The Anthelmintic Potential of Ethyl Acetate Fraction of Berenuk Fruit (*Crescentia cujete* L.) Against *Haemonchus contortus* Mortality In Vitro

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Abstract

This study aims to determine the anthelmintic potency of the ethyl acetate fraction of berenuk fruit (*Crescentia cujete* L.) on the mortality of the *Haemonchus contortus* worms in vitro, with the hypothesis that the optimal concentration of the ethyl acetate fraction obtained, Lethal Concentration 50 (LC_{50}), and Lethal Time 50 (LT_{50}). Method used in the research was a post-test only control group design. There were five treatments and each treatment carried out in four repetitions. Twenty samples of *H. contortus* used in each treatment for all replications. Observation and recording of *H. contortus* mortality carried out at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes, and when all worms in the petri dish died. Data analysis using ANOVA, followed by Duncan's Test and Probit analysis. The results showed that the ethyl acetate fraction of berenuk fruit had anthelmintic activity. The optimal concentration found in 0.5% ethyl acetate fraction concentration, the LC_{50} at each observation time successively is 1.39%; 0.97%; 0.70%; 0.48%; 0.39%; 0.34%; 0.31%; 0.28%; 0.27%; 0.26%; 0.25%; and 0.24%, while LT_{50} at a concentration of 0.125%; 0.25%; and 0.5% respectively are 1 hour 17 seconds, 41 minutes 42 seconds, and 20 minutes 58 seconds.

Keywords: *Haemonchus contortus*, ethyl acetate fraction, berenuk, anthelmintic.

Introduction

Haemonchosis is a helminthiasis disease that attacks ruminants and is caused by nematode worms of the genus Haemonchus. The most common Haemonchus species is *Haemonchus contortus* with a predilection for the abomasum of goats and sheep (Noviana et al., 2017). According to FAO (1991), the prevalence of haemonchosis in Indonesia is 89.4%. Nugroho (2013) reported that the prevalence of haemonchosis in Subang District, Banyumas was dominated by *H. contortus* with a prevalence of 58.26-66.21%. Mariyam et al. (2018) reported the prevalence of *H. contortus* in Pegirian RPH Surabaya in September-November 2017 of 47.2%. Arifin et al. (2019) the prevalence of haemonchosis in Kalipuro District, Banyuwangi in January-February 2019 was 15%.

Disadvantage caused by this worm reach 7,000,000 per year and will increase if no serious control measures are taken (Rachmat et al., 1998 and Ahmad et al., 2006). The resulting losses are in the form of weight loss, anemia and even death in livestock (Subekti et al., 2013). Control that is usually done is by giving synthetic worm medicine. However, in its administration it is often misused and results in drug resistance (Fitri et al., 2011). So it is necessary to develop and discover drugs derived from plants (herbal medicines).

Berenuk (*Crescentia cujete* L.) is a species of plant from the Bignoniaceae family that grows in the tropics (Mahbud et al., 2011). Rismayani (2013) stated that Indonesia is a country that has the potential to be overgrown with berenuk because it is still in the Southeast Asia and South Asia region. This plant is widely available in various regions in Indonesia and is common to find. Therefore, the availability of berenuk in Indonesia is quite abundant, but its use is still rare (Bahroni and Istianah, 2017). Berenuk contains tartaric acid, citric acid, tannins, -sitosterol, stigmasterol, and amirina, triacontanol stearic acid, palmitic acid, quercetin, apigenin (Kaneko et al., 1998; Ogbuagu 2008; Dawodu et al., 2016).

In this study, a test will be carried out to prove the anthelmintic potential of giving the ethyl acetate fraction of berenuk fruit to the mortality of

worms. Phytochemical screening of the ethyl acetate fraction of berenuk fruit that was carried out by Billacura and drawerapag (2017) proved that the ethyl acetate fraction of berenuk fruit contains tannins, flavonoids, alkaloids, and can be used as an anthelmintic in *Eudrilus eugenia* worms. Tannins are complex phenolic compounds from the polyphenol group that have a function to capture and precipitate proteins (Lenny, 2006). The way tannins work as anthelmintics is by interfering with the formation of energy by inhibiting oxidative phosphorylation which causes death (Patel et al., 2010). Alkaloids have a way of working that is almost the same as saponins, namely they can inhibit the work of the cholinesterase enzyme which functions to decompose acetylcholine which is a carrier of nerve impulses / neurotransmitters (Sandika et al., 2012), while the way flavonoid compounds work is by causing protein denaturation in worm tissue, resulting in death. worms (Faradila, 2013).

Methods

This type of research is true experimental with a posttest only-control group design approach. The sample used was *Haemonchus contortus* worm taken from goat abomasum with the criteria that the worm was still active. The sample size in this study was 20 worms per petri dish with 4 replications for each treatment so that the total sample size was 400 *H. contortus* worms. The tools used in this study were a petri dish, pipette, tweezers, stirring rod, water bath, incubator, 250 mL erlenmeyer flask, 500 mL beaker, 100 mL measuring cup, digital scale, thermometer, gloves, extraction and fractionation tools, stopwatch, and documentation camera. The materials used in this study were berenuk fruit that fractionated in several concentrations, distilled water, 0.9% physiological NaCl solution, Tween 80, 96% ethanol, ethyl acetate, levamisole as a drug control, and the worm *Haemonchus contortus*.

Berenuk fruit was dried and mashed into powder, then macerated using 96% ethanol for approximately three days and stirring occasionally for the first six hours. The maceration results filtered with cotton and filter paper. Next, the residue macerated again until it turns brown. The filtrate obtained then combined and concentrated using a rotary evaporator at a temperature of 40-50°C until a thick extract obtained (Khoirani, 2013). The viscous extract obtained fractionated with ethyl acetate as solvent. The filtrate evaporated with a rotary evaporator to obtain the ethyl acetate fraction of the berenuk fruit (Sudarmika et al., 2021).

In this study, there were 3 concentrations of the fruit ethyl acetate fraction used as the treatment group, namely the concentration of 0.125%; 0.25%; 0.5%. Positive control used levamisole 10 mg/mL and physiological NaCl 0.9% as negative control so that the worms could survive even though they were outside the host's body (Jeyathilakan et al., 2010). Each group was given Tween 80 1% as an emulsifier. Each treatment group replicated 4 times with 20 worms in each petri dish. Observations on worm mortality seen by paying attention to the presence or absence of movement or response of the worms. If the worms still remained silent while touched with objects or poured with 50°C water, then they could be declared dead. If there was movement, it indicated that the worms were only experiencing paralysis (Ali et al., 2012). Observations made every 5 minutes for 60 minutes and when all the worms in the petri dish died (approximately 125 minutes).

Calculation of LC_{50} and LT_{50} of the ethyl acetate fraction of worms to determine the concentration and length of time for which ethyl acetate fraction of worms kills 50% of worms which will be analyzed using the probit test. The data on the number of worms mortality analyzed using the One Way ANOVA test and continued with Duncan test.

Results and Discussion

In this study, the results obtained in the form of data on the number of worm mortality and the length of time for worm mortality. Based on the mortality data, the mean and standard deviation of the percentage of worm mortality will be obtained which can be seen in Table 1.

The results of observations at 5 minutes showed that K+, P2, and P3 had been able to cause mortality in worms, while K- and P1 had not shown worm mortality. This shows that the onset of action on K+, P2, and P3 starts at 5 minutes. The results of observations at 10 minutes showed that there was worm mortality in P1 so that the onset of action in P1 started at 10 minutes and there was an increase in the number of worm mortality in K+, P2, and P3. The K- has not shown the mortality of worms. Observations at 55 minutes showed that K- started to cause worm mortality. This could be due to the use of the in vitro method, which although this method designed to adapt to the condition of the host's body as much as possible, but still has different physiological conditions (Mahardika et

al., 2017). Data from Table 1 can be graphed the percentage of the average number of deaths *Haemonchus contortus* worms in each treatment group can be seen in Figure 1.

Table 1. Average and Standard Deviation Percentage of *H. contortus* Worms that Died Every 5 Minutes After Treatment in All Treatments

Treatment	Observation Time (Minutes)											
	5	10	15	20	25	30	35	40	45	50	55	60
K-	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	0,00^{a} ± 0,00	1,25^{a} ± 2,50	1,25^{a} ± 2,50
K+	23,75^{c} ± 4,78	58,75 ^{d} ± 13,76	73,75^{d} ± 14,93	82,50^{e} ± 6,45	88,75^{e} ± 4,78	98,75^{d} ± 2,50	100,00^{d} ± 0,00	100,00^{d} ± 0,00	100,00^{d} ± 0,00	100,00^{d} ± 0,00	100,00^{d} ± 0,00	100,00^{d} ± 0,00
P1	0,00^{a} ± 0,00	1,25^{ab} ± 2,50	2,50^{a} ± 2,88	7,50^{b} ± 6,45	11,20^{b} ± 4,71	15,00^{b} ± 9,12	22,50^{b} ± 13,22	28,75^{b} ± 16,52	32,50^{b} ± 15,54	36,25^{b} ± 12,47	38,75^{b} ± 11,08	41,25^{b} ± 11,08
P2	1,25^{a} ± 2,50	6,25^{b} ± 6,29	8,75^{b} ± 4,78	17,50^{c} ± 2,88	26,25^{c} ± 8,53	35,00^{c} ± 9,12	43,75^{c} ± 14,93	53,75^{c} ± 11,81	58,75^{c} ± 8,53	63,75^{c} ± 11,08	68,75^{c} ± 7,50	71,25^{c} ± 6,29
P3	10,00^{b} ± 4,08	22,50^{c} ± 10,40	32,50^{c} ± 11,90	55,00^{d} ± 10,80	71,25^{d} ± 13,15	82,50^{d} ± 15,54	88,75^{d} ± 8,53	93,75^{d} ± 6,29	100,00^{d} ± 0,00	100,00^{d} ± 0,00	100,00^{d} ± 0,00	100,00^{d} ± 0,00

Note: different lowercase superscripts ($^{a\ b\ c\ d\ e}$) in the same column show significant differences ($p<0.05$)

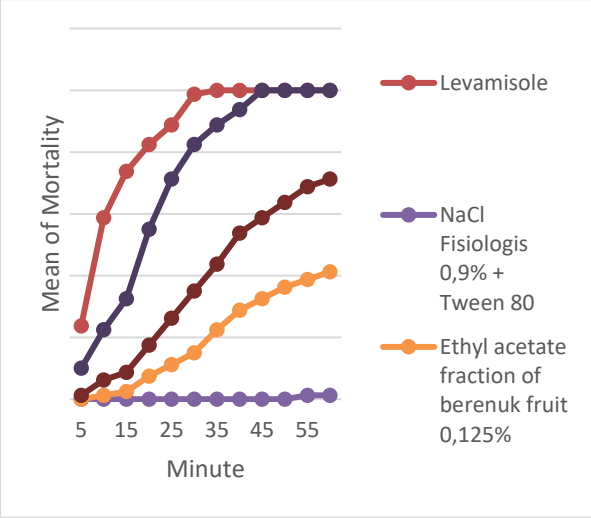

Figure 1. Graph of the average percentage of mortality of *H. contortus* worms.

Based on Figure 1, it can be seen that the mortality of the worm *H. contortus* in the treatment of the 0.5% concentration of ethyl acetate fraction of worms has the ability to be faster than the treatment of the ethyl acetate fraction of the worms with concentrations of 0.25%, 0.125%, and Tween 80 1%.

Anthelmintic activity obtained from the results of research observations at a time of 125 minutes when all worms in each treatment had experienced total mortality. Based on these data, the percentage of anthelmintic potency of the ethyl acetate fraction of fruit berenuk compared with levamisole can be seen in Table 2.

Table 2. Percentage of Anthelmintic Potency of Fruit Ethyl Acetate Fraction Compared with Levamisole

Treatment	Percentage of Anthelmintic Potency
Levamisole	100%
P1	26,04%
P2	36,23%
P3	75,75%

The calculation of the percentage of anthelmintic potency in Table 2 used to see the anthelmintic effectiveness of the ethyl acetate fraction of berenuk fruit with levamisole as a

standard drug that can cause mortality in *H. contortus* worms in vitro. The increase in the calculation of the percentage of anthelmintic potency can be interpreted that the higher the concentration of the ethyl acetate fraction of fruit berenuk, the anthelmintic effect caused will also be higher and closer to the effect of levamisole.

A concentration of 0.5% (P3) gave the best results in this study in causing the number of mortality of *H. contortus* worms compared to other concentrations, followed by a concentration of 0.25% (P2), and finally by a concentration of 0.125% (P1). Based on the calculation of the percentage of anthelmintic potency, a concentration of 0.5% has the closest result to the effect of levamisole with a value of 75.75%. This means that the concentration of 0.5% ethyl acetate fraction of worms can cause death of 75.75% of worms in the time of death of 100% of worms in levamisole positive control. The percentage of anthelmintic potency data in Table 2 presented in the form of a diagram as shown in Figure 2.

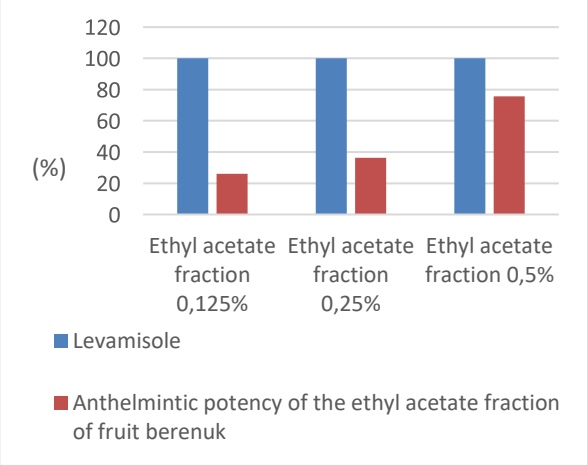

Figure 2. Comparison diagram of the percentage of anthelmintic potency of the ethyl acetate fraction of fruit berenuk compared to levamisole.

Based on Figure 2, it shows that the anthelmintic effectiveness of the ethyl acetate fraction of berenuk fruit worms is lower than levamisole because in the same period of time levamisole is able to kill more worms than the ethyl acetate fraction of berenuk fruit worms. Although the anthelmintic effect of the berenuk fruit ethyl acetate fraction is smaller than that of levamisole, the ethyl acetate fraction has the potential to be developed as an alternative medicine in the treatment of haemonchosis. This indicated by the percentage of the anthelmintic potency ratio of the 0.5% ethyl acetate fraction of the berenuk fruit with the effectiveness of the levamisole positive control. In addition, levamisole has side effects that can harm livestock if given in excess and not according to the rules for use.

The phytochemical test of berenuk fruit proven to contain three compounds, namely tannins, flavonoids, and alkaloids. If it related to the observation of worm mortality and the active substance test, the anthelmintic power of the ethyl acetate fraction of berenuk fruit comes from the content of active substances in it, namely tannins, alkaloids, and flavonoids.

The data then processed using a probit analysis test to determine the killing potency in the form of Lethal Concentration 50 (LC_{50}) and Lethal Time 50 (LT_{50}) fractions of ethyl acetate of berenuk fruit. Lethal Concentration 50 (LC_{50}) is the concentration at which the extraction solution is capable of causing the death of the population up to 50% (Noerbaeti and Ambon, 2012). The calculation of LC_{50} can be seen in Table 3.

Table 3. Results of Probit LC_{50} Analysis of the Ethyl Acetate Fraction of Berenuk Fruit (*Crescentia cujete* L.) at Every 5 Minutes of Observation

Observation Time (Minutes)	LC_{50}
5	1,39%
10	0,97%
15	0,70%
20	0,48%
25	0,39%
30	0,34%
35	0,31%
40	0,28%
45	0,27%
50	0,26%
55	0,25%
60	0,24%

Based on the results of the probit analysis of the concentration of the ethyl acetate fraction of berenuk fruit against the treatment time, the results showed that the longer the duration of the treatment, the smaller the concentration of the ethyl acetate fraction of berenuk fruit in killing 50% of the total population of *H. contortus* worms in vitro. In addition, the onset of action from each treatment time successively accelerated along with

the concentration of the ethyl acetate fraction of the berenuk fruit.

Lethal Time 50 (LT_{50}) is the time required to kill 50% of experimental animals under certain conditions (Ahmad et al., 2008). The calculation of the probit test to determine the LT_{50} value of the fruit ethyl acetate fraction can be seen in Table 4.

Table 4. Results of Probit LT_{50} Analysis of the Ethyl Acetate Fraction of Berenuk Fruit (*Crescentia cujete* L.) against *Haemonchus contortus* worms.

Treatment	Mortality Percentage 50%		
	Time (Minutes)	Lower Limit (Minutes)	Upper Limit (Minutes)
P1	60,281	55,692	65,665
P2	41,698	37,613	45,180
P3	20,968	16,612	23,969

Based on the data above, the LT_{50} results in the 0.5% concentration of ethyl acetate fraction showed a faster time to kill worms by 50% of the total population compared to concentrations of 0.25% and 0.125%. This indicates that the higher the concentration of the ethyl acetate fraction of the worms, the more active content contained in the fruit and the faster the time needed to cause death in *H. contortus* worms.

Conclusion

The conclusion based on the results of research and discussion in this study is that the ethyl acetate fraction of berenuk fruit with a concentration of 0.5% is the optimal concentration in this study in providing anthelmintic effect. In addition, the LC_{50} values obtained at treatment times of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 12 55, and 60 minutes respectively were 1.39%; 0.97%; 0.70%; 0.48%; 0.39%; 0.34%; 0.31%; 0.28%; 0.27%; 0.26%; 0.25%; and 0.24%, while the LT_{50} at each concentration of 0.125%, 0.25%, and 0.5% respectively were 1 hour 17 seconds, 41 minutes 42 seconds, and 20 minutes 58 seconds.

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