

In vitro anthelmintic activity of pineapple peel juice (*Ananas comosus* (L.) Merr.) against *Paramphistomum* sp.

Siska Yunita Damiyati^{a,*}, Iman Surya Pratama^a, Galuh Tresnani^b

^aDepartment of Pharmacy, Faculty of Medicine, Mataram University, Mataram 83125, Indonesia

^bDepartment of Biology, Faculty of Mathematics and Natural Science, Mataram University, Mataram 83125, Indonesia

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Abstract

Pineapple peel (*Ananas comosus* (L.) Merr.) has a potential natural-based anthelmintic agent. This study aimed to determine the effective concentration of pineapple peel juice against *Paramphistomum* sp. in vitro. Adults *Paramphistomum* sp. were divided into 5 test groups, the control group contained Albendazole 10% w/v, the nontreatment group contained NaCl 0.9% w/v and the treatment group contained pineapple peel juice with concentration of 12.5, 20, and 25% w/v. The number of live flukes and the motility scores were recorded every 15 minutes for 5 hours of incubation. The Survival Index (SI) and Relative Motility (RM) of flukes were calculated and statically analyzed using SPSS version 23 software. SI values of flukes in the 12.5, 20, and 25% w/v concentration of treatment group were 46.3, 50.0, and 38.9% respectively. RM values of flukes in the 12.5, 20 and 25% w/v concentration of treatment groups were 39.1, 42.0, and 34.1 respectively. RM values of flukes in the 12.5, 20 and 25% w/v concentration of treatment groups were 39.1359, 42.0249, and 34.1174 respectively. This research showed that, pineapple peel juice 25% w/v was effective against *Paramphistomum* sp. with survival index and relative motility values comparable to Albendazole 10% w/v ($p < 0.05$).

Keywords: Pineapple Peel Juice; Anthelmintic; *Paramphistomum* sp

1. Introduction

Paramphistomiasis is an infection caused by the flukes *Paramphistomum* sp. from Paramphistomatidae family [1]. These flukes infect ruminants such as cattle, sheep, and goat, particularly in ruminants with poor body conditions [2]. The infection of *Paramphistomum* causes tissue damage because the flukes penetrate the small intestinal wall and migrate into the rumen of livestock [3]. Paramphistomiasis is distributed all around the world with the highest prevalence reported in tropical and subtropical regions, particularly in Africa, Asia, Australia, Eastern Europe, and Russia [4].

The prevalence of paramphistomiasis in West Nusa Tenggara including the one recorded by Denpasar Veterinary Center was found to be 24.27% in 2016. The identification result showed that the highest prevalence of trematodosis occurred in Central Lombok and Mataram [5].

Paramphistomum infection is frequently overlooked although the flukes have a major impact on the health and productivity of livestock [6]. The infection of these flukes causes losses including inhibiting ruminants' growth, damaging tissues or organs, and reducing body weight. This impact can cause heavy losses for farmers, including a decrease in economic value of livestock and an increase in production

costs due to drug use [7,8,9]. Non-pharmacological control of paramphistomiasis is carried out by maintaining the hygiene of feed and cowshed, drying trenches, avoiding livestock grazing during rainy season, and caring for livestock in dry season [10]. Pharmacological control is carried out by administering synthetic anthelmintics such as Albendazole, Niclosamide, Resorantel, or a combination of Oxytocanide-Levamisole [11,12,13].

Several studies have reported resistance to the use of synthetic anthelmintic including Albendazole due to frequent and inappropriate use [14]. Medicinal plants can be used as an alternative to overcome the limitations of synthetic anthelmintics. Medicinal plants are easy to obtain, process and administer to livestock [15]. Secondary metabolites of plant work synergistically with various mechanisms, thereby reducing the risk of side effects and resistance [16,17]. Garlic (*Allium sativum*), goldenseal (*Hydrastis canadensis*), and papaya (*Carica papaya*) are some of the natural anthelmintics that are widely used by the community [18]. Pineapple peel or *Ananas comosus* (L.) Merr. has the potential as a natural anthelmintic [19].

According to Widodo, pineapple peel juice at a dose of 40g per kg of body weight was able to reduce the number of eggs of *Paramphistomum* sp. in Bali cattle with an egg reduction rate of 100% comparable to Albendazole as a positive control with a 95% confidence level [20]. Research on the effectiveness of pineapple peel against *Paramphistomum* adults is still limited

* Corresponding author.

Email: siskayunita38ixa@gmail.com

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[21]. This study aimed to determine the effective concentration of pineapple peel juice (*Ananas comosus* (L.) Merr.) against *Paramphistomum* sp. in vitro.

2. Materials and Methods

2.1. Collection of adult *Paramphistomum* sp.

Adult *Paramphistomum* were collected from Majeluk Slaughterhouse, Mataram City, Lombok, West Nusa Tenggara, Indonesia. Flukes were collected from the rumen and reticulum of the cattle. The flukes were collected in a jar containing a warm 0.9% (w/v) NaCl solution. Two hours after collection, the flukes stored at 37°C were then selected for identification and in vitro anthelmintic test. The selected flukes of this study were actively mobile and had a relatively uniform size.

2.2. Morphological identification

Adult *Paramphistomum* were identified by observing the shape, color, size, oral sucker, acetabulum, testes, and caecum. The identification was carried out by a whole-mount preparation. Flukes were clamped between two glass objects and fixed with 70% (v/v) alcohol. The specimens were immersed in natural dye from red beetroot 100 g/100 mL and synthetic dye from Acetocarmine 1 g/100 mL for 24 hours.

Beetroot dye was made following the preparation by Al-Amura et al. with a few modifications, 100 g of beetroot pieces were boiled in 100 mL of water for 1 hour, then the solution was filtered [22]. Acetocarmine[®] dye was prepared by dissolving 1g Acetocarmine[®] powder in 100 mL water and filtered using filter paper [23]. The specimen was washed with acid alcohol (2 mL HCl in 100 mL 70% (v/v) alcohol)

The specimens were dehydrated into 70, 80, 90, and 96% (v/v) alcohol. The specimens were cleared with methyl salicylate and mounted with Entellan[®]. The morphology was identified according to the literature by Mehlhorn et al. [24].

2.3. Preparation of pineapple peel juice

The pineapples were obtained from Lendang Nangka Village, Masbagik Sub-district, East Lombok District, West Nusa Tenggara Province. The sample was identified in the Advanced Biology Laboratory of Plant Ecology and Biosystematics, Department of Mathematics and Natural Sciences, Mataram University. Based on determination letter No. 10/UN18.7/LBL/2020, the sample has been confirmed to be Pineapple (*Ananas comosus* (L.) Merr.).

Pineapple was peeled and the peels were weighed, sorted, cleaned, and cut. The pineapple peel pieces were then blended with water to obtain a juice solution with a concentration of 25% w/v. Pineapple peel juice 25% w/v was then diluted to a concentration of 20% w/v and 12.5% w/v [52] Pineapple Peel Juice is further abbreviated as PPJ.

2.4. In vitro anthelmintic test

These tests were divided into 5 test groups, the treatment group contained pineapple peel juice (12.5, 20, and 25% w/v), the negative control group contained NaCl 0.9% w/v and the positive control group contained Albendazole 10% w/v. Each petri dish was filled with the solution of each test group and

incubated for 30 minutes at 37 °C. The flukes were incubated in petri dishes containing the solutions at 37 °C. Each test group contained 3 flukes.

The flukes' mortality was observed one by one in each fluke every 15 minutes for 5 hours of incubation. The flukes' motility at each incubation period was assessed using the criteria from Lorsuwannarat et al [25]. Flukes without motility were stained with 1% w/v methylene blue dye diluted in 0.9% w/v NaCl for 2 minutes. The in vitro test was carried out with 3 replications.

2.5. Data analysis

Relative Motility of flukes were determined using the following formula [25]:

$$MI \text{ (Motility Index)} = \frac{(\sum nN)}{(\sum N)}$$

$$RM \text{ (Relative Motility)} = \frac{(MI \text{ test} \times 100)}{(MI \text{ control})} \quad (1)$$

n = Motility Score

N = Numbers of flukes in each group

The survival index of flukes was calculated by the following formula [25]:

$$SI \text{ (Survival Index)} = \frac{(\text{Numbers of live flukes})}{(\text{Numbers of all flukes})} \quad (2)$$

The data were analyzed statistically using SPSS version 23. A normality test was performed to find out whether the data were normally distributed or not. Hypothesis testing with One Way ANOVA was carried out if the data were normally distributed then the analysis was continued with the Post Hoc LSD test. Kruskal Wallis test was carried out if the data were not normal and followed by the Post Hoc Mann-Whitney U Test.

3. Results and Discussion

3.1. Morphological identification

Based on the macroscopic observations in figure 1, the flukes were oval, pink, 7-10 mm long, and 2-3 mm wide. These observations indicated that the flukes had a similar morphology to *Paramphistomum* sp. [24,12,26]. Microscopic identification was carried out by observing the morphology of the whole preparations of flukes stained by red beets as natural dyes and acetocarmine as synthetic dyes.



Fig. 1. Morphological identification of *Paramphistomum* sp.

Based on figure 2 (a) and (b), preparations with 40x magnification showed the morphology of *Paramphistomum* sp. according to references by Mehlhorn et al. The oral suction cavity was located at the anterior end, while the acetabulum was located at the posterior end [26]. The anterior cecum of the flukes branched into two [27]. On two lateral sides, there were vitellary glands, while in the middle near the cecum there were genital pores [24,11]. Two testes were located in the middle of the fluke's body [28].

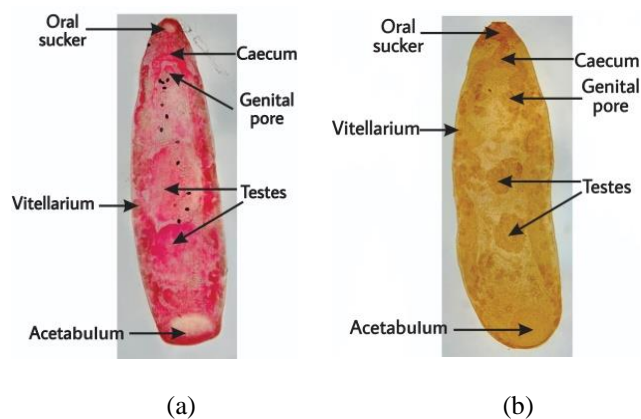


Fig. 2. Morphological identification of whole mount of *Paramphistomum* sp.: (a) Red Beet staining; (b) Acetocarmine staining

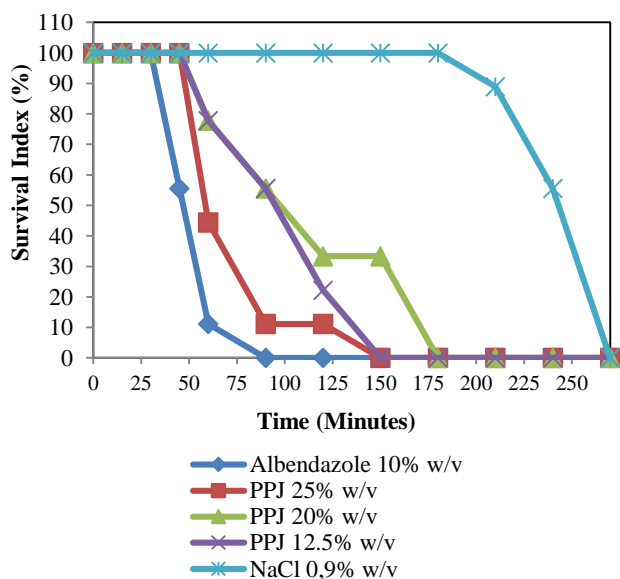


Fig. 3. Survival index mean

Beetroot dye with a concentration of 100 g/100 mL was made by boiling process for 1 hour. The whole-mount specimen that had been soaked for 24 hours produced a yellow color. This color came from the pigment betaxanthin as part of betalain in red beet [29]. Betalain has a carbonyl group which is in an acidic condition will react with amino protein groups and will form salts with alkaline proteins [30].

Betaxanthin is formed through a condensation reaction between amino acids or amines with the aldehyde group of betalamic acid. Betalamic acid is the main chromophore of betalain which plays an important role in the staining process [31]. Betalamic acid causes the water extract of red beet to have an acidic pH. This allows alkaline structures such as cytoplasm,

nerve fibers, muscle fibers, mucin, keratin, and red blood cells to be stained [30].

Specimen with 1% w/v Acetocarmine dye produced carmine red color as shown in figure 2 (b). The mechanism of Acetocarmine is the fine carmine particles enter the cell, then the molecules are aggregated into larger particles. This aggregation occurs due to the low pH in the cell proteins. The diffusion process is further inhibited which leads the carmine molecules to be trapped in the cell [32].

Red beet and Acetocarmine were able to stain all the main morphologies of *Paramphistomum* sp. Therefore, natural red beet dye has the potential as a dye for whole mount preparations of *Paramphistomum* sp. Beetroot has been used by industry as a natural food coloring agent [33]. Some studies also suggest that beetroot has the potential to dye textiles, wood, and leather. The use of natural dyes is believed to be environmentally friendly and non-carcinogenic so that it can reduce health and environmental problems due to the use of synthetic dyes [34,35].

3.2. In vitro anthelmintic activity

Albendazole 10% w/v was used as a positive control because it is a broad-spectrum anthelmintic drug that can kill various types of trematodes [36]. A concentration of 10% w/v was chosen according to Purnamasari, Albendazole 10% w/v was effective against adult *Paramphistomum* [37]. There is a recommendation from Hossain et al. and Saowakon et al. to increase the concentration of Albendazole, because the concentrations of 100 µg/mL and 10 mg/mL used in both studies had a weak anthelmintic effect [38,39].

Physiological saline solution was used as a negative control for isotonic properties of flukes so that it did not damage the cell membrane [40]. The PPJ treatment concentrations of 12.5, 20, and 25% w/v were chosen because they were ovicidal to *Paramphistomum* sp. [20]. The parameters measured in this study were the survival index and the relative motility of flukes. Survival Index (SI) is the percentage of flukes that live at a certain time after being given treatment [41].

The SI values are shown in figure 3. The lowest and highest fluke survival rates were shown by positive control and negative control, respectively. Albendazole as a positive control has been known to have an anthelmintic effect with the following mechanisms: it binds to β-tubulin so that it inhibits polymerization and formation of fluke tubules, inhibits glucose intake, occurs glycogen depletion which slowly results in fluke death [36,42].

The highest survival in the NaCl 0.9% w/v solution group showed no anthelmintic effect. Among the three PPJ treatment groups, flukes in the PPJ 20% w/v group had the longest survival. Flukes in the 25% w/v and 12.5% w/v PPJ groups experienced death simultaneously at the 150th minute. Even though, the decline in survival at PPJ 25% w/v was steeper than PPJ 20% w/v.

The fluke mortality was identified by the flukes stained with 1% methylene blue. Living eukaryotic cells were able to reduce methylene blue enzymatically which caused the color to fade and the cells were not stained. The dead cells were unable to reduce methylene blue, it caused the oxidation of methylene blue and the cells were stained blue to black [43].

The SI values of each group were then compared statistically. The results are summarized in the graph in figure

4. The graph shows that there is a significant difference between positive and negative controls ($p < 0.05$), with the positive control SI value being lower than the negative control. This shows that the fluke survival in the positive control group was lower than the negative control. The SI value in the PPJ group was lower and significantly different from the negative control, meaning that the fluke survival in each treatment group was lower than the negative control ($p < 0.05$).

The 25% w/v PPJ group was not significantly different from the positive control. This suggests that the effect produced by the PPJ 25% w/v group was comparable to that of the positive control. The Relative Motility (RM), was calculated from the score of the motility of the flukes after being exposed to the test solution. The smaller the RM value, the stronger the drug activity [41].

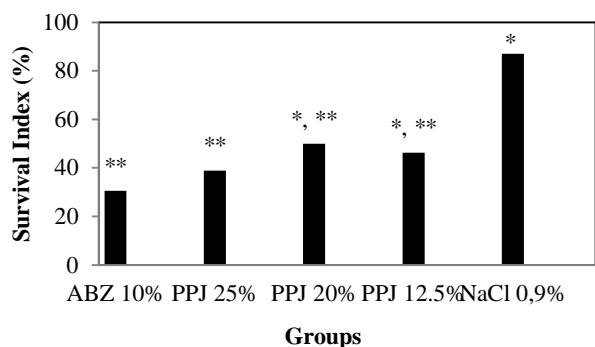


Fig. 4. SI mean of each group: * significantly different from Albendazole 10% w/v, ** significantly different from NaCl 0.9% w/v ($p < 0.05$)

The RM value is shown in figure 5. Flukes in the negative control group showed active motility from 0-240 minutes. Meanwhile, the motility of the flukes weakened after being exposed to Albendazole and all PPJ concentrations. Flukes at the three concentrations of PPJ lost their motility and died simultaneously at 150 minutes, however, the reduction in RM per unit time was different. The most passive and most active motilities among the PPJ treatments were shown by PPJ 25% w/v and PPJ 20% w/v.

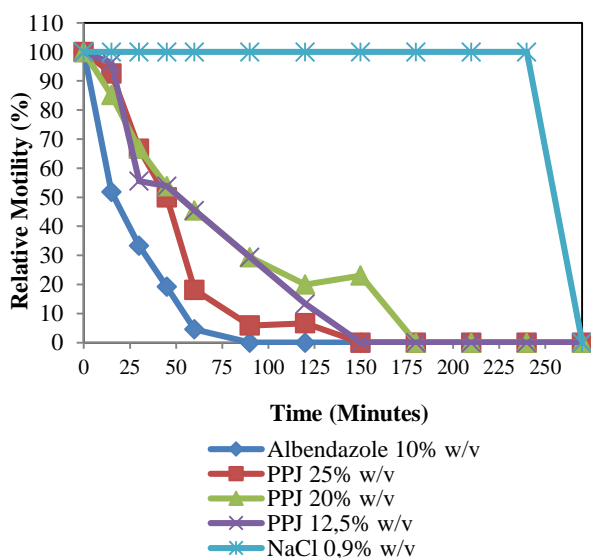


Fig. 5. Relative motility mean

The RM value of each test group was then processed statistically. The statistical test results are presented in figure 6.

Based on the graph, there is a significant difference between the positive and negative control groups ($p < 0.05$), with the positive control SI value being lower than the negative control. This shows that the motility of flukes in the positive control group was more passive than the negative group. The RM value in the PPJ group was lower and significantly different from the negative control, meaning that the fluke survival in each treatment group was lower than the negative control ($p < 0.05$). All PPJ groups were not significantly different from the positive controls so that the effects produced by the two groups were comparable.

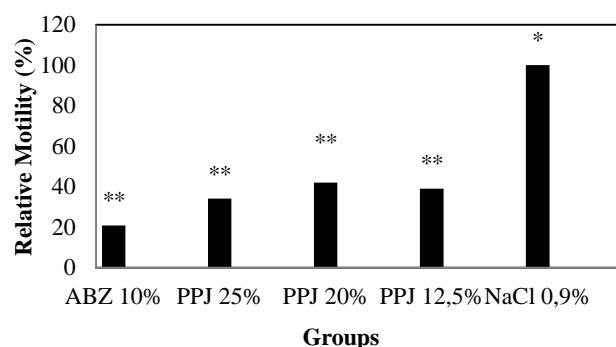


Fig. 6. RM mean of each group: * significantly different from Albendazole 10% w/v, ** significantly different from NaCl 0.9% w/v ($p < 0.05$)

Based on the statistical analysis, it shows that Albendazole and PPJ have anthelmintic effects ($p < 0.05$). Saowakon et al. explained the anthelmintic mechanism of Albendazole with damage to adult Paramphistomum flukes. Albendazole binds to β -tubulin and interferes with the formation of flukes' microtubules. Gradually this causes damage to the surface of the tegument, therefore water enters the surface of the tegument and causes an osmotic imbalance. Blebbing occurred in papillae around oral suction vanity [39].

Albendazole also damages the folds on the surface of the flukes' body. Damage to the folds of the tegument on the acetabulum can interfere with the attachment of flukes to the rumen tissue of the host. Tegument damage can also interfere with flukes' physiological processes such as protection, secretion and synthesis [44,39].

The secondary metabolite compounds have been determined qualitatively by Damiyati et al. Based on the previous study, PPJ contains tannins, alkaloids, flavonoids, saponins, and triterpenoids that have anthelmintics activity [52]. Tannins bind to proteins in the flukes that interfere with motility, absorption of nutrients and cause death [45]. Flavonoids denature proteins in the fluke's body tissue which cause paralysis and death of flukes [46].

Alkaloids act on the central nervous system causing paralysis and death of flukes [47]. Saponins can increase the permeability of cell membranes resulting in vacuolization and disintegration of the tegument [48]. The mechanism of triterpenoid is to interfere with the surface of the tegument membrane, mitochondrial dysfunction, and inhibition of the lipase enzyme [49].

PPJ 25% w/v is potential as an alternative treatment for intestinal flukes because the effect is comparable to Albendazole ($p < 0.05$). In this study, increasing the concentration did not cause an increase in the anthelmintic effect, but the effect was the same at each concentration. This is presumably due to the selection of the concentration range

which was still too narrow so that the optimal effect was only seen in the 25% PPJ.

Based on an in vivo study by Widodo, PPJ 20% was known to be the most effective concentration in reducing the number of eggs, meanwhile in this study PPJ 25% was the most effective concentration against adult *Paramphistomum* sp. The difference in concentration is thought to be because, in the in vivo test, the activity of bioactive compounds is affected by the biological activity of cattle. Physicochemical factors that can affect drug levels such as digestive enzymes, pH, and ruminal flora. Ruminal flora plays an important role in drug bioavailability because it can change the efficacy of drugs through the biotransformation process [50].

In addition, the content of tannins in PPJ can affect digestive enzymes in cattle. Tannins are large molecules that can bind to proteins in digestive enzymes to form complexes. The higher the concentration of the tannins, the more tannin-protein complexes formed causing low solubility. This may cause the tannins in a high concentration of PPJ to be difficult to penetrate the membrane [51]

4. Conclusion

The concentration 25% w/v Pineapple Peel Juice is effective to kill *Paramphistomum* sp. with survival index and relative motility values comparable to Albendazole 10% w/v. SI values of flukes in the 12.5, 20, and 25% w/v concentration of treatment group were 46.3, 50.0, and 38.9% respectively. RM values of flukes in the 12.5, 20 and 25% w/v concentration of treatment groups were 39.1, 42.0, and 34.1 respectively.

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