UNIVERSITI PUTRA MALAYSIA

THE EFFECTS OF METHANOL EXTRACTED *ARECA CATECHU* (BETEL NUT) AND LEVAMISOLE ON *IN-VITRO* SURVIVAL RATE OF STRONGYLES IN GOATS

NURUL FARLIANA BINTI MAT DESA

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A Project Report Submitted to the

Faculty of Veterinary Medicine, University Putra Malaysia

in Partial Fulfillment of the Requirement for the

Degree of Doctor of Veterinary Medicine University Putra Malaysia

Serdang Selangor Darul Ehsan.

MARCH 2016

It is hereby certified that we have read this project paper entitled "The Effects of Methanol Extracted *Areca catechu* and Levamisole on in *In-vitro* Survival Rate of Strongyles in Goats", by Nurul Farliana Binti Mat Desa and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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DEDICATIONS

I dedicate this thesis with love and appreciation to:

My parents and siblings

NORFAH HAMID and MAT DESA SAIDON

NURUL FATIN AZRINA and NURUL SYAMIMIE

My furry friends

ABBY, BABU, NAOMEI, AFIL'S FAMILY

My friends and FYP mates

IDA AMALINA MAHADI

NURUL HAIRUNNISA

AISYAH PAUZI

NUR HUSNA ATIKA AZHAR

My Coursemates

DVM 5, BATCH 2016

ACKNOWLEDGEMENTS

All praises be to Allah Almighty, I wish to express my gratitude to my supervisor, Dr. Rozaihan Mansor for her guidance, help and precious time spent for the discussion and correction of my project and thesis.

I would like to acknowledge of my co-supervisor Prof. Madya Dr. Shaikh Mohamed Amin Babje for his contribution towards better understanding of my project, guidance and willingness to spend time with me to finish my final year project.

I also would like to recognize Prof, Rehana Abdullah Sani, Dr. Nurul Hayyan Basripuzi, Prof. Ariff Omar, staff and post-graduate students of Parasitology Laboratory, Pharmacology Laboratory and Faculty of Veterinary Medicine Universiti Putra Malaysia, for their kindness and help till the completion of my project.

I appreciate and thanks to my family especially my parents for their continuous dua', love, patience and support from the beginning up until the end of my project.

Last but not least, I would like to acknowledge all of my friends fo their help and support. May our friendship last till jannah.

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ABSTRAK

Abstrak daripada kertas kerja projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek.

KESAN EKSTRAK METANOL*ARECA CATECHU* (PINANG) DAN LEVAMISOL KE ATAS KADAR KEMANDIRIANSTRONGIL SECARA IN-VITRO PADA KAMBING

Oleh:

Nurul Farliana Binti Mat Desa

2016

Supervisor: Dr. Rozaihan Mansor

Co-Supervisor: Prof. Madya Dr. Shaikh Mohamed Amin Babje

Penggunaan meluas antelmintik kimia dalam pengurusan gastroenteritis parasit (PGE) telah mewujudkan rintangan, sekali gus mengurangkan keberkesanannya. Kajian ini telah dijalankan untuk membandingkan keberkesanan ekstrak *Areca catechu* (pinang) dan levamisole terhadap kadar kemandirian larva strongil peringkat ketiga (L3) pada kambing dan untuk menentukan konsentrasi efektif ekstrak pinang (EP) untuk aktiviti larvacidal terhadap strongil L3 dalam keadaan *in-vitro*. Bahan tinja segar kambing yang dijangkiti secara semula jadi telah dikumpulkan dan larva peringkat kedua dituai selepas 7 hari untuk menganggarkan bilangannya. Ekstrak metanol pinang pada 300, 600 dan 1200 µg/ml, levamisol pada 10 mg/ml dan PBS ditambah 0.1% DMSO telah disediakan untuk menentukan kesan aktiviti larvisid mereka. EP pada 1200 µg/ml menunjukkan 61.70% daripada L3 mati dalam tempoh 24 jam selepas ekstrak didedahkan, pada masa yang sama levamisol pada 10 mg/ml menunjukkan kadar kematian 100% daripada L3 dalam tempoh 2 jam selepas ekstrak didedahkan ($p < 0.05$). Peratusan kematian tertinggi strongil L3 (43.40%) dimiliki oleh *Oesophagostomum* sp, diikuti oleh 40.90% daripada *Haemonchus* sp dan 15.70% daripada *Trichostrongylus* sp. Walaupun kesan EP tidak efektif seperti levamisole, ia mempunyai pengaruh besar dalam aktiviti antelmintik terhadap larva strongil pada kambing dan boleh digunakan sebagai alternatif antelmintik pada masa hadapan.

Kata kunci: Pinang (*Areca catechu*), Strongil, Larvasid, Metanol.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999-Project

THE EFFECTS OF METHANOL EXTRACTED *ARECA CATECHU* (BETEL NUT) AND LEVAMISOLE ON *IN-VITRO* SURVIVAL RATE OF STRONGYLES IN GOAT

By:

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2016

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Co-Supervisor: Prof. Madya Dr. Shaikh Mohamed Amin Babje

The extensive use of chemical anthelmintics in parasitic gastroenteritis (PGE) management has created resistance, thus reducing their effectiveness. The present study was conducted to compare the effectiveness of *Areca catechu* (betel nut) extract and levamisole on survival rate of the third stage larvae (L3) strongyles of goat and to determine effective concentration of betel nut extract (BNE) for larvacidal activity against of L3 strongyles in *in-vitro* condition. Fresh fecal materials of naturally infected goats were collected and the second stage larvae were harvested after 7 days to estimate their numbers. Methanolic extract of betel nut at 300, 600 and 1200 µg/ml, Levamisole at 10 mg/ml and PBS plus 0.1% DMSO were used to determine their larvacidal effects. BNE at 1200 µg/ml showed 61.70% of L3 died within 24-hours post exposure while Levamisole at 10 mg/ml showed 100% mortality rate of L3 within 2-hours post exposure ($p<0.05$). The highest L3 strongyles death percentage (43.40%) belongs to *Oesophagostomum* sp, followed by 40.90% of *Haemonchus* spand 15.70% of *Trichostrongylus* sp. Although the effects of BNE is not as effective as levamisole, it has a considerable anthelmintic activity against strongyles larvae in goats and can be used as an alternative anthelmintic in future.

KEYWORDS: Betel nut (*Areca catechu*), Strongyles, Larvacidal, Methanol.

Chapter 1

INTRODUCTION

1.1 Background

Parasitic gastroenteritis (PGE) is the second most important disease of small ruminant in Malaysia causing high morbidity and mortality of the animals (Sani and Chandrawathani, 1996). Goats are more susceptible compared to sheep (Basripuzi *et al.,* 2012; Sani *et al*., 2004) and the common nematodes (mostly strongyles) commonly found in small ruminants are *Haemonchus contortus, Trichostrongylus* and *Oesophagostomum*. *H.contortus* was reported to be the most prevalent species in small ruminant farms in Peninsular Malaysia (Basripuzi *et al.,* 2012; Khadijah *et al.,* 2006a and 2006b).

Because of the extensive use of chemical anthelmintics such as levamisole and mebendazole, their effectiveness is restricted by the development of antihelmintic resistance (Waller, 2002). Thus, increase the usage of organic compounds from plants asa good substitute or alternative to commercial chemical dewormers in order to slow down the development of current anthelmintic drug resistance.

The betel nut (*Areca catechu*) is widely distributed in Southern and Southeast Asia including China, India, Indonesia, Malaysia, Philippines and New Guinea (Amudhan Begum and Hebbar, 2012). Modern investigations demonstrate that betel nut has various pharmacological activities including antiparastic, antioxidant, antibacterial, antifungal, anti-inflammatory, analgesic, anti-allergic, effects on digestive, nervous and cardiovascular system and regulatory effects on blood glucose and lipids (Peng *et* *al.,* 2015). Alkaloid is the primary active ingredients in plant-derived medicines (Amirkia and Heinrich, 2014) and it has been reported that arecoline is the main alkaloid in betel nut (Peng *et al.,* 2015; Huang and Michael, 1989). Additionally, arecoline served as the paralyzing agent (antihelmintic alkaloids) in *Areca catechu* targeting the acetylcholine receptors of the parasites which disrupts their nervous systems (Bahmani *et al.,* 2014; Peng *et al.,* 2015). Besides, the betel nut also contains polyphenolic compound such as tannins which possess antioxidant activity (Hannan *et al.,2012).*

1.2 Justification

PGE in small ruminants is the most common disease that cause high morbidity and mortality, thus causing economic losses to farmers. The abundance of betel nut in our country will provide an oppurtunity to explore its effectiveness as an alternative anthelmintic. Moreover, it can reduce the problem of drug resistance induced by the chemical anthelmintic drugs. This will helps the farmers to strategize the use of chemical drugs and natural remedies that would bemore economical to them.

1.3 Study Objectives

a) To compare the efficacy of processed mature betel nut and levamisole on survival rate of strongyles in goats.

b) To determine the effective concentration of processed mature betel nut extraction against larvacidal activity of strongyles in *in-vitro* condition.

1.4 Study Hypothesis

The hypothesis of this study is *Areca catechu* (Betel nut) extracts has an anthelmintic effectagainst the survival rate of L3 strongyles of small ruminants

Chapter 2

LITERATURE REVIEW

2.1 Strongyles in Small Ruminants

Gastrontestinal (GI) strongyles infections remain as one of the main constraints to goat production worldwide (Rinaldi *et al.* 2009). The hot, wet, tropical climate all year round favours the continuous GI strongyles infections in sheep and goats (Chandrawathani and Nurulaini, 2012). In Sani and Gray's study (as cited in Basripuzi, Sani and Ariff, 2012), the significant species of nematodes in Southeast Asia include *Haemonchus contortus, Trichostrongylus, Strongyloides and Oesophagostomum.* All grazing sheep and goats are infected with a community of these strongylid nematodes whose combined clinical effects is commonly known as parasitic gastroenteritis (PGE) (Zajac, 2006).

Strongyle infections, namely *Haemonchus* spp. is the major cause of helminth disease in South East Asia causing severe losses in terms of mortality and morbidity (Chandrawathani and Nurulaini, 2012). *H. contortus* adults are abomasal parasites of small ruminants that feed on host blood and it is the one of the most prolific nematodes (Zajac, 2006). Haemonchosis can cause fatal anemia and hypoproteinemia which are very common clinical signs in sheep and goats (Dorny, Symoens, Jalila, Vercruysse and Sani, 1994).

Trichostrongylus spp. larvae spend a variable period of time developing in the abomasal gastric glands. In heavy infections, sheep or goats may develop diarrhea, anemia, hypoproteinemia or in severe cases, death can occur (Zajac, 2006). According to Soulsby's study (as cited in Dorny *et al., 1994) Oesophagostomum*spp. is another serious pathogen for sheep and goats because it can cause severe diarrhoea containing mucus and blood. It is normally found in the large intestine (Zajac, 2006).

2.2 *Areca catechu* (Betel nut)

Betel nut is a seed of the *Areca catechu* tree, under the family of Arecaceae (palm family) which contains over 200 genera and 2600 species (Ali and Khuwaja, 2011). The nut found within the fruit is mottled brown and has grayish white markingsand can be used by man either fresh or dried by sun-drying or roasting. It has characteristic of an astringent and has slightly bitter taste when consumed at different stages of maturity according to preference (Lingapa, Nappalli, Sujatha and Shiva, 2011).Chewing betel nut along with betel leaf (*Piper betle*), lime and sometimes tobacco and spices is an ancient and popular practice among 200-400 million people (Gupta and Warnakulasuriya, 2002).

The plant is reported to have multiple therapeutic properties like antiparasitic effects, anti-depressive effects, anti-fatigue effects, antioxidant effects, antibacterial and antifungal effects, antihypertensive effects, anti-inflammatory and analgesic effects, anti-allergic effects and promotion of digestive functions (Peng *et al.,* 2015). The major constituents of the nut are carbohydrates, fats, protein, crude fibre, polyphenols (flavonols and tannins), alkaloids (arecoline, arecaidine, arecaine, arecolidine, guvacine, isoguvacine, guvacoline and coniine) (Lingapa *et al.,* 2011).Among the alkaloids, 0.12-0.24% of arecoline in ripe nut has been reported to be themain alkaloid having cholinergic muscarinic agonist activity and is present in the seed as salt of tannic acid. About 8-18% polyhenolic compounds of the nut are tannins and the average tannin content in sun dried nut is 25% (Hannan, Karan and Chatterjee, 2012).

The areca nut is a traditionalmedicine commonly used to kill parasites including tapeworms, ascaricide and pinworms (Peng *et al.,* 2015). According to Feng's study (as cited in Peng *et al.,* 2015), the water extracts of areca nut (WEAN) can effectively kill tapeworms related to the paralytic effects of the WEAN against the scolex of tapeworms. This is because arecoline in the WEAN was found to be the active constituent against tapeworms and the mechanism was correlated with paralytic effect (disruption of nervous system) based on Zheng's study (as cited in Peng *et al.,* 2015). As an anthelmintic alkaloid, arecoline is known as paralyzing agent that target acetylcholine receptors of the organisms (Bahmani *et al.,* 2014).

Tannin has an anthelmintic effect to form complexes with parasite proteins (Alonso-Díaz, Torres-Acosta, Sandoval-Castro and Hoste, 2011). According to Hoste's study (as cited in Alonso-Díaz *et al.,* 2011), tannin seem to affect the biological processes of nematodes depending on where and how it bind with various nematode structures such as cuticle, digestive or reproductive tracts. Other than that tannin also possess antioxidant activity and have excellent antioxidant and anti-inflammatory capacity against oxidative stress-induced damage (Huang, Chi and Liu, 2010).

2.3 The Methanolic Extraction Method

Arecoline and tannins are the main chemical constituents of Areca nut that can serve as anthelmintic in this study. Methanol was chosen as a solvent for betel nut extraction because of being the highest in polarity and it can dissolve most of the chemicals present in plant compared to other solvents (Giri, Roy and Babu, 2013). The process of extraction requires the betel nut to be completely dried by sun drying, oven drying or both and later ground into the powder form before being placed into a container containing1 litre of 99.8% of methanol.

According to Hannan *et al.,* (2012) percentage of tannin content (TC) and total phenolic content (TPC) was found higher in 100% methanol extract (ME) than in petroleum ether, ethyl acetate and in water extract when extracted successively by maceration.

2.4 Levamisole Usage in Endoparasite Management

Commercial broad-spectrum anthelmintics that are currently available belong to five different groups include benzimidazoles (white drenches), imidazothiazole (yellow drenches), macrocyclic lactones (clear drenches), amino-acetonitrile derivatives and spiroindoles (Mark, 2014). Levamisole is a member of imidazothiazole other than pyrantel and morantel that is also known as nicotinic agonist drug that exert their effect on the nervous system of the parasite (Köhler, 2001).It works well against adult worms but less against the immature stages (e.g. L4) and it is particularly effective against the lungworms (Handbook for the Control of Internal Parasites of Sheep and Goats, 2012).

Recent studies by Evans and Martin, 1996; Martin et al., 1996 and Martin et al., 1998 (as cited in Köhler, 2001), stated the surface of somatic muscle cells of nematodes possess nicotinic acetylcholine receptors (nAChR) that can be opened by the nicotinic anthelmintics. Binding of these compounds to the recognition site of the excitatory receptors induces depolarization and spastic paralysis of the nematode muscle, that result in parasite expulsion (Köhler, 2001). Because of levamisole also acts as a nicotinic agonist in mammals, it has a rather narrower therapeutic index than other popular broad spectrum anthelmintic. Therefore some care is required when medicating a flock of animals with a large range of body weights (McKellar and Jackson, 2004).

Signs of toxicity in animals include salivation, slow heart rate and muscle tremors with occasional death (Handbook for the Control of Internal Parasites of Sheep and Goats, 2012).In goats, levamisole is excreted faster, which requires administering a higher dose than in sheep (Junquera, 2015).

According to Martin and Robertson, (2007), the development of resistance to cholinergic agonists may be produced by four general mechanisms. First, changes in drug translocation (e.g. increased metabolism or excretion of the drug). Second, changes in receptor number (reduced number of receptors). Third, receptor modification (e.g. change in receptor binding sites due to amino acid substitution). Fourth, post-receptor modification (e.g. change in the downstream pathway to contraction).

2.5 The Effects of Betel Nut and Levamisole as Anthelmenthic

Based on the studies done by Jaishwal and Singh (2008), arecoline (active compound of *Areca catechu* seed) act as molluscicidal by inhibiting the acetylcholinesterase (AchE), acid and alkaline phosphatase (ACP/ALP) activity in the nervous tissue of freshwater snail, *Lymnaea acuminuta* as (as cited in Patil, Rakesh, Dhabale and Burade, 2009). Furthermore, arecoline acts as a vermifuge, causing the detachment of scolex from the intestinal mucosa by inducing muscular paralysis of worms and increase intestinal motility to expel detach worms alive (Laudato and Capasso, 2013). Since, this experiment was done in-vitro, therefore the expulsion of live worms cannot be appreciated.

According to Thompson and Geary (1995), tannin may acts as anthelmintic activity due to its capacity to bind to proteins which resulted in nutrient reduction availability, thus cause the larvae starve and die. Another studies done by Alonso-Díaz *et al.* (2011), reported that tannin-rich plant extracts are more potent inhibitors of the exsheathment of *H.contortus* L3 larvae than inhibit their motility.

In Robertson and Martin's study (as cited in McKellar and Jackson, 2004), they reported that levamisole acts as a cholinergic agonist at nicotinic neuromuscular junctions in nematode parasites, first by opening and then blocking the acetycholine receptor-mediated cation channels. This mechanism will induces depolarization and spastic paralysis of the nematode muscle that aid in parasite expulsion (Köhler, 2001).

Chapter 3

MATERIALS AND METHODS

3.1 Material Preparation

Plant materials: Mature *Areca catechu* fruits (betel nut) were purchased from the owner of the *Areca catechu* plants, located in my hometown of Sungai Karangan, Padang Serai, Kedah. Only normal looking fruits, without any damage were chosen for this experiment. The husks of the fruits were removed and the nuts were exposed before cutting them into small pieces and dried under the sun for 3 days. The dried nuts were grounded to powder by using a mechanical grinder and stored at room temperature.

3.2 Fecal Egg Count (FEC) by using McMaster Chamber Technique

Fecal samples of naturally infected goats were collected via rectum in the morning at De Kebun Farm located in Puchong, Selangor. About 3gm of faeces were emulsified in saturated sodium chloride (NaCl) solution in a plastic beaker to break up the faeces. Later, saturated NaCl solution was added up to 30ml and mixed well before being filtered through tea sieve into a clean beaker. The fecal solution was mixed well and pipetted into the two chambers of McMaster slide. The ova/oocysts in both chambers were calculated separately. The FEC of strongyles ova were calculated to give a rough estimation of worm burden count of animals in the herd.

Formula:

$$\frac{\text{No.of ova or oocysts in both chambers}}{\text{Wt.of faeces (g)}} \times \frac{\text{Vol.NaCl (ml)}}{2(0.15)} = \text{---------ova/g}$$

3.3 Fecal Culture and Harvesting L3

Fecal culture was performed to harvest larval stage 3(L3) from the culture and to identify the species of L3 strongyles under the light microscope. First, we need to place approximately 30g of fecal material into several small glass jars. Distilled water were added into the glass jars to maintain its moisture before being covered with the gauze and kept at room temperature for 7 days. After 7 days, L3 stongyles can be harvested by filling in the warm distilled water into the glass jars up to the edge of glass mouth. Then, the petri dishes were inverted and covered the mouth of glass jars before turn them over. Lastly, we can observed the active L3 strongyles swimming by observing under the dissecting microscope and the species of L3 strongyles were recorded. The L3 strongyles stock solution can be kept in the falcon tube at 4°C until future use.

3.4 Identification of L3 strongyles

About 20µl of L3 stock solution was placed on the petri dish before one drop of Lugol's iodine was added and covered with a cover slip. The slide was examined under the microscope with the magnifcation of 400x to determine the species of L3 strongyles. These steps were repeated multiple times to give the average total number of each species of L3 strongyles present in the whole stock solution. In 30ml of L3 stock solution, it consists of 30,000 of L3 strongyles comprising 30% *Oesophagostomum* spp. , 40% *Haemonchus* spp. and 30% *Trichostrongylus* spp.

The morphology of L3 strongyles species can be differentiated based on its anterior (head) and posterior (tail) parts. Reffering to Manual of Veterinary Parasitological Laboratory Technique (1986), Haemonchus spp. had a narrow rounded head with short cone tail as shown in Figure 1. While, Trichostrongylus spp. had a tapered head with medium cone tail as shown in Figure 2. Lastly, Oesophagostomum spp. had a broad rounded head with elongated filamentous tail as shown Figure 3.

3.5 *Areca catechu* (Betel Nut) Methanolic Extract

First, about 100g of betel nut powder was mixed with 1L of 99.8% of methanol solution in the glass jar. The mixture were kept at room temperature for 3 days. Later, the mixture were filtered with filter paper No. 1 Whattman England to get pure betel nut extractsolution. The extract solution were concentrated by using rotary vacuum evaporator at 40°C with 60 rpm to facilitate the evaporation process of methanol. Finally, the semi-solid form of betel nut extract were kept in the glass bottle in the freezer until further use.

3.6 Preparation of Betel Nut Extract (BNE) and Levamisole Concentration

Betel nut extract (BNE) and Levamisole were diluted with Phosphate Buffer Saline (PBS) containing 0.1% Dimethyl Sulfoxide (DMSO) to get 300µg/ml, 600µg/ml and 1200µg/ml of BNE and 10mg/ml of Levamisole concentration.

3.7 Motility and Mortality Assessment of L3 Strongyles

20µl of L3 stock solution containing 20 live L3 strongyles were added into 30 petri dishes. Each treatment groups had 6 duplicate petri dish. 3ml of BNE at 300µg/ml, 600µg/ml and 1200µg/ml were added into 18 petri dishes. 3ml of Levamisole at 10mg/ml was added into 6 petri dishes as the positive control. 3ml of PBS containing 0.1% DMSO was added into 6 petri dishes as the negative control.

Petri dishes were shaken manually for 30 seconds after starting the treatment and were kept at room temperature. Each petri dishes were examined for 2, 4, 6 and 24 hours post exposure to determine the motility and mortality of L3 strongyles. The motility of L3 strongyles can be confirmed with the coiling shape of L3 strongyles with minor movements of its head and tail (Figure 4). While the dead L3 strongyles can be confirmed when L3 strongyles appear straight in shape with the absence of minor movements (Figure 5). The mortality index formula was use to determine the mortality percentage of L3 strongyles:

$$\text{Mortality index} = \frac{No.dead\ L3\ strongyles}{Total\ number\ of\ L3\ strongyles\ in\ petri\ dish} \text{ x } 100\%$$

Figure 1: *Haemonchus* sp. with narrow rounded head and short cone tail

(400x magnification)

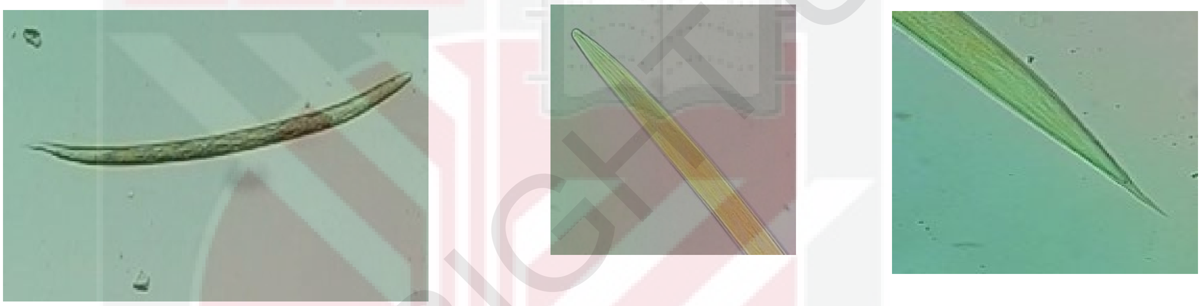
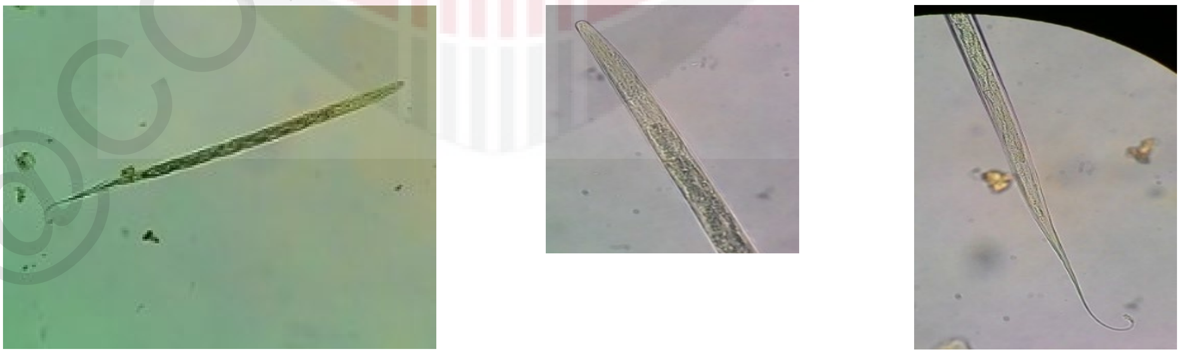


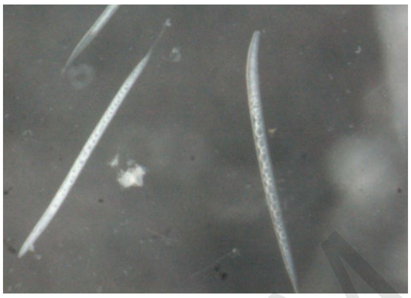
Figure 2:*Trichostrongylus* sp. with tapered head and medium cone tail

(400x magnification)



Oesophagostomum sp. with broad rounded head and elongated filamentous tail

(400x magnification)



Alive L3 strongyles(coil
n appearance)

Figure 5: Dead L3 strongyles
(straight in appearance)

3.8 Statistical Analysis

The statistical analysis was performed by using SPSS version 22 statistical software. Since, the data was not normally distributed $p>0.05$, Kruskal-Wallis and Mann-Whitney Test were used to determine the significant difference between the death percentage of L3 strongyles with the different concentration of treatment groups (BNE at 300 µg/ml, 600 µg/ml amd 1200 µg/ml and Levamisole at 10 mg/ml).

Chapter 4

RESULTS

4.1 Larvae Stock Solution

Before the experiment was conducted, the percentage of three species of L3 strongyles were calculated by taking the average of each species present in 20µl of larvae stock solution. For each 20µl of larvae stock solution, it comprised of 20 L3 strongyles. In 30ml of stock solution contains 30,000 of L3 with 40% of *Haemonchus* spp, 30% of *Trichostrongylus* spp. and 30% of *Oesophagostomum* spp.

4.2 Relation between Death Percentage of Larvae and Treatment Groups

Ranks

	GROUP	N	Mean Rank
DEATH_PERCENTAGE (%)	LEV 10mg/ml	24	108.50
	PBS+0.1%DMSO	24	12.50
	BNE 300 µg/ml	24	53.31
	BNE 600 µg/ml	24	60.81
	BNE 1200 µg/ml	24	67.38
	Total	120	

Table 1a: Kruskal-Wallis test

Test Statistics[a,b]

	DEATH_PERCENTAGE (%)
Chi-Square	94.803
df	4
Asymp. Sig.	.000

Table 1b: Kruskal-Wallis test

The results (Table 1) showed p-value is 0.000, which is less than 0.05. Thus, death percentage is significantly different between treatment groups of levamisole, PBS+ 0.1% DMSO and betel nut extracts (BNE). The mean rank of Levamisole 10mg/ml is the highest followed by BNE 1200µg/ml, BNE 600Mg/ml, BNE 300µg/ml and PBS+DMSO which are 108.50, 67.38, 60.81, 53.31 and 12.50. While, the results of test for death percentage between times were not significantly different($p > 0.05$).

4.3 Mean of Death Percentage of Larvae by Treatment Groups

Treatment Groups	n	x̄ ± s.e		
PBS + 0.1% DMSO	6	3.13 ± 0.786	a	
BNE 300µg/ml	6	41.88 ± 1.643	**b**	
BNE 600µg/ml	6	45.21 ± 2.341	**b**	**c**
BNE 1200µg/ml	6	50.21 ± 2.700		**c**
LEV 10mg/ml	6	100	d	

Table 2: Mann-Whitney test results between treatment groups

- abcd means in some columns are significantly different at $p < 0.05$
- same alphabets (e.g. aa or bb) are not significantly different at $p > 0.05$

Interpretations :-

The mean difference between PBS + 0.1% DMSO and BNE at 300µg/ml is significantly different ($p < 0.05$).

The mean difference between PBS + 0.1% DMSO and BNE at 600µg/ml is significantly different ($p < 0.05$).

The mean difference between PBS + 0.1% DMSO and BNE 1200µg/ml is significantly different ($p < 0.05$).

The mean difference between PBS + 0.1% DMSO and LEV 10 mg/ml is significantly different ($p < 0.05$).

The mean difference between BNE 300µg/ml and BNE 600µg/ml is not significantly different since ($p > 0.05$).

The mean difference between BNE 300µg/ml and BNE 1200µg/ml is significantly different ($p < 0.05$).

The mean difference between BNE 600µg/ml and BNE 1200µg/ml is not significantly different ($p > 0.05$).

The mean difference between BNE 300µg/ml and LEV 10 mg/ml is significantly different ($p < 0.05$).

The mean difference between BNE 600µg/ml and LEV 10 mg/ml is significantly different ($p < 0.05$).

The mean difference between BNE 1200µg/ml and LEV 10 mg/ml is significantly different since sig (0.000) < α (0.05).

To simplify:-

- There is a significant difference in death percentage between Levamisole 10mg/ml with PBS+0.1% DMSO and all BNE concentration.
- There is a significant difference in death percentage between PBS+0.1% DMSO and all BNE concentration indicates BNE has an anthelmintic effect against L3 strongyles.
- There is a significant difference in death percentage between BNE at 300µg/ml and BNE at 1200µg/ml indicates the higher the concentration, the higher the death percentage of L3 strongyles.

4.4 Different Concentration of Betel Nut Extracts

Different concentration of betel nut extracts showed significant difference in the percentage of strongyles dead over time. The higher the concentration of betel nut extracts, the higher the percentage of dead strongyle over 24-hours post exposure. Betel nut concentration at 300 μg/ml killed 49.2%, 600 μg/ml killed 54.2% and 1200 μg/ml killed 61.7% of L3strongyles in 24-hours post exposure (Figure 6).

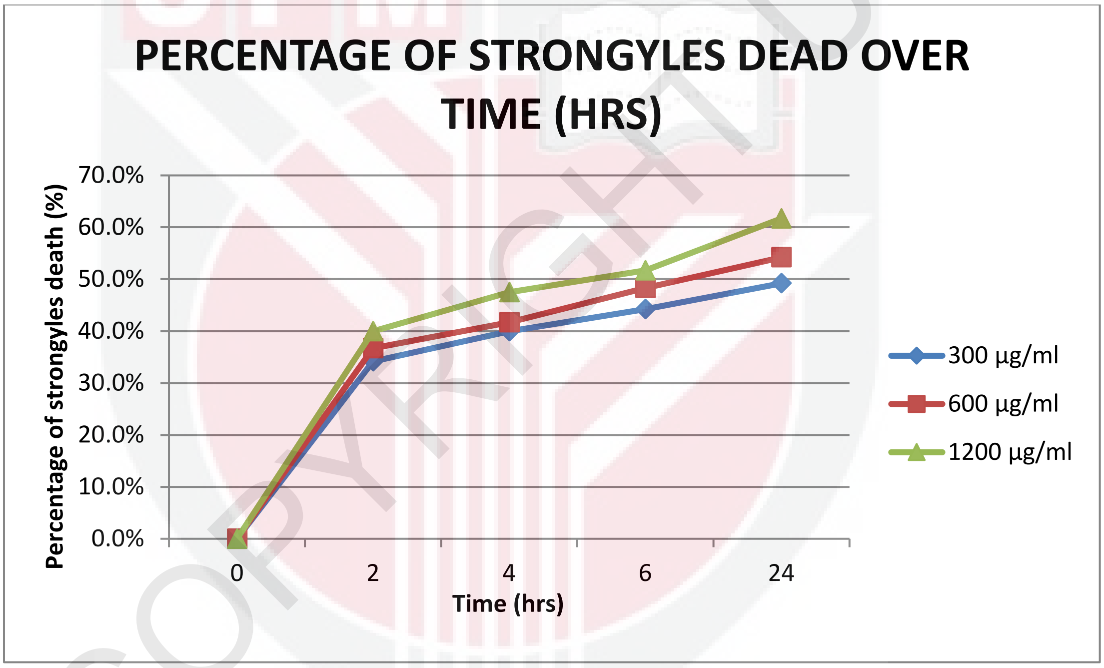


Figure 6: Percentage of strongyles dead over time of different betel nut extract.

4.5 Comparison Effects of Betel Nut Extract at Highest Concentration of BNE (1200µg/ml) and Levamisole (10mg/ml)

The result showed there was a significant difference between BNE at1200 µg/ml and Levamisole 10mg/ml on the survival rate of strongyles over 24-hours post exposure ($p<0.05$). Levamisole at concentration 10mg/ml killed 100% of strongyles just within 2-hours post exposure, while the BNE at 1200 µg/ml killed 40% of strongyles in 2 hours, 47.5% in 4 hours, 51.7% in 6 hours and 61.7% in 24-hours post exposure (Figure 7).

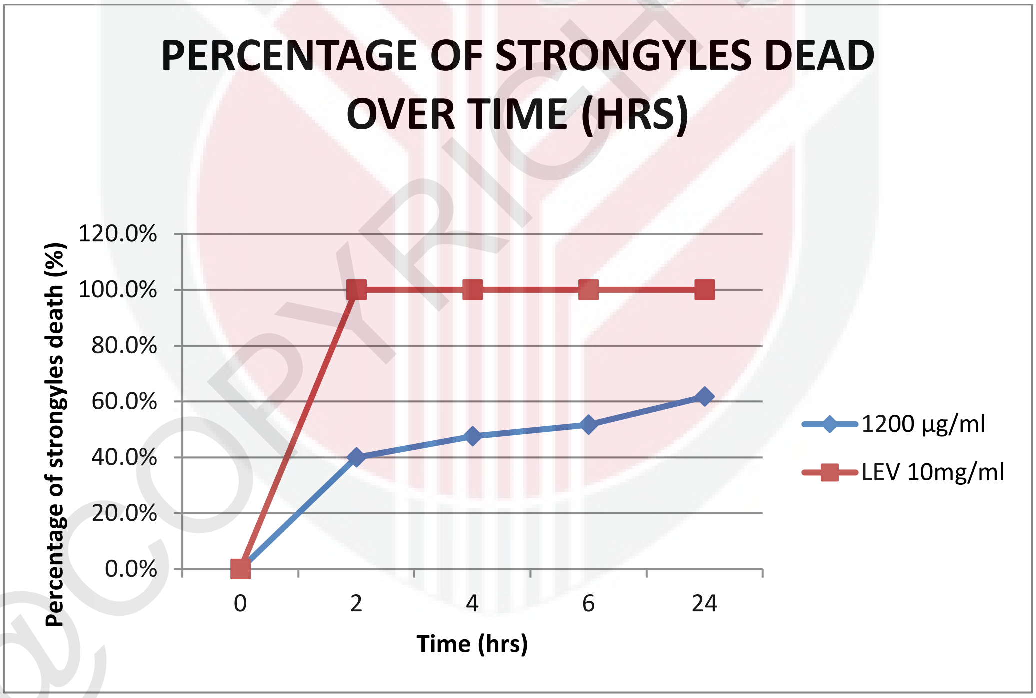


Figure 7: Percentage of strongyles dead over time between highest concentration of betel nut extract and levamisole

4.6Susceptible Species of L3 Strongyles Towards Betel Nut Extract

The rough estimation for the mortality percentage for each species of strongyles were calculated based on the percentage of each species of L3 strongyles used in this experiment. 40% of *Haemonchus contortus*, 30% of *Trichostrongylus* sp. and 30% of *Oesophagostomum* sp were used in this experiment for each petri dish. The highest mortality percentage about 43.40% belongs to *Oesophagostomum* spp, followed by 40.90% of *Haemonchus* spp.and 15.70% of *Trichostrongylus* spp (Figure 8)

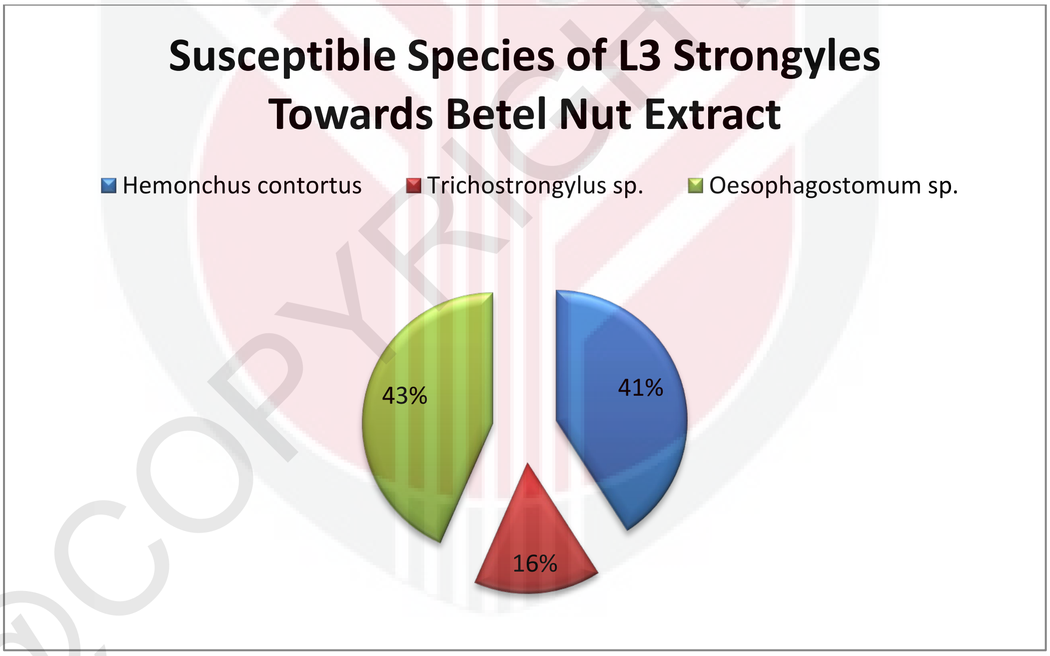


Figure 8: Percentage of total each species of L3 strongyles dead post betel nut extracts exposure

Chapter 5

DISCUSSION

In this study, betel nut extracts were choosed to be an alternative anthelmintic to small ruminants. The concentration used in this study were refered to previous studies done by Alonso-Díaz *et al.* (2011) on*in-vitro* test of anthelmintic activity of tropical tannin rich plant extract against *H.contortus*. Phosphate buffer solution (PBS) plus 0.1% of Dimethyl Sulfoxide (DMSO) were used as a standard diluent in all treatment group concentation especially in BNE. This is because normally DMSO was used to dissolve the residues of methanol, acetone and chloroform extracts (Chowdhury, Kubra and Ahmed, 2014).

The results from Mann-Whitney test showed the mean rank for Levamisole at 10 mg/ml is 36.5, while the mean rank for PBS+0.1% DMSO,BNE at 300µg/ml, 600µg/ml and 1200µg/ml are 12.5. In this case, the Levamisole 10 mg/ml had the highest death percentage compared to PBS+0.1% DMSO and all BNE concentration. The p-value is less than 0.05, thus there is a significant different in death percentage between Levamisole 10mg/ml with PBS+0.1% DMSO and all different concentration of BNE. Therefore, the null hypothesis will be rejected to accept the alternative hypothesis that state the BNE has anthelmintic effect against L3 strongyles.

The p-values of PBS+0.1% DMSO and all different concentrations of BNE are less than 0.05 and this showed there is a significant difference among the treatment groups.

In this case, all betel nut extracts had the highest death percentage with mean rank of 36.50 compared to PBS+0.1% DMSO which is 12.50. This indicates the BNE has a considerable anthelmintic activity when compared with PBS + 0.1% DMSO that serve as the negative control.

The p-value between BNE at 300µg/ml and BNE at 1200µg/ml is 0.022, which is less than 0.05. Hence, there is a significant different between both of treatment groups. In this case, BNE 1200µg/ml had the highest death percentage with mean rank of 29.06 compared to BNE 300µg/ml which is 19.94. It can be concluded that the higher the concentration of BNE, the higher the death percentage of L3 strongyles.

In 24-hours post exposure, the highest mortality percentage were found in BNE at 1200 µg/ml followed by BNE at 600 µg/ml and BNE at 300 µg/ml (61.7%, 54.2% and 49.2%, respectively) compared to positive control, Levamisole at 10 mg/ml which cause 100% mortality within 2-hours post exposure.

The significant result between Levamisole at 10 mg/ml and BNE at 1200 µg/ml showed levamisole can kill all L3 strongyles in the shortest time compared to the highest concentration of BNE at 1200 µg/ml. This might be because of the wide range in concentration unit between levamisole and BNE. Based on this result, BNE shows its anthelmintic effect against L3 strongyles eventhough it is not as potent as levamisole.If we take the effect of BNE at 1200 µg/ml in 24-hours post exposure that kill 61.7% of L3 strongyles as a cut off point of its effect, the other 40% may be supported by other parameters such as good nutrition and environment of the animals. In future, if we extend the time to 48 hours may be 100% mortality of L3 strongyles could be achieved at 1200 µg/ml of BNE.

Furthermore, I choosed 1200 µg/ml as the highest concentration for BNE because BNE has its toxic effects. According to Porter's studies (as cited in Peng *et al.,* 2015) arecoline has also shown genotoxic, mutagenic and carcinogenic effects which is closely associated with oral mucosal fibroblast (OMF) in human. According to Dasgupta *et al.,*(2006), arecoline cause hepatotoxicity, arrest the splenic lymphocytes cell cycle and kidney problem in albino mice (as cited in Peng *et al.,* 2015). Therefore, it is important to find potential strategy to balance the pharmacological and toxic effects of arecoline for the future perspective.

Chapter 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Betel nut extract (BNE) concentration at 1200 µg/ml showed a significant anthelmintic effect with 61.7% L3 mortality within 24-hours post exposure. While, Levamisole at concentration of 10 mg/ml showed highest anthelmintic effect with 100% L3 mortality within 2-hours post exposure. Therefore, this study can be concluded that BNE has a considerable anthelmintic effect against L3 strongyles, eventhough it is not as potent as levamisole. Further research on BNE should be done to define its better larvacidal effect as an alternative anthelmintic against strongyles in small ruminants.

6.2 Recommendations

As for the recommendations for future study are,

- Future research of betel nut extract (BNE) at concentration higher than 200µg/ml to determine the most effective concentration of BNE that expressed lethal effect on the L3 strongyles.
- *In-vitro* studies of BNE by using different extraction techniques like aqueous extraction or by using different solvents such as acetone, chloroform or ethyl ether.
- Study other active ingredients from BNE that possess as anthelmintic activity against L3 strongyles.
- Study extensively the possible toxicology properties of BNE on animals to determine its therapeutic index.

- *In-vivo* studies on animal model to evaluate the effectiveness of BNE in expelling the adult strongyles together with ova and to determine its anthelmintic effects on different species of animals.

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