

Tannins Quantification in *Terminalia catappa* Leaves Extract and Antihelminthic Potential Evaluation

L.M. Azrul^{1,2*}, R. Nurulaini⁴, M.A. Adzemi², H. Marina³, A.W.M. Effendy^{1,2}

¹Department of Agrotechnology, Faculty of Agrotechnology and Food Science,

²Natural Product Laboratory, Institute of Marine Biotechnology,

³Department of Aquaculture, Faculty of Fisheries and Agro-Industry,

Universiti Malaysia Terengganu, 21030, Kuala Terengganu, Terengganu, Malaysia.

⁴Haematology and Parasitology Unit, Veterinary Research Institute, Jalan Sultan Azlan Shah, 31400, Ipoh, Perak, Malaysia

*Corresponding Author

(Received 25 Dec. 2013; Revised 29 Jan -12 May 2014; Accepted 24 May 2014)

ABSTRACT

Folin-Ciocalteu method was conducted to measure the total phenolics content in *T. catappa* leaves extract. Polyvinylpyrrolidone (PVPP) solution was used to bind the tannins, so that the non-tannins (NT) content value can be detected. Thus, the total tannins (TT) content can be measured. Next, larvae at infective stage (L3) from selected species (n= ±50) were distributed in well in a 96 multiwells plate and incubated with diluted crude extract of *T. catappa* at a ratio of 1:1 at 20°C for 3 hours and 5 hours. Control was conducted using PBS as positive control and distilled water as negative control. After incubation, larvae motility were observed and counted using inverted microscope. All the non-motile L3 were identified in order to ensure survivability of the larvae. Total phenolics (TP) value for *T. catappa* obtained was 1.156% in 100 mg samples. The non-tannins (NT) content value detected is 0.0085%. The total tannins (TT) content calculated were 1.1475%. Reduction percentage for *T. colubriformis*, *C. curticei* and *H. contortus* was 70%, 63% and 73% respectively while at 5 hour's incubation, reduction percentage for each species was at 77%, 67% and 80% respectively.

Keywords: *Terminalia catappa*; Phenolics and tannins; Larvae survivability.

INTRODUCTION

Goats are one of major important animals in livestock industry in worldwide (Wahab, 2003). Unfortunately, nowadays, a range of diseases become a main factor that reduces number of goats' production. There are a lot of problems and diseases in goats (Wahab, 2003; Sani and Gray, 2004). According to Department of Veterinary Services of Malaysia, parasitic worm infection is one of major cause for reduced productivity in livestock industry. To overcome this problem, the conventional method is by using the chemical known as anthelmintic or dewormer that based on drugs (Waller, 1994; 1997). However, according to Hammond et al. (1997), these manufactured anthelmintics had some serious disadvantages, such as high cost and

risk of misuse leading to drug resistance, environmental pollution and food residues. According to this phenomenon mainly for the resistance, naturally produced plant anthelmintics offer an alternative that can overcome some of these problems. The studies about the alternative anthelmintic are becoming urgent because of the rapid escalation of anthelmintic resistance worldwide (Jackson and Coop, 2000; Waller, 1997). Besides that, the use of plant as anthelmintic is both sustainable and environmentally acceptable (Hammond, et al., 1997; Githiori, 2004). Traditional knowledge about the anthelmintic plants was widely explored nowadays. In this study, potential plant that claimed to have anthelmintic potential based on ethnobotanical practice was used as an experimental plant namely *Terminalia catappa* or locally known as Ketapang (Wiart, 2000). Tannins which is one of the plant secondary metabolites is believe to be responsible for the anthelmintic potential (Hoste, et al., 2008). Unfortunately there is no quantification study for this plant variety in Malaysia. Thus, this paper focusing on quantification of anthelmintic property and the anthelmintic efficacy of *T. catappa* by *in vitro* bioassay against three selected species of nematode infective larvae (L3) namely *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Cooperia curticei*.

MATERIALS AND METHODS

Plant material: Leaves sample of *T. catappa* were freshly collected from coastal area of Teluk Ketapang, Terengganu (N 05°22.629'; E 103°07.146'), according to the standard agronomy procedure and was identified by Mr. Embong, M. (biological senior staff) from Department of Biological Sciences, Universiti Malaysia Terengganu. Leaves were put into a dark color plastic bag and immediately brought into the laboratory for the next process and labeled as UMT-TC0911.

Drying process: Leaves were dried in the oven with the drying temperature not more than 55°C (Makkar, 2000). Leaves were dried until the dry weight became stable.

Crude powder preparation: Oven-dried leaves were ground using laboratory grinder. All the plant samples were ground as small as it can pass through the filter in the filtration process. Filtration process was involved three different levels of sieves with three different size of mesh, which is 1.0mm, 0.5mm and 0.25mm respectively (Harinder, et al., 2007). After pass through the last filtration process, crude powder was ready for the next process.

Tannins extraction: The extraction was done according to the methodology that drafted in FAO/IAEA Working Document (Makkar, 2000). The dried samples of *T. catappa* leaves were ground into dust crude powder form. Then, 200mg of dust crude were put into the beaker and 10ml aqueous acetone 70% was added. Ultrasonic treatment was done to the samples by suspended the beaker in the ultrasonic water bath for 20min at room temperature. After that, the samples were transferred into the centrifuge tube and centrifuged for 10min at 3000rpm and 4°C. Thereafter, supernatant was collected and directly kept on ice. The supernatant was considered as tannins-containing extract (Makkar, 2000).

Measurement of total phenolics and total tannins content: Measurement of total phenolics and total tannins compound were done using Folin-Ciocalteu method and expressed as tannic acid (TA) equivalent (Makkar, et al., 1993).

Analysis of total phenolics: Tannins-containing extract was transferred into the test tube with three different quantities which is 0.02ml, 0.05ml and 0.1ml. Distilled water (dH₂O) was used to make the volume up to 0.5ml. Then, 0.25ml Folin-Ciocalteu reagent and 1.25ml sodium carbonate solution were added. Vortex the tube and absorbance will be recorded at 725nm, after stayed at room temperature for 40min.

The amount of total phenols were calculated based on standard calibration curve and was expressed on a dry matter basis as TP.

Removal of tannins from tannins-containing extract: Polyvinylpyrrolidone (PVPP) was used in this process and was acted as tannins binder. A total of 100mg PVPP were put into the test tube and 1ml dH₂O were added. The test tube was vortex and kept at 4°C for 15min. Thereafter, test tube was again vortex before it was centrifuged at 3000rpm for 10min and the supernatant were collected. The supernatant has only simple phenolics other than tannins. So, the phenolics content in this supernatant was calculated according to Folin-Ciocalteu method (Makkar, 2000). This non-tannins compound was expressed on a dry matter basis as NT.

Measurement of total tannins content: Total tannins compound can be expressed as TT. After total phenolics and non-tannins compound were calculated, the amount of total tannins can be calculated by minus the amount of non-tannins from the amount of total phenolics.

In vitro bioassay: For the targeted species of nematodes larvae, the samples were obtained in pure form. Then, each species was distributed (n= ±50) in each well. Vehicle solution which is distilled water was used as negative control while PBS as a positive control. After the larvae were inserted into the well, it was mixed up with the *T. catappa* crude aqueous extract (CAE) which freshly prepared before. Two sets of well were prepared for each species as it have to incubated for two different incubation period which is set to be at 3hrs and 5hrs. At the end of the incubation period, wells were taking out and being gently shaken to make sure the larvae is not in dormant phase. Observation was done focusing on motility of the larvae using inverted microscope. Larvae motility was observed and survived larvae were counted using inverted microscope at 40X objective power.

Statistical analysis: All the data were analyzed by one-way ANOVA to determine the significance between the experimental solution and the control and also with another two species of infective larvae used before proceed with the Dunnett Test.

RESULTS

Standard calibration curve: Standard calibration curve was prepared first according to the protocol (Table 1) and was used for calculation of total phenolics and total tannins content. By using the standard calibration curve, absorbance value for 0.1ml of tannin-containing extract = 1.801abs

From graph, $y = mx + c$

$x = 1.801 / 0.082 = 21.9634\mu\text{g tannic acid equivalent in 2ml solution}$

In 1ml = $(21.9634 / 0.1\text{ml}) \times 1\text{ml} = 219.634\mu\text{g TA} = 0.2196\text{mg TA}$

For 200mg leaf sample was extracted in 10ml solvent

Therefore, 100mg leaf sample has $0.2196 \times 5 = 1.0982\text{mg TA}$

In leaf contains 95% dry matter (DM),

Total phenols in DM = $1.0982 / 0.95 = 1.156\%$

Removal of non-tannins content: Content of non-tannin phenols; 1000 μl of the supernatant after polyvinyl pyrrolidone (PVPP) treatment in the assay mixture gives 0.066abs:

$y = mx + c$

$x = 0.066 / 0.082$

= $0.8049\mu\text{g tannic acid equivalent from standard curve} = 0.0008\text{mg TA}$

In 10mg leaf sample has 0.00074mg TA, Therefore, 100 mg leaf sample has $0.0008 \times 10 =$

0.0080mg TA

If leaf contains 95% dry matter (DM),

Non-tannin compound in DM = $0.0080 / 0.95 = 0.0085\%$

Measurement of total tannins content: Percentage of tannins as tannic acid equivalent on a dry matter (DM) basis,

$Z = \text{Total phenols in DM (X)} - \text{Non-tannin compound in DM (Y)}$

$Z = X - Y = 1.801\% - 0.085\% = 1.1475\%$ in dry matter (DM) basis

Observation on larvae survivability: After 3 hours, reduction percentage of *H. contortus*, *T. colubriformis* and *C. curticei* were 73%, 70% and 63% respectively while at 5 hours incubation, reduction percentage for each species were 80%, 77% and 67% respectively (Table-2). There was no significant difference ($P > 0.05$) among them as the reduction percentages between these three species were slightly different (Table 3). The control wells showed no significant reduction.

DISCUSSION

Based on the results, it was found that the quantity of total tannins (TT) in 100mg sample dust crude of *T. catappa* is 1.1475% in 95% dry matter (DM) basis. There are wide ranges of phenolics and tannins content of the plant fed to ruminants (Lowry, 1992). Previous studies reported that tannins content in acacia (*Acacia auriculiformis*) and mango (*Mangifera indica*) leaves were in range of 4-10% (Wina, 1992; Hassanpour, 2011). Cassava (*Manihot esculenta*) leaves and leucaena (*Leucaena leucocephala*) leaves were reported to contain a medium level of tannins (1-4%) (Ademola, et al., 2005; Ademola and Idowu, 2006) and another plant species contain low level of tannins (0-1%). Eventough the total tannins content in *T. catappa* used in this study was in medium range, there is a potential of *T. catappa* to be used as an alternative anthelmintics in the future since in Indonesia, studies were conducted on plants with tannin that only content less than 1% were succeed (Wina, 1992).

The reduction percentages of larvae are shown in Table 2. After 3hrs incubation, the reduction rate for *H. contortus*, *T. colubriformis*, and *C. curticei* were 73%, 70% and 63% respectively while for 5hrs of incubation, the reduction were 80%, 77% and 67% respectively. The reduction percentages were slightly different between both incubation periods. Decreasing trend can be seen in both periods; with the number of motile L3 at 5hrs incubation is less than motile L3 after 3hrs incubation. Significant difference ($P > 0.05$) was determined for all three targeted species compared to the control when analyzed with Simple T-Test. Longer incubation period will reduce more number of motile L3. These results proved that there is anthelmintic activity in *T. catappa*; by inhibiting the motility and survivability of larvae as mentioned previously in ethno-veterinary reports.

Once the reduction percentage was achieved more than 50%, it can be claimed that the plant extract used against targeted larvae have anthelmintic activity which successfully inhibited the larvae survivability by showing no motility after incubation period (Alonso-Diaz, et al., 2008). However in some condition, by showing no motility, the larvae might be still alive because in the incubator with the incubation temperature at 20°C, the larvae could be in dormant phase (Nenni, 2006). Therefore, before the larvae was counted under inverted microscope, the wells have to be well shake to ensure all the dormant larvae are not in dormant phase anymore, so that all the survived larvae can be counted by observing the larvae motility.

CONCLUSION

By using Folin-Ciocalteu method, tannins from *T. catappa* leaves was successfully extracted and the quantity of total tannins could be measured. Then, for anthelmintic potential of *T. catappa*, *in vitro* anthelmintic efficacy was also successfully evaluated

in this study. The inhibition of the larvae motility and survivability rate at more than 50% can be the scientific proof for the anthelmintic potential.

Acknowledgement: Authors thanks to few particular persons regarding their helps in conducting this research and clarify the quantification part, namely Ms. Rawaidah and Dr. Jasnizat from Institute of Marine Biotechnology, Universiti Malaysia Terengganu, Ms. Imelda from Veterinary Research Institute, Malaysia.

REFERENCES

- Alonso-Diaz, M.A., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., Aguilar-Caballero, A.J., Hoste, H., (2008): *In vitro* larval migration and kinetics of exsheathment of *H. contortus* larvae exposed to four tropical tanniferous plant extracts. *Veterinary Parasitology*, 153(3-4): 313-319.
- Githiori, J.B., (2004): Evaluation of anthelmintic properties of ethnoveterinary plant preparations used as livestock dewormers by pastoralists and small holder farmers in Kenya, Ph. D Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.
- Hammond, J.A., Fielding, D., Bishop, S.C., (1997): Prospects for plant anthelmintics in tropical veterinary medicine. *Veterinary Res. Communications*, 21: 213-228.
- Harinder, P.S., Makkar, H.P.S., Siddhuraju, P., Becker, K., (2007): Plant secondary metabolites. Humana Press Inc., Springer Science+Business Media, LLC, New Jersey, United States.
- Hassanpour, S. and Mehmandar, F.B. (2011): Anthelmintic effects of *Acacia mearnsii* (Wattle tannin) in small ruminants; a review. *J. of Comp. Clin. Pathol. Res.*, 1/1:1-8.
- Hoste, H., Torres-Acosta, J.F., Alonso-Diaz, M.A., Brunet, S., Sandoval-Castro, C., Houzangbe-Adote, S., (2008): Identification and validation of bioactive plants for the control of gastrointestinal nematodes in small ruminants. In: I. Vythilingam (Ed.), Proceedings of 5th International Workshop: Novel Approaches to the Control of Helminth Parasites of Livestock 2008.
- Jackson, F., Coop, R.L., (2000): The development of anthelmintic resistance in sheep nematodes. *Parasitology*, 120: (Suppl.): 95-S107.
- Lowry, J.B., Petheram, R.J., Tangendjaja, B., (1992): Plants fed to village ruminants in Indonesia. Technical Reports No. 22. ACIAR. Canberra.
- Makkar, H.P.S., (2000): Quantification of tannins in tree foliage: A laboratory manual. FAO/IAEA Edition, Vienna.
- Makkar, H.P.S., Bluemmel, M., Borowy, N.K., Becker, K., (1993): Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J. Sci. Food Agric*, 61: 161-165.
- Nenni, M., (2006): Famacha; What's Famacha? Hoosier Hummer Newsletter (online). <http://www.shagbarkridge.com/info/famcha.html> [Accessed on 15th June 2011]
- Sani, R.A., Gray, G.D., (2004): Worm control for small ruminants in South East Asia. In: Worm control for small ruminants in Tropical Asia (Edited by R.A. Sani, G.D. Gray, R.L. Baker), ACIAR Monograph 113: 3-21.
- Wahab, A.R., (2003): Cacing dalam kambing; masalah dan penyelesaian. Siri Syarahan Umum Pelantikan Profesor Univesiti Sains Malaysia: 1-5 (In Malay).
- Waller, P.J., (1994): The development of anthelmintic resistance in ruminant livestock. *Acta Tropica*, 56(2-3): 233-243.
- Waller, P.J., (1997): Sustainable helminth control of ruminants in developing countries. *Vet Parasitol*, 71: 195-207.
- Wiar, C., (2000): Medicinal Plants of Southeast Asia. Pelanduk Publications (M) Sdn. Bhd: 69-73.
- Wina, E., (1992): Nutritive value of calliandra, gliricidia and leucaena as supplement for sheep fed elephant grass Proc. Pengolahan and Komunikasi Hasil Penelitian Teknologi and Tanaman Pakan Balitnak: 13-19 (In Indonesia).

Table-1: Mixing solution from standard protocol that used for preparation of standard calibration curve.

Tube	Tannic acid Solution (0.1 mg/ml)	Distilled water (ml)	Folin reagent (ml)	Sodium carbonate solution (ml)	Tannic acid (μg)
Blank	0.00	0.50	0.25	1.25	0
T1	0.02	0.48	0.25	1.25	2
T2	0.04	0.46	0.25	1.25	4
T3	0.06	0.44	0.25	1.25	6
T4	0.08	0.42	0.25	1.25	8
T5	0.10	0.40	0.25	1.25	10

Table-2: Number of L3 (n) from three different species following incubation with experimental solution (*Terminalia catappa* crude aqueous extract).

Species	Before	After 3hrs	After 5hrs
<i>H. contortus</i>	1200	320	240
<i>T. colubriformis</i>	1200	360	280
<i>C. curticei</i>	1200	450	400
Control	1200	1200	1100

Table-3: Comparison of P-values of pre and post incubation period for three species of infective nematode larvae.

Species	Category	Compare to	Standard Error (SE)	P-value	Significance
<i>H. contortus</i>	Before	After 3hrs	8.48528	0.000	$P < 0.05$
		After 5hrs	21.46625	0.001	$P < 0.05$
<i>T. colubriformis</i>	Before	After 3hrs	16.09969	0.000	$P < 0.05$
		After 5hrs	10.73313	0.000	$P < 0.05$
<i>C. curticei</i>	Before	After 3hrs	12.58571	0.000	$P < 0.05$
		After 5hrs	12.58571	0.000	$P < 0.05$