

The population, protein profile and ultrastructure of *Ascaridia galli* in chicken treated using *Areca catechu* crude aqueous extract

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ABSTRAK

Penelitian ini bertujuan untuk mengetahui populasi, profil protein dan ultrastruktur cacing dewasa dalam usus halus ayam kampung yang diberikan infusa biji buah pinang. Sebanyak 50 ekor ayam betina berumur 6 minggu dibagi dalam 5 kelompok. Kelompok A (kontrol negatif) tidak diberi perlakuan dan tidak diobati. Kelompok B, C dan D masing-masing diberi perlakuan dosis 26 mg/mL, 53 mg/mL dan 79 mg/mL. Kelompok E (kontrol positif), diberi Pyrantel®. Semua ayam dinekropsi 14 hari setelah perlakuan. Cacing dewasa dikoleksi dan dihitung. Cacing yang digunakan pada SDS-PAGE dan Scanning Electron Microscopy (SEM) adalah cacing hasil koleksi dari jejunum ayam kelompok A, B dan C. Jumlah cacing tertinggi ditemukan pada bagian jejunum. Hasil elektroforesis menyatakan bahwa dosis 53 mg/mL menghasilkan lebih sedikit pita protein dibandingkan kontrol negatif (21 banding 12) sedangkan pada SEM ditemukan kerusakan kutikula dan abrasi bagian anterior bibir pada dosis 53 mg/mL. Kesimpulan: infusa biji buah pinang menunjukkan potensi aktifitas anthelmentik dengan mengurangi jumlah, mengurangi profil protein dan merusak cacing dewasa *A. galli* pada usus halus.

Kata kunci: Ascaridia galli, biji buah pinang, SEM, SDS-PAGE

ABSTRACT

The study aimed at investigating the population, the protein profile and the ultrastructure of adult worms in the intestine of domestic chicken treated using *Areca catechu* crude aqueous extract. Fifty domestic female chickens of 6 weeks of age were assigned to 5 groups. Group A (negative control) was not given any treatment and any drug. Groups B, C and D were given the treatment at the doses of 26 mg/mL, 53 mg/mL and 79 mg/mL, respectively. Group E (positive control) was given Pyrantel®. Necropsy was conducted to all of the chickens 14 days after the treatment. Adult worms were collected and counted. The worms used in Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Scanning Electron Microscopy (SEM) were those collected from the jejunum of the chickens in the groups A, B and C. The biggest number of the worms was found in the jejunum. The results of electrophoresis showed that the dose 53 mg/mL gave fewer protein bands than the negative control (21:12 ratio), while the results of the SEM showed that there was cuticle damage and anterior labia abrasion at the dose of 53 mg/mL. The *Areca catechu* crude aqueous extract showed anthelmintic

activity potential by reducing the number of the adult worms, lowering their protein profile and damaging the *A. galli* worms in the intestine.

Keywords: *Ascaridia galli*, *Areca catechu*, SEM, SDS-PAGE

INTRODUCTION

Ascaridia galli is the most common species of nematodes found in poultry all over the world. Alkharigy *et al.* (2018) suggest that the prevalence of *A. galli* worms in Tripoly, Libya is up to 22%. *Ascaridia galli* infects millions of poultry that it inflicts economic loss both in commercial farm and people's farm (Balqis *et al.*, 2017). Helminthiasis control in chicken is still carried out using chemoprophylaxis method (synthetic anthelmintics). The treatment of the synthetic anthelmintics is expensive and it inflicts following losses: environmental pollution, influencing hospes' health, and causing resistance (Vercruyssa *et al.*, 2011).

One of the methods to overcome the problem is to find alternative anthelmintics resulting from natural substances with efficacious anthelmintics against *Ascaridia galli*, such as ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) (Bazh and El Bahy, 2013); pineapple leaves (*Ananus comosus*) (Akter *et al.*, 2016); squash seeds (*Cucurbita pepo*) and pomegranate peels (*Punica granatum*) (Aziz *et al.*, 2018); *Acanthus ilicifolius* (Husori *et al.*, 2018); and *Iris kashmiriana* Linn. (Khan *et al.*, 2018).

In general, the parameters of in vivo study include the decrease in egg per gram (EPG) value (Bazh and El Bahy, 2013; Khan *et al.*, 2018; Das *et al.*, 2015), the measurement of mean body weight (Akter *et al.*, 2016; Khan *et al.*, 2018) and hematological value (Akter *et al.*, 2016; Saha *et al.*, 2015). The population, the ultrastructure and the protein profile of intestinal adult worms treated with *Areca catechu* crude aqueous extract have not been established up to the present.

The study aimed at investigating the population, the protein profile and the ultrastructure of adult worms in the intestine of domestic chicken treated using *Areca catechu* crude aqueous extract.

MATERIALS AND METHODS

All stages of the research were approved by the Ethical Committee of Gadjah Mada University (number 00126/04/LPPT/XI/2018).

Preparation of The Extract

The *Areca catechu* were obtained from a garden in Muntilan area of Magelang district of Central Java Province, Indonesia. They were sliced into small pieces and dried up (Mubarokah *et al.*, 2019). The doses of 26 mg/mL, 53 mg/mL and 79 mg/mL were prepared from the *Areca catechu* crude aqueous extract. The dose of 26 mg/mL was prepared from 2.65 grams of the sliced *Areca catechu* by adding 100 mL of aquades. And then, it was heated at 90 °C for 15 minutes. After that, it was cooled and filtered using filtering paper and kept in a refrigerator and used on next day (Widiarso *et al.*, 2018).

Experimental Chickens

The animal experiments were carried out according to Aziz *et al.* (2018) with modifications. There were 50 female chickens of 6 weeks of age were used in the study. Adaptation period lasted for 14 days and on the day 15 the chickens were sterilized from protozoa and parasite infections treated with Coxy® and Levamid®. Each chicken underwent worm's egg examination for each gram of its feces to make sure that it was free of any worm infection. They were raised individually in battery equipped cage, fed using commercial feed that contains coccidiostat (BR 2® medicated) and given drinking water ad libitum. They were assigned to 5 groups. The chickens in group A were not given any treatment and any drug. The chickens in group B were given the treatment at the dose of 26 mg/mL of the *Areca catechu* crude aqueous extract. The chicken in group C were given the treatment at the dose of 53 mg/mL. The chicken in group D were given the treatment of 79 mg/mL. The chicken in group E were given Pyrantel® at the dose of 50 mg/mL. Necropsy was conducted to all of the chickens 14 days after the treatment. The whole intestine was removed and dissected longitudinally. The adult worms in duodenum, jejunum, and ileum were collected in petri dish containing physiological NaCl. The population of the *A. galli* in the intestine was counted on the basis of its location.

The Examination of Worms' Protein Profile

The worms used in the study were those collected from the jejunum of the chickens in the treatment groups A, B and C. In short, the specimens were washed several times using Phosphate Buffered Saline (PBS) to remove impurity and chicken's tissue remnants. They were crushed in 250 µl of PBS. The dissolved protein was insulated by centrifuging it at 1200x g for 5 minutes and then electrophoresis was carried out. Modified SDS-PAGE was carried out following Laemmli (Laemmli, 1970). The dissolved protein and the marker (Thermo scientific, Lithuania) were boiled for 2 minutes in sample buffer at 4:1 ratio, containing 50% of glycerol, 10% of SDS, 2.5% of DTT and 0.05% (w/v) of aqueous bromophenol blue as marker dye. The electrophoresis was carried out at 100 V in vertical slab gel system for 3 hours. Subsequently, the gel was stained using 0.2% (w/v) coomassie blue.

Scanning Electron Microscopy

The worms used in the study were those collected from the jejunum of the chickens' intestine. In short, the fixed specimens were washed using cacodylate buffer for 2 hours, and then the pre-fixation in 2.5% glutaraldehyde was carried out for 48 hours, the fixation in 2% tannin acid was carried out by washing the specimens 4 times with cacodylate buffer and distilled water, and finally, they were dehydrated in a series of ascending alcohols followed by drying in tertiary butanol. Dried specimens were coated using copper for 15 minutes and then observed in a SEM (JEOL JSM- 5319LV, JEOL USA, Inc.) (Sambodo *et al.*, 2018).

Statistical Analysis

The data of the worms' population were analyzed using ANOVA test with IBM SPSS 16.0 software. The results of the analysis were expressed in mean ± standard error. Meanwhile, the results of the SDS PAGE and SEM were descriptively analyzed. (Sambodo *et al.*, 2018).

RESULTS AND DISCUSSIONS

The Population of *A. galli* Worms

The populations of adult worms in each location are summarized in Table 1. The results of variance analysis showed that the treatment and location had a very significant effect ($P < 0.01$) on the number of worms. There was a very

significant interaction ($P < 0.01$) between the treatment and the location and the number of the worms. The results of Duncan test of Alpha 0.05 show that there was significant difference ($P < 0.05$) in the number of the worms among the three locations.

All of the doses of the *Areca catechu* crude aqueous extract show anthelmintic activity potential, except the dose of 26 mg/mL. It is believed that the death of the adult worms in the study results from the presence of tannin contained in the *Areca catechu* crude aqueous extract. Various simple phenolic acids, including gallic acid, caffeic acid and flavonoid increase the tannin that is anthelmintic in nature (Mondal *et al.*, 2015). The tannin may directly interact with the surface of the worms' body that directly or indirectly causes the damage of the worm by increasing protein nutrition and improving hospes' immune system (Min *et al.*, 2003). It hampers fumarate reductase and succinate dehydrogenase enzymes that ATP synthesis in mitochondria is hampered. The hampered fumarate reductase and succinate dehydrogenase result in the obstruction of terminal electron acceptor that the formation of the succinate dehydrogenase is hampered and subsequently it results in ATP deficit. Consequently, it causes the death of the worms (Dhanraj and Veerakumari, 2016). The damaged membrane of the worms because of the tannin results in the paralysis and finally the death of the worms. Additionally, the tannin resulting from polyphenol is also able to precipitate protein in copolymer form that is insoluble in water (Susanti and Prabowo, 2014). The results of the study are consistent with those of the study by Akter *et al.* (2016) showing that the death of 87.5% of *A. galli* worms is caused by the treatment of pineapple leaf extract. Herawati *et al.* (2018) suggests that areca nut powder is able to eliminate 80% of the *A. galli* worms in chicken's intestine.

The biggest number of the *A. galli* worms is found in the jejunum, while the smallest one is found in the ileum. Based on the location of the worms, the results of the study are consistent with Ferdushy *et al.* (2012) suggesting that the location of the majority of the *A. galli* worms is anterior jejuno-ileum. Additionally, Prastowo *et al.* (2017) suggest that the biggest population of the adult *A. galli* treated using areca nut powder and *Binahong* (*Bassela rubra linn*) is found in jejunum. The location of the worms depends on infection period, the presence of other infections

by other worm species and by bacteria (Ferdushy *et al.*, 2012; Roepstorff *et al.*, 1997).

The Protein Profile of The Worms

The protein profile of the worms in the treatment groups is illustrated in Figure 1. The negative control and the dose of 26 mg/mL give 21 protein bands with molecular weights of 131.6 kDa, 120.2 kDa, 109.8 kDa, 87.6 kDa, 66.8 kDa, 63.8 kDa, 58.3 kDa, 53.3 kDa, 46.5 kDa, 44.4 kDa, 40.6 kDa, 35.4 kDa, 32.4 kDa, 29.6 kDa, 22.5 kDa, 20.6 kDa, 19.7 kDa, 15,7 kDa, 14.3

kDa, 12.5 kDa, and 10.5 kDa, while the dose of 53 mg/mL gives 12 protein bands.

The difference may also result from the difference in protein concentration of each species of the worm used in the study. Kusnoto *et al.* (2001) suggest that the difference in the protein expression may relate to the genetics and the content of homogenate protein.

The decrease in the number of the protein bands at the dose of 53 mg/mL may result from the presence of the tannin in the *Areca catechu* crude aqueous extract. The tannin in its condensed

Table 1. The Population of Adult Worms According to Their Predilection Post Treatment

Site	Treatments				
	Control (-)	Doses of 26 mg/mL	Doses of 53 mg/mL	Doses of 79 mg/mL	Control (+) Pyrantel pamoat 50 mg/mL
Duodenum	27.6±3,98 ^a	19.4±11,99 ^d	0.7±1.16 ^g	0±0.00 ^j	0±0.00 ^m
Jejenum	52.3±9,31 ^b	37±21.79 ^e	1.3±2.21 ^h	0±0.00 ^k	0±0.00 ⁿ
Ileum	3.4±1.51 ^c	2.4±0.95 ^f	0±0.00 ⁱ	0±0.00 ^l	0±0.00 ^o

^{a-o}Different superscripts in a column and row indicate significantly difference (P<0.01)

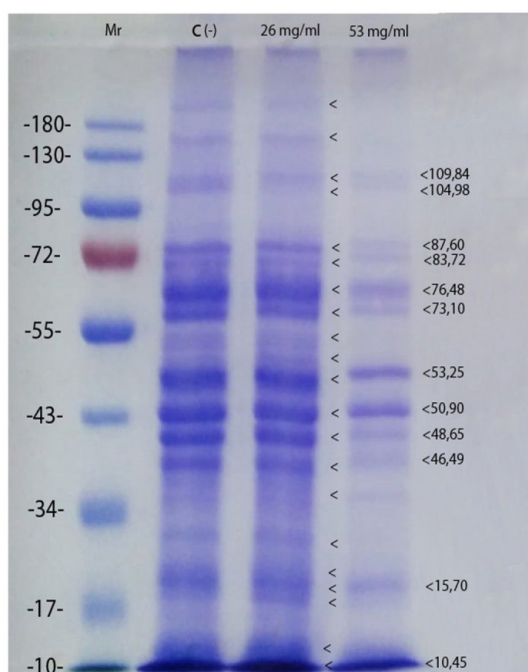


Figure 1. Elektroferogram SDS PAGE of *A. galli*. Lane 1 marker protein, lane 2, 3 and 4 parasite sample proteins. (Mr: Marker; C(-): control negative)

form has affinity to proline-rich protein, while nematode's cuticle is known to have proline-rich structure covering its body (Hoste *et al.*, 2006). The proposed nematode controlling mechanism is that the tannin interferes with the physiological process of the nematode by directly binding the protein of the nematode and then blocking the physiological process of the parasite and/or modifying the immunity response of the hospes in eliminating infective larvae and adult worms (Zhong *et al.*, 2014). The results of the negative control and the dose of 26 mg/mL are different from those of the study by Hargiyanto (2011) showing that there are 12 protein bands resulting from whole *A. galli* worm with the molecular weights of 293.8 kDa, 236.9 kDa, 115.6 kDa, 75.1 kDa, 60.6 kDa, 42.3 kDa, 23.8 kDa, 16.7 kDa, 12.5 kDa, 8.1 kDa, 5.7 kDa and 4.3 kDa.

Scanning Electron Microscopy

Figures 2-4 illustrate the ultrastructure of the worms treated using *Areca catechu* crude aqueous

extract. The results of the micrography of the SEM show that there is a significant change in the ultrastructure of the *A. galli* after the exposure of the worms to the *Areca catechu* crude aqueous extract at the doses of 26 mg/mL and 53 mg/mL *in vivo*.

The change proves that there is an interaction between the *Areca catechu* crude aqueous extract and the cuticle that serves very important function for the nematode, especially for motility and absorption of certain nutrition. Hassain *et al.* (2009) suggest that *in vitro* test of the *A. galli* worms treated using *C. micrantha* indicates drastic change in mouth and cuticle. The change in the mouth relates to labia in which inflammation and damage take place on papilla sensory. Morphological damage takes place to the three labia (1 on dorsal part and 2 on subventral part). Morphologically, the labia become rounded or square, while the smallest labial accessory is separated from the 2 labial subventral parts. However, only a part of the labial accessory in the

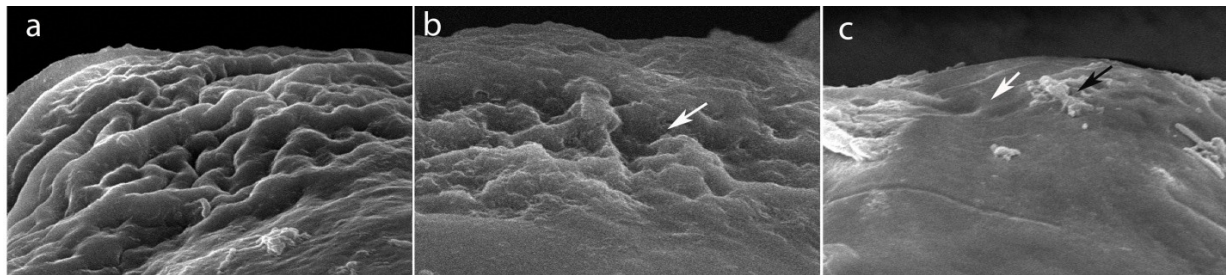


Figure 2. The ultrastructure of Worm Anterior. a. Negative control: amphids structures were seen intact, b. Doses of 26 mg/mL: amphids structures were disfigurements (white arrow), c. Doses of 53 mg/mL: amphids structures were lost (white arrow) and adhesion (black arrow)

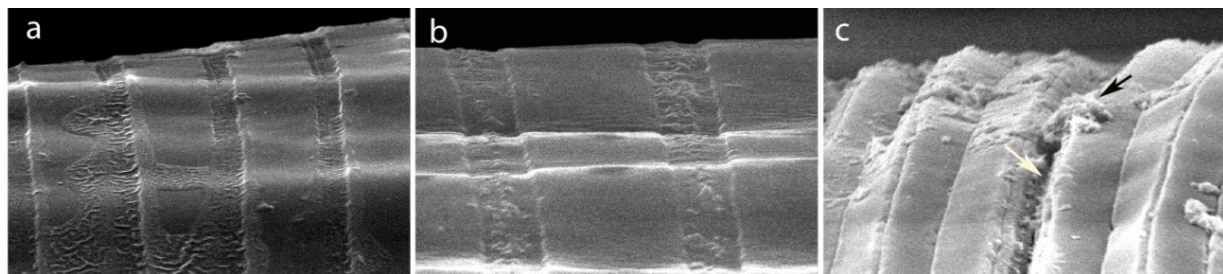


Figure 3. Cuticle ultrastructure of middle body of worm. a. Negative control: striations of annuli transversal were regular, b. Doses of 26 mg/mL: striations of annuli transversal were regular, c. Doses of 53 mg/mL: striations of annuli transversal were irregular (white arrow) and abrasion (black arrow) takes place to cuticle

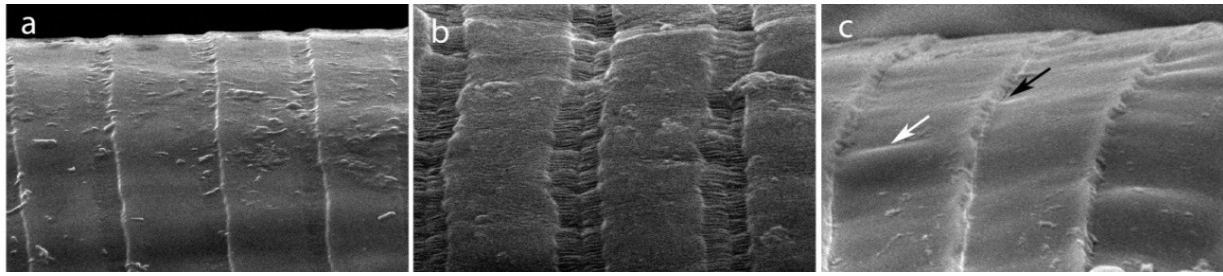


Figure 4. The Ultrastructure of the Cuticle of Worm Genital Sex Opening. a. Negative control: striations of annuli transversal were regular, b. Doses of 26 mg/mL: striations of annuli transversal were regular, c. Dosis 53 mg/mL: siltation (black arrow) and loosening (white arrow) take place to striation of annuli transversal groove

dorsal part is separated, while the remaining is still attached to its main part. The opening and the cavity of the mouth are clogged up by granulation tissue attached around the labia that results in the widening of the labia. Additionally, the formation of amphid vanishes because of the compressed labia. The cuticle groove around the labia vanishes because of the inflammation. Additionally, Lalchhandama (2010) suggests that the amphid represents the main chemosensory organ of the nematode and plays a very important role in its capability to recognize the behavior of the hospes and to control its development. The neurons found in the amphid are responsible for the majority of the sensory function such as detecting liquid chemoattractant, strong scent, heat receptor and chemotaxis (the movement resulting from stimuli) during the development of the larvae so that it can recognize the right hospes. The damage of the labia is usually caused by the damage of the amphids.

According to Hassain *et al.* (2009), *C. micrantha* has also significant impact on the cuticle structure that it influences the function of the cuticle and results in the disruption of nutrition uptake and finally causes worm's death. The cuticle is a functional part of protecting and selecting in absorption function so that it becomes the main target of anthelmintic drug. Normal cuticle has annuli of continuous and well-ordered grooves that look like a ring, which stretch to posterior part. In preanal sucker part, the annuli do not connect to its irregular form (Lalchhandama, 2010). Additionally, the change in the cuticle into longitudinal and transversal wrinkles after the exposure to walnut extract rich of in vitro condensed tannin in *Trichostrongylus colubriformis* has been reported by Hoste *et al.*

(2006).

The affinity of the condensed tannin to protein is determined by the molecular mass and the molecular configuration of the tannin and also those of the protein. The tannin-protein bond is usually reversible. The pH (the acidity or the alkalinity), the treatment of detergent (surfactant) or phenol or other organic solvents can cause dissociation of a complex bond. The condensed tannin has special affinity to proline-rich protein such as gelatin, prolamin and the protein resulting from grains (Hoste *et al.*, 2006). Also, it can interact with multiple lipid layers by binding cholesterol that may be found in the cuticle of some nematodes. The primary interaction of the condensed tannin may take place with cuticlins through the insertion of the condensed tannin into lipid layer (glycosylation). The secondary interaction with collagen proline-rich layer will result in further interference of hypodermal cells (Ropiak *et al.*, 2016).

CONCLUSION

Areca catechu crude aqueous extract has a significant anthelmintic activity potential in reducing the number, lowering the protein profile and causing serious damage to adult *A. galli* worms in chicken's intestine.

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