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In Vitro and *in Vivo* Anthelmintic Activities of Aqueous Leaf Infusion of *Azadirachta indica* against *Haemonchus contortus*

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ABSTRACT

Anthelmintic resistance of *Haemonchus contortus* worm has become a major problem in ruminant production. Traditional medicinal and potential tropical plants with high tannin contents have a great potency as an alternative source of bio-anthelmintic. The study was carried out to assess the *in vitro* egg hatches inhibiting (EHI) and adult worm motility (AWM) tests and *in vivo* anthelmintic activities of aqueous leaf infusion of *Azadirachta indica* against *H. contortus*. Leaf infusion at doses of 2%, 4%, and 6% were used for *in vitro* treatments, and albendazole at a dose of 2 mg/mL was used as a positive control and 0.9% of sodium chloride was used as a negative control. The EHI assay was conducted two times i.e., before treatment and 24 h after treatment. The AWM were monitored 15 and 30 min, and 1, 2, 3, 4, 5, 6, 7, and 8 h post treatment. Sixteen Javanese Thin Tail ewes at the ages of ±12 months that naturally positive of *H. contortus* with about 1.000 EPG were selected and allocated randomly to 4 groups (n= 4). They were subsequently received the ensuing treatments i.e., *A. indica* infusion at doses of 0% (A) as a control, 6% (B), 8% (C), and 5 mg/BW albendazole (D) at a single oral dose as a positive control. Experimental ewes in Groups B and C were given oral infusion weekly during 4 weeks of treatment. EPG's fecal examinations were conducted weekly. The result showed that the leaf infusion of *A. indica* containing condensed tannin (CT) at a dose of 6% significantly inhibited egg hatching (P<0.05) with a better effect compared to doses of 0%, 2%, and 4%. In AWM test, three doses of infusion significantly difference each other, as well as both negative and positive controls (P<0.05). After treatment, both of the *A. indica* containing CT levels and albendazole reduced EPG count significantly (P<0.05). Meanwhile, significant difference was not detected in dose of 8% aqueous leaf infusion of *A. indica* and albendazole treatments on weeks 3, 4, 5, and 6 consecutively. In conclusion, the aqueous leaf infusion of *A. indica* is fully potential as a bio anthelmintic against *H. contortus*.

Keywords: Haemonchus contortus, Azadirachta indica, anthelmintic activity, leaf extract, sheep

INTRODUCTION

Haemonchus contortus called abomasum parasite is one of the nematodes which may lead to anemia that causes the decline in ruminant production resulting in economic losses (Martins *et al*., 2017; Pathak & Tiwari, 2013; Gilleard, 2006). Anthelmintic treatment is applied to control this worldwide major pathological constraint. On the other hand, anthelmintic resistance of nematodes has become a major problem in many parts of the world (Ferreira *et al*., 2013; Costa *et al*., 2008; Gilleard, 2006). Because of this concern, there is a great effort to explore novel approaches for solving the problem of anthelminthic resistance by screening traditional medicinal and potential tropical plants with high tannin contents (Pathak *et al*., 2016; Hoste *et al*., 2015; Nawaz *et al*., 2014).

Neem tree (*Azadirachta indica*) popularly known as a tropical traditional medicine plant has been explored for its medicinal application. Neem leaf has been investigated to contain chemically and structurally complex active substances, i.e., nonisoprenoids include polyphenolics such as flavonoids and tannins (Adjorlolo *et al*., 2016; Costa *et al*., 2008; Subapriya & Nagini, 2005). Condensed tannin (CT) was tested for anthelmintic activity against *H. contortus* and it was shown that CT was effective as an anthelmintic both in goat and sheep (Hoste *et al*., 2016; Nawaz *et al*, 2014; Hamad *et al*., 2013; Costa *et al*., 2008). However, the data

on the anthelminthic activities of neem leaf reported on Javanese Thin Tail sheep, the Indonesian local sheep, are limited. Accordingly, the objectives of this study were to determine the effect of water-based leaf infusion supplementation of *A. indica* on *in vitro* egg hatches inhibitory effect and adult worm motility test. In addition, *in vivo* test was also conducted to measure the effect of *A. indica* infusion on the number of eggs per gram of feces as compared to albendazole in Javanese Thin Tail ewes.

MATERIALS AND METHODS

Preparation of the *A. indica* Water Extract

The mature and immature leaves (mix) of *A. indica* were collected from plants cultivated at the Research Unit for Natural Product Technology of the Indonesian Institute of Sciences, Yogyakarta, Indonesia. By following the preparation of Ferreira *et al*. (2013) with a slight modification, the water-base leaf extract was prepared by a simple infusion method. The fresh leaves were chopped about ± 1 cm and then dried in a freeze drying for three days at -20°C. The dried leaves were then ground and sheaved into 80 meshes of powder, then analyzed for CT content according to Abdulrazak & Fujihara (1999). Two grams of triturated leaves were added with 20 mL of distilled water and the mixture was maintained at 2 h, and then the mixture was ultrasonicated at 4°C for 5 min twice by an ultrasonicator. The suspension was filtered through gauze and centrifuged at 372 g for 5 min (Odhong *et al*., 2014). The resulted supernatants were diluted in 0.9% of sodium chloride to a final volume of 5 ml each with a concentration of 20%, 40%, and 60% (with consecutive concentrations the dried leaves i.e. 2%, 4%, and 6%) of the leaf infusion for *in vitro* test. On the other hand, the stock solution was prepared in 6% and 8% concentrations to be used for the infusion in *in vivo* test.

In Vitro Egg Hatch Inhibiting Test

H. contortus eggs used to perform the *in vitro* Egg Hatch Inhibiting Test (EHIT) (Coles *et al*., 1992; Coles *et al*., 2006) were obtained from female *H. contortus* worm isolated from the abomasum of infected Javanese Thin Tail ewe (>1.500 EPG). The infected ewes were slaughtered in Tegal Senggotan small ruminant abattoir in Bantul Regency. The *A. indica* infusion was added to the test tube exactly at the concentrations of 2%, 4%, and 6% in a total volume of 3 mL of 0.9% of sodium chloride, and each concentration was replicated three times. Albendazole (2 mg/mL) was used as a positive control and 9% of sodium chloride was used as a negative control. Three adult female *H. contortus* worms were subsequently added and pulverized to each of those test tubes. The experiment was executed for 24 h at room temperature (25-32°C). The assay was replicated two times for 0 and 24 h and the number of eggs were counted by following the method reported by World Association for the Advancement of Veterinary Parasitology (WAAVP), and the data were presented as % inhibition of eggs hatch, compared with two controls.

In Vitro Adult-Worm Motility Test

In vitro adult-worm motility test was conducted by following the method of Eguale *et al*. (2007) with modifications. Adult *H. contortus* worms were collected from abomasum of ewe slaughtered at Tegal Senggotan small ruminant abattoir in Bantul Regency. Adult worms were collected and washed and then were immediately placed in 0.9% of sodium chloride. The *A. indica* infusion was poured into the petri dishes at the concentrations of 2%, 4%, and 6% each in a total volume of 5 mL of 0.9% of sodium chloride. Whilst sodium chloride (0.9%) without infusion of *A, indica* was used as a negative control and albendazole (2 mg/mL) was used as a positive control. Each treatment was replicated 3 times. Meantime, 5 adult female worms were put into and maintained in one petri dish. Similar to the *in vitro* egg hatching inhibiting assay, the present experiment was conducted for 8 h at room temperature (25-32°C). The motility of each adult worm was monitored and the percentage of motile worms was calculated after 15 and 30 min, and on 1, 2, 3, 4, 5, 6, 7, and 8 h post treatment for each treatment.

In Vivo Anthelmintic Test

Sixteen female Javanese Thin Tail ewes at the age of ± 12 months that were naturally positively infected by *H. contortus* with EPG more than 1.000 were selected at the Research Unit for Natural Product Technology, Indonesian Institute of Sciences, Gunungkidul, Indonesia. The experimental ewes were randomly allocated into 4 groups (n= 4) and maintained in a sheep house and received the following treatments:

A : administered with 0.9% of NaCl as a control (negative control)
B : administered with *A. indica* infusion at a dose of 6%
C : administered with *A. indica* infusion at a dose of 8%
D : administered with Albendazole at a dose of 5mg/kg BW (positive control)

The experimental ewes were adapted to the experimental condition for 14 d before the onset of treatments. In the present experiment, the treatments were given for 4 weeks after adaptation period. Groups B and C were administered with 6% and 8% (w/v) of *A. indica* infusion orally. The calculation of the dose of *A. indica* infusion was referred to Beriajaya & Haryuningtyas (2005) by observing the average values of abomasum fluid. Meanwhile, albendazole was given to group D as a single oral dose (Jamra *et al*., 2014). The experimental ewes were fed with *Penissetum hybrid* and wheat bran provided with water *ad lib*.

Fecal EPG Examination

Eggs per gram fecal (EPG) examinations were implemented on week zero before treatment and on week 1, 2, 3, and 4 post treatment. Fecal samples of each ewe were collected directly from the rectum and the eggs were counted under microscope with 100x magnification by following the McMaster modified technique according to Coles *et al*. (1992).

Experiment Ethical Aspects

The experiment was carried out in accordance to the animal welfare standards. Design methods of this study had been approved by The Committee of Ethical Clearance for Pre-clinical Research of The Integrated Laboratory of Research and Testing, Universitas Gajah Mada, Yogyakarta, Indonesia with the Reference number of 00152/04/LPPT/XII/2017.

Data Analysis

The egg hatches tested and *in vivo* data were analyzed by using one-way ANOVA. Meanwhile the adult worm mobility tested was analyzed by factorial ANOVA, and both were continued by Duncan test to compare means. The analyses were performed by using Costat Version 6.

RESULTS

Condensed Tannin Content of *Azadirachta indica*

The concentrations of CT in freeze-dried young and mature leaves of *A. indica* were 3.99±1.59% and 4.15±1.18%, respectively. There was no significant difference in CT contents (%) of young and mature leaves. Therefore, the maturity of leaf did not affect the CT content. The averages of mixed *A. indica* freeze dried leaves CT content were 4.07±0.11% with 36.38±0.08% of dry matter.

In Vitro Egg Hatch Test

Egg hatches test showed that the leaf infusion of *A. indica* containing CT at a concentration of 6%, significantly inhibited worm-egg hatching (P<0.05) compared with the concentrations of 2% and 4%, as were shown in Figure 1. Leaf infusion containing 6% CT showed a good anthelmintic activity with a similar activity with the albendazole as a positive control. Both of leaf infusion with a concentration of 6% CT and albendazole significantly inhibited egg worm hatching (P<0.05). There was no statistically significant difference in the activity of 2% and 4% levels of *A. indica* leaf infusions in inhibiting worm-egg hatching compared with negative control treatment.

In Vitro Adult-Worm Motility Test

Figure 2 shows the average efficacy of the *A. indica* infusion containing CT to inhibit adult *H. contortus* motility (%) during the 8 h of immersion. There was a statistically significant interaction of *A. indica* concentrations with the duration of treatment (P<0.05). Three doses of infusion significantly difference each other, as well as both of controls (P<0.05). The minimum adult *H. contortus* motility inhibition was found in negative control with a mortality rate was not higher than 30% over 8 h of observation (P<0.05). On the other hand, albendazole showed the best efficacy in inhibition of motility by nearly 90% mortality (P<0.05). The dose of *A. indica* infusion at 2% did not have CT level to reach LD_{50}, except both of the 4% and 6% levels after 8 and 5 h

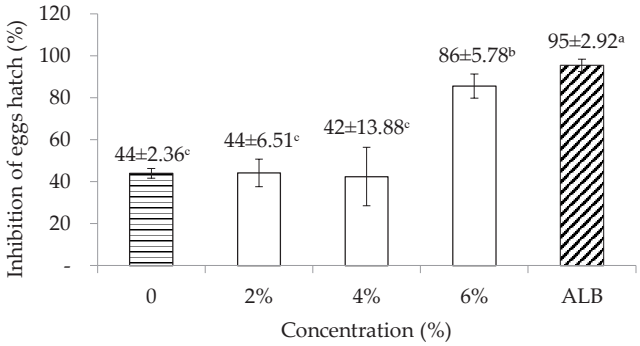

Figure 1. *In vitro* inhibitory effect of different leaf infusion concentrations of *A. indica* at ▤ 0%, □ 2%, □ 4%, & □ 6% (w/v) on egg hatch test against *H. contortus*. ALB (▨): albendazole 2 mg/mL. Means with different superscripts differ significantly (P<0.05).

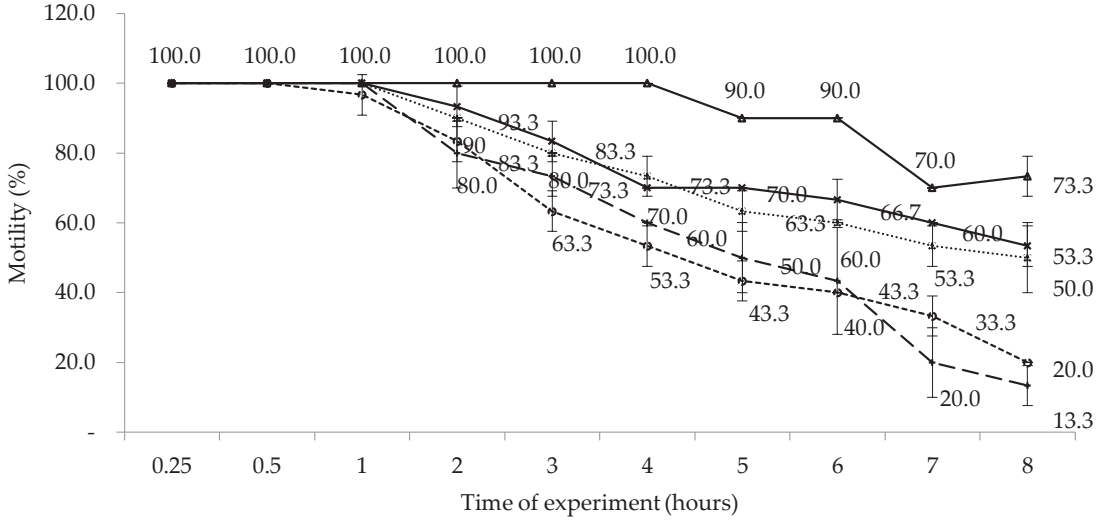

Figure 2. *In vitro* inhibitory effect of different leaf extract concentrations of *A. indica* (—▲— 0%; —×— 2%; ···×··· 4%; - -●- - 6%) on adult worm motility test against *H. contortus*. ALB (— ← –): albendazole 2 mg/mL.

treatment, respectively. The effectiveness of *A. indica* leaf infusion at a dose of 6% and albendazole 8 hours after treatment were not significantly difference, which can reached LD_{50} after 5 h of immersion.

In Vivo Anthelmintic Test

Reducing of the EPG counted data are shown in Figure 3. The data showed that there was no statistically significant difference in EPG data during weeks 0-2 pre-treatment, which was an adaptation period prior to treatment. Thereafter, one week post-treatment (on week 3) both of *A. indica* containing CT levels and albendazole reduced EPG count significantly (P<0.05). There were significant differences in EPG in weeks 3, 4, and 5 in ewes treated with various doses of *A. indica* leaf infusion (6% and 8%) and albendazole compared to control ewes. However, in week 6 of observation, the EPG counts in control ewes and ewes treated with 6% of *A. indica* leaf infusion were similar while those treated with 8% of *A. indica* infusion and albendazole were still different from control (P<0.05).

The anthelminthic effects of 8% *A. indica* leaf infusion was similar to those of positive control (albendazole). This condition was indicated by the non-significant difference in EPG in ewes treated with 8% *A. indica* leaf infusion level and albendazole in weeks 3, 4, 5, and 6 of treatment.

There was no significant difference in EPG count in ewes treated with 6% *A. indica* leaf infusion and albendazole on week 4 of observation. However, observation on weeks 3, 5, and 6 post treatment, the anthelmintic effects of 6% *A. indica* leaf infusion were significantly lower (P<0.05) than those of control positive albendazole, as were shown by the higher (P<0.05) EPG in ewes treated with 6% *A. indica* leaf infusion compared to those treated with albendazole.

Doses of 6% and 8% of *A. indica* leaf infusions had similar anthelmintic effects. There was no significant difference in EPG count in ewes treated with doses of 6% and 8% of *A. indica* leaf infusions observed on 3, 4, 5, and 6 weeks post treatment. However, as was stated above, in week 6 of observation, the EPG counts in control ewes and ewes treated with 6% of *A. indica* leaf infusion were similar while those treated with 8% of *A. indica* infusion and albendazole were still different from control (P<0.05).

DISCUSSION

In this study, *A. indica* leaf was chosen based on the reported bio-anthelmintic potency and availability. Benzimidazoles (including albendazole) is well known for its broad-spectrum anthelmintic activity which highly effective (>95%) against ruminant parasites. However, the uses of albendazole lead to the resistance of nematodes to the albendazole, especially *H. contortus* (Rezansoff *et al*., 2016). The resistance has been widespread globally due to the frequent use and the time of their availabilities (Besier *et al*., 2016; Martins *et al*., 2017). Based on these resistance factors, searching of the bio-anthelmintic plant extracts may contribute to the novel development of traditional plant-based remedies that could provide a lower risk of resistance than the chemical anthelmintic (Ferreira *et al*., 2013; Besier *et al*., 2016). Aqueous infusion, based on this research with *A. indica*, and according to the previous study using the other traditional plant containing CT (Vieira *et al*., 2017; Nawaz *et al*., 2014; Ferreira *et al*., 2013; Eguale *et al*., 2007), tannin clearly inhibited egg hatching and adult worm motility. These facts then allowed that aqueous extract method could be used in the preparation of bio anthelmintic source in anthelmintic research work. The efficacy of 6% *A. indica* leaf infusion was better than

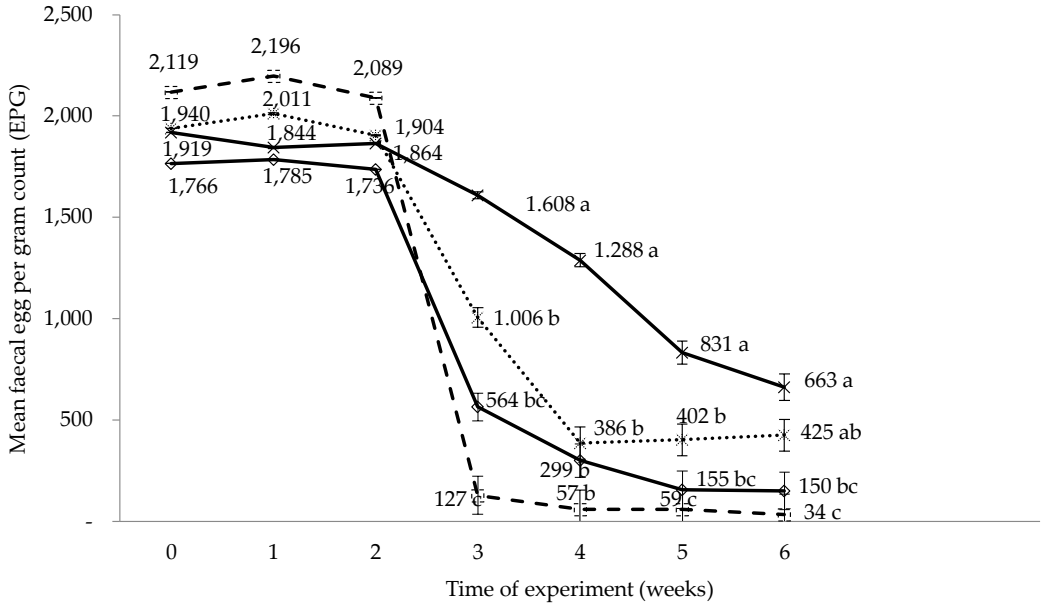

Figure 3. *In vivo* anthelmintic activity of different leaf extract concentrations of *A. indica:* 0% (Control negative, —×—), 6% (····※····), 8% (—◇—), and Albendazole 5 mg/BW (Control positive, - - ▫ -), on mean faecal egg per gram count of Javanese thin tail ewes. Means with different superscripts ($^{a, b, c}$) at same time of experiment were differ significantly (P<0.05).

those of 0%, 2%, and 4%. The dose of 6% indicated a substantial potency of *A. indica* as a good bio-anthelmintic activity to inhibit worm-egg hatching. Similar to this study for about 5%-6% levels of leaf simple infusion reported egg-hatching inhibition until 84.91% in *Annona muricata* at 5% (w/v) against *H. contortus* (Ferreira *et al*., 2013).

This study did not only conduct *in vitro* study, but also *in vivo* experiment. *In vitro* model gave advantage that compounds of plant contacted and affected the target process freely and directly e.g., egg stage of *H. contortus*. However, effectiveness of plant material that is tested *in vitro* does not always show an equally effective result *in vivo* (Ferreira *et al*., 2013). This study used Javanese thin-tailed sheep, a native breed of small ruminant in Indonesia. Javanese thin-tailed sheep is considered to be a more suitable breed for grazing system (Budisatria *et al*., 2007). On the other hand, small ruminant with grazing behavior may be susceptible to infection, especially *H. contortus,* one of the most important representatives of the order Strongylida in tropical and subtropical areas (Gasser *et al*., 2016).

EPG count data on *in vivo* adaptation period were not different due to the implementation of the similar feed ration to all of treatments. The non-significant EPG count was also contributed by feeding a cultivation grass type such as *P. hybrid* that might be expected to have a fewer larvae of parasites contamination than a field grass. Owing to the fact that *P. hybrid* cultivation area has a limited contact with susceptible animals, different from the non-rotational pasture system. This condition leads to a slowing down of the ewe-infection dynamics (Besier *et al*., 2016; Hoste *et al*., 2006; Paolini *et al*, 2003).

The test based on EPG count confirmed a statistically significant reduction in one weeks post-treatment (on week 3). Both levels of *A. indica* infusion could produce a similar result with albendazole. The anthelmintic efficacy of *A. indica* against *H. contortus* is related to the presence of CT. Recently, the novel research involving the supplementing of forages containing CT to ruminants has been focused on the utilization of anthelmintic activities (Nauman *et al*., 2013). Condensed tannins of the leaf extract damage the cuticle of *H. contortus* (Tresia *et al*., 2016) which can bind the parasite's cuticle protein (Kerboeuf *et al*., 2008), that may lead to the reduction of the flavonoid diffusion and increase the exposure to the compound (Tresia *et al*., 2016). The fatal intracellular consistency occurred due to the inhibition of enzyme secretion by CT that would cause a paralysis of parasites (Tresia *et al*., 2016; Kerboeuf *et al*., 2008; Hoste *et al*., 2006).

Nevertheless, the effectiveness of treatments did not persist during a long period, which was found the similarity of efficacy at 6% on week 6 compared to negative control. The reduction on EPG count in ewes treated with negative control might be affected by nutrition factor, as a consequence of implementation of forages and wheat bran which had a 19.15% dry matter (DM) of crude protein (Jayanegara *et al*., 2017). Indirect effect on *H. contortus* biology is the presence of adequate dietary nutrient of the digestive content that improves the host's immune response systems against the parasites (Hoste *et al*, 2016). There is a significant relationship between nutrition and infection (Pathak & Tiwari, 2013). The host ability to control any parasites can be affected by the enhanced nutrition and the levels of protein and energy (Pathak & Tiwari, 2013; Maherisis *et al*., 2011; Athanasiadou *et al*., 2001). Most of the CT at 6% and 8% *A. indica* present in feed in the rumen of ewes would be expected to form complexes with the dietary protein leading to an improvement of host's immune response against *H. contortus* (Hoste *et al*, 2016; Athanasiadou *et al*., 2001).

CONCLUSION

The aqueous leaf infusion of *A. indica* at doses of 6% and 8% showed *in vitro* and *in vivo* anthelmintic activities against *H. contortus* by reducing egg hatch, adult-worm motility, and the count of egg per gram feces as from one weeks post-treatment (on week 3) on *in vivo* test. The infusion of *A. indica* is fully potential as a bio anthelmintic against *H. contortus*.

CONFLICT OF INTEREST

The authors ensure that have no financial and personal conflict of interest.

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