Anthelmintic effects of ethanolic extracts from pomegranate peels, mangosteen peels and tamarind seeds on gastrointestinal nematode egg counts in lambs

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Abstract

The anthelmintic drug resistance problems in animal husbandry are widely spread all over the world. The gastrointestinal nematodes in sheep economically impact on the decreasing of live-weight gain and body condition scores and have been recently resistant to many anthelmintics, especially albendazole, levamisole, macrocyclic lactone, moxidectin and ivermectin. Therefore, the herbal medicine is the one of alternative anthelmintic ways to solve chemical resistance and economic problems in small ruminant farms. This study was aimed to investigate the effects of pomegranate (*Punica granatum* L.) peels, mangosteen *(Garcinia mangostana)* peels and tamarind *(Tamarindus indica)* seeds extracts on gastrointestinal nematode egg counts in lambs comparing with albendazole and ivermectin. The results were shown that ivermectin could not decrease egg counted number significantly, but albendazole could significantly decrease percent of the egg per gram (EPG) when compared with pre-treatment (day 0) at day 1 (68±9%), day 3 (53±10%) and day 7 (62±8%). At day 1, the ethanolic extract of pomegranate peels and tamarind seed were significantly effect on gastrointestinal nematode egg count (47±8% and 48±10%, respectively). EPG were not affected by mangosteen peels extract. Serum alanine aminotransferase (ALT) (8-17.8 IU/L), aspartate aminotransferase (AST) (88.5-127.3 IU/L), creatinine (0.7-0.9 mg/dl) and blood urea nitrogen (BUN) (11-18.5 mg/dl) value after treatment were also normal and the lambs did not express any clinical renal or hepatic symptoms. It is concluded that the ethanolic extracts of pomegranate peels, and tamarind seeds possess significant anthelmintic activities at day 1 and could be a potential alternative for management of gastrointestinal roundworm problem in lambs.

Keywords: pomegranate peels, mangosteen peels, tamarind seeds, gastrointestinal nematode, lamb

ผลของสารสกัดด้วยเอทานอลจากเปลือกทับทิม เปลือกมังคุด และเมล็ดมะขามที่มีต่อจำนวนไข่พยาธิตัวกลมในทางเดินอาหารของลูกแกะ

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บทคัดย่อ

ปัญหาการดื้อยาถ่ายพยาธิในปศุสัตว์นั้นได้แพร่กระจายไปทั่วโลก ซึ่งปัจจุบัน พยาธิตัวกลมในทางเดินอาหารของแกะดื้อต่อยาถ่ายพยาธิหลายชนิด โดยเฉพาะอัลเบนดาโซล (albendazole) เลวาไมโซล (levamisole) มาโครไซคลิก แลคโตน (macrocyclic lactone) มอกซี่เดคติน (moxidectin) และไอเวอเมคติน (ivermectin) ปัญหาดังกล่าว ส่งผลกระทบเชิงเศรษฐกิจเนื่องจากลูกแกะมีน้ำหนักตัวและคะแนนร่างกายของลูกแกะลดลง ดังนั้น การใช้ยาสมุนไพรจึงเป็นทางเลือกหนึ่งในการแก้ไขปัญหาการดื้อยาและปัญหาทางเศรษฐกิจในฟาร์มสัตว์เคี้ยวเอื้องขนาดเล็ก การทดลองนี้ ได้มีการศึกษาเกี่ยวกับ ผลของสารสกัดจากเปลือกผลทับทิม เปลือกผลมังคุด และเมล็ดมะขามที่มีต่อจำนวนไข่พยาธิตัวกลมในการเดินอาหารของลูกแกะ เปรียบเทียบกับอัลเบนตาโซลและไอเวอเมคติน จากผลการทดลองพบว่า ไอเวอเมคตินไม่สามารถลดจำนวนไข่พยาธิได้อย่างมีนัยสำคัญทางสถิติ แต่อัลเบนดาโซลสามารถลดเปอร์เซนต์ของ egg per gram (EPG) เมื่อเปรียบเทียบกับ pre-treatment (day 0) ในวันที่ 1 (68±9%) วันที่ 3 (53±10%) และวันที่ 7 (62±8%) วันที่ 1 พบว่าสารสกัดเอทานอลของเปลือกทับทิมและเมล็ดมะขามมีผลต่อจำนวนไข่พยาธิตัวกลมทางเดินอาหารอย่างมีนัยสำคัญทางสถิติ (47±8% และ 48±10% ตามลำดับ) ส่วนเปลือกมังคุดไม่มีผลต่อจำนวนไข่พยาธิ ดังกล่าว ค่า serum alanine aminotransferase (ALT) (8-17.8IU/L) aspartate aminotransferase (AST) (88.5-127.3 IU/L) creatinine (0.7-0.9 mg/dl) และ blood urea nitrogen (BUN) (11-18.5 mg/dl) หลังจากการให้ยาทุกกลุ่ม มีค่าปกติ และลูกแกะไม่แสดงอาการผิดปกติทางคลินิกที่เกี่ยวข้องกับไตและตับ สรุปได้ว่า สารสกัดด้วยเอทานอลจากเปลือกทับทิมและเมล็ดมะขามมีคุณสมบัติเป็นยาถ่ายพยาธิได้อย่างมีนัยสำคัญทางสถิติในวันที่ 1 และสามารถใช้เป็นทางเลือกในการจัดการปัญหาพยาธิตัวกลมในทางเดินอาหารในลูกแกะได้

คำสำคัญ : เปลือกผลทับทิม เปลือกผลมังคุด เมล็ดมะขาม พยาธิตัวกลมในทางเดินอาหาร ลูกแกะ

Introduction

For many years, the resistance to the anthelmintics of gastrointestinal nematodes is widely spread in sheep populations. Much surveillance reveals the severity of anthelmintic drug resistance in many areas all over the world. In New Zealand, the parasitic nematodes of sheep were resistance to albendazole, levamisole, macrocyclic lactone and ivermectin (McKenna 2010; Waghorn et al 2006). The sheep flocks in São Paulo state, Brazil (Verissimo et al 2012) and the southeastern United States (Howell et al 2008) were resistant to albendazole and ivermectin, moxidectin, and levamisole. The first report from Costa Rica in 2011 showed that *Haemonchus* spp., *Strongyloides* spp., and *Trichostrongylus* spp. were resistant to albendazole and ivermectin (Maroto et al 2011). The benzimidazole resistance in sheep nematodes also happened in Northeast Spain (Calvete et al 2012), Norway (Domke et al 2012), Australia (Dobson et al 2011) and Canada (Falzon et al 2013). High use of albendazole could cause drug residue problem in lamb productions. From all farms throughout Southern Greece, 27.6% of goat, sheep, and cow raw milk were positive for the residue levels of albendazole and some of their metabolites (albendazole sulphoxide and albendazole sulphone). And 11.4% of samples were found the residues concentration exceeded the established Maximum Residue Limits (Tsiboukis et al 2013). Albendazole sulphoxide (ABZSO), the metabolite in sheep plasma, could be measured at the highest concentrations for up to 60 h (7.5 mg/kg) after oral administration (Moreno et al 2004). Low effective anthelmintics could cause the economic impact on live-weight gain and body condition scores of lamb (Miller et al 2012). There was a reduction in lamb carcass weight and grades. The albendazole resistance could result 14% reduction in carcass value (Sutherland et al 2010).

In the sustainable control of sheep nematodes, the combination of anthelmintics with new, similar spectrum activities and different mechanisms of action are widely used for the control of sheep nematodes (Bartram et al 2012) For instance, the combination of derquantel-abamectin could retard the development of a resistant parasite population by reducing the number of multi-resistant genotypes (Little et al 2011). However, this method increased economic cost of farm management on long time and may become unsustainable method. For this reason, it is necessary to establish alternative strategies of sheep farm management. Hence, traditional plants used for medicine is the alternative anthelmintic way to solve chemical resistance and economic problem in sheep husbandry systems (Boonmasawai 2012). The ethnoveterinary medicine can be used the high effective and low cost parts from traditional plants in local area, especially the waste products such as fruit peels and seed. These experiments would investigate the pharmacological activities of pomegranate *(Punica granatum* L.*)* peels, mangosteen *(Garcinia mangostana)* peels and tamarind *(Tamarindus indica)* seeds on gastrointestinal nematode egg counts in lambs. Feeding dry pomegranate peel 3 g/kg body weight could effectively reduce gastrointestinal nematodes egg per gram (EPG) in sheep (Akhtar and Riffat 1985). Alcoholic extracts of pomegranate rinds also showed moderate *in vitro* activities against human *Ascaris lumbricoides* (Raj 1975). Mangostin, a major bioactive xanthone isolated from the pericarp and fruit of *Garcinia mangostana* had *in vitro* effects (IC_{50}= 2.9-15.6 μg/ml) on the *Schistosoma mansoni, Echinostoma caproni*, and *Fasciola hepatica*. 400 mg/kg and 800 mg/kg of the mangostin showed effects on *S. mansoni* and *E. caproni in vivo* (Keiser et al 2012). Tamarind is a local plant in Thailand which are widely used in livestock based on Aryuravedic medicine (Sharma et al 2012) and African medicine (Havinga et al 2010). The alcohol and aqueous extract of bark of *Tamarindus indica* effected on paralysis and death of *Pheretima posthuma* (earthworms) and *Tubifex tubifex* (sewage worms) in India (Das et al 2011). Therefore, ethanolic extracts from these waste products of three fruits were used to determine the anthelmintic activities

in lambs comparing with albendazole and ivermectin. The serum samples were also collected from all lamb to evaluate the biochemistry values of liver and kidney functions before and after treatment.

Materials and Methods

Plant extract preparation

Pomegranate *(Punica granatum* L.*)* peels, mangosteen *(Garcinia mangostana* L.*)* peels and tamarind *(Tamarindus indica* L.*)* seeds were collected from the local markets in Nakhon Pathom, Rayong and Petchaboon provinces, Thailand, respectively. The pomegranate and mangosteen peels were dried at room temperature for 48h and ground to 60 mesh size powder. Then the crude peel powders were dried in70 ^{o}C hot air ovens for 24h. Tamarind seed kernels were roasted and ground to 1.5 mm powder by Hammer mills at Faculty of Pharmacology, Silapakorn University, Nakhon Pathom, Thailand. All crude powders were extracted with ethanol (1000g: 1.5 L) in dry and dark place at room temperature for 5 days and the waste particles were by filtering through Whatman no. 41 filter papers. The filtrate was concentrated in a rotary evaporator at 40 ^{o}C and dried in freeze dry system at vacuum pressure 138 x 10^{-3} mbar and condenser temp -46 ^{o}C for 5 h. The dry extract powders were kept at -20 ^{o}C until use.

Experimental Procedure

The protocols in experimental procedures were under documentary proof of ethical clearance from the Faculty of Veterinary Science-Animal Care and Use Committee, Mahidol University (FVS-ACUC: MUVS-2011-24). A total of 42 mix breed, Santa Inês, Dorper, Thai local breed (50:25:25), 4-5 months lambs were randomly selected from a herd of more than 200 animals in Small ruminant R&D Center, Kasetsart University (KamphaengSaen Campus), Nakhon Pathom, Thailand. All animals were freely grazing in the morning every day and naturally infected with gastrointestinal nematodes eggs and did not receive any anthelmintics at least 3 months.

In experimental design, the lambs were divided in to 6 groups of 6 animals each (3 male and 3 female) and randomly assigned to different treatments. Group 1 was given a single dose of albendazole (7.5 mg/kg), group 2 was subcutaneously injected by single dose of ivermectin (200 μg/kg). Group 3 received single doses of pomegranate peel extracts (300 mg/kg; equivalent dry fruit peel powder 3 g/kg) (Akhtar and Riffat 1985) in 5 ml sterile water each dose. Group 4 was given a single dose of mangosteen peel extracts (200 mg/kg; equivalent dry fruit peel powder 3 g/kg) in 5 ml sterile water each dose. Group 5 was given tamarind seed extract (15 mg/kg; equivalent dry fruit peel powder 3 g/kg) in 5 ml sterile water each single dose. And group 6 was not given any anthelminthic (control group).

Sample collections

Fresh fecal samples were collected from all groups, starting from day 0 (pre-treatment) and day 1, 3, 7, 14 and 30 (post-treatments) to evaluate the presence of strongyle type egg count numbers in universal egg count slides by using modified McMaster technique (Zajac and Conboy 2006). The fresh feces from each lamb (2 g/sample) in clean container were filling with 28 ml of saturated sodium chloride solution and strain through cheesecloth. The mixture solution was filled in universal egg count slide chamber for 10-15 minutes. The equation that is used for calculation the strongyle-type egg numbers examining under light microscope is : The eggs per gram (EPG) = the number of counted egg x 100 x fecal consistency score. Fecal consistency score of all fecal samples in our experiments was 1 (1= normal formed pellets) (Le Jambre et al 2007). To evaluate the renal and hepatic functions, the biochemistry values of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and blood urea nitrogen (BUN) in blood serum at day 0 (pre-treatment) and days 1, 3 and 7 (post-treatments) were determined by

Laboratory of Veterinary Science, Mahidol University.

Statistical analysis

Data are expressed as mean ± SD. All statistical analyses were performed using SPSS version 17.0. One-way analysis of variance (ANOVA) and the t-test were used for the comparison of mean values of the eggs per gram (EPG) in each group. All tests were considered to be statistically significant at $p< 0.05$.

Results

At Small Ruminant R&D Center, a herd of lamb stayed in elevated housing and slatted floors. The lambs were practically fed by standard commercial concentrate, hay racks with forage and day time grazing. The lambs were fed water *ad libitum* throughout the experimental period. The single dose of albendazole per oral or ivermectin injection repeatedly every 3 months were used to prevent gastrointestinal round worm infections from natural environment. The fresh fecal samples were collected from lamb in the morning to measure EPG before and after treatment (Table 1). The average sum EPG of gastrointestinal nematodes that naturally infected in all groups is 37,100 (31,800-40,900).

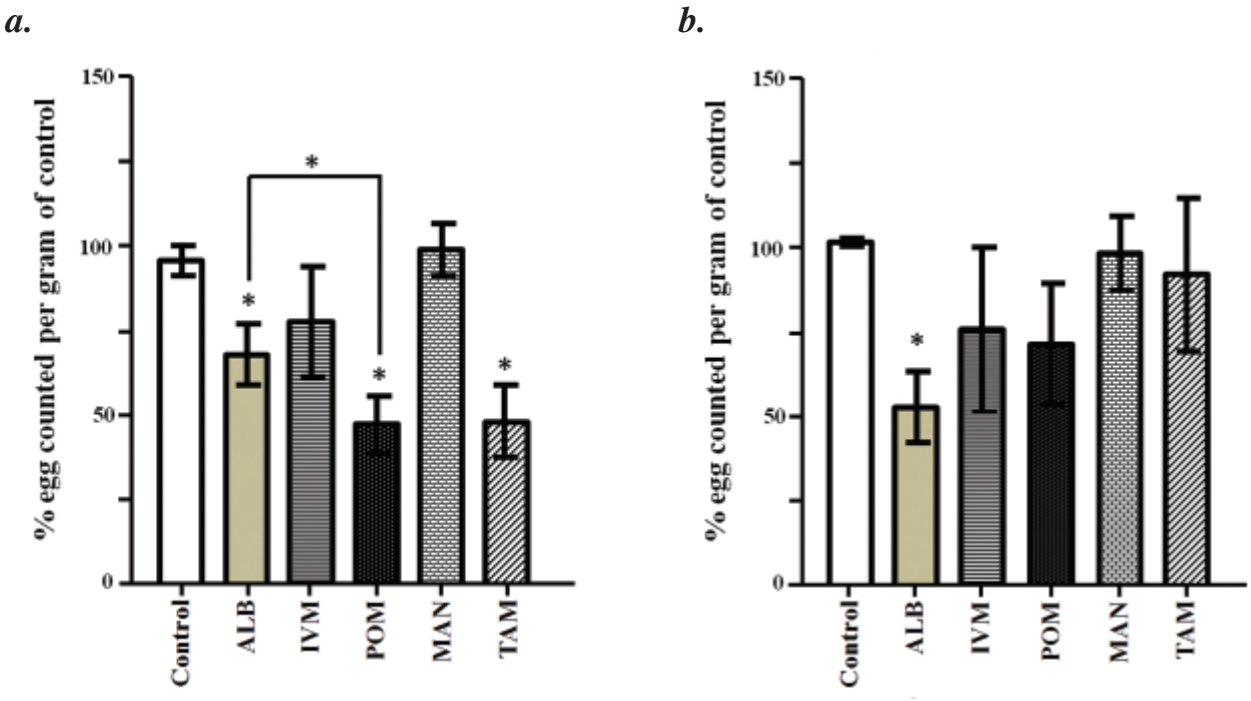
In our studies, the EPG from each group were calculated as percent of control for comparing the effects in pre- (day 0) and post-treatment (day 1, 3 and 7) (Fig 1). Data were shown that ivermectin could not significantly decrease egg counted number. In contrast, oral albendazole could significantly decrease percent EPG when compared with pre-treatment condition at day 1 (68±9%), day 3 (53±10%) and day 7 (62±8%) (Fig 1*a*-1*c*). At day 1, the ethanolic extract of pomegranate peels that dissolved in clean water were significantly effect on gastrointestinal nematode egg count (47±8%) and the effects of pomegranate peels were significantly different from albendazole (Fig 1*a*). Tamarind seed extracts could significantly reduce EPG number in lamb at day 1 (48±10%), and the EPG were reversely increase at day 3 (92±22%) and day 7 (84±28%). The crude alcoholic extracts of mangosteen peels did not significantly decrease the EPG in all treatment days.

The effects of treatments on liver and kidney functions at day 0 (pre-treatment) and days 1, 3 and 7 (post-treatment) were represented in table 2 and 3. The ALT values after ivermectin (16.5±2.2) and mangosteen peels (17.8±2.3) treatments at day 1 slightly increased but did not exceed the normal range. In all groups AST (88.5-127.3 IU/L), creatinine (0.7-0.9 mg/dl) and BUN (11-18.5 mg/dl) levels were also normal. In our studies, the lambs did not express any clinical renal or hepatic symptoms. Two lambs died by accidental traumatic conditions. The average weights after all herbal and drug treatments were slightly the same among groups.

Table 1 Sum of egg counted number per gram (sum EPG) x 100 from feces after treatment with albendazole, ivermectin, and ethanolic extract of pomegranate peels, mangosteen peels and tamarind seed at day 0 (pre-treatment) and day 1, 3, and 7 (post-treatment) in lamb. D = day, n = number of lamb in each group, control = non-treatment group. $^{*}p< 0.05$ when compared with pre-treatment (control) values.

Treatment	n	Weight (mean : kg)		sum EPG x 100			
		D0	D7	D0	D1	D3	D7
Control	6	12.40	12.70	371	364	381	409
Albendazole	6	12.43	12.93	366*	268*	224*	240
Ivermectin	6	12.36	12.86	388	347	299	372
Crude extracts							
Pomegranate peels	6	11.57	11.43	375	155*	235	324
Mangosteen peels	6	11.43	12.43	409	403	401	397
Tamarind seeds	6	12.21	12.79	318	133*	220	131

Figure 1 Percent of egg counted per gram of control in lamb at day 1 (*a*), day 3 (*b*) and day 7 (*c*) after treatment with albendazole, ivermectin, crude extracts from pomegranate peels: POM, mangosteen peels: MAN and tamarind seeds: TAM. Control = non-treatment group. $^{*}p< 0.05$ when compared with pre-treatment (control) values or among each group.



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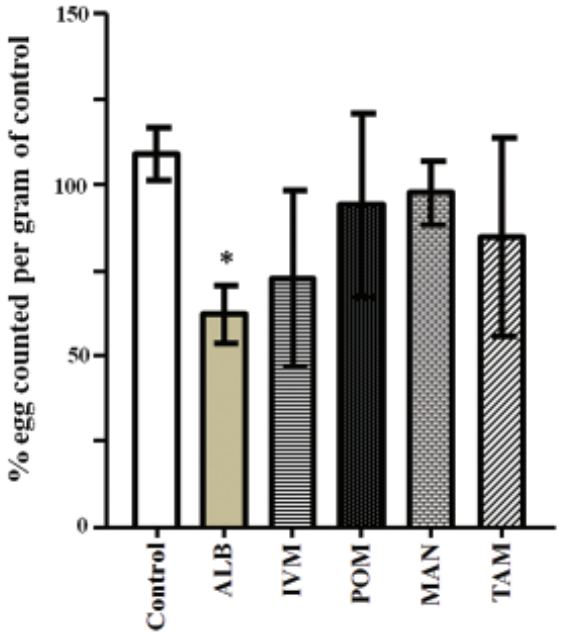

Table 2 The values of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of lambs (mean ± SD) pre- (day 0) and post- (day 1, 3 and 7) treatment with albendazole, ivermectin, crude extracts from pomegranate peels, mangosteen peels and tamarind seeds. D = day. Normal ALT: 22-38 IU/L, AST: 70-390 IU/L (Peter 2007).

Treatment	ALT (IU/L)				AST (IU/L)			
	D0	D1	D3	D7	D0	D1	D3	D7
Albendazole	10.5±2.7	10.7±3.0	9.0±2.5	12.0±2.7	95.5±8.1	109.5±8.5	99.5±7.1	100.8±6.5
Ivermectin	13.3±3.0	16.5±2.2	14.3±2.9	11.5±1.7	95.8±12.0	105.0±14.1	97.3±11.1	91.0±6.0
Crude extracts								
Pomegranate peels	8.0±1.8	9.5±3.1	9.3±2.7	10.0±2.3	127.3±30.2	100.5±9.6	100.5±9.8	99.8±7.7
Mangosteen peels	13.8±4.4	17.8±2.3	16.8±3.1	17.8±1.5	90.5±7.0	113.3±8.0	102.8±4.2	100.8±5.3
Tamarind seeds	9.3±1.7	11.8±3.5	9.5±2.0	10.3±1.5	92.3±5.5	94.8±4.7	91.3±6.1	88.5±7.3

Table 3 The values of serum creatinine and blood urea nitrogen (BUN) of lamb (mean ± SD) pre-treatment (day 0) and post-treatment (day 1, 3 and 7) with albendazole, ivermectin, crude extracts from pomegranate peels, mangosteen peels and tamarind seeds. Normal creatinine: 1.2-1.9 mg/dl, BUN: 8-20 mg/dl (Pugh 2002). D = day

Treatment	Creatinine (mg/dl)				BUN (mg/dl)			
	D0	D1	D3	D7	D0	D1	D3	D7
Albendazole	0.8±0.1	0.9±0.1	0.7±0.1	0.8±0.1	11.0±1.1	15.6±0.9	11.7±1.0	13.5±0.4
Ivermectin	0.9±0.1	0.8±0.1	0.8±0.1	0.8±0.02	14.1±1.0	18.5±1.7	15.8±2.0	16.7±2.1
Crude extracts								
Pomegranate peels	0.8±0.1	0.9±0.1	0.9±0.1	0.9±0.03	13.5±1.8	17.2±1.5	14.9±2.8	15.7±1.3
mangosteen peels	0.9±0.1	0.9±0.02	0.9±0.1	0.8±0.05	12.5±2.1	16.9±2.9	12.3±1.2	13.7±2.0
Tamarind seeds	0.7±0.2	0.9±0.1	0.9±0.1	0.8±0.05	12.7±1.8	14.2±1.7	12.2±1.0	12.4±1.6

Discussion

The modified McMaster technique has been used practically to monitor gastrointestinal nematode eggs in fecal samples to assess anthelmintic efficacy in sheep (Morgan et al 2005; Nicholls and Obendorf 1994; Rinaldi et al 2011). The usually found gastrointestinal parasites of Santa Inês breed sheep were *Strongyloides* spp., *Trichuris* spp., and *Moniezia* spp. The helminthes identified from the fecal samples and intestinal contents were: *Haemonchus contortus, Cooperia pectinata, Cooperia punctata, Trichostrongylus colubriformis, Moniezia expansa, Skrjabinema ovis, Oesophagostomum* spp. and *Trichuris* spp. (de Souza Mde et al 2012). The *Trichostrongylus* spp. was one of predominant species in German sheep farm (Idris et al 2012). In Thailand, the endoparasites found in sheep are *Strongyloides-papillosus, Cooperia, Haemonchus, Oesophagostomum, Trichostrongylus* spp., *Moniezia benedeni, Paramphistomum* spp. and *Coccidia*. The common anthelminthic that had been always used in Southern area were fenbendazole, albendazole, ivermectin and levamisole since 1985 (Kochapakdee and Saithanoo 2004). Before treatments,lambs in our experiments were injected consecutively 3 times every 3 months with ivermectin and recently resist to these drug. Comparing with sheep farms in Great Britain and Ireland, lambs were treated 3.6 times annually on average (Morgan et al 2012). The most gastrointestinal nematodes in sheep from in the Slovak Republic (Cernanska et al 2006) and Netherlands (Borgsteede et al 2010) were also resistant to ivermectin. However, albendazole (7.5 mg/kg) could significantly decrease EPG at 1, 3 and 7 day. The factor of albendazole effectiveness may be the discontinues of using albendazole in sheep farm throughout the year.

The comparative studies between albendazole, ivermectin and crude extracts from 3 waste products of fruits reveal that pomegranate peel (300 mg/kg; equivalent dry fruit peel powder 3 g/kg) and tamarind seed (15 mg/kg; equivalent dry fruit peel powder 3 g/kg) had significantly effects on percent of gastrointestinal nematode egg counted numbers in sheep at first 24h. The effects of ethanolic extracts of pomegranate peel treatment (47±8%) had the most efficiency comparing with albendazole (68±9%) and tamarind (48±10%).

The previous report showed that the single dose of pomegranate peel extracts (300 mg/kg; equivalent dry peel powder of 3 g/kg) could significantly decrease faecal egg counted number of gastrointestinal round worm at day 1, 3 and 7 in does. These effects were and not significantly difference from ivermectin injection (Boonmasawai et al 2013). There has not been any studies about the ethanolic extracts from these plants in lamb before, but from other reports, there are many botanical extracts from various species of plants that have anthelmintic efficacy on gastrointestinal nematode eggs and larvae in sheep such as *Andrographis paniculata* Wall., *Anisomeles malabarica* L., *Annona squamosa* L., *Datura metel* L., *Solanum torvum* Swartz (Kamaraj et al 2011) and *Myracrodruon urundeuva* (de Oliveira et al 2011). The ethyl acetate, acetone and methanol extracts (50 mg/ml) of *Annona squamosa, Eclipta prostrata, Solanum torvum, Terminalia chebula,* and *Catharanthus-roseus* have *in vitro* ovicidal and larvicidal activities on *Haemonchus contortus* (Kamaraj and Rahuman 2011). The aqueous methanol extract from the stem-bark of *Combretum molle* (500 and 1000 mg/kg) caused faecal egg count (FEC) reduction of *Haemonchus contortusin* lambs (Simon et al 2012).

The biochemistry values of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and blood urea nitrogen (BUN) in blood serum at day 0 (pre-treatment) and days 1, 3 and 7 (post-treatments) were not significantly different from normal values of small ruminant. The lambs from all groups had no lesion or symptoms. However, the no observed adverse effect level (NOAEL) of tamarind seed polysaccharide in the Sprague-Dawley rat diet was 120,000 ppm (equivalent to 10,597 mg/kg/day for male and 10,691 mg/kg/day for female rats) (Heimbach et al 2013).The NOAEL of pomegranate seed oil was 50,000 ppm (4.3 g /kg /day in rat) (Meert et al 2009). And the oral LD_{50} of the pomegranate fruit extract with 30% punicalagins in rats and mice was greater than 5 g/kg body weight (Patel et al 2008). Therefore, the herbal use is seemed to have high safety and efficacy in animal treatment. We suggest that the ethanolic extracts from pomegranate peels and tamarind seed have interesting trend for safely and economically used as alternative anthelmintic drugs in sheep farms. The data of composition and pharmacokinetic of all crude extracts must be investigated further to determine the dosage and dosing interval for more powerful anthelmintic activities.

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