In vitro anthelmintic activity of kersen leaf (Muntingia calabura) infusion against to Haemonchus contortus worm

A A Sakti¹, Kustentinah²*, R W Nurcahyo³, E Baliarti⁴ and B Suwignyo⁵

¹Research Unit for Natural Product Technology, Indonesian Institute of Sciences, D.I. Yogyakarta, Indonesia
²Department of Animal Nutrition and Feed Science, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia
³Department of Parasitology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
⁴Department of Animal Production, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia

Corresponding author’s e-mail: kustentinah@ugm.ac.id

Abstract. The problem caused by the gastrointestinal parasite has caused economic losses in the centres of ruminant livestock throughout the world. Parasitic resistance to synthetic antiparasitic, led researchers to explore alternative herbs as bio-anthelmintic. This study aims to determine the in vitro effect of M. calabura leaf infusion on egg hatchability (EHI) and mortality of adult female H. contortus worms (AWM), as a parameter of the antiparasitic properties of the leaves of the plant. The leaf infusion of M. calabura at doses of 2%, 4% and 6% was used for both treatments and albendazole at a dose of 2 mg/mL was used as a positive control. The negative control was sodium chloride at 0.9% (w/v). The EHI assay was conducted two times, before and 24 h after treatment, while AWM was monitored 15 and 30 min, and 1, 2, 3, 4, 5, 6, 7, and 8 h post test. The result showed that M. calabura leaves contain secondary metabolites, one of which was condensed tannins detected in this study. The leaf infusion of M. calabura at a concentration of 6% significantly inhibited EHI and AWM, higher than the negative control (P<0.05). While, it was not significantly different from albendazole 2 mg/ml on EHI test. The results conclude that the leaf infusion of M. calabura is fully potential as a bioanthelmintic against H. contortus worm.

1. Introduction
The gastric worm nematodes called Haemonchus contortus is one of the parasites found in the small ruminant abomasum. They can cause anemia to decrease performance and can lead to livestock deaths [1–4]. Regular administration of anthelmintics and pasture rotation can be done to prevent infestation of H. contortus worms by cutting their life cycle, which is through the grass. However, administration of broad-spectrum synthetic anthelmintics such as albendazole is more commonly given [5] as well as natural anthelmintics from the leaves of tropical plants containing condensed tannin (CT) [6,7]. Meanwhile, the resistance of H. contortus to several commercial anthelmintics has been found, as a consequence of the routine administration of synthetic anthelmintics, thus the similar dose has been inefficient [8,9]. The use of natural anthelmintics from plant leaves containing antiparasitic compound is considered appropriate, and continues to be developed as an alternative to natural anthelmintics.
against gastrointestinal nematodes [10]. Positive effects of plants containing tannins for livestock include antiparasites, anticoccidial and reducing methane [11-13].

In Indonesia, the leaves of the kersen plant (Muntingia calabura) are used in part by the community as bio-feed additive for sheep and goats, although it has not been widely explored widely regarding its anthelmintic properties. Muntingia calabura leaves contain tannins and show properties that can protect digestive health in experimental animals [14]. The study of the antiparasitic properties of M. calabura leaf infusion on H. contortus worms needs to be done, to determine its potential as a natural anthelmintic and can be used as a reference for the production of M. calabura leaf infusion in the anthelmintic herbal industry. This is considered to be able to provide an alternative solution to overcome resistance to anthelmintics, and its residual hazards, towards the goal of food safety for livestock products. This study aims to determine the in vitro effect of M. calabura leaf infusion on egg hatchability and mortality of adult female H. contortus worms, as a parameter of the antiparasitic properties of the leaves of the plant.

2. Materials and methods
The research was conducted at the Laboratory of Bio-Feed Additive Technology, Research Division for Natural Product Technology, Indonesian Institute of Sciences (LIPI) and at the Laboratory of Animal Nutrition, Department of Animal Nutrition and Feed Science, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia.

2.1. Preparation of the M. calabura infusion
Fresh M. calabura leaves were collected from the collection plants cultivated at LIPI, Yogyakarta. The selected M. calabura leaves were mature leaves, which have higher condensed tannin (CT) levels than immature ones (P<0.05) based on CT level analysis according to [15]. Infusion preparation was conducted by following the method according to [10] with several modifications. The fresh M. calabura leaves collection was cut to ± 1 cm in size, then dried in a freeze dryer (Lyovac GT2) at -20°C for 3 days. Then, the dried leaves were mashed using an electric blender (Philips) and filtered using a 80 mesh sieve shaker (Retsch AS 200).

Infusa was made by soaking 2 grams of M. calabura leaf flour in 20 ml of distilled water for 2 hours and being ultrasonicated for 5 minutes at 4°C using ultrasonicator (Elmasonic S-100H) twice. Then, the mixture was filtered through gauze and the filtered liquid was centrifuged (Sentrofriger BL II) at 372 g for 5 minutes [16]. The leaf infusion for in vitro (EH1 and AWM tests) was obtained by diluting the supernatants in 0.9% sodium chloride to 5 ml final volumes with a concentration of 20%, 40% and 60%. The consecutive concentrations the dried leaves were 2%, 4% and 6% (w/v).

2.2. Egg Hatch Inhibiting (EH1)
The in vitro EHI study was conducted referring to [17-18] and the number of eggs were counted by following World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods [17]. Egg samples were obtained from female H. contortus worms collected from abomasum of thin-tailed ewes which was slaughtered in the small ruminant abattoir in Bantul Regency. The selected ewes were infested > 1,500 worm eggs per gram of faeces (EPG). A total of 15 test tubes were prepared which were divided into 5 groups with 3 replications in each group. Furthermore, 3 ml of 0.9% sodium chloride was poured in group A as a negative control. Sequentially, groups B, C, and D were filled with 2%, 4% and 6% infusion of the previous preparation of 3 ml each as 3 treatment groups, whereas group E, albendazole (Wormzol-B, Medion) 2 mg / ml was used as a positive control. Three female H. contortus worms were inserted into each test tube throughout the treatment and then crushed to dissolve into each solution. The observation of the number of worm eggs in each replication was carried out in the 0 minute to find out the initial number of eggs in each sample, and at 24 hour as the final egg count data, and all work was done at room temperature (25-32°C) for 24 hours. Eggs that failed to hatch, were marked by the sturdy layer of the egg membrane, and it was observed that there was no movement of larvae in the cell fluids.
2.3. Adult-Worm Motility (AWM) Test

The adult *H. contortus* worms used in this study were female with red and white physical characteristics color on the body, whose length reaches 3 cm [1]. As in the EHT test, worms of AWM test were obtained from abomasum of thin-tailed ewes slaughtered in the abattoirs in Bantul Regency. The *in vitro* study of *M. calabura* leaves infusion was carried out to determine its effect on the existence of adult female *H. contortus* worms by observing of worm mortality (%), following the procedure of [19] with some modifications [11]. Adult female worms were collected using loop then rinsed with 0.9% sodium chloride to separate from polluter, and were stored in other 0.9% sodium chloride solution. Meanwhile, infusion of 2%, 4% and 6% with 0.9% sodium chloride diluent were poured in each petri disk as much as 5 ml as B, C, and D treatment groups respectively. All groups were replicated by 5 replications. Subsequently, each petri dish was filled with 5 worms and incubated at room temperature (25-32°C) for 8 hours. The observation on worm motility were carried out at 15 and 30 minutes, and continued on 1, 2, 3, 4, 5, 6, 7 and 8 hour post treatment by nudging worms with the loop. If the worm did not move for 10 seconds of sighting, it was declared dead.

3. Results and discussions

3.1. Condensed Tannin Content of *M. calabura* Leaves

*Muntingia calabura* L. (*Muntingiaceae*) has leaves, roots, flowers and barks that have been used traditionally to treat various diseases [14] CT 2.96% of dry matter [20]. The CT content (± Standard Deviation) of immature and mature *M. calabura* leaves were 2.87 ± 0.44% dan 4.21 ± 0.45% with dry matter content (DM) were 29.88 ± 0.84% dan 30.12 ± 0.97%, respectively. There was a significant deference in CT content levels of immature and mature *M. calabura* leaves (P<0.05). The result showed that the leaf maturity of *M. calabura* leaves significantly affected the level of active compounds in the leaves, especially CT levels. Based on these results, it was shown that *M. calabura* leaf contain secondary metabolites, one of which was CT detected in this study. The result was in accordance with the results of [21] study on phytochemical screening in *M. calabura* leaf. Furthermore, the results showed that mature *M. calabura* leaves had higher CT levels than young leaves. It was thought to be due to the number of glandular trichomes in older leaves more than young leaves, where the glandular trichome serves as a storage of secondary metabolites [22].

3.2. In Vitro EHI Test

The effect of *M. calabura* leaf infusion on different concentration levels on the egg hatch inhibiting test is shown in Figure 1. After 24 hour of incubation compared with the observation of the number of worm eggs at the beginning, it was seen that the infusion of *M. calabura* leaves at a concentration of 2% and 4% was not able to show a better hatchability than negative controls (P>0.05). Meanwhile, *M. calabura* leaf infusion at a concentration of 6% significantly inhibited *H. contortus* eggs hatching 52.32% higher than the negative control (P<0.05). In fact, the efficacy of 6% *M. calabura* leaf infusion was not significantly different from albendazole 2 mg/ml.

The efficacy of *M. calabura* leaves in EHI test was not significantly different compared to albendazole. It indicated that the 6% *M. calabura* leaf infusion was not only able to inhibit the hatching of *H. contortus* eggs, but also able to compete with broad-spectrum commercial worm drugs such as albendazole at the level of 2 mg/ml. The ability of hatch inhibition against *H. contortus* eggs is possible due to the role of the pharmacological action of secondary metabolites contained in *M. calabura* leaves. Plants containing CT compounds are known to have a direct influence on nematodes, and indirectly by increasing host immunity [2,23]. The egg phase is one of the important phases to be inhibited, considering that *H. contortus* eggs are released through sheep faeces, will hatch into stage 1 larvae (L1s) [1].
Figure 1. *In vitro* inhibitory effect of *M. calabura* leaf infusion at different concentration on *H. contortus* egg hatch test. Different superscripts show significant differences (P<0.05).

3.3. *In Vitro AWM Test*

The ability to defeat adult female *H. contortus* worms of *M. calabura* leaves infusion on the in vitro test is shown in figure 2. The results showed that the concentration of 6% containing *M. calabura* leaf infusion had better efficacy than other concentrations, even in negative controls (P<0.05). It shows that the higher the infusion concentration, the more effective adult *H. contortus* killing. However, the highest concentration (6%) has not been able to compare with the ability of albendazole in killing *H. contortus* (P>0.05). The efficacy of 6% *M. calabura* leaf infusion was still less than 16% compared to positive control, in killing *H. contortus*.

Figure 2. *In vitro* inhibitory effect of *M. calabura* leaf infusion at different concentration on *H. contortus* adult worm motility test. Different superscripts show significant differences (P<0.05). ALB: Albendazole
Based on the results of this research, we conclude that aqueous leaf infusion at doses of 6% (w/v) of *M. calabura* containing CT can inhibit egg hatching and adult worm motility of *H. contortus* worm. It was in accordance with the result of the previous study using the other traditional plant containing CT [7, 9-11, 19].

4. Conclusion

The leaf infusion of *M. calabura* at doses of 6% (w/v) showed *in vitro* anthelmintic activities against *H. contortus* by reducing egg hatch and adult-worm motility. The infusion of *A. indica* is fully potential as a bioanthelmintic against *H. contortus* worm.

Acknowledgment

The author would like to express full thanks to the BPTBA-LIPI Biofeed Laboratory, IMT Laboratory of the Faculty of Animal Husbandry, UGM, and the Parasitology Laboratory of the Faculty of Veterinary Medicine, UGM for supporting this research.

References

[1] Gasser R B, Schwarz E M, Korhonen P K and Young N D 2016 *Adv. Parasitol.* **93** 519 - 547
[2] Hoste H, Torres-Acosta J F J, Quijada J, Chan-Perez I, Dakheel M M, Kommuur D S, Muller-Harvey I and Terrill T H 2016 *Adv. Parasitol.* **93** 239 - 351
[3] Van den Brom R, Moll L, Kappert C and Vellema P 2015 *Vet. Parasitol.* **209** 278 – 280
[4] Pathak A K and Tiwari S P 2013 *Vet. World.* **6** 22 – 26
[5] Besier R B, Khan L P, Sargison N D and Van Wyk J A 2016 *Adv. Parasitol.* **93** 181 – 238
[6] Pathak A K, Dutta N, Banerjee P S, Goswami T K and Sharma K 2016 *J. Parasit Dis.* **40** 100 – 105
[7] Nawaz M, Sajid S M, Zubair M, Hussain J, Abbasi Z, Mohi-Ud-Din A and Waqs M 2014 *Global Vet.* **13** 996 – 1001
[8] Martins A C, Bergamasco P L F, Felippielli G, Tebaldi J H, Moraes M F D, Testi A J P, Lapera I M and Hoppe E G L 2017 *Semina: Ciencias Agr.* **38** 231 – 238
[9] Vieira T M, Fonseca L D, Bastos G A, Vasconcelos V O, Silva M L F, Morais-Costa F, Ferreira A V P, Oliveira N J F and Duarte E R 2017 *Vet. Res. Commun.* **41** 99 – 106
[10] Ferreira L E, Castro P M N, Chagas A C S, França S C and Beleboni R O 2013 *Exp. Parasitol.* **134** 327 – 332
[11] Sakti A A, Kustantinah and Nurcahyo R W 2018 *Trop. Anim. Sci. J.* **41** 185 – 190
[12] Adiwimarta K, Indarto E, Zuprizal, Noviandi C T, Dono N D and Mukti F A 2017 *The 7th Int. Sem. on Tropical Animal Production* (Yogyakarta: Universitas Gadjah Mada) p 178 – 184
[13] Kustantinah, Dono N D, Zuprizal, Indarto E, Wisnu B and Iskandar A 2014 *The 16th AAAP Congress* (Yogyakarta: The Asian-Australian Assosiation of Animal Production Societies) 339
[14] Zakaria Z A, Balan T, Suppaiah V, Ahmad S and Jamaludin F 2014 *J. Ethnopharmacolog.* **151** 1184 – 1193
[15] Abdulrazak S A and Fujihara T 1999 *Animal Nutrition: A Laboratory Manual* (Shimane: Shimane University) 24 – 28
[16] Odhong C, Wahome R G, Vaarst M, Nalubwama S, Kiggundu M, Halberg N and Githigia S 2014 *Afr. J. Biotechnol.* **13** 4667 – 4672
[17] Coles G C, Bauer C, Borgsteede F H M, Geerts S, Klei T R, Taylor M A and Waller P J 1992 *Vet. Parasitol.* **44** 35 – 44
[18] Coles G C, Jackson F, Pomroy W E, Prichard R K, Von Samson-Himmelstjerna G, Silvestre A, Taylor M A and Vercruysse J 2006 *Vet. Parasitol.* **136** 167 – 185
[19] Eguale T, Tilahun G, Debella A, Feleke A and Makonnen E 2007 *J. Ethnophar.* **110** 428 – 433
[20] Pratiwi D, Herdian H., Sakti A A and Setiawan Y 2013 *Prosiding Seminar Ilmu Pengetahuan
Buhian W P C, Rubio R O, Valle Jr. D L and Martin-Puzon J J 2016 *Asian Pac. J. Trop. Biomed.* 6 682 – 685

Kuntorini E M, Fitriana S, and Astuti M D 2013 *Prosiding Semirata* (Lampung: Universitas Lampung) 291 – 296

Sakti A A, Kustantinah, Nurcahyo R W, Perdani L, Ekaningrum M 2018 *Materials Science Forum (Yogyakarta)* (Switzerland: Trans Tech Publications) 78 - 84