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Anthelmintic Potential of *Annona muricata* Leaf Extract against Parasitic Nematode *Haemonchus contortus*

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Abstract: Gastrointestinal infections caused by the parasitic worm *Haemoncus contortus* are the main cause of economic losses in the agricultural sector. *H. contortus* causes small ruminants such as goats to experience acute anemia and diarrhea, weight loss, decreased productivity, and eventually mortality, which impacts economic losses. Synthetic antiparasitics have been used extensively around the world to combat *haemonchosis*. However, the emergence of resistance to anthelmintics, accompanied by the unavailability of an effective vaccine, results in difficulties in controlling this disease. Because of the high cost of treatment and the emergence of resistance and toxicity due to the residue of antiparasitic drugs, it is necessary to develop natural anthelmintics that are easy to obtain, economical, and have minimal risk. This study aims to determine the anthelmintic effect of *Annona muricata* leaf extract on mice infected with *H. contortus* larvae. First, an in vitro study was conducted to see if the soursop leaf extract from Lombok would show a different effect from the previous research. In the in vivo test, the impact of *A. muricata* leaf extract was measured from the number of *H. contortus* eggs found in the feces and the percent reduction of adult worms collected from mice's stomachs. The result shows that the higher the concentration of soursop leaf extract, the higher the inhibition effect on larvae motility. The combination of *A. muricata* and albendazole had the strongest antiparasitic effect, shown by the lowest amount of eggs in feces and the highest reduction of adult worms in the stomach. However, administering *A. muricata* leaf extract can also reduce the number of eggs and adult worms in the stomach. Therefore, it can be concluded that *A. muricata* is a potential anthelmintic candidate for overcoming the *H. contortus* infection.

Keywords: Animal Model; *Annona muricata*; *Haemonchus contortus*; Natural Anthelmintic; Sheep.

Introduction

Gastrointestinal tract infections caused by parasitic worms are the most infectious diseases that cause economic losses in the agricultural sector [1]–[3]. Among several types of parasitic worms that commonly infect ruminants, *Haemonchus contortus* is one of the most pathogenic and economically detrimental (Figure 1) [4], [5]. *H. contortus* generally infects small ruminants such as goats and sheep. These worms feed into abomasums and live by sucking the blood of their hosts. *H. contortus* can cause livestock to experience acute anemia, hemorrhagic gastroenteritis, edema, diarrhea, weight loss, decreased production of milk, meat, wool, and even livestock mortality, ultimately impacting economic losses [6], [7]. To treat haemonchosis, synthetic antiparasitic drugs such as ivermectin and albendazole have been used extensively throughout the country. Residues that accumulate on ruminants and their products due to the long-term use of synthetic anthelmintics trigger resistance and toxicity [8], [9]. The emergence of anthelmintic resistance and lack of vaccines have resulted in difficulties in controlling the disease. Therefore, it is necessary to develop safer natural anthelmintics to control *haemonchosis* in ruminants.

Indonesia is a country with abundant plant diversity. Plants contain a variety of bioactive compounds that are beneficial for life. Several plant extracts have been shown to have anthelmintic activity against *H. contortus*, including plants from the Annonaceae group [10]–[12]. The soursop plant (*Annona muricata* L.) is the most common *Annonaceae* in the Lombok area (Figure 2). Based on phytochemical tests, soursop leaves contain bioactive compounds: phenols, flavonoids, alkaloids, saponins, terpenoids, tannins, and acetogenins [13], [14]. These secondary metabolites allow soursop leaves to have antioxidant, antibacterial, anticancer, and antiparasitic effects [15]–[17]. Goel et al. (2023) found that quercetin, a flavonoid derivative, has anthelmintic activity at any stage of *H. contortus* development [18]. Condensed tannins have also been shown to be effective as anthelmintics [19]–[21]. Therefore, soursop leaves have great potential as a natural anthelmintic that is easy to obtain, economical, and has minimal risk.

Figure 1. (a) *H. contortus* inside sheep abomasum (29); (b) *H. contortus* infective larvae observed under a microscope (30).

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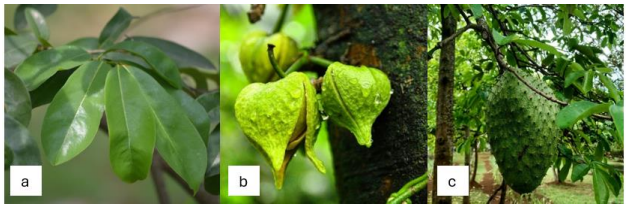

Figure 2. (a) Leaves, (b) Flower, and (c) Soursop fruit [22].

Previous research has proven that soursop leaf extract can effectively inhibit egg hatching and larval movement of *H. contortus* [11], [12]. This inhibition is associated with the presence of acetogenin [12]. However, this research is only limited to in vitro testing. Therefore, in vivo testing is essential. In vivo testing should be carried out on target livestock, but testing on livestock will require higher costs and longer research time. So, in this study, an in vivo test of soursop leaf extract was conducted on an animal model of mice (*Mus musculus*) infected with *H. contortus* infective larvae. According to Sommerville (1977), the stomach of mice is an appropriate place to develop *H. contortus* worms [23]. However, pharmacological effects testing of plant extracts is often performed on Mongolian gerbils (*Meriones unguiculatus*) [24]–[26]. In addition, mice are very commonly used as model animals to test the effectiveness of synthetic antiparasites and develop vaccines against *H. contortus* [27]–[29].

According to this description, in vivo testing of the anthelmintic activity of soursop leaf extract is critical to control *H. contortus* infection in the future. Therefore, this study aims to determine the anthelmintic effect of *A. muricata* leaf extract on mice infected with *H. contortus* larvae. This research is expected to be the basis of knowledge for future study and eventually culminate in the production of natural anthelmintic drugs to overcome antiparasitic resistance issues.

Research Methods

Extraction and Phytochemical Screening

Soursop leaf extraction begins with the making of simplicia, which is done by drying the soursop leaves in the sun for 3-5 days or until they are completely dry. The dried soursop leaves are blended into powder. Soursop leaf extraction uses an infusion technique using aquades as solvents. One hundred grams of fine powder of soursop leaves are dissolved with 1 liter of boiling aquades. After 30 minutes, the infusion mixture is blended and filtered using a 500-micrometer sieve and centrifuged at 2318g for 5 minutes [12]. The infusion of soursop leaves was diluted to obtain several concentration variations for treating the tested animals. In addition, this extract is used for phytochemical screening to confirm the presence of bioactive compounds phenolics, alkaloids, and acetogenins using FeCl3, Dragendorff, and Kedde reagents, respectively.

H. contortus Larvae Collection

H. contortus infective larvae were isolated from the feces of positively infected goats and cultured for 1 week. The fecal samples were mixed with a 0.8% saline solution and homogenized to obtain sufficient oxygenation for better egg hatching. The stool culture was then incubated at 25°C in a dark room for 7 days. The growing infective larvae are collected and stored at 4°C for further use [18], [30].

Motility Test of L3 *H. contortus* Larvae

The motility test was carried out to determine the inhibition of soursop leaf infusion against the movement of *H. contortus* larvae. Twenty grams of feces containing *H. contortus* eggs were homogenized with the rest of sterile wood shavings in a ratio of 1:2. The culture was incubated for seven days at room temperature. The larvae are then collected after watering with warm water (37°C) through spontaneous migration. A total of 50 L3 larvae were distributed on microdilution plates, and an infusion of soursop leaves was added with concentrations of 50%, 25%, 10%, and 5%. The microdilution plate was incubated for 24 hours at 27°C, and the number of motile and non-motile larvae was counted. Positive control uses levamisole and aquades for negative control [12].

Infection of Mice and Administration of Soursop Leaf Extract

In this study, 15 3-month-old mice were divided into five groups [30] (Yang et al., 2017). Group 1 was infected, without treatment (n=3); group 2 was infected, given synthetic antiparasitic drugs (n=3); group 3 was infected, given soursop leaf extract 1 g/kg bw (body weight) (n=3); group 4 was infected, given soursop leaf extract 50 mg/kg bw (n=3); and group 5 was infected, given a combination of soursop leaf extract and albendazole (n=3). Each group was orally infected with infective larvae. Infection was carried out once at the beginning of the experiment, with a total of 50 infective larvae per mouse. Groups 2 and 5 were given albendazole antiparasitic. The administration of soursop leaf extract and albendazole was carried out 7 days post-infection (dpi).

Counting the Number of *H. contortus* Eggs and Adult Worms

All groups of mice were kept and observed after infection and treatment. Fecal samples were observed under a microscope for the presence of *H. contortus* eggs. The method used to examine feces is the formalin-ether technique. The feces dissolved with 10% formalin are filtered into a centrifugation tube, and ether is added. The solution is centrifuged for 2 minutes at 1500 rpm. The sediment is dripped on the glass of the object and then observed under a microscope [31]. Examinations are carried out three times per stool sample. The number of eggs is calculated as the number per gram of feces. Mice that have been given treatment are then anesthetized and dissected to have their stomachs removed. Adult worms *H. contortus* were collected from the stomachs of mice and counted in number.

Percent Parasite Reduction

All mice were euthanized and necropsied to remove their stomachs. This stage was carried out based on research

by Yang *et al*. [30]. *H. contortus* larvae were collected from the stomach and counted. The % reduction was calculated.

Data Analysis

The number of worm eggs per gram of feces is expressed as the mean. The efficacy of the extract against *H. contortus* is calculated as a percentage decrease in the number of parasites:

$$\% \text{ reduction} = 100x \frac{(C-T)}{C}$$

Where C is the average number of worms that are not treated (negative control), and T is the average number of worms in the group that is given treatment [26].

Results and Discussion

Phytochemical Screening

Phytochemical analysis aims to confirm bioactive compounds' anthelmintic activity in soursop leaf infusion. Phytochemical analysis using FeCl3, Dragendorff, and Kedde reagents showed positive results on phenolics and alkaloids but negative results on acetogenin (Table 1). The color changes to blackish-green in the phenolic test, while in the alkaloid test, a white precipitate is formed. Acetogenin is known to have a strong antiparasitic effect by causing nerve damage in mice and humans [32]. This result aligns with previous research where acetogenin was not detected in the infusion of soursop leaves with water solvents [12]. Other studies have detected acetogenins from leaves and seeds of the Annonaceae family [33]. Souza et al. (2008) demonstrated the antiparasitic effect of acetogenin against *H. contortus* [34]. Water as an infusion solvent will be safer to administer to tested animals or target livestock.

Table 1. Phytochemical screening

Compounds	Reagent	Result
Phenolic	$FeCl_3$	+
Alkaloid	Dragendorff's reagent	+
Acetogenin	Kedde's reagent	-

Motility Test of *H. contortus* Larvae

The motility testing of L3 larvae was carried out on several different dilution variations of soursop leaf infusion, namely 50%, 25%, 10%, and 5%. The injection of soursop leaf water at 50%, 25%, 10%, and 5% dilution inhibits the movement of L3 by 87.32%, 83.02%, 70.54%, and 30.25%, respectively (Figure 3). The positive control triggers 96.37% inhibition, while inhibition in the negative control is 2.56%.

In vitro tests are very common in detecting anthelmintic candidates before in vivo testing on animal models or livestock. The advantage of in vitro testing is that the tested extract directly contacts the target parasite. However, extracts or compounds that are effective in in vitro tests are not necessarily also effective in in vivo tests. The study's results showed that the higher the concentration of soursop leaf infusion given, the higher the inhibition against the motility of L3 *H. contortus* larvae.

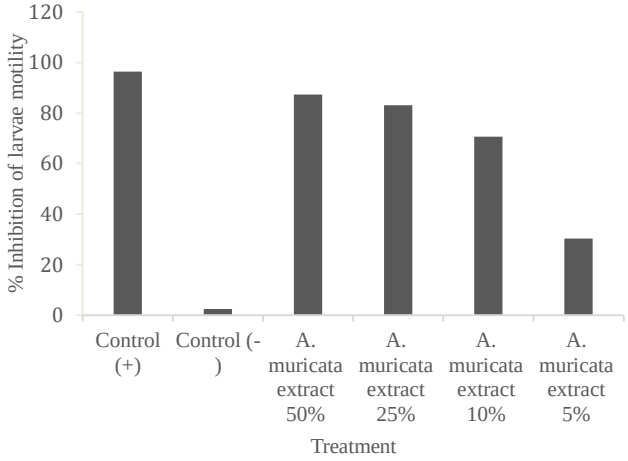

Figure 3. Graph of % inhibition of larvae motility by infusion of soursop leaf

Detection of *H. contortus* Eggs in the Feces.

The effect of soursop leaf infusion treatment on mice infected with *H. contortus* was evaluated by counting the number of *H. contortus* eggs in the feces of mice. Examinations are carried out three times per stool sample. The number of eggs is calculated as the number per gram of feces. The number of eggs in each treatment group is presented in Table 2. The highest number of eggs was found in group 1 (negative control), namely mice that were infected and not given soursop leaf infusion. Almost no eggs were found in the feces of group 2 (positive control), namely mice that were infected and given albendazole antiparasitic. Meanwhile, the number of eggs in the feces of mice given soursop leaf infusion of 1 g/kg bb (group 3) and 50 mg/kg bb (group 4) did not differ significantly. The combination of soursop leaves and albendazole showed better effectiveness in reducing the number of *H. contortus* eggs in mice feces (Figure 4). *H. contortus* eggs detected in the feces of the test animals are presented in Figure 5. The egg examination was conducted to determine the effect of soursop leaf infusion on test animals infected with *H. contortus*. The number of eggs detected in mice given soursop leaf infusion was lower than that of negative controls. This suggests that infusion of soursop leaves can inhibit the growth of *H. contortus* in tested animals' bodies.

Table 2. The number of *H. contortus* eggs found in the feces of mice treated

Group	Treatment	The number of eggs (per gram feces)
1	Infected mice + albendazole (positif control)	0.3
2	Infected mice without treatment (negative control)	6.3
3	Infected mice + 1 g/kg bb extract	2.3
4	Infected mice + 50 mg/kg bb extract	2.7
5	Infected mice + extract + albendazole	1.3

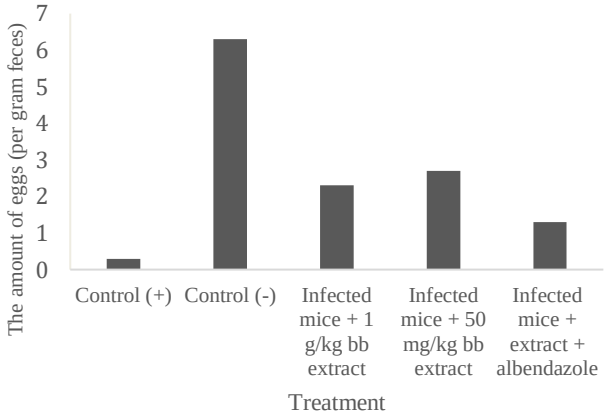

Figure 4. Graph of the amount of *H. contortus* eggs per gram of feces

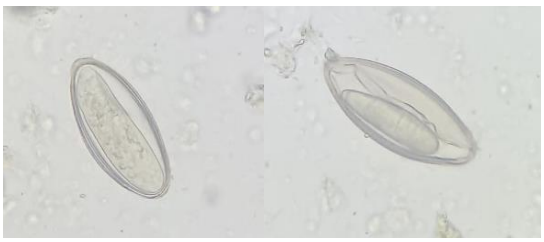

Figure 5. *H. contortus* eggs in the feces of tested animals

Percent Parasite Reduction

The percentage of *H. contortus* reduction can be known based on the number of larvae collected from the stomachs. The rate was obtained by comparing the average number of larvae in infected mice and those not given soursop leaf infusion. Data analysis showed that positive controls had the % reduction percentage at 82.5% (Figure 6). In the mice that were given the treatment, the highest percentage reduction of *H. contortus* was demonstrated in mice that were given a combination of soursop leaves and albendazole which was 57.5%. Meanwhile, in mice that were only given 1 g/kg bb and 50 mg/kg bb soursop leaf infusion, the percentage reduction of *H. controtus* was obtained by 42.5 and 17.5, respectively. This result aligns with the amount of eggs detected from the feces of treated animals. The infected mice, given the combination of extract and albendazole, showed the lowest amount of eggs and the highest percentage of parasite reduction (after positive control). The percent parasite reduction of *H. contortus* treated with leaves extract of *A. muricata* has not been done previously.

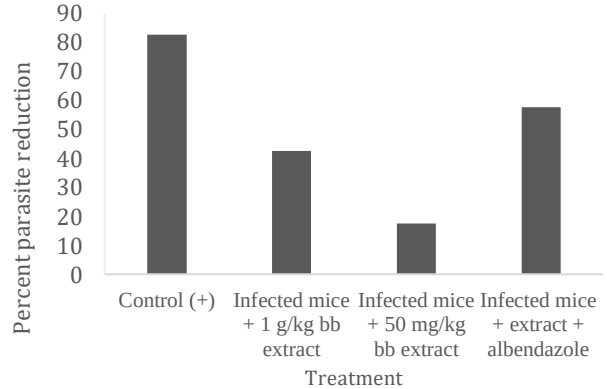

Figure 6. Graph of the percent *H. contortus* reduction

Conclusion

In the in vitro test, *Annona muricata* aqueous extract significantly affects larvae motility. The higher the extract concentration, the higher its inhibition of larvae movement. In addition, lower egg accumulation and adult worm reduction are found in mice's feces treated with *Annona muricata* and albendazole. According to this research, it can be concluded that *Annona muricata* leaf is a potential candidate for natural anthelmintics. However, more studies need to be done on the purification, identification, and quantification of the active compounds responsible for this anthelmintic activity.

Author Contribution

B.Y.H.Pratiwi and B. Y. H. Pratiwi and Novitarini designed and experimented together. Analysed the data, developed the figure, and wrote the manuscript.

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