



EVALUATION OF *In Vitro* ANTHELMINTHIC EFFECT
OF CRUDE LEAVES EXTRACT OF *Piper betle* L.
(SIREH) ON COMMONEST GASTROINTESTINAL
HELMINTHS OBTAINED FROM GOATS

BY

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A thesis submitted in fulfilment of the requirement for the
degree of Master of Health Science (Biomedical Science)

Kulliyyah of Allied Health Science
International Islamic University Malaysia

JULY 2019

ABSTRACT

Livestock industry such as goat farming has been providing the farmers in developing countries with steady income. However, farm mismanagement, poor welfare and climate factor has contributed to the increase infections of helminths in goats which challenged the demands-supply chain. The condition worsened when they showed signs of resistance towards majority of commonly available anthelmintic drug. Improving animal husbandry and plant-based treatment could become an alternative in treating helminths infections in goats. Thus, the study is aimed to identify the occurrences of gastrointestinal helminths in goats' faecal samples and determines the anthelmintic potential of *Piper betle* L. Following the physical screening, 120 goats' faecal sample from three different farms in Kuantan, Pahang were observed for helminths under microscope. Subsequently, the species of helminths were confirmed using real-time PCR and high-resolution melt assay (qPCR-HRM) and the predominant species was determined. Then, aqueous and methanol extracts from the *Piper betle* L. leaves that were prepared for the anthelmintic assay were screened for their phytochemical compounds using liquid and gas chromatography-mass spectrometry (LCMS) and (GCMS) respectively. In addition, their feed nutritional assessments were identified. Finally, the anthelmintic effect of *Piper betle* L. leaves extracts were examined on the predominant species using egg hatch assay (EHA) and larval paralysis assay (LPA). This study revealed 74.17% (89/120) samples of positive gastrointestinal helminths species. *Haemonchus contortus* was predominant among the six different species discovered. Besides, the study also revealed multiple infections of internal helminths in a sample. The diagnosis of Helminthiasis is often unrecognized in animal. It is usually suspected on the basis of physical inspection. However, this study has found no direct relationship between physical screening and microscopic observation. Goats with perfect body score were actually severely infected when their faecal samples were studied. Thus, the appearance of helminth under microscopic and further confirmation with molecular assay not only confirms the occurrence but also the species. Both GCMS and LCMS revealed 'Phenols' as majority bioactive compound that is responsible for the anthelmintic property. Nevertheless, LCMS has found many more compounds with different possible uses that could be utilised further. All treatment concentration of *Piper betle* L. has effectively induced anthelmintic activity. However, 100% mortality on the death of *Haemonchus contortus* eggs and larvae was successfully achieved at the concentration of 5.0 mg/ml within 24 hours for EHA and concentration of 7.5 mg/ml within 12 hours for LPA. In conclusions, *Piper betle* L. leaves indeed possess the anthelmintic properties where it successfully eliminated the predominant species of gastrointestinal helminths from goats. Nevertheless, these strategies represent possible therapeutic treatment in the future.

خلاصة البحث

يوفر قطاع الماشية مثل تربية الماعز للمزارعين في البلدان النامية دخلاً ثابتاً، ومع ذلك فإن سوء إدارة المزارع وسوء الرعاية وعوامل المناخ قد تسببت في ارتفاع حالات الإصابة بالديدان في الماعز والتي أثرت على سلسلة العرض والطلب لها. تزداد الحالة سوءاً عندما يظهر الماعز علامات للمقاومة تجاه غالبية العقاقير المتوفرة المضادة للديدان، ولذلك فإن تحسين طرق تربية الحيوانات والأخذ بالعلاجات النباتية قد يكون بديلاً لعلاج التهابات الديدان الطفيلية في الماعز. بناءً على ذلك هدف هذا البحث إلى تحديد حالات تفشي الديدان المعوية في العينات البرازية للماعز والتعرف على قدرة أوراق باير بيتل ل. المضادة للديدان. بعد الفحص البدني تمت معاينة 120 عينة براز تحت المجهر للبحث عن الديدان الطفيلية من ثلاث مزارع مختلفة في كوانتان في ولاية باهانج. تم في وقت لاحق تأكيد أنواع الديدان باستخدام تفاعل البلمرة التسلسلي الفوري وفحص الذوبان عال الدقة (qPCR-HRM) وتحديد الأنواع السائدة في العينات. بعد ذلك تم فحص المركبات الكيميائية النباتية للمستخلصات المائية والميثانولية لأوراق باير بيتل ل. التي تم تحضيرها لاختبار خواصها المضادة للديدان باستخدام كروماتوغرافيا السائل والغاز-مطياف الكتلة (LCMS) و (GCMS). بالإضافة إلى ذلك تم تحديد التقييم الغذائي لعلف الماعز. وأخيراً تم فحص التأثير المضاد للديدان لمستخلصات أوراق باير بيتل ل. على أنواع الديدان السائدة باستخدام فحص تفقس البيوض (EHA) وفحص شلل اليرقات (LPA). كشف هذا البحث أن 74.17% (120/89) من العينات كانت إيجابية في احتوائها على أنواع الديدان المعوية. كانت ديدان المحجنية الملتوية هي السائدة بين الأنواع الستة المختلفة المكتشفة. إلى جانب ذلك كشف البحث أيضاً عن عدوى متعددة للديدان المعوية في عينة واحدة. تشخيص الإصابة بالديدان الطفيلية في الحيوانات غالباً ما يكون صعباً، ويشتهب عادة بالاعتماد على الفحص البدني. ومع ذلك لم يجد هذا البحث أي علاقة مباشرة بين الفحص البدني والمراقبة المجهرية حيث كانت الماعز الحاصلة على درجة مثالية في الفحص البدني مصابة للغاية بالديدان عند دراسة عيناتها البرازية. وهكذا فإن ظهور الديدان تحت الفحص المجهرى وبالتأكيد الإضافي بالفحص الجزيئي لا يؤكد وجودها فحسب، بل يدل أيضاً على نوعها. كشف كل من GCMS و LCMS أن "الفينول" هو المركب الحيوي السائد في المستخلصات والمسؤول عن الخاصية المضادة للديدان. ومع ذلك فقد وجد LCMS العديد من المركبات بتطبيقات مختلفة محتملة والتي يمكن استخدامها بشكل أكبر. تمكنت مستخلصات باير بيتل من القضاء بشكل فعال على بيوض ويرقات ديدان المحجنية الملتوية بنسبة إبادة بلغت 100% والتي تحققت بتركيز 5.0 ملغ/مل في غضون 24 ساعة لـ EHA وبتركيز 7.5 ملغ/مل في غضون 12 ساعة لـ LPA، علماً بأن جميع التركيزات العلاجية الأخرى قد تسببت في نشاط مضاد للديدان. ختاماً، تمتلك أوراق باير بيتل ل. بالفعل خصائص مضادة للديدان حيث نجحت في القضاء على الأنواع السائدة من الديدان المعوية في الماعز، وبذلك فإن هذه الاستراتيجيات تمثل علاجاً محتملاً في المستقبل.

APPROVAL PAGE

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to my family and friend.

ACKNOWLEDGEMENTS

All praise to Allah, the Almighty God who have given me the best of patience, time and health along the way in completing my research project. This thesis is the result of contribution from many people who deserve special recognition from me.

Firstly, I wish to express my deepest thanks to my supervisor, Asst. Prof. Dr. Mardhiah Muhammad and my co-supervisor, Assoc. Prof. Dr. Afzan Yusof for their continuous support, encouragement, constructive ideas, thoughtful comments and valuable guidance, and for that, I will be forever grateful. It has been an honour for me to be under their supervision, otherwise this project will not be accomplished.

Not to forget my special thanks to Site Kasmadiana, for her assistance in laboratory work related and her unconditional moral support. I would also like to convey my appreciation to the laboratory officers and staffs at Kulliyyah of Allied Health Science and Centre Research and Animal Facility (CREAM) for their kindness and technical support in completing my research project.

Last but not least, to my dear parents, it is my utmost pleasure to dedicate this work to them, who granted me the gift of their unwavering beliefs in my ability to accomplish this project. Thank you for your support and patience.

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LIST OF SYMBOLS

%	Percentage
cm	Centimetre
ha	Hectare
ml	Millilitre
μl	Microliter
bp	Base pair
°C	Celsius
rpm	Revolutions per Minute
min	Minutes
mg	Milligram
sec	Seconds
ng/μl	Nanogram per Microliter
kV	Kilovolt
mAH	Milli amp hour
m	Meter
mm	Millimetre
°C/min	Celsius per Minutes
ml/min	Millilitre per Minutes
mg/ml	Milligram per Millilitre
gm	Gram
MJ/kg	Megajoules per Kilogram
±	Plus or Minus

LIST OF ABBREVIATION

App.	Approximate
T _m	Temperature
spp.	Species
HC	Hydroxychavicol
EU	Eugenol
GDP	Gross Domestic Product
HDI	Human Development Index
TDN	Total Digestible Nutrient
NFE	Nitrogen-free Extract
TST	Targeted Selective Treatment
EHA	Egg Hatch Assay
LPA	Larval Paralysis Assay
GCMS	Gas Chromatography-Mass Spectrometry
LCMS	Liquid Chromatography-Mass Spectrometry
qPCR	Quantitative Polymerase Chain Reaction
HRM	High Resolution Melt
BLAST	Basic Local Alignment Search Tool
MAFF	Ministry of Agriculture, Food and Fisheries
W.A.A.V. P	World Association of the Advancement in Veterinary Parasitology
NIST-MS	National Institute of Standard and Technology-Mass Spectral
FAO	Food and Agriculture Organization (United Nations)
WHO	World Health Organization
DVS	Department of Veterinary Service
CV	Coefficient of Variance

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Livestock and its product have become one of the fastest-growing segments in agriculture economy, which contribute to the household livelihood, food security and nutrition in many developing countries (Nabarro & Wannous, 2014) such as India (Dash, 2017) and Thailand (Singhapreecha, 2014). This is due to its relatively low-cost breeding stock, high production rates, and the ability of the animal to survive on native pasture condition that was not suitable for crops (Nakavisut & Anothaisinthawee, 2014). In Malaysia, this sector is considered important because of its contribution toward development and sustainability of economy. The agriculture sector grew about 8.1% in which the livestock sector contributed 11.6% to the Malaysian Gross Domestic Product (GDP) (DVS, 2017). Hence, there is a high demand for the dietary animal protein including their wastes in many parts of the world (Henchion et al., 2017).

However, this demand could not be supplied because of a decline in the production of the livestock due to gastrointestinal parasitic infection which has becomes worldwide problems for farmers (Owhoeli et al., 2014). The infection is prevalence in countries where the sanitation is poor, standard of living is low and farm management is bad (Jitapalapong et al., 2012). Studies had shown that goats have numerous internal parasites; with common infection of nematode species *Haemonchus contortus* and *Trichostrongylus* spp. (Waller et al., 2006; Mortensen et al., 2003).

These infections were normally treated with a variety of commercially available anthelmintic drugs such as Albendazole, Mebendazole, Levamisole, and Ivermectin. These drugs intended to treat, control or prevents the helminths parasite infection (Ford & Roach, 2013). However, continuous and uncontrolled drug administrations eventually lead to anthelmintic resistance of the commercial drugs, thus increasing the occurrence of internal helminths infection (Kupcinskis et al., 2015). The ineffectiveness of commercial anthelmintic drug has triggered farmers to seek alternative treatments that originated from natural resources (Mali & Mehta., 2008).

Natural resources such as plant gives a great opportunity for treatment by providing different therapeutic actions which originates from the plant properties itself, for example anthelmintic, antibiotics, antimicrobial and insecticides properties (Urie, 2006). Research on herbal plants (Yuan et al., 2016; Liaqat et al., 2017) proved that plant-based remedy was environmentally friendly, non-toxic and low resistances in diseases treatment.

Piper betle L. is originated from the central and eastern part of Peninsular Malaysia and locally known as ‘Sirih’ and ‘Betel’ in English (Pin et al., 2010). The leaves of *P. betle* L. possess many beneficial properties that have positive impact on public health (Hossain et al., 2017). Studies by Ibrahim et al. (2016) and Foo et al. (2015) confirmed that the *P. betle* L. leaves possess the antimicrobial properties, while studies by Sazwi et al. (2013) and Uddin et al. (2015) indicated the presence of antioxidant activities potential. However, most of these studies only indicated the phytochemical properties presence in *P. betle* L. leaves without the evidence of biological actions. Thus, further investigation regarding the plant biological action is needed to prove the availability of certain phytochemical properties.

1.2 STATEMENT OF THE PROBLEM

Gastrointestinal helminths are said to be the most devastating diseases that exist in the ruminant especially goat which is often overlooked. This is because the clinical symptoms are rarely apparent, that make it harder for early treatment (Adebisi et al., 2007). The infections from parasitic worm results in food deprivation, blood loss and toxic in the host which left untreated, could results in serious diseases, disability, and death (Hotez et al., 2008; Wang et al., 2008).

Generally, helminths infection in ruminant is treated and controlled with medicine called anthelmintic drug, for example, Benzimidazoles, Imidazothiazoles and Macrocyclic lactones (Bishop, 2001). However, the treatment and control are challenged when drug resistance is presented immediately after drug administration. Earliest report on anthelmintic drug resistances was published in mid-fifties by Drudge et al (1957) which demonstrated varying degrees on resistance development of Phenothiazine against difference strains of nematode in sheep. Due to the continuous resistance in livestock treatment, various antihelminth drugs were constantly being developed to counter the problem. Still, this problem is recurrent and conclusive solution has never been encountered. In Malaysia, especially in Kelantan, a study revealed that anthelmintic resistance has caused great concern because some drug such as Ivermectin, Levamisole, and Closantel showed long-term ineffectiveness on gastrointestinal nematode and this has been a concern among farmers (Basripuzi et al., 2012).

The cost of anthelmintic treatments is high, thus making countries with high occurrences of helminthiasis to provide inadequate dose of treatment that eventually lead to drug resistance (Cooper et al., 2007). Sometimes the infected animal would be killed for an immediate solution. Therefore, the occurrence of these helminths would

never reduce and the cycle continues which impact the agriculture sector, economy and also environment. These have made treatment of helminths infections difficult and over time, it becomes a severe problem worldwide because productivity as well as the reproductive performance of the ruminant was affected (Sharma et al., 2016). Therefore, alternative treatments against gastrointestinal helminths infection that focus on natural resources are sought. Obviously, treatment that are safe, cost effective, and easily accessible would be preferred and this is common practice among most farmers in India (Mann et al., 2008; Kumar et al., 2014). Consequently, the benefits of natural remedies have caused the increases of interest in the importance of seeking for medicinal plants.

There have been few controlled trials in plants that investigate the efficacy of anthelmintic compounds. Study on *Cassia tora* leaves indicated that phenolics like flavonoids and tannins as well as anthraquinones were present and may act as an active compound for anthelmintic activity against the earthworm *Pheretima posthuman* (John et al., 2009). Besides that, studies on the anthelmintic activity of Fenugreek seed (Khadse & Kakde, 2010) as well as *Cissus quadrangularis* L. and *Schinus mole* L. (Zenebe et al., 2017) demonstrated successful inhibition of helminths tested. However, literature revealed no sufficient study regarding anthelmintic activity of *P. betle* L. in Malaysia. Hence, the present study is focused on evaluating the *in vitro* anthelmintic effect of crude leaves extracts of *P. betle* L. (Sirih) on gastrointestinal helminths obtained from goats.

1.3 RESEARCH OBJECTIVES

1.3.1 Main Objective

The main objective of this study is to evaluate the *in vitro* anthelmintic effect of crude leaves extracts of *Piper betle* L. (Sireh) on gastrointestinal helminths obtained from goats.

1.3.2 Specific Objective

The specific objective of this research is:

- i. To determine and cultivate the gastrointestinal helminths collected from goats' faecal sample
- ii. To identify the species of helminths using quantitative real-time polymerase chain reaction-High resolution melt assay (qPCR-HRM)
- iii. To identify the phytochemical compound in *Piper betle* L. (Sireh) leaves extract using Liquid Chromatograph-Mass Spectrometer (LCMS) and Gas Chromatography-Mass Spectrometry (GCMS)
- iv. To measure the anthelmintic effect of crude leaves, extract of *Piper betle* L. (Sireh) on *Heamonchus contortus* using Egg Hatch Assay (EHA) and Larva Paralysis Assay (LPA).

1.4 RESEARCH QUESTION

The question that could be established from this for this research is:

Is there an anthelmintic effect of crude leaves extract of *Piper betle* L. (Sireh) on gastrointestinal helminths obtained from goats?

1.5 RESEARCH HYPOTHESIS

The hypothesis of research:

There is an anthelmintic effect of crudes leaves extract of *Piper betle* L. (Sireh) on gastrointestinal helminths obtained from goats.

CHAPTER TWO

LITERATURE REVIEW

2.1 CLASSIFICATION OF HELMINTHS

Helminths parasites are generally larger and some may grow to relatively massive sizes and several meters long, bilaterally symmetrical with three germ layers. They can be divided into two main groups, first, Nematelminths or Nematode (roundworm), and second, Platyhelminthes which include of Cestode (tapeworm) and Trematode (flukes) (Janggi, 2005). The differentiation on each of the helminths group can be determined by observing the external and internal morphology of egg and larvae through a microscope or by observing the adult stage which can be identified with the naked eye (VanMeter & Hubert, 2015).

Nematelminths Nematode or roundworm has an elongated, cylindrical and unsegmented body structure without sucker and hooks. It usually tapers at both ends with an anterior mouth and longitudinal digestive tracts. They also have a body cavity called pseudocoelom that acts as hydrostatic skeleton providing rigidity. As well as a complete set of alimentary canals with the presence of anus and separate sexes (Lee, 2002). Meanwhile, Platyhelminthes Trematodes or flukes are generally flat with leaf-like, unsegmented body structure and a ventral sucker to hold the organism in place. Absent of body cavity with the presence of incomplete alimentary canal with no anus. Trematodes are hermaphrodite except for Schistosomes that have separate sexes. Each type of Trematode name is usually given based on the tissue where the adult worm lives such as lung fluke or liver fluke (Watson, 2014).