Effects of alcoholic extract from pomegranate (Punica granatum L.) peels on gastrointestinal nematode egg counts in doe

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Abstract

Anthelmintic drug resistant is one of the major problem that quietly reflecting the small ruminant production qualities. Because this problem recently spread out in many areas all over the world, the alternative anthelmintic methods are recently requiring, especially using local economic plants or remedies for reducing the impact. The pomegranate (Punica granatum L.) is one kind of fruit that is used as an anthelmintic plant in Ayurveda remedies for a long time. We investigated the anthelmintic activity of alcoholic extract from pomegranate peels against female goat gastrointestinal nematodes and the toxic effects after treatments. The results were shown that albendazole and pomegranate peel powder could not affect percent of strongyle egg per gram (EPG) when comparing with pre-treatment control data. In contrast, ivermectin injections could significantly affect a first 24h (32 ± 5%), and had continuously effect at day 3, 7 and 14 (31 ± 17%, 51 ± 13% and 33 ± 11%, respectively). Alcoholic extract of pomegranate represented significantly anthelmintic effects at day 1, 3 and 7 (45 ± 11%, 57 ± 14% and 64 ± 7%, respectively) with non-significantly different when comparing with ivermectin. We also preliminary investigated the effects of alcoholic extract from mangosteen (Garcinia mangostana) peels, it could not effect on egg count number per gram when comparing with pre-treatment control. The results suggested that alcoholic crude extracts from pomegranate peels could inhibit gastrointestinal strongyle-type egg production and may be promising used as an alternative anthelmintic drugs in small ruminant farms.

Keywords: pomegranate peel, alcoholic extract, doe, anthelmintic effects, gastrointestinal nematode
ผลของสารกัดด้วยผลักอดผ้าถุงเปลือกผลิตภัณฑ์อ่อนวัยของ
ฟาร์มด้ำกในทางคืนอาการของแพะเพศเมีย

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2 ภาควิชาวิทยาศาสตร์และสหศาสตร์ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล
3 ภาควิชาวิทยาศาสตร์และสหศาสตร์ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล

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บทความย่อ

ปัจจุบันการคืนอาการแพะในฟาร์มผลิตภัณฑ์อ่อนวัย ใช้วิธีที่นิยมที่จะส่งผลต่อผลิตภัณฑ์ได้ยาก
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Introduction

Gastrointestinal (GI) nematode infections, especially *Haemonchus, Trichostrongylus* and other *Strongyloides* species remain the major problem that cause decreasing qualities of small ruminant productions. The hosts were usually infected nematode larvae from the fields through grazing management methods. Recently, the main controlling methods of gastrointestinal roundworms in goat farms were based on chemical anthelmintic treatments. There are three groups of broad spectrum anthelmintic that always have been used, benzimidazole (albendazole, fenbendazole and oxfendazole), imidazothiazole (levamisole), avermectins and milbemycins (ivermectin, doramectin and moxidectin). The prevalence and severity of resistance problem of these anthelmintic drugs have been reported in many countries (Wolstenholme et al 2004). The alternative anthelmintic methods are recently required to get rid of resistant and chemical residue problems in small ruminant farms. The concept of medicinal plants is interesting approach, especially using local economic plants or remedies (Iqbal et al 2005). There are few present in vivo studies that evaluate the anthelmintic activity of herbal extracts in goat, such as sisal waste (*Agave sisalana*), but it had low efficacy (Botura et al 2011). One of the tropical fruit that are widely consumed in Southeast Asia because of their potential antioxidant and anti-inflammatory properties is pomegranate or *Punica granatum* L. (Punicaceae) (Bachoual et al 2011). Moreover, pomegranates have anti-proliferative, anti-invasive, and anti-metastatic and apoptotic effects in some human cancer, such as breast cancer (Dikmen et al 2011) and prostate cancer (Faria and Calhau 2011). Important constituents from pomegranate fruit extract are ellagic acid, ellagitannins, punicalagins, punicic acid, flavonoids, anthocyanidins, anthcyanins, estrogenic flavonols and estrogenic flavones (Jurenka 2008). Pomegranate peel composed of 67% of the total phenolic content and that is the highest amount of total phenolic in whole fruit (Gözlekçi et al 2011). Major constituents in pomegranate pericarp (peel) are phenolic punicalagins; gallic acid and other fatty acids; catechin, quercetin, rutin, and other flavonols; flavones, flavonones; anthocyanidins (Jurenka 2008). The alcoholic extracts of pomegranate peels have protective effects oxidative stress in rat (Cekmen et al 2012; Kumar et al 2013), broad-spectrum activities on both Gram-positive and Gram-negative bacteria (Fawole et al 2012). The pharmacological effects of pomegranate peels which have been the waste for many tons each year from fruit juice industry were also very interesting as the anthelmintic herbal drug. The previous reports showed that the extracts of pomegranate peels also have anthelmintic activities by inhibit the hatching of *Haemonchus contortus* eggs (Prakash et al 1980). The studies of Akhtar and Riffat showed that extracts from pomegranate peels equivalent of 3 g/kg of dry peel weight effectively reduced gastrointestinal nematodes egg per gram (EPG) in sheep (Akhtar and Riffat 1985). Moreover, there are not any continuous studies about anthelmintic effects of pomegranate peels for long times. In our studies, the dry pomegranates peels were extracted by ethanol. Then dry peel powder and crude extracts were investigated the anthelmintic activities of gastrointestinal roundworms in mature female goats comparing with albendazole and ivermectin. The gastrointestinal strongyle fecal egg count which is the simple, conventional method and has well related with total GI strongyle worm burden in goats (Rinaldi et al 2009) was used to determine the anthelmintic activities of fruit peel extract. The blood samples were collected from all doe for testing effects on liver and kidney functions before and after treatment. In this study, the effects of alcoholic extract from mangosteen (*Garcinia mangostana*) peels were also preliminary investigated on EPG number.
Materials and Methods

Plant extract preparation

Pomegranate (*Punica granatum* L.) peels were obtained from local market in Nakhon Pathom province, Thailand. And the mangosteen (*Garcinia mangostana*) peels were obtained from fruit garden in Rayong province, Thailand. The pomegranate and mangosteen peels were dried in 70 °C ovens for 24h, and powdered to get 60 mesh sizes. The crude peel powders were extracted by ethanol (1000g: 2 L) in dark place at room temperature for 5 days. The extract was filtered through Whatman no. 41 filter paper for removal of peel particles. The filtrate was concentrated in a rotary evaporator at 40 °C and dried in freeze dry system at vacuum pressure 138x10⁻³ mbar and condenser temp -46 °C for 5 h. The dry powders and crude extracts were kept at -20 °C until used.

Experimental procedure

All animal care and used protocol in experimental procedures were reviewed and accepted by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC) from Mahidol University (Protocol: MUVS-2011-03). The total of 20 mixed local breed, Bohr and Anglo-Nubian (50:25:25), female, 1-2 years goats were randomly selected from a herd in Small ruminant R&D Center, Kasetsart University (Kamphaeng Saen Campus), Nakhon Pathom, Thailand. Before the studies (Pre-treatment), all doe were freely grazing in the morning every day and naturally infected by gastrointestinal nematodes eggs with non-anthelmintic treatments for 3 months. In experimental design, the doe were randomly divided in to 4 groups of 5 animals each and assigned to different treatments. Group 1 was orally given a single dose of albendazole (7.5 mg/kg). Group 2 was subcutaneously injected by single recommended dose of ivermectin (200 µg/kg). Group 3 received crude pomegranate peel powder (3 g/kg) (Akhtar and Riffat 1985) in 10 ml water solution each dose per oral. Group 4 was orally given a single dose of pomegranate peel extract (300 mg/kg; equivalent dry peel powder of 3 g/kg) in 5 ml sterile water each dose.

Sample collections

Blood samples were collected at day 0 (Pre-treatment) and days 1, 3 and 7 (Post-treatments) from all doe. Sera were determined value of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and blood urea nitrogen (BUN) by The Center for Veterinary Diagnosis, Mahidol University. Fresh fecal samples were also collected, 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 by using modified McMaster technique (Zajac and Conboy, 2006). Two gram of fresh feces from each sample was fillingwith 28 ml of saturated sodium chloride solution then mixed well and strain through cheesecloth. The mixture was filled each chamber of universal egg count slides at least 10-15 minute before examining under light microscope. The equation that is used for calculation the strongyle-type egg numbers in 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). Fecal egg count percent reduction (FECR) was also calculated from post-treatment group means according to the standard equation (Yazwinski et al 2013): FECR = [(day 0 mean - day X mean) / day 0 mean] x 100

Statistical analysis

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

The doe in this study stay in farm house with elevated floor at Small Ruminant R&D Center, Kasetsart University (KamphaengSaen Campus), Nakhon Pathom, Thailand. The animals are fed with standard commercial ration (concentrates) foods. In addition, the doe are freely grazing in the morning and given dry grass in the afternoon. Every three months, the doe will be routinely deworm by using single dose of albendazole per oral or ivermectin injection to prevent gastrointestinal round worm infections from grazing environment. The strongyle-type egg count numbers of female goats are presented in Table 1. Fecal egg count percent reduction (FECR) was also calculated from post-treatment group data at day 1, 3, 7, 14 and 30 (Table 2). All doe were naturally having gastrointestinal nematodes eggs 10,900-12,000 EPG. In our studies, the egg numbers from fecal sample were calculated as percent of control in each group. Data were shown that albendazole and crude pomegranate peel powder orally given could not decrease egg counted number significantly. In contrast, ivermectin injection could significantly decrease percent EPG when compared with pre-treatment control (day 0) at day 1 (32 ± 5%), day 3 (31 ± 17%), day 7 (51 ± 13%) and 14 (33 ± 11%). The FECR of ivermectin was 68, 69, 49, 67 and 51, respectively. Alcoholic extract of pomegranate peels that easily dissolve in clean water were also orally given single dose at day 1, 3 and 7, represents significantly affects (45 ± 11%, 57 ± 14% and 64 ± 7%, respectively). Pomegranate peel extracts had 55, 43 and percent reduction of worm egg number at day 1, 3 and 7. At any rate, there are not significant different between ivermectin and alcoholic extract of pomegranate in % EPG of control (Figure 1). We also preliminary investigated the effects of dry peel powder and crude alcoholic extract from mangosteen (Garcinia mangostana) peels at day 1, 3, 7, 14 and 30, but there are non-effects on percent of EPG of control when compared with pre-treatment control (data not shown).

Table 1  Sum of egg counted number per gram (EPG) x 100 from feces after treatment with albendazole, ivermectin, crude powder and alcoholic extract of pomegranate peels at day 0 (pre-treatment) and day 1, 3, 7 and 14 (post-treatment). D = day, POM = pomegranate, Ext = extracted, n = number of animal in each group. * p<0.01, ** p<0.05 and *** p<0.001 when compared with pre-treatment (control) values.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Weight (mean : kg)</th>
<th></th>
<th>Sum egg counted number per gram (EPG) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre - treatment</td>
<td></td>
<td>D0</td>
</tr>
<tr>
<td>Albendazole</td>
<td>5</td>
<td>26.8</td>
<td>27.5</td>
<td>118</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>5</td>
<td>25.8</td>
<td>26.8</td>
<td>111</td>
</tr>
<tr>
<td>Crude-POM</td>
<td>5</td>
<td>24.9</td>
<td>25.5</td>
<td>120</td>
</tr>
<tr>
<td>Ext-POM</td>
<td>5</td>
<td>27.6</td>
<td>29.6</td>
<td>109</td>
</tr>
</tbody>
</table>
For basic testing toxic effects of pomegranate peels on liver and kidney functions within 7 days, blood samples were collected at day 0 (pre-treatment) and days 1, 3 and 7 (post-treatment) for determined standard serum chemistry values of every groups (Table 3 and 4). After crude pomegranate peel powder treatments, ALT (normal: 15-52 IU/L) were 20.5 ± 4.2, 17.3 ± 2.6, 19.5 ± 3.5 IU/L at day 1, 3 and 7. AST (normal: 66-230 IU/L) were 80.0 ± 4.1, 77.0 ± 4.7 and 75.3 ± 3.0 IU/L. Creatinine (0.7-0.8 mg/dl) and BUN (10.9-17.3 mg/dl) were also not exceed from the normal range levels. The levels of ALT in alcoholic extract of pomegranate peels treatment groups at day 1, 3 and 7 were 18.3 ± 1.4, 17.5 ± 2.1 and 20.3 ± 2.6 IU/L, respectively. AST values were 74.5 ± 5.3, 73.3 ± 10.1 and 75.3 ± 7.7 IU/L. Creatinine (0.5-0.7 mg/dl) and BUN (10.6-15.4 mg/dl) were all in normal range. The serum chemistry values of all doe in other treatments by albendazole and ivermectin were also normal. During study, the doe have well healthy without any clinical symptom. Three months after single herbal treatment, 80% of doe in each group had pregnancy and continuously give normal births.

Table 2  Fecal egg count reduction (FECR) percentages after treatment with albendazole, ivermectin, crude powder and alcoholic extract of pomegranate peels at day 1, 3, 7, 14 and 30. D = day, POM = pomegranate, Ext = extracted, n = number of animal in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>D1</th>
<th>D3</th>
<th>D7</th>
<th>D14</th>
<th>D30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albendazole</td>
<td>5</td>
<td>29</td>
<td>35</td>
<td>-32</td>
<td>-17</td>
<td>-56</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>5</td>
<td>68</td>
<td>69</td>
<td>49</td>
<td>67</td>
<td>51</td>
</tr>
<tr>
<td>Crude-POM</td>
<td>5</td>
<td>53</td>
<td>38</td>
<td>5</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>Ext-POM</td>
<td>5</td>
<td>55</td>
<td>43</td>
<td>36</td>
<td>29</td>
<td>20</td>
</tr>
</tbody>
</table>

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Table 3  Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of doe (mean ± SD). Normal ALT: 15-52 IU/L, AST: 66-230 IU/L. D = day, POM = pomegranate, Ext = extracted

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT (IU/L)</th>
<th>AST (IU/LX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D1</td>
</tr>
<tr>
<td>Albendazole</td>
<td>20.3 ± 1.1</td>
<td>19.0 ± 0.5</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>21.8 ± 2.7</td>
<td>21.8 ± 2.5</td>
</tr>
<tr>
<td>Crude-POM</td>
<td>18.8 ± 2.9</td>
<td>20.5 ± 4.2</td>
</tr>
<tr>
<td>Ext-POM</td>
<td>16.5 ± 1.2</td>
<td>18.3 ± 1.4</td>
</tr>
</tbody>
</table>

Figure 1  The effects of albendazole, ivermectin, crude powder and alcoholic extract of pomegranate peels on % number of roundworm eggs (EPG) in doe. POM = pomegranate, Ext = extracted, * p< 0.01 ** p< 0.05 and *** p< 0.001 when compared with pre-treatment values.
Discussion

After 1900s the investigations about non-chemical gastrointestinal control initially were important principles in small ruminant farm managements (Hoste and Torres-Acosta 2011) because of broad-spectrum anthelmintic resistance occurring worldwide (Waller 1997). Nowadays, many in vitro studies about ovicidal and larvicidal activities of crude extracts from plants were investigated such as *Maesa lanceolata* (Myrsinaceae), *Plectranthus punctatus* (Lamiaceae) (Tadesse et al 2009), *Combretum molle* (Combretaceae) (Ademola and Eloff 2010), *Vernonia amygdalina* (Asteraceae) (Ademola and Eloff 2011a), *Cassia alata* (Caesalpinaceae) (Ademola and Eloff 2011b). At any rate, these crude extracts may be low efficacy in vivo treatments or high economic costs. In our in vivo studies, we were searching for appropriate plants which were cheap and easy to find in local area of Thailand. In our studies, the doe have naturally strongyle-type gastrointestinal round worms infections (average EPG = 11,500 eggs). But the prevalence study in 2011 for intestinal parasite infections in 12 goat herds in NakhonPathom province reveal that herd prevalence was 100% while individual prevalence was 79.47%. The most common intestinal parasite is strongyle group and the average number of EPG was 1,176 (Ratanapob et al 2012). The seasonal change, humidity, stage of infection, grazing with other animal such as dairy cow and other farm management may be the factors influencing the high EPG in experiments and needed the further study. Report from goat farm in Kashmir valley, India showed that the gastro-intestinal nematodes (GINs) fecal egg counts: and average worm burden were significant differences between the seasons. The mean prevalence of GIN infection was maximum in the summer and lowest in winter (Tariq et al 2010). The fecal egg counts from small ruminants in the Kenyan Central Highlands were higher at 2-3 months after onset of rains because of suitable climatic conditions for the development of free-living stages of the nematodes. The free-range grazing could increase risk of nematode infection and re-infection compared with zero-grazing system. The zero-grazing system is easier to control helminthes because this system could decrease risk of exposure gastrointestinal nematode larvae (Odoi et al 2007).

From EPG data, this farm may have albendazole-resistant problems because of the using the same anthelmintic agent for a long time. The benzimidazoles resistance problems in small ruminant herds were also found in many other country such as Southeastern United States (Howell et al 2008), Switzerland, Southern Germany (Scheuerle et al 2009), India (Godara et al 2011).

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Serum creatinine and blood urea nitrogen (BUN) value of doe (mean ± SD). Normal creatinine: 0.9-1.8 mg/dl, BUN: 10-20 mg/dl. D = day, POM = pomegranate, Ext = extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td><strong>Creatinine (mg/dl)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>D0</strong></td>
</tr>
<tr>
<td>Albendazole</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Crude-POM</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Ext-POM</td>
<td>0.5 ± 0.1</td>
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and Norway (Domke et al 2012). Those mean the alternative drug is required for solution. In our studies, the effects of treatment reveal that alcoholic extracts of pomegranate peels could significantly decrease EPG of gastrointestinal roundworms in goat comparing with pre-treatment control (day 0) within 24h with 55% reduction of worm egg number. Other in vivo studies in plants, crude ethanolic extracts (CEE) from the aerial parts of *Artemisia absinthium* L. (Asteraceae) or Tethwen tree 2.0 g/kg body weight were associated with significant reduction in fecal egg count in sheep about 90.46% at on day 15 (Tariq et al 2008). In addition the study by Macedo et al (2010) showed that anthelmintic efficacy of *Eucalyptus staigeriana* (Myrtaceae) essential oil was 76.57% at day 15 after treatments (Macedo et al 2010). The inhibitions of pomegranate extract in our studies are not significantly difference from ivermectin effects. While the dried pomegranate fruit peel powder could not significantly decrease eggs counted number at any time.

The effects of pomegranate on liver and kidney functions within 7 days were also investigated. The ALT, AST, creatinine and BUN of all samples were in normal ranges and no toxic symptoms were obtained in all treatment groups. The toxicity testing studies of pomegranate peels recently have not any report, but the oral LD50 of the pomegranate fruit extract; standardized to 30% punicalagins in rats and mice was found toxic effects at greater than 5 g/kg body weight (Patel et al 2008), represent that pomegranate constituents commonly used in traditional medicine without toxic effects. We suggest that the alcoholic extracts from pomegranate peels have interesting trend for further study to inhibit gastrointestinal roundworm egg productions in large number and another species in small ruminants and may be safely and economically used as an alternative anthelmintic drugs in goat farms.

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