus spp*. are the most helminths species involved (Rey, 1991). More than 95% of small ruminants living in the tropical countries have been infected by helminths (Githigia *et al*., 2001). Anthelmintic drugs are the method mostly used by farmers to control parasitism in their livestock (Charles *et al*., 1989). Tropical/sub-tropical climate, with high temperatures and rainfall prevailing continuously throughout the year, have caused farmers practicing frequent and indiscriminate use of anthelmintics for helminthiasis control (Sani *et al*., 1995). However, the effectiveness of these drugs has reduced because of resistant strains (Echevarria, 1996). This

condition is more severe with sheep and goat nematodes (Andrews, 2000; Zajac and Gipson, 2000). There has also been an increase in farms reporting that the anthelmintic drugs are no longer effective (Sani *et al*., 1995) indicating drug resistance. Other alternative to control gastrointestinal worms was suggested by introducing integrated systems such as pasture management and strategic deworming (Sani *et al*., 1995).

2.2 Neem (*A. indica*) Leaves as Anthelmintic

Neem has been reported to have multiple medicinal applications such as an anti-inflammatory, antipyretic, analgesic, immuno-stimulant, hypoglycemic, anti-fungal and antibacterial (Conrick, 1994; Nogueira and Neves, 1996). Neem is also known for its activity against insects and parasites of both plants and animals (Mitchell *et al*., 1997). Anthelmintic potentials of *A. indica* has been widely studied previously. Neem contains several biologically active constituents such as azadirachtin, meliantriol and salanin (Priya *et al*., 2015). Azadirachtin-A, the substance believed to act against the parasites, present in the leaves only contains 0.59 mg/100 g (Sundaram, 1996).

Neem leaves have been reported to be safe, ecofriendly, cheap and palatable to animals (Chandrawathani *et al.* 2000). Consumption of dried neem leaves in cattle caused a reduction in the number of eggs per gram (EPG) of faeces against intestinal nematodes (Pietrosemoli *et al*., 1999). In contrast, some studies reported non-significant findings in sheep fed with dried neem leaves compared to the control group (Githiori *et al*., 2004; Costa *et al*., 2006). *Ad libitum* feeding of fresh neem leaves

resulted in 82% reduction in worm eggs of the animals (Chandrawathani *et al.,* 2000) and a further trial on a limited number of sheep showed that neem produced a significant reduction in worm burdens (Chandrawathani *et al*., 2002). Rob *et al*. (2004) observed that water extracts of neem was 53.72% effective against *H. contortus* in sheep. Brelin (2002) found that fresh neem leaves significantly reduced *H. contortus* in the abomasum of the treated sheep. Arunachal *et al*. (2002) noted that neem leaves, seeds and bark were 53%, 49% and 38% effective against GI helminths in sheep, respectively. Amin *et al*. (2008) reported that 10% water extract of neem leaves given to cattle had reduced significantly ($p<0.01$) the egg count at 62.23%, 65.77%, 56.70% and 48.05% on 3^{rd}, 10^{th}, 17^{th} and 28^{th} day, respectively. Rahman (2002) reported that water extract of neem leaves showed 62% efficacy against GI nematodes of goats after 21 days treatment.

2.3 Use of Levamisole as Anthelmintic in Livestock

Levamisole is an agonist on nicotinic acetylcholine receptors at the synaptic and extra synaptic nicotinic acetylcholine receptors on nematode muscle cells and produce contraction and spastic paralysis (Martin, 1997). This mechanism has made levamisole an anthelmintic and it has been used widely in livestock farm. However, anthelmintic resistance in commercial drugs has emerged in parasites of small ruminants in Malaysia (Dorny *et al*., 1994). Rahman (1994) reported that 10 goat farms surveyed in Penang Malaysia had levamisole resistant parasite populations. According to

Chandrawathani *et al.,* (1999), resistance to levamisole and other chemical products that commonly used by farmers was detected. The authors reported that from the 39 farms studied, resistance to levamisole was detected the highest in 15 farms, compared to other anthelmintic groups namely benzimidazoles (3 farms), moxidectin (3 farms), ivermectin (4 farms), closantel (5 farms) and to the combination drugs (1 farm).

Chapter 3

MATERIALS AND METHOD

3.1 Materials Preparation

Plant materials:

Two kilograms (kg) of the neem leaves was collected at Taman Warisan, Putrajaya. The leaves were collected in the same tree at the same level. The neem leaves were then washed and dried using clean towel, and left at room temperature for 1 day. On the next day, the leaves were dried in the hot oven at 40°C for 3 days. The dried leaves were then separated from its twigs and then grounded into fine powder by using an electrical grinder. Total fine powder from the two kg wet weight was about 550g.

3.2 Faecal Culture and Harvesting L3

Faecal samples were collected rectally from 22 sheep from Hulu Langat farm, Selangor with a history of GI parasitism. Faecal samples were then processed using McMaster procedure for egg count of GI parasites expressed in EPG. The EPG of GI parasites were between 2,500 and 13,500. Faecal samples were then be maintained in distilled water and incubated at room temperature for 7 days. The L3 were then recovered by spontaneous migration using warm water (37^{o}C). All the L3 collected were stored in the Falcon tube and stored in refrigerator within 4^{o}C to inhibit the growth of L3.

3.3 Neem Leaves (*A. indica*) Chloroform Extract (ACE)

100g of the neem leaves fine powder was placed in 1 litre of glass jar followed with 1 litre of 100% chloroform. The glass jar was shaken using rotary shaker with magnetic stirrer inside. The mixture was continuously shaken and kept at room temperature for 3 days consecutively and then filtered with filter paper No. 1 Whattmann England. The filtrate was poured into a boiling flask and evaporated under vacuum using Rotary Vacuum Evaporator at 40^{o}C and speed 60 rpm to remove the chloroform. The concentrate was left at room temperature for 24 hours under fumigation hood to remove all chloroform from the concentrate.

3.4 Preparation of Levamisole

10 mg/ml of levamisole concentration was used as positive control in this *in vitro* experiment. 32 g/L of levamisole was diluted using deionized water to make 10 mg/ml concentration.

3.5 Treatment and Motility Assessment

Experiment was done on 30 petri dishes that contained 100 L3 each. Three serial concentrations (5, 10, 15mg/ml) of ACE were prepared by diluting the concentrate of the extract mixed in 0.01% DMSO and deionized water. Then, ACE (5, 10, 15mg/ml) was added into petri dish with six replicates for each concentration. Another group of 6 petri dishes with 10 mg/ml levamisole was the positive controls, and another 6 dishes added with 0.01% DMSO and deionized water were the negative controls.

Subsequently, the number of alive larvae was recorded after 2, 4, and 6 hours and 24 hours after treatment. Coiled and movable L3 were considered alive. All observations were done using stereo microscope.

3.6 Data Analysis

The data were tabulated in Microsoft Excel including the number of active and dead L3 for each time of observation (2, 4, 6 hour, 24 hour) for all five groups. Results were expressed as percentage (%) inhibition of L3 mortality calculated using the formula:

$$\text{Percentage inhibition (\%) of L3 mortality} = \left[\frac{\text{Number of dead L3}}{\text{Total number of L3 counted}}\right] \times 100$$

The L3 mortality percentages for all respective five groups were calculated and expressed in mean $\pm$ standard error of the mean (Mean $\pm$ SEM). The mean percentage inhibition of L3 mortality in ACE or levamisole in 3 times repetitions was calculated to determine the anthelmintic activity. The Shapiro-Wilk test for normality showed that the data of mean percentage of L3 mortality for all five groups were not normally distributed ($p<0.05$). Therefore, non-parametric tests were used for the statistical analysis. Comparison of the mean percentage of L3 mortality for ACE groups against the positive controls was done using Kruskall- Wallis and Mann-Whitney tests. The significance level was set at $p<0.05$. All statistical tests were done in IBM Statistical Software for Social Sciences (SPSS) ver. 22. The results from all treated groups and positive controls were compared with the negative controls.

Chapter 4

RESULTS

4.1 L3 Mortality

There was an increased trend in the average percentage of L3 mortality over time for all ACE treated groups (Table 1 and Figure 1). The ACE groups with 5, 10 and 15 mg/ml recorded 68%, 83% and 79%, respectively, of L3 mortality in 2-hour post-exposure. Up to 24 hours, the highest 93% L3 mortality was in ACE group of 5 mg/ml, whereas ACE groups of 10 and 15 mg/ml recorded 91% each.

Table 1: Average of percentage of L3 mortality at ACE concentrations of 5, 10 and 15 mg/ml, and at 2, 4, 6 and 24 hour of observation

Group	ACE Concentration	n	L3 mortality (%)			
			Time			
			2 hour	4 hour	6 hour	24 hour
1	5 mg/ml	6	68	80	84	93
2	10 mg/ml	6	83	85	85	91
3	15 mg/ml	6	79	82	85	91

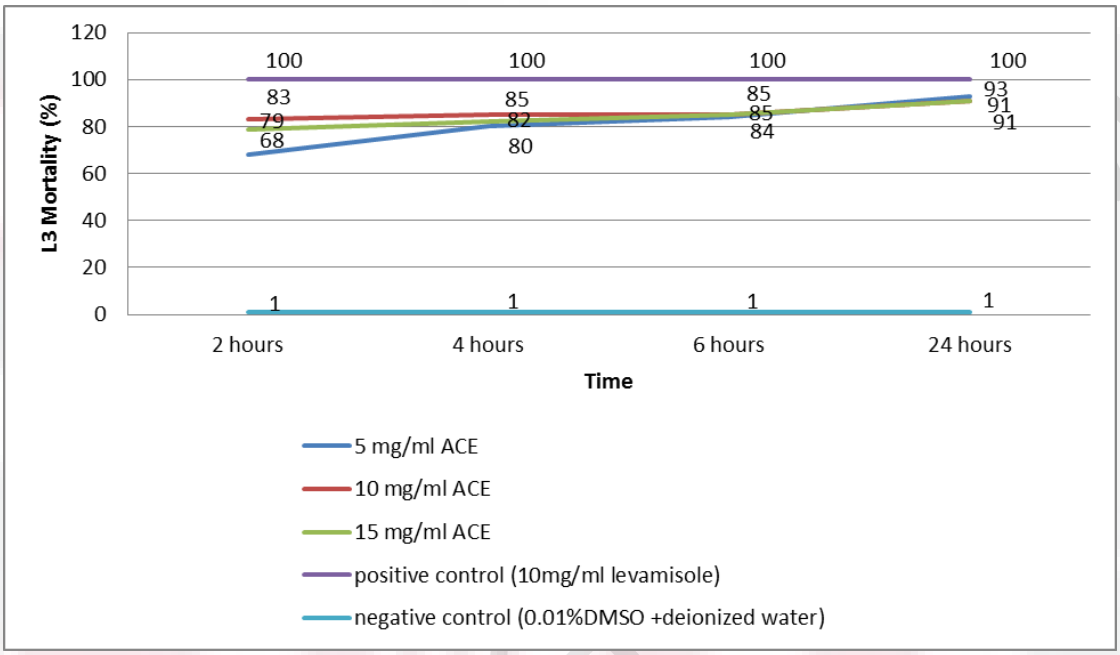

Figure 1: Average percentage of L3 mortality for the three groups treated with the neem leaves chloroform extract (ACE) (5, 10, 15 mg/ml), positive control and negative control groups

Table 2 shows that the L3 mortality was the highest (100%) in positive controls, followed by 10, 15, 5 mg/ml of the ACE groups, and the negative control. Among the three groups, 10 mg/ml ACE concentration recorded the highest average of L3 mortality. No significant difference in L3 mortality between ACE groups ($p>0.05$) was found. Kruskal-Wallis test showed that there was statistically significant difference in L3 mortality between all ACE and levamisole treated groups compared with the negative controls ($p<0.05$). Mann Whitney test showed that there was significant difference in the mean percentage of L3 mortality between all ACE and levamisole treated groups compared with the negative control group ($p<0.05$).

Table 2: The mean and standard error of the mean (SEM) of percentage of L3 mortality in three groups treated with the neem leaves chloroform extract (ACE) (5, 10, 15 mg/ml), positive control and negative control groups

Group	Treatment	n	L3 mortality (%)
			Mean ± SEM
1	5 mg/ml	6	81.04 ± 2.388^{ab}
2	10 mg/ml	6	86.50 ± 0.799^{ac}
3	15 mg/ml	6	84.17 ± 1.299^{abc}
4	Positive Control (10 mg/ml Levamisole)	6	100^{d}
5	Negative Control (0.01% DMSO + deionized water)	6	$0.83 \pm .155^{e}$

^{abcde} – average percentage of L3 mortality were significantly different at $p<0.05$

4.2 L3 mortality between ACE (10 mg/ml) and levamisole (10 mg/ml)

ACE treated group with 10 mg/ml was selected for comparison with the positive control group levamisole (10mg/ml) based on the highest L3 mortality recorded among the three ACE groups. Results showed that levamisole killed all L3 (100%) whereas ACE recorded 91% of L3 mortality over 24-hour exposure (Table 3 and Figure 2) and the difference was statistically significant ($p<0.05$).

Table 3: Average percentage of L3 mortality at 2, 4, 6 and 24 hours of observation for ACE treated (10mg/ml) and the Levamisole (10 mg/ml) groups

Groups	L3 mortality (%)			
	Time			
	2 hour	4 hour	6 hour	24 hour
ACE (15 mg/ml)	83	85	85	91
Levamisole (10mg/ml)	100	100	100	100

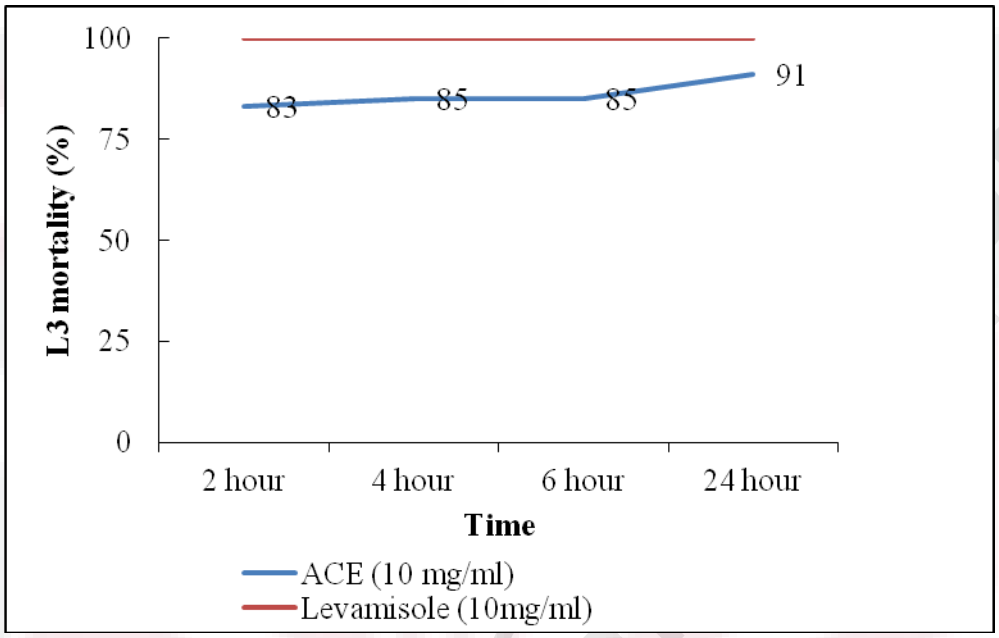

Figure 2: L3 mortality (%) of ACE (10 mg/ml) and Levamisole (10 mg/ml) groups

Chapter 5

DISCUSSION

The findings from the present study showed that ACE could cause mortality of the L3 of strongyles from sheep indicating *in vitro* anthelmintic efficacy although not 100% within 24 hours of observation. Levamisole is still a potent anthelmintic drug.

This study also found that the lowest concentration 5 mg/ml of ACE resulted in L3 mortality similar to the higher ACE tested concentrations (10 and 15 mg/ml) after 24-hours exposure. The explanation could be that the possibility that the dilution had changed into toxic form due to exposure to room temperature after certain period of time. In this case, the L3 mortality could be due to the toxic effect (Tiwari *et al.*, 2011) of the ACE solution instead of the bioactive compound in ACE that reacts on the L3. Other possibility could be due to the 'lock and key' mechanism as described for levamisole preveiously (Martin, 1997), in which the bioactive compound in ACE might have similar mechanism that binds with the L3 receptors. Once all the receptors have been fully bind with the bioactive compound or other physiological compounds, adding more bioactive compound would not cause changes anymore. Therefore, the highest L3 mortality will occur at certain concentration of ACE only.

In this study, it was found that L3 mortality varied with ACE concentration tested and time of observation. The reason could be due to that each species of strongyles has different type of receptors. Therefore, only certain receptors within each strongyles

species will combine with the bioactive compound, Azadirachtin in ACE. As a result, not all L3 will die during the period of observation. Strongyles species which receptors do not bind with Azadirachtin would still be alive. According to Sundaram (1996), Azadirachtin-A is a major bioactive compound in the neem leaves. Pessoa (2001) reported that Azadirachtin (1%) inhibited 68% of egg hatching in *H. contortus*. Azadirachtin interferes with the parasite's central nervous system by inhibiting excitatory cholinergic transmission and blocks the calcium channel resulting in expulsion of parasites from host body (Qiao *et al.*, 2013; Veerakumari and Priya, 2006). Other than the bioactive compound, ACE also contains few secondary metabolites such as Tannin and Glycosides (Imaran *et al*., 2010; Al Rofaai *et al.*, 2011**)**. These secondary metabolites have synergistic effect on L3 and may cause L3 mortality.

Neem has potential as herbal anthelmintic due to the fact that neem leaves are palatable to animals, and its availability as it can grow in our tropical region (Chandrawathani *et al*., 2013). Neem as natural product could potentially reduce chemical pollution (since chemical anthelmintic are readily available in the market) and could reduce cost for farmers to control GI parasitic problem in livestock. Interest in natural products as an alternative source of anthelmintic drugs has developed, stemmed from issues such as high costs of drugs, drug residues in animal and animal related products in food, and environmental pollution (Vieira *et al*., 1999).

Chapter 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, the present study revealed that ACE at different concentrations showed *in vitro* anthelmintic activity against L3 of strongyles from sheep and at 10 mg/ml concentration of ACE showed the highest anthelmintic activity. ACE also showed lower anthelmintic activity compared to levamisole. However, ACE exhibited considerable anthelmintic activity of more than 90% L3 mortality within 24 hours post treatment. These study findings indicate neem has potential as herbal anthelmintic for sheep.

6.2 Recommendations

The present study has assessed the anthelmintic activity of ACE on L3 mortality using *in vitro* method. More studies are needed before neem can be conclusively recommended for use in the control of helminthiasis due to strongyles infection in the farms.

Below are the recommendations for future studies:

- Repeat the experiment using lower concentration than 5 mg/ml of ACE e.g. 1 - 2.5 mg/ml, to determine the anthelmintic efficacy on L3 of strongyles.

- Further studies on mechanism of action of Azadirachtin as anthelmintic whether it has agonist or antagonist mechanism towards L3.
- Further studies on toxicity of neem and its level in animals as certain bioactive compound might counter react with the normal physiology of the target host body system.
- Further studies done *in vivo* on the most effective methods of administration of neem as an herbal anthelmintic to animals, for example by oral or injectable.

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