In Vitro Anthelmintic Activity of *Limonia acidissima*, L. Leaves Aqueous Extract on *Haemonchus contortus* (Rudolphi, 1803)

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ABSTRACT
Mortality, loss of production, slowed growth, poor weight gain, and even death are all common economic losses caused by haemonchosis. Due to the emergence of anthelmintic resistance and the expensive expense of chemical anthelmintic treatments, medicinal plants have been investigated as potential anthelmintics. This study aimed to examine the in vitro anthelmintic activity of *Limonia acidissima* leaves aqueous extract on *Haemonchus contortus*. Phytochemical analysis of *L. acidissima* was conducted to measure some secondary metabolites. The efficacy of six extract concentrations (2.5, 5, 7.5, 10, 12.5, and 15 %) was tested through an adult worm motility test while ivermectin 0.1% was positive control and NaCl 0.62% was a negative control. Histology preparation was conducted using the paraffin method and hematoxylin-eosin staining. The secondary metabolites measurement show that tannin is 1.83 %b/v, saponin is 0.23 %b/v, and flavonoid is 0.85 %b/v. These bioactive compounds are believed to have a role in causing the death of worms. The results of adult worm motility revealed that the highest mean inhibition of adult worm motility is at a concentration of 15% and the lowest one is at a concentration of 0.25%. While ivermectin 0,1% showed inhibited motility after 2 hours incubation and in contrast NaCl 0,62 % showed no effect on motility test. All concentrations of *L. acidissima* aqueous extract showed significantly inhibited the motility of adult worms at a 1% level significance. Duncan's post hoc test showed that *L. acidissima* aqueous extract had slow onset of activity as compared to ivermectin 0.1%. Each concentration caused damage to the cuticle and muscular layer of *H. contortus*. The increase in the level of concentration is directly proportional to the increase in damage to the integumentary structure. The overall findings of this study have shown that *L. acidissima* leaves aqueous extract has an anthelmintic activity and further evaluation of these plants should be carried out.

Keywords: *Anthelmintic, Haemonchus contortus, In Vitro, Limonia acidissima.*

1. INTRODUCTION

Haemonchosis is a disease caused by infection with parasitic worms from the Nematoda class, namely *Haemonchus contortus* that sucks blood in the abomasum of small ruminant animals such as goats and sheep [1]. *Haemonchus contortus* is a blood-sucking parasite that causes weight loss, anemia, hypoalbumin anemia, lipoprotein anemia, and death, especially in cattle [2]. In addition, acute haemonchosis is characterized by anemia, edema, submandibular form, and ascites, the most easily recognized being lethargy, dark stools, and hair loss [3]. In cases of acute haemonchosis, synthetic anthelmintics can be used, namely albendazole, benzimidazoles, levamisole, pyrantel, morantel, oxantel, monepantel, tribendimidine, piperazine, and ivermectin [4].

In addition to being relatively expensive, the use of synthetic anthelmintics is thought to cause resistance if used for a long period with inappropriate doses [5]. The use of herbal anthelmintics can be an alternative that is cheap, safe, and can overcome the problem of resistance with the presence of multitarget compounds against parasites.

The Rutaceae family's wood-apple (*Limonia acidissima* L.) is well-known for its traditional

applications. Alkaloids, phenolic compounds, triterpenoids, tannins, and steroids, among other phytoconstituents, have been identified from *L. acidissima* and are known to have potential anthelmintic activity. [6]. Research related to testing the anthelmintic activity of this plant is still very rare, especially in Indonesia because wood-apple is a tropical plant that only grows in certain areas in Indonesia.

In light of the preceding rationale, the goal of this work was to determine the in vitro anthelmintic activity of *L. acidissima* leaves aqueous extract against *H. contortus.*

2. METHODOLOGY

2.1. Limonia acidissima Leaves Collection and Preparation

Limonia acidissima leaves were collected from Bima, West Nusa Tenggara. Fresh leaves were washed and then dried. Cut the leaves until smooth and then sieved. Simplicia is stored in a container until ready to use.

2.2. Extraction

The extraction was conducted according to [7] with slight modifications. A total of 22.5 g of Simplicia were soaked in a beaker containing 150 mL of distilled water (15% stock solution). For 15 minutes, the beaker was incubated in a water bath at 90°C. The solution was then filtered via filter paper and diluted into 2.5, 5, 7.5, 10, and 12.5 % solutions, which were then stored until ready to use.

2.3. Phytochemical Analysis

Some secondary metabolites (tannin, flavonoid, and saponin) of *L. acidissima* leaves aqueous extract were analyzed using the UV-Vis spectrophotometry method.

2.3.1. Determination of Total Tannin Content (Chanwitheesuk et al., 2005)

Take 0.2 mL of sample, extract for 20 hours with 10 mL diethyl ether, filter, and evaporate the remaining diethyl ether. To a volume of 10 mL, add aquadest to the sample. Take 1 mL of sample solution, add 0.1 mL Folin Ciocalteu reagent, vortex, and set aside for 5 minutes. Vortex 2 mL of 20% sodium carbonate and set aside for 5 minutes. Dilute 5 times with aquadest in a volume of 10 mL. After 30 minutes of incubation at room temperature, measure the absorbance at = 760 nm.

2.3.1.1. Preparation of Tannic acid Standard Curve

Carefully weigh the standard of Tannic Acid (Standard weight of tannic acid: 0.0100 g). Add and vortex 10 mL Folin Ciocalteu reagent, then wait 5 minutes. Adjust the volume with a 20% Sodium Carbonate solution until it reaches 100 mL. Dilute using the concentration standard curve as a guide. After 30 minutes of incubation at room temperature, the absorbance was measured at = 760 nm.

2.3.2. Determination of Total Saponins (Ing–Luen et al., 2009)

Take 0.2 mL of sample and mix it with 2 mL of 25% H_2SO_4. Autoclave for 120 minutes at 110°C. Using ether for extraction. The filtrate should be dried. After adding 1 mL of aquadest, vortex for 5 minutes to extract. Add 50 liters of anisaldehyde, shake, and set aside for 10 minutes. Add 2 mL of 50 percent sulfuric acid to the mixture. Heat for 10 minutes in a water bath at 60°C. Using a measuring flask, fill a measuring flask with water to a capacity of 10 ml. 5 times dilution At a wavelength of 435 nm, measure the absorption.

2.3.2.1. Preparation of Saponin Standard Curve from Quillaja bark

Mix the 10 mg saponin standard and 5 ml of water, then vortex for 5 minutes. Shake in 50 liters of anisaldehyde, then set aside for 10 minutes. Add 2 mL of 50 percent sulfuric acid to the mixture. Heat for 10 minutes in a water bath at 60°C. Using a measuring flask, fill a measuring flask with water to a capacity of 10 ml. Standard dilute from 200,100,50,25,12.5, 6.25 µL. At a wavelength of 435 nm, measure the absorption.

2.3.3. Determination of total flavonoid (Zhishen et al., 1999)

Fill a 10 ml test tube with 0.25 ml of the sample and 0.3 mL sodium nitrite (5% sodium nitrite). Add 0.6 ml of 10% aluminum chloride after 5 minutes, then wait 5 minutes to add 2 ml of 1 M sodium hydroxide. Using a measuring flask, fill up to 10 ml with aquadest. As needed, dilute. Transfer to a cuvette and retain the absorbance at 510 nm.

2.3.3.1. Preparation of Flavonoid Standard Curve

10.0 mg standard Quercetin + 0.3 mL sodium nitrite (5% sodium nitrite) After 5 minutes, add 0.6 ml 10% aluminum chloride and wait 5 minutes before adding 2 ml 1 M sodium hydroxide. Using a measuring flask, fill 10 ml with aquadest. Transfer to a cuvette and maintain absorbance at 510 nm.

2.4. Adult Worm Collection

Parasites will be obtained from the abomasum of goats slaughtered in Kentungan slaughterhouse, Sleman, Yogyakarta. Abomasum will be handled in the laboratory

by opening along the major curvature and the worms are collected using a small paintbrush. Parasites were collected into containers containing 0.62% physiological saline solution [7].

2.5. In Vitro Anthelmintic Assay (Adult Worm Motility Test)

Solutions of aqueous extract were prepared at six different concentrations (2.5, 5, 7.5, 10, 12.5, and 15 %). Ten actively moving adult worm was then placed into each petri dish. NaCl 0,62% and ivermectin 0.1 % were also prepared and were used as negative and positive controls. The test was repeated three times for all treatments. Observations were made by recording the time of death of the treatment worms at the 2 and 4 hours after treatment. Worms are considered dead if the worms do not move for 30 seconds after the worm's body parts are touched using a surgical needle and shaking the petri dish. The dead worms were fixed in 10% formaldehyde and stored in the refrigerator until used. The lethal concentration 50 (LC_{50}) was calculated using the Reed and Muench method [7, 8].

2.6. Histological preparation

The preparation of histology of the integumentary tissue of *H. contortus* was carried out according to [9] with modifications. Preparation of integument tissue preparations using a procedure that includes fixation using 10% formaldehyde for 24 hours, dehydration with graded alcohol concentration and cleared in toluol/xylol. Worms were embedded in paraffin, then sections were sliced at 5-7 µm in the transverse plane using a rotary microtome. Tissue staining was performed with hematoxylin and eosin (HE).

2.7. Statistical analysis

The data was given as a mean approximately standard deviation. The differences from the effect of each treatment were analyzed by one-way analysis of variance (ANOVA) at a 1% level of significance ($p<0.01$) followed by Duncan's post hoc test method (Duncan Multiple Range Test) using IBM SPSS Statistics 23.

3. RESULT AND DISCUSSION

3.1 Phytochemical Analysis

The phytochemical analysis using UV-Vis spectrophotometry of the leaf's aqueous extract of L. acidissima revealed the presence of tannins, saponins, and flavonoids (Table 1).

Table 1. The secondary metabolites assayed in *L. acidissima* leaves aqueous extract

No	Metabolites	Result (%b/v)
1	Tannins	1.83
2	Saponins	0.23
3	Flavonoids	0.85

These bioactive compounds are believed to have a role in causing the death of worms. The action of the plant metabolites as anthelmintics can be additive, synergistic, and/or antagonistic. These metabolites can act at one or more target sites on the worm which is each secondary metabolite has a specific mechanism of action.

3.2 In Vitro Anthelmintic Assay

The results of the in vitro test after 2 and 4 hours of *L. acidissima* aqueous extract administration (Table 2) revealed that the highest mean inhibition of adult worm motility, both at an exposure time of 2 and 4 hours, is at a concentration of 15% and the lowest one is at a concentration 0.25%. While ivermectin 0,1% as positive control showed inhibited motility after 2 hours incubation and in contrast NaCl 0,62 % as a negative control showed

Table 2. The average of death worm

No	Treatments	Death worm	
		2 hours	4 hours
1	NaCl 0.62%	0 ± 0.00^{a}	0 ± 0.00^{a}
2	LAE 2.5%	0 ± 0.00^{a}	0.33 ± 0.58^{a}
3	LAE 5%	1.67 ± 0.58^{ab}	3.68 ± 0.58^{b}
4	LAE 7.5%	3.33 ± 1.53^{bc}	6 ± 1.00^{c}
5	LAE 10%	5 ± 1.00^{cd}	7.67 ± 0.58^{d}
6	LAE 12.5 %	6 ± 1.73^{d}	8.33 ± 0.58^{de}
7	LAE 15 %	7.33 ± 1.53^{d}	9.33 ± 0.58^{ef}
8	Ivermectin 0.1%	10 ± 0.00^{e}	10 ± 0.00^{f}

LAE = *L. acidissima* leaves aqueous extract.
*The same letter superscript indicates insignificant ($p<0.01$)

no effect on motility test until the end of the experiment. All concentrations of *L. acidissima* aqueous extract showed potential anthelmintic activity.

Adult worm inhibited motility at a concentration of 15% with an exposure time of 4 hours showing the largest mean±sd, which is 9.3±0.58, and at a concentration of 0.25% showing the lowest mean±sd, which is 0.3±0.58. The LC50 value with an exposure time of 4 hours was 7.3%. This value indicates that at a concentration of 7.3% *L. acidissima* leaves aqueous extract was able to kill the worm up to 50% of the population.

The results of one-way analysis of variance (ANOVA) showed that the concentration level of *L. acidissima* aqueous extract on the exposure time of 2 and 4 hours was significantly different from the inhibition of adult worm motility that means *L. acidissima* aqueous extract at the various level of concentrations affected the inhibition of adult worm motility.

Duncan's new multiple range tests revealed that the concentration of 15% was significantly different to positive control at 2 hours post-exposure but not at 4 hours post-exposure, it shows that *L. acidissima* aqueous extract had slow onset of activity as compared to ivermectin 0.1%.

3.3 Histological Preparation

Histological preparation showed changes in *H. contortus* after in vitro exposure to *L. acidissima* leaves aqueous extract at 15% concentration, NaCl 0.62% as a negative control, and ivermectin 0.1% as a positive control.

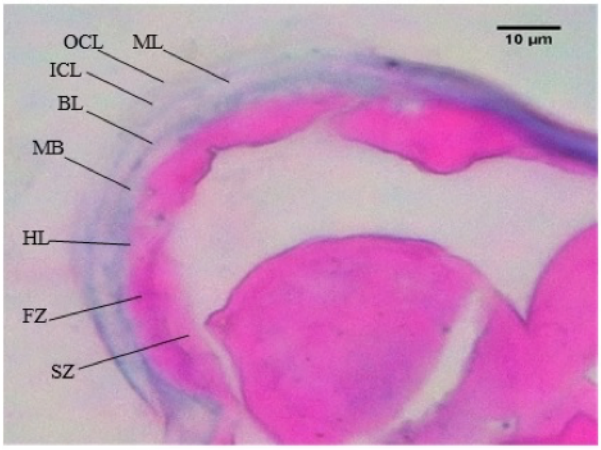

Figure 1. Tranverse section of *Haemonchus contortus* at NaCl 0,62% (1000x magnification) showing the outer cortical layer (OCL), inner cortical layer (ICL), median layer (ML), basal layer (BL), membrane basalis (MB), hypodermis layer (HL), fibrillar zone (FZ) and sarcoplasmic zone (SZ) of muscle cell.

Histological observations of *H. contortus* at NaCl 0,62% as negative control (Figure 1), the cuticle was seen covering the outer surface of the body of the worm that the cuticle layer was intact and thick. The hypodermis layer below the cuticle layer is still attached to the cuticle and the muscular layer (fibrillar and sarcoplasmic zone) looks elongated.

The thick cuticle is the body wall's outermost layer. It's an extracellular secretion that isn't alive. The cuticle serves as a multipurpose exoskeleton. It serves as a highly impenetrable barrier between the animal and its surroundings. It is necessary for body shape and integrity, and it plays an important role in locomotion through attachments to body wall muscles. The cuticle is secreted by the syncytial hypodermis (epidermis), which is thinner than the cuticle. The integument is made up of the hypodermis and cuticle [10].

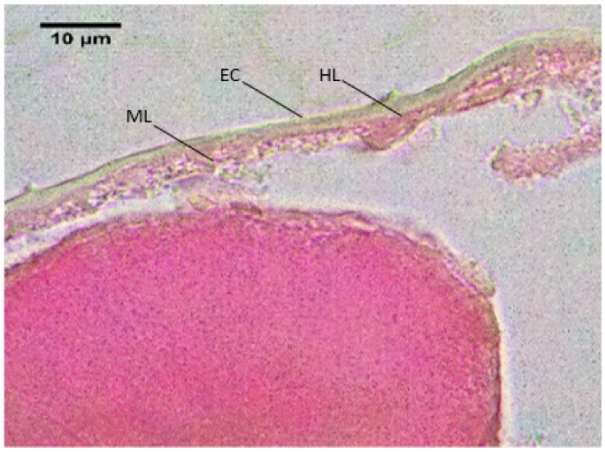

Figure 2. Tranverse section of *Haemonchus contortus* at 15% concentration of LAE (1000x magnification) showing the epicuticle (EC), hypodermis layer (HL), and muscular layer (ML).

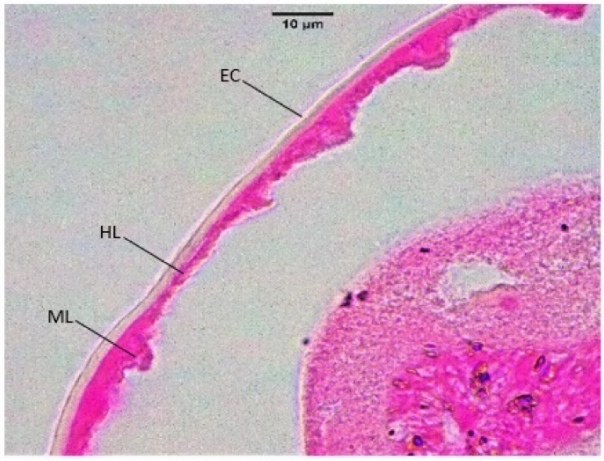

Figure 3. Tranverse section of *Haemonchus contortus* at ivermection 0,1% NaCl 0,62% (1000x magnification) showing the epicuticle (EC), hypodermis layer (HL), and muscular layer (ML).

The fibrillar or contractile zone (FZ) of the muscle cell is located on the inner of the hypodermis' basal membrane and houses the cell's contractile myofibres. The fibers stain dark pink and form a broad outline

around this section of the cell, making it easy to spot. The nucleus and most of the cytoplasm (or sarcoplasm) reside in a massive, bulging cell body or sarcoplasmic zone (SZ) that reaches deep into the pseudocoel but is less visible. [10].

Meanwhile, histological observations of *H. contortus* at a concentration of 15% (Figure 2), there was a similarity with ivermectin 0.1% (Figure 3) as a positive control. The cuticle layer was eroded so that it looked thinner than the cuticle layer of *H. contortus* at 0.62% NaCl and the muscular layer look stringy and wrinkled. These situations made the muscular layer of *H. contortus* at a concentration of 15% looked shorter than at negative control.

Limonia acidissima leaves aqueous extract at various levels of concentrations caused damage to the integumentary structure of *H. contortus*, especially in the cuticle and muscular layer. The increase at the level of concentration is directly proportional to the increase in damage to the cuticle and muscular layer

Given the overall results, L. acidissima leaves aqueous extract to have in vitro anthelmintic potential on H. contortus. The bioactive compounds responsible for these activities could be secondary metabolites that are present in the extract such as tannins, saponins, and flavonoids. While the in vitro activities are comparable to those of a commercial anthelmintic, an in vivo test is required to fully comprehend the potential of L. acidissima leaves in the treatment of haemonchosis.

AUTHOR'S CONTRIBUTION

Muh. Andhi Hardianto and Slamet Widiyanto conceived of the presented idea. Muh. Andhi Hardianto developed the theory and collected the data. Slamet Widiyanto encouraged Muh. Andhi Hardianto to investigate the worm's media (a preliminary study) and supervised the findings of this work. The findings were considered by all authors, and they all contributed to the final publication.

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