IN VITRO ANTI-PARASITIC ACTIVITIES OF POMEGRANATE, PUNICA GRANATUM AGAINST PARASITIC NEMATODES OF RUMINANTS

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ABSTRACT. Parasitic nematode infection in animal is one of the main causes for the mortality of animals and most of the treatment relies on the use of the anthelmintic drugs to overcome such a problem. However, the heavy use of anthelmintic contributed to the problem of multidrug resistance. This study was carried out to investigate the infectiveness of *Punica granatum* (aqueous leaf and peel extracts) as an alternative treatment. This study utilised the *in-vitro* assay technique (motility assay and larval migration inhibition assay) to determine the effect of the extracts on the survival of L_3 stage of parasitic nematodes. The results showed that incubation of L_3 in different concentrations (5, 10, 20, 50 and 100 mg/ml) of extracts paralysed and killed the worms after 24 hours and 48 hours of incubation periods. The same results were obtained from larval migration inhibition assay, showing that both extracts of *Punica granatum* inhibited the migration of the L_3. After 2 hours of incubation in the leaf extract (5 mg/ml), the migration of nematode larvae was inhibited to 56±12.29% as compared to the control. While for the peel extract the percentage of migration was reduced to 53 ±3.33%. Further reduction of the migration was observed at 10, 20 and 50 mg/ml of leaf extract.

Keywords: Punica granatum, anti-parasitic, nematodes, *in vitro*, phytochemical

INTRODUCTION

Infection of parasitic nematodes in ruminants is a global issue. In tropical, developing countries, gastrointestinal nematode infection remains one of the main causes of impaired production in small ruminants and can lead to the fatalities of the animals (Aumont *et al*., 1997; Jabbar *et al*., 2006). Previous studies showed that the infected animals decreased in growth rate, fecundity and affected the production of milks (Carles, 1992; Thumbi *et al.,* 2013a; Thumbi *et al.,* 2013b) and indirectly raised the rate of death. Current efforts was focused on the use of the integrated system for the control of gastrointestinal parasites, such as pasture management and strategic de-worming (Sani *et al.,* 1995), but intensive anthelmintic treatment is still the most ideal method by farmers to regulate internal parasites in their flock (Chandrawathani *et al.,*

1999). In response to the use of commercially anthelmintic drugs, some helminth species had developed resistance at partial of full scale (Waller, 1997; Jackson and Coop, 2000; Wolstenholme *et al.,* 2004). The usage of anthelmintic drugs has resulted in higher or more frequent doses to kill or control nematode infections (Jackson and Coop, 2000; Kaplan, 2004) and at the same time, increased the cost of production. Thus, there is a need to develop a new, safer, effective and economical treatment to overcome such a problem.

Therefore, by using ethnobotanical knowledge, the usage of anthelmintic drugs could be reduced and it may provide new solutions to treat parasitic nematodes in livestock. Previous studies showed the use of plant extracts in alternative medicines for treatment against parasitic nematodes and playing an important role in today's traditional medical practice especially in the tropics (Waller *et al.*, 2001). According to Azaizeh *et al.* (2003), 80% of the global population depends on plant remedies for their primary health. Therefore, because of the medicinal properties of the plants, this *in vitro* study was carried out to investigate the effectiveness of *Punica granatum* against parasitic nematodes of ruminants. The leaves and peels of this plant is traditionally used to treat diarhoea and gastroinfectional diseases (Chooi, 2004).

MATERIALS AND METHODS

Plant collection and preparation of the aqueous extract

The *Punica granatum* leaves were collected in Taman Sri Kuching, Kuala Lumpur and in residential areas at Seberang Jaya, Sungai Ara and Bukit Gedung, Pulau Pinang. About 4 kg of *Punica granatum* leaves were collected. For the peel, the *Punica granatum* fruits were bought at fruit stores in Sungai Dua, Pulau Pinang and Chow Kit Market, Kuala Lumpur. Both leaves and peels were washed with running tap water and left to dry in an oven at 40 °C for approximately 2 days. The dried plant materials of both leaves and peel were ground into fine powder. 100 g of the ground plant material was weighed and dissolved in 500 ml distilled water in a conical flask. The conical flask containing the dissolved plant material was then kept for 24 hours at 50 °C. After 24 hours, the dissolved plant materials were filtered then centrifuged for 15 minutes at 2500 RPM. The aqueous filtrate was then kept in universal bottles to be freeze-dried. After freeze-drying, the aqueous filtrate formed into powder form. Stock solution of different concentrations (5 mg/ml, 10 mg/ml, 20 mg/ml, 50 mg/ml and 100 mg/ml) were prepared for both *Punica granatum* leaves and peels (Al-Rofaai *et al*., 2012).

Phytochemical tests

Five different tests (alkaloids, flavonoids, tannins, tripertenes and steroids) were applied as described by Raaman (2006), Ayoola *et al.* (2008), Evans and Trease (2009), and Pushker *et al.* (2011).

Test for alkaloids

Two g of iodine and 6 g of potassium iodide were dissolved in 100 ml distilled water to prepare the Wagner's reagent. Two drops of Wagner's reagent were added to 1 ml of aqueous extract. Formation of reddish-brown precipitate indicates the presence of alkaloids.

Test for flavonoids

One ml of 10% ammonia was added to 1 ml of aqueous extract. The formation of yellow coloration indicates the presence of flavonoids.

Test for saponins

Two ml of aqueous extract were mixed with 5 ml of distilled water in a test tube. The test tube was shaken vigorously and observed for a stable persistent froth. Frothing which persisted for 15 minutes indicates the presence of saponins.

Test for tannins

One ml of aqueous extract was added with 2 drops of 1% ferric chloride solution. The formation of blue-black precipitate indicates the presence of tannins.

Test for tripertenes and steroids

One ml of aqueous extract was dissolved in 1 ml of chloroform, added with 1 ml of concentrated sulfuric acid. A reddish brown colouration formed at the interface indicates the presence of terpenoids.

Fecal sample collection, nematodes culture and identification

Faecal samples were freshly collected from a goat farm at Kampung Tobiar at Permatang Buloh, Penang. The faeces were collected soon after it was deposited. The faeces were kept in a labeled specimen bottle and placed in an ice box and all the collected samples were brought immediately to the laboratory.

The larvae from the goat faeces were obtained by culturing the eggs in the faeces by using the Harada and Mori (1955) technique. Filter papers were cut into squares of 15×15 cm. The faeces were crushed by using a pestle and mortar and distilled water was added to keep it moist. Then the moist crushed faeces were spread evenly on the centre of the filter paper, leaving about 2 cm were left at each end of the filter paper to make it easier to roll. The filter paper was then rolled and inserted into a test tube. Distilled water was slowly added into the test tube until it touches the bottom of the filter paper. The test tube was left to stand for 7 to 10 days and the water level was checked daily. The test tube was covered with cotton wool to prevent other microorganisms from contaminating the water. After 7 to 10 days, the filter paper was slowly removed from the test tube using forceps. The water filled with larvae was transferred into a universal bottle. A smear was prepared on a glass slide, covered with a cover slip and examined under a compound microscope of 10×10 and 40×10 magnifications for species identification. 50 third-stage (L_3) larvae were randomly selected and were identified using morphological identification keys according

to Dickman and Andrew (1933) and Gordon (1933).

Larval motility assay and larval inhibition migration assay

Two assays were applied to investigate the infectiveness of leaf and peel extracts against a mix species of L_3 recovered from the faecal sample prepared earlier.

Table 1. Motility scale of L_3 larvae

Scale	Motility
0	Dead
1	Paralysed
2	Slow moving
3	Active

*5 third-stage (L_3) larvae for each concentration were scored individually. The score then were converted to a numerical value and expressed as a percentage of the maximum score (15 = 5 L_3 larvae ×3)

For larval motility assay, the scale from 0 to 3 was used (Table 1). Five third-stage (L_3) larvae were placed in 1 ml distilled water in a 24-well test plate and 1 ml of *Punica granatum* leaf extract at different concentrations (5 mg/ml, 10 mg/ml, 20 mg/ml, 50 mg/ml and 100 mg/ml) were added into each well and left to incubate at 27 °C for 24 hours. The motility of L_3 nematode larvae were recorded for every 6 hours up to 48 hours. Four replicates were run for each concentration and a positive control. Distilled water was used as the positive control. The experiment was repeated 3 times. The same procedure was applied for the peels extract.

For larval migration inhibition assay, the standard method as described by Molan *et al.* (2000) was used with some modification. Five third-stage (L_3) larvae were placed in 1 ml distilled water in a 24-well test plate and 1 ml of *Punica granatum* leaf extract at different concentrations (5 mg/ml, 10 mg/ml, 20 mg/ml, 50 mg/ml and 100 mg/ml) were added into each well and were left for incubation at 27 °C for 2 hours. After 2 hours of incubation, the incubated L_3 larvae were transferred onto muslin cloth (34 µm) and left to incubate for another 2 hours at 27 °C to enable the active larvae to migrate through the muslin cloth for counting. The number of L_3 larvae which had migrated through the muslin cloth was counted under the dissecting microscope. Two replicates were run for each concentration with a negative and a positive control. 100% methanol was used for the negative control, while distilled water was used for the positive control. The experiment was repeated 3 times. The same procedure was applied for the peel extract.

Statistical analysis

The relationship between aqueous extracts and L_3 activity were analysed using the Mann-Whitney test ($p < 0.05$) on the IBM Statistical Package for Social Science (SPSS) Statistics version 20.

RESULTS

The phytochemical constituents of leaves and peels of *Punica granatum* were tested and the results are shown in Table 2. The phytochemical test revealed that the leaves of *Punica granatum* contained alkaloids, flavonoids, tannins, tripertenes and steroids. The peels of *Punica granatum* contained

Table 2. Phytochemical constituents test indicated the present of some important compound in the extracts.

Phytochemical Constituent	Aqueous Extract	Test Result
Alkaloids	Leaves	Positive – Reddish-brown precipitate formed
	Peel	Positive – Reddish-brown precipitate formed
Flavonoids	Leaves	Positive – Yellow coloration formed
	Peel	Positive – Yellow coloration formed
Saponins	Leaves	Negative – No frothing
	Peel	Positive – Persistent frothing
Tannins	Leaves	Positive – Blue black precipitate formed
	Peel	Positive – Blue black precipitate formed
Tripertenes & Steroids	Leaves	Positive – Yellow layer formed
	Peel	Positive – Yellow layer formed

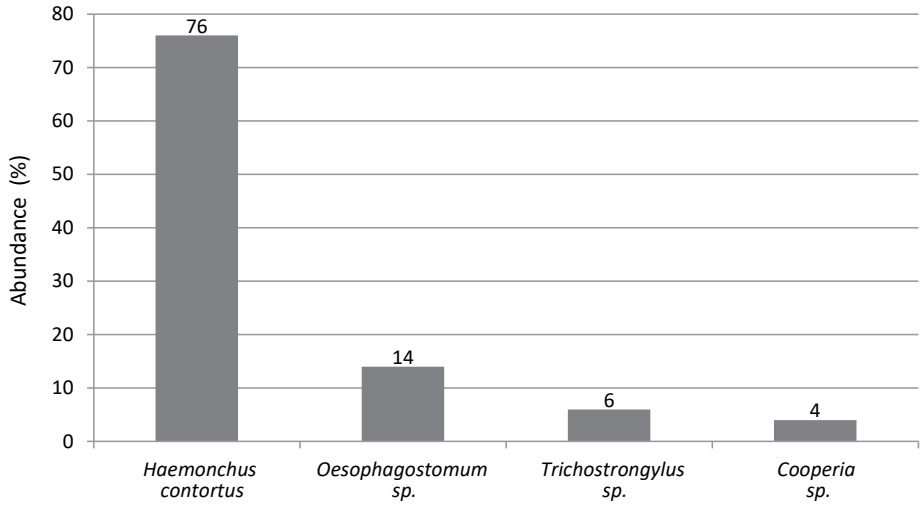
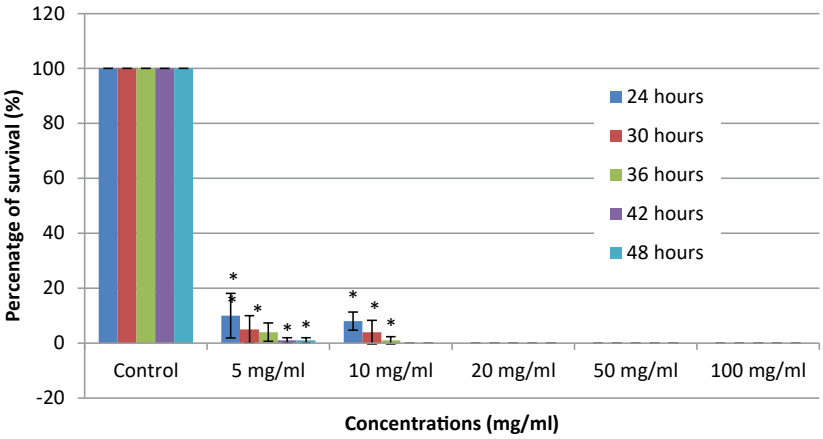


Figure 1. Species abundance of parasitic nematodes obtained from the fecal culture after 7 days incubation at room temperature.

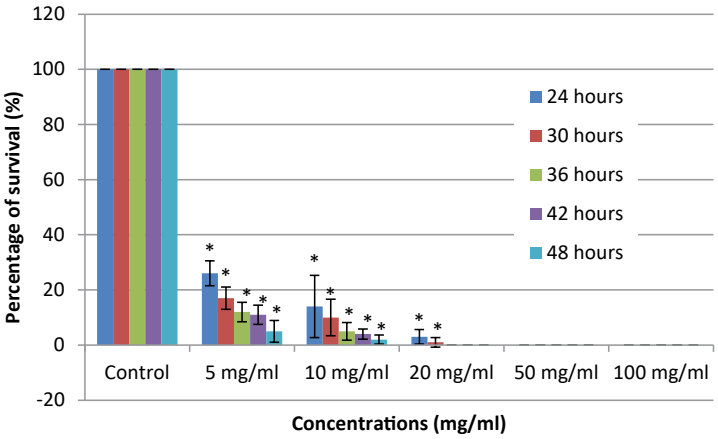
alkaloids, flavonoids, saponins, tannins, tripertenes and steroids.

For the larvae culture, there were four nematode larvae species that had been identified: *Haemonchus contortus*, *Oesophagostomum* sp., *Trichostrongylus* sp. and *Cooperia* sp. *Haemonchus contortus* was found to be the most dominant in the collected samples with the abundance of 76%, followed by *Oesophagostomum* sp. (14%) and *Trichostrongylus* sp. (6%), while *Cooperia* sp. was found to be the least dominant with the abundance of 4%, as shown in Figure 1.



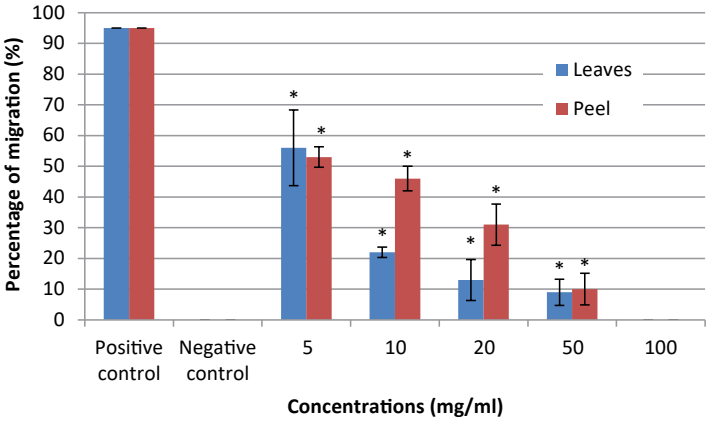
* significant different as compared to the control group ($p<0.05$).

Figure 2A. Larva motility assay of leaves aqueous extract againts L_3 of parasitic nematodes at different concentrations. Graph represented the mean ± standard error based on 3 different experiments.



*significant different as compared to the control ($p<0.05$)

Figure 2B. Larva motility assay of peels aqueous extract againts L_3 of parasitic nematodes at different concentrations. Graph represented the mean ± standard error based on 3 different experiments.



*significant different as compared to the control ($p<0.05$)

Figure 3. Larva migration inhibition assay of leaves and peels aqueous extracts against parasitic nematodes at different concentrations. Graph represented the mean ± standard error based on 3 different experiments.

In the motility assay, incubation of the L_3 at different concentrations of aqueous leaf extract significantly reduced the motility of the L_3 as compared to the control group. At 5 mg/ml of *Punica granatum* leaf extract, the motility of nematode larvae were reduced from 10±8.08% after 24 hours incubation to 1±1% at 48 hours of incubation. At 10 mg/ml of leaf extract, the motility of nematode larvae reduced from 8 ± 3.33% at 24 hours incubation to 0% at 48 hours incubation. For the 20 mg/ml, 50 mg/ml and 100 mg/ml of *Punica granatum* leaf extract, 24 hours of incubation was enough to kill all the tested L_3 nematode larvae (Figure 2A). The same observations were found with the peel extract against L_3 larvae in the motility assay (Figure 2B). At 5 mg/ml of *Punica granatum* peel extract, the motility of nematode larvae was reduced from 26±4.51% at 24 hours to 5±3.93% at 48 hours incubation. In 10 mg/ml of peel extract, the motility of nematode larvae reduced from 14±11.26% at 24 hours to 2±1.67% at 48 hours incubation. However, for the peel extract, higher concentration is required to kill the L_3 as compared to the leaf extract. Statistical analysis showed that there is a significant difference between leaf and peel extract of *Punica granatum* ($p < 0.05$).

For the larval inhibition migration assay, there were 2 controls that were used; positive control at which the larvae were incubated in distilled water, and negative control at which the larvae were incubated in 100% methanol. The percentage of migrated larvae in positive control and negative control were 95% and 0%, respectively. In this study, significant inhibitions of the migration of the L_3 were observed as compared to the control groups at different elevated concentrations of leaf and peel extracts. After 2 hours of incubation (5 mg/ml) in the leaf extract, the migration of nematode larvae was inhibited to 56±12.29% as compared to the control. While for the peel extract, the percentage of the migration was reduced to 53±3.33%. Further reduction of the migration was observed at 10, 20 and 50 mg/ml of leaf extract. The same results were observed for the peel extract (Figure 3). Meanwhile for both extracts, at 100 mg/ml the migrations of the l L_3 were totally inhibited. The leaf and peel extracts of *Punica granatum* were significantly inhibited the migration of L_3 nematode larvae at the concentration of 5 mg/ml, 10 mg/ml, 20 mg/ml, 50 mg/ml and 100 mg/ml ($p<0.05$).

DISCUSSION

Haemonchus contortus is a common species affecting ruminants in Malaysia. *Haemonchus contortus* females are capable of producing thousands of eggs up to 5,000 –15,000 eggs per day which leads to rapid larval pasture contamination (Hansen and Perry, 1994). Besides that, the pre-patent period of this nematode species is only 19 to 21 days, but can be shorter in goats with a weak immune system (Junquera, 2014). This might give some advantages for the parasite to increase their population in the paddock. Compared to *H. contortus*, the female *Cooperia* produces only 1,000 to 3,000 eggs per day (Hansen and Perry, 1994). Even though the pre-patent period of this species is short of 14 to 21 days, it is not very pathogenic compared to other nematode species. High productivity of eggs and short pre-patent period can increase nematode population

in a goat farm. Optimum conditions in a goat farm can cause the eggs and larvae to develop within a short period of time. Based on the observations during sampling, most paddocks were not well managed. The problem that might occur is that animal fecal can be found all around the paddock. This could contribute to contamination of the grass with infected faeces. From this study, the eggs per gram (epg) counted in the collected faecal samples showed a high average of 7,033 epg. Generally, an epg count of 500 or more is considered highly infected and requires treatment (Hutchinson, 2003).

The assays showed that the L_3 were affected after incubation with the extracts at different elevated concentrations. Some L_3 were shown to be paralysed and inactive due to the presence of the extracts. Some of them were dead after 48 hours incubation. This *in vitro* results demonstrated the ability of *Punica granatum* extract as the candidate for alternative treatment of parasitic nematode infection in ruminants. The phytochemical constituent tests showed the presence of important compounds that might directly affect the motility and the fitness of the L_3. The study by Shah *et al.* (2012) suggested that antioxidant properties and high content of flavonoids and tannins in the extracts play a major role, exhibiting anti-protozoa, anti-cestodial, anti-nematodial and anti-bacterial effects. Flavonoids are also well-known for its antioxidant properties by uncoupling oxidative phosphorylation and hindering energy production in helminths (Higdon, 2005). The suggested mechanism of saponin action was in the changes of cell membrane permeability and pore formation, resulting in disintegration of teguments of the parasites (Al-Rofaai *et al.*, 2012). The mode of action of tannin as an anthelmintic is attributed to its capacity to bind onto some proteins of metabolism or the larva's organs and muscles, thereby causing a change in function and resulting in the paralysis or death of the L_3 larvae (Al-Rofaai *et al.*, 2012).

CONCLUSION

This study has revealed the potential use of *Punica granatum* against parasitic nematodes of ruminants. The results also revealed the presence of medicinally important constituents in *Punica granatum*. Many studies have confirmed that these constituents contribute to the treatment of the ailment. Looking into this possibility, this project should be further carried out to isolate, separate, identify, and characterise the constituents that are responsible for the anthelmintic properties of this plant. Further work could be done to test the effectiveness of *Punica granatum* against parasitic nematodes in *in vivo*.

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