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Ovicidal Effect of Tannins-containing Extract of *Sesbania grandiflora* Leaves against Goat's Parasitic Nematode Egg

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Authors' contributions

This work was carried out in collaboration among all authors. Author MAL designed the study following discussion with authors KP, SP and WA helped in preparation for plant extract solution. Author SJ helped in reviewed the parasitological examination for the trial results. Manuscript have been prepared and reviewed by author MAL with the helps from all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In this study, sesbania (*Sesbania grandiflora*) leaves were used as an experimental material. Tannins was measured using Folin-Ciocalteu method. The ovicidal effect of this plant leaves was determined against goat's parasitic nematode egg using egg hatch assay.
Study Design: Completely Randomized Design (CRD).
Place and Duration of Study: Nematode eggs collection and *in vitro* trial were conducted at Animal Physiology Laboratory, Faculty of Agriculture of Kasetsart University in Bangkok, Thailand between June 2016 and December 2016.

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Methodology: Two type of extracts; aqueous and ethanolic extracts were prepared prior to the *in vitro* trial. Six increasing 2-fold concentration of both extracts; from 0.075 to 2.4 mg/ml were used against mixed sample of nematode's egg from naturally infected goats. Gridded petri dishes containing a mixture of egg suspension with tested solution; both extracts and control were incubated for 27°C for 24hrs and 48hrs, respectively. At the end of each incubation period, hatched and unhatched eggs were counted and egg hatching inhibition rate was determined.
Results: Total tannins in this plant was measured at 1.08% of 95% dry matter (DM). Both extracts showed significant inhibition effect for both incubation periods ($P<0.05$). Highest concentration used for aqueous and ethanolic extracts showed more than 80% and 60% of hatching inhibition after 48hrs, respectively. Significantly higher inhibition rate was observed for aqueous extract compared to ethanolic extract ($P<0.05$). For aqueous extract, the maximum inhibition rate was observed at 85.33% for the highest concentration after 48hrs which is almost similar to anthelmintic efficacy of oxfendazole used as positive control (96.67%).
Conclusion: This study showed that *S. grandiflora* leaves can be used as natural anthelmintic for goats since their ovicidal effect against nematode's egg was scientifically proven.

Keywords: Sesbania grandiflora; goat's parasitic nematode egg; egg hatch assay; egg hatching; inhibition rate; ovicidal effect.

1. INTRODUCTION

Small ruminants as goats and sheep are part of major commodities in global livestock industry [1]. Besides being as one of the food resources for human, livestock sector is also supporting the livelihoods of more than 500 million poor small holder farmers in developing countries around the world [2]. The consistently increasing growth of human population has been led to the increasing demand for animal products [3]. Asian countries dominate the global goats production as reported by Food and Agriculture Organization (FAO) of the United Nation [1]. Unfortunately, a range of diseases remain a major factor that can reduce goat population due to the increasing of goat's mortality and morbidity [4].

Gastrointestinal parasitic infection is one of the important factors that can contribute to unsatisfactory productivity as this infection can affects goats severely either acute or chronic [5]. This infection is a serious problem worldwide which leads to the economic losses in goat husbandry such as decreased feed efficiency, increased time for selling, decreased carcass value and causing lethargic event [6]. Helminthiasis also can increase the mortality rate and can cause the slow growth of young animals and affecting the performance of adult animals [7]. Common species of parasitic nematode in goats as reported previously are dominated by few species such as *Haemonchus contortus, Trichostrongylus colubriformis* and also few coccidia species [8]. Conventionally, manufactured anthelmintics are used to treat this infection. Anthelmintics are synthetic drugs used to control gastrointestinal parasites in humans and animals [9]. By having same drug from time to time, this allows parasites to develop resistance gene allele in their body system. This phenomenon are occurs worldwide and known as anthelmintic resistance [10]. As a result of this, farmers are encouraged to use the ethnoveterinary medicine based on ethnobotanical knowledge of potential local plants [3,11]. Plants leaves such as leucaena (*Leucaena leucocephala*), neem (*Azadirachta indica*), cassava (*Manihot esculenta*) and sesbania (*Sesbania* sp.) are among the plants traditionally used as a sole diet or as a supplemental feed in ruminants [12].

Sesbania grandiflora is native to Asian region and can be easily found in many Asian countries [12]. This plant was reported to be used in farms as one of the protein resources to obtain better nutrient intake and optimum growth for small ruminants [13-15]. The characteristics of this plant that grows fast is suitable and practical to be used as a fodder for ruminants [12]. Thus, in this current study, tannins content in *S. grandiflora* were measured using quantification method and then followed by *in vitro* anthelmintic assay to determine the ovicidal effect against nematode's egg.

2. MATERIALS AND METHODS

2.1 Plant Collection and Preparation

Sesbania grandiflora as plant samples were collected around Kasetsart University, Bangkhen campus in Bangkok (13°51'04.8"N, 100°34'02.7"E) and Kamphaeng Saen campus in

Nakhon Pathom (14°01'10.9"N, 99°48'47.1"E). Plant species was confirmed by the botanist from the Department of Botany, Faculty of Science, Kasetsart University, Bangkok, Thailand. Drying process and plant preparation were done at the same location. Leaves were air-dried in a covered area at the Green House, Department of Botany, Faculty of Science, Kasetsart University, Bangkok, Thailand. Air-drying method was chosen since oven-drying could increase the amount of neutral detergent fiber (NDF) value [16]. Leaves were dried until the dry weight became stable. Air-dried leaves were ground using laboratory grinder. All plant samples were ground finely till it could pass through sieves. Sieving process involved three different types of sieves with different mesh sizes; 1.0 mm, 0.5 mm and 0.25 mm, respectively. The crude powder obtained after the sieving process was then subjected to analysis and extraction.

2.2 Tannins Quantification

Measurement of total phenolics and total tannins compounds were conducted based on Folin-Ciocalteu method [17]. The standard calibration curve was prepared prior to the quantification of phenolics and tannins compounds based on Table 1 [17]. Tannins extract was transferred into the test tube in three different quantities; 0.02 ml, 0.05 ml and 0.1 ml. Distilled water (dH_2O) was then added to the volume of each test tube up to 0.5 ml. This followed by adding 0.25 ml Folin-Ciocalteu reagent and 1.25 ml sodium carbonate solution before the tubes were vortexed and left at room temperature for 40 min. The absorbance was then recorded at 725 nm using a spectrophotometer. The amount of total phenolics were calculated based on standard calibration curve and was expressed on a dry matter (DM) basis as TP.

Polyvinylpolypyrrolidone (PVPP) was used in this process where it acted as tannins binder. A total of 100 mg PVPP were put into the test tube and 1 ml of distilled water (dH_2O) was added. The test tube was later vortexed and kept at 4°C for 15 min. The test tubes were vortexed again before it was centrifuged at 3000 rpm for 10 min. The supernatant was later collected. This supernatant contains only simple phenolics other than tannins. This non-tannins compound was expressed on a dry matter basis as NT. Total tannins compound can be expressed as TT. After total phenolics and non-tannins compounds were calculated, the amount of total tannins can be obtained by subtracting the amount of non-tannins from the amount of total phenolics as shown in the following formula [17].

Total tannins (TT) = TP – NT;

Where:

TT = Total tannins content
TP = Total phenolics content
NT = Non-tannins content

An amount of 0.5 ml of tannins extract was transferred to the test tube and diluted with 70% acetone solution. Then, 3 ml of butanol-HCL reagent and 0.1 ml of ferric reagent were added. Test tube was then vortexed. The mouth of test tube was covered and put in the boiling water for 60 min. After that, test tube was cooled before absorbance was recorded at 550 nm. Condensed tannins (CT) content in a unit of percentage (%) in dry matter (DM) were calculated by the formula below [17].

CT = (550 nm x 78.26 x Dilution factor) / (% dry matter)

2.3 Aqueous Extract Preparation

An amount of 100 g *S. grandiflora* leaves dried powder was mixed with 600 ml distilled water (dH_2O) in a beaker and continuously shaken with electric shaker for 4hrs. The

Table 1. Mixing solution 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

Source: [17]

suspension was filtered through filter paper and kept in deep freezer for 24hrs and before lyophilized. The lyophilized dry powder of aqueous extract was stored in a dark vial bottle and kept in desiccators until used [18].

2.4 Ethanolic Extract Preparation

An amount of 100 g *S. grandiflora* leaves dried powder were mixed with 1000 ml of 30% ethanol for 2hrs at 60°C. The suspension was filtered through filter paper and then lyophilized. The lyophilized dry powder of ethanolic extract was stored in a dark vial bottle and kept in desiccators until used [18].

2.5 Nematode's Egg Preparation

Fresh macerated fecal samples were crushed and placed into a glass jar. Fecal matter was well-pressed to the bottom of the jar and water was sprayed onto it. The glass jar was stored at room temperature for at least seven to 10 days with daily watering. Eggs were collected immediately after fecal egg count (FEC) procedure. Due to similarity in shape of nematode's eggs, eggs sample was categorized as a mixed sample of strongyle nematode eggs [19].

2.6 Egg Hatch Assay

Anthelmintic test on egg stage was conducted according to the modification of egg hatch assay (EHA) as recommended by the authority of the World Association for the Advancement of the Veterinary Parasitology (WAAVP) [20]. About 10 g of fresh fecal sample was mixed with distilled water (dH_2O). The mixture was strained and centrifuged for 2 min at 2000 rpm. Cover slip was put on the top of the glass over the meniscus of the mixture for 5 min for collection of the eggs. Eggs suspension containing 100 eggs/0.2 ml was put in a gridded petri dish together with *S. grandiflora* aqueous extract. Mixture was tested with six increasing 2-fold concentration of *S. grandiflora* aqueous extract from 0.075 to 2.4 mg/ml. Oxfendazole was used as a positive control. Petri dish was covered with aluminium foil and incubated at 27°C. Two sets at triplicate were prepared for 24hrs and 48hrs. Another trial with *S. grandiflora* ethanolic extract was prepared with same procedure. At the end of each incubation period, petri dishes were taken out and Lugol's iodine solution was dropped into each mixture to stop further hatching of the eggs. Hatched larvae; dead or alive and unhatched eggs were counted under a dissecting microscope with 40x magnification. Egg hatching inhibition (EHI) rate was determined by the formula below [19].

EHI (%) = (Final number of eggs – Number of larvae) / (Initial number of eggs x 100)

2.7 Statistical Analysis

All data in plant analysis works were analyzed with descriptive statistic and presented in percentages on a dry matter basis. Data for egg hatch assay were presented in mean ± S.D. One-way analysis of variance (ANOVA) with Tukey post hoc test were used to analyze the data. P-values less than 0.05 were considered to be statistically significant ($P<0.05$).

3. RESULTS AND DISCUSSION

3.1 Tannins Quantification

Folin-Ciocalteu method based on FAO/IAEA Working Document [17] was used in laboratory work to measure the total phenolics and total tannins in the experimental plant sample and presented in Table 2. The results are expressed as tannic acid equivalent percentage on dry matter as explained in previous studies [17,18].

Table 2. Measurement of tannins content in *Sesbania grandiflora* leaves

Constituents	Total content on 95% DM (%)
Total phenolics (TP)	1.0958
Non-tannins (NT)	0.0116
Total tannins (TT)*	1.0842
Condensed tannins (CT)	1.0302

*$TT = TP – NT$

For quantification study, it was found that the quantity of total tannins (TT) in 100 mg sample dust crude of *S. grandiflora* is 1.0842% in 95% dry matter (DM) basis. There are wide ranges of phenolics and tannins content of the plant fed to the ruminants [21]. A study in Indonesia reported that tannins content in acacia (*Acacia auriculiformis*) and mango (*Mangifera indica*) leaves were in the range of 4-10% [22]. Cassava (*Manihot esculenta*) and leucaena (*Leucaena luecocephala*) leaves were reported to contain a medium level of tannins (1-4%) [23,24], while other plant species contain low level of tannins (0-1%) [22]. Thus, quantity of total tannins and

condensed tannins were in a medium range. Despite that the tannins content in *S. grandiflora* was in a medium range, *S. grandiflora* is still can be a potential alternative anthelmintic since in previous works, the anthelmintic test on plants with tannins that only contain less than 1% were successful [22,25].

The quantification of chemical compounds in natural plant products are depend on some factors which limit their natural characteristics and quality. The nature and amount of the targeted properties are dependent on various factors that must be controlled as much as possible to optimize the result. One of the factors is stress and this could naturally change the phytochemical contents. The metabolic state of the plant will be changed when stress occurs in any manner [17,18]. This problem can occur before and after harvesting plant part for phytochemical analysis. Once the senescent process has occurred, it could lead to the death of inner cells and the cellular integrity become lost. The enzymes come in contact with substrates to which they are not normally exposed in living cells. In addition, it also increases the oxidation process, which is a problem with phenolics since these are prone to oxidation. Phenolics oxidize to quinones and followed by polymerization reaction [17,18].

3.2 Egg Hatch Assay

For this assay targeting the egg stage of parasitic helminth, aqueous and ethanolic extracts of *Sesbania grandiflora* leaves showed significant inhibition effect for both incubation periods. Significantly higher inhibition rate ($P<0.05$) was observed for aqueous extract compared to ethanolic extract (Table 3). Overall, results showed that the increasing trend of egg hatching inhibition rate was proportional to the increasing concentration of plant extracts used and incubation time.

Highest concentration used for aqueous and ethanolic extracts showed more than 80% and 60% hatching inhibition after 48hrs incubation, respectively. For aqueous extract tested, minimum inhibition rate was recorded at 26.33% for lowest concentration after 24hrs incubation and the maximum inhibition rate was observed at 85.33% for highest concentration after 48hrs incubation period which is almost similar with the efficacy of oxfendazole used as positive control (96.67%). For ethanolic extract, it was showed a lower inhibition effect compared to aqueous extract as minimum and maximum inhibition rates were recorded at 22.33% and 65.33% for both 24hrs and 48hrs incubation periods, respectively.

Table 3. Egg hatching inhibition after incubation with aqueous and ethanolic extracts of *Sesbania grandiflora* leaves

Experimental solution (mg/ml)	Mean of Egg hatching inhibition (%) ± S.D*	
	24hrs	48hrs
Aqueous extract		
Control (-)	1.73 ± 0.42^{a}	1.73 ± 0.52^{a}
Oxfendazole (+)	96.33 ± 2.31^{b}	96.67 ± 2.08^{b}
0.075	26.33 ± 2.08^{c}	30.33 ± 1.15^{c}
0.15	29.67 ± 1.53^{c,d}	32.33 ± 2.08^{d}
0.3	34.67 ± 0.58^{d}	41.67 ± 2.08^{e}
0.6	40.33 ± 1.53^{e}	49.67 ± 4.51^{f}
1.2	44.33 ± 2.08^{e}	64.33 ± 2.08^{g}
2.4	55.33 ± 2.52^{f}	85.33 ± 2.52^{h}
Ethanolic extract		
Control (-)	1.73 ± 0.42^{a}	1.73 ± 0.42^{a}
Oxfendazole (+)	96.33 ± 2.31^{b}	96.33 ± 2.31^{b}
0.075	22.33 ± 1.53^{c}	25.67 ± 1.15^{c}
0.15	23.33 ± 2.08^{c}	31.67 ± 0.58^{d}
0.3	28.67 ± 0.58^{d}	35.67 ± 0.58^{e}
0.6	33.33 ± 1.15^{d,e}	45.33 ± 1.53^{f}
1.2	37.33 ± 2.52^{e}	54.33 ± 3.06^{g}
2.4	42.33 ± 1.53^{f}	65.33 ± 1.15^{h}

**One-way analysis of variance (ANOVA) with Tukey post hoc test means with different superscripts within a column for each group differ significantly at P<0.05*

In vitro ovicidal effect expressed by tannins-containing plants are directly related to the presence of tannins and condensed tannins [25]. Tannins was successfully detected and measured in previous experiment in this study and was found to be similar with previous phytochemical results on *S. grandiflora* which stated that this plant was proved to have tannins and condensed tannins, ranged at medium level [25,26]. Due to molecular weight of tannins compound, tannins are soluble in water and polar organic solvents [27]. Previous studies with variety of plants in different type of extraction such as aqueous, alcoholic, hydro-alcoholic or essential oils have shown anthelmintic effects on different stages of parasitic helminths [19,20]. For anthelmintic assay against egg stage, both of the extracts used were significantly reduced the egg hatching under 24hrs and 48hrs incubation periods. Lowest and highest concentrations in aqueous extract showed higher inhibition rates for both incubation periods compared to ethanolic extract. These results can be related to previous study that anthelmintic potential of different plant extracts; aqueous, ethereal and alcoholic have shown anthelmintic effects in decreasing order, respectively [28]. Anthelmintic effect on egg hatching can be described as an effect on inhibiting larvae formation within eggs [29]. Tannins can entering into the eggshell and exhibit the anthelmintic activity [30]. This mechanism of action was similar to the action of anthelmintic drugs from benzimidazoles group which known to have ovicidal effects on helminths eggs [31]. Another anthelmintic effect on egg was reported the efficacy of tannins in blocking the eclosion of larvae that already formed within eggs [29]. Larvae that already formed within the eggs are failed to complete the eclosion process and this was caused by the ability of tannins to bind with protein. When tannins bind to the lipoproteins at the membrane of eggshell, the required change on eggshell not occur and prevent the larvae to emerge from the eggs [30]. Egg hatching enzymes such as lipases, aminopeptidase and metalloproteases are also affected by tannins which can cause the egg hatching inhibition [32].

4. CONCLUSION

For quantification study, it can be concluded that tannins which is claimed to be responsible as anthelmintic property was successfully measured using Folin-Ciocalteu method. By using this method, tannins from *Sesbania grandiflora* leaves was extracted and total phenolics content were then measured before the quantification of total tannins was successfully obtained. Tannins and condensed tannins measured were at the medium range; at 1.0842% and 1.0302%, respectively. These were considered safe to be used in goats with no toxicological effects. Aqueous extract showed higher inhibition effect at 85% of inhibition rate on egg hatching assay. Dose was clearly observed to be the important parameters in *in vitro* anthelmintic assay. Direct effect of tannins can be related to the direct contact of tannins within plant extract with the targeted parasites.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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