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In vivo study of pomegranate (*Punica granatum*) peel extract efficacy against *Giardia lamblia* in infected experimental mice

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ABSTRACT

Objective: To investigate the efficacy of pomegranate (*Punica granatum*) peel extract as an alternative treatment on the white laboratory mice against giardiasis.
Methods: Experimental animals were divided into five groups, including Group A: control (infected untreated), Group B: infected and fed with pectin 7 days before infection, Group C: infected and fed with pectin starting from 7th day of infection, Group D: infected and fed with pomegranate peel extract 7 days before infection, and Group E: infected and fed with pomegranate peel extract starting from 7th day of infection.
Results: Results from this study revealed that the prevention rate in the experimental groups reached approximately 50% by the 10th day of using pomegranate peel extract. Moreover, stool cyst counts of groups showed a significant reduction in the shedding of cysts approximately 75.6% by day 20 post-infection. ELISA test showed a reduction in *Giardia* antigen in the stools of the experimental groups which received pomegranate peel extract. The cure rate of these groups was approximately 97.4% by 28th day of infection.
Conclusions: Our present findings indicated that the pomegranate peel extract proved to be valuable in prevention and treatment of *Giardia lamblia* infection. Further studies are required to determine the effective dose of pomegranate peel extract against *Giardia lamblia* infection.

1. Introduction

Giardia lamblia (*G. lamblia*) (syn. *Giardia intestinalis*, *Giardia duodenalis*) is a flagellated protozoan that infects the human small intestine, causing a disease called giardiasis. It is one of the common zoonotic infectious parasites widespread all over the world [1]. Globally, it is estimated that approximately 280 million people per year are infected with *Giardia* [2]. It is also an opportunistic parasite and it is the main cause of acute and chronic diarrhea and malnutrition in poor communities, with a prevalence range between 10% and 50% in developing countries [2–5]. Epidemics in developed countries might be attributed to inappropriate water treatment, water contamination with human or animal faeces, particularly from surface water collections and lakes [6]. It is reported that the incidence of this parasite among children in temperate countries has reached 25%, and the proportion rises remarkably in the tropics with up to 50%–80% of the population carriers of the parasite. In Saudi Arabia, incidence rates of 28% make the parasite *G. lamblia* the most prevalent parasite among children [7]. Furthermore, *Giardia* is the most common human intestinal parasite in many developed countries. Giardiasis is transmitted by the ingestion of cysts present in food and water; water dissemination being easier due to cysts resistance to chlorination [8,9]. Direct transmission from person to person is another infection mechanism, through direct faecal-oral contact among members of the same family [10], particularly in collective institutions, such as children in daycare centers and orphanages [11], and by sexual practices (oral-anal contact) [12]. Despite its significance and scope giardiasis is still considered a neglected disease by the World Health Organization. Since it occurs primarily in the poorest

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The experiments were conducted in accordance to Saudi Arabian rules for animal protection and approved by state authorities. Informed written consent was obtained from outpatients.

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regions, giardiasis most likely has a negative impact on child health, pregnancy and worker productivity, imparing the ability of those infected individuals to achieve their full potential, thus affecting their socio-economic development [4,13]. The symptomatology of human giardiasis is extremely variable; many individuals have the asymptomatic form while others have nausea, abdominal pain, chronic or acute diarrhea, which may last several months, thus causing malabsorption and weight loss [14–16]. Several studies have reported genetic variations among *Giardia* samples isolated from human beings. These differences are thought to significantly influence giardiasis epidemiology and control, especially for host susceptibility, drug sensitivity, antigenicity, and *in vivo* and *in vitro* development [17].

Eventhough some advances have been observed in isolating and characterizing *Giardia* samples, few studies have been undertaken regarding this parasite's chemotherapy [18]. Some studies have reported resistance to different drugs which have been used for the treatment of this disease and the number of cases is likely to increase [19–21]. Numerous chemotherapeutic drugs such as furazolidone, quinacrine, paromomycin, benzimidazole compounds, 5-nitroimidazole compounds, nitazoxanide, have been used as a therapy for giardiasis. In Saudi Arabia, metronidazole and nitroimidazole are the common treatment for giardiasis. A short period of treatment is most effective with a cure rate of approximately 90% after a week [22]. However, most drugs used have considerable adversarial side effects and they are contraindicated [23,24]. Furthermore, *Giardia* appears to have a great capability to fight against these drugs [21,24]. Moreover, the Food and Drug Administration US refuses to ratify metronidazole due to the serious side effects as being carcinogenic factors in mice and rats and mutagenic in bacteria [25]. Further problems caused are severe inflammation of the pancreas and brain [26], and it may also lead to embryo abnormalities [22]. Consequently, there is a need for more studies into medicinal plants or herbs for alternative regimens against giardiasis. A recent study by Mahmoud *et al.* [27] confirmed the efficiency of ginger and cinnamon extracts as a promising alternative therapy to the commonly used antigiardial drugs. Pomegranate, *viz.* *Punica granatum* (Punicaceae) (*P. granatum*), described as nature's power fruit, a plant used in folkloric medicine for the treatment of various diseases [28], is widespread in the Mediterranean region. Pomegranate contains very high levels of antioxidant compounds with anti-inflammatory properties [28,29]. In addition, it has antifungal action against *Trichophyton rubrum* [30]. In spite of the many studies which have been conducted to examine the efficacy of pomegranate for treating diseases and microbial infections, to-date still much remains unknown about its effects on parasitic infections. Various studies indicate that pomegranate extract shows positive effects in the suppression of intestinal cestodes and trematodes [31–34] and anti-protozoan activities [35,36]. Recently, some studies have reported that pomegranate peel is a promising treatment for *Cryptosporidium parvum* and it is effective as an anti-coccidial as well as a anthelmintic that does not induce side effects [37,38]. Furthermore, ELISA was recently used as a diagnostic method for detection of different soluble antigen rather than cysts detection [39].

The main aim of this study was to determine the efficacy of pomegranate (*P. granatum*) from Saudi Arabia as an alternative and safe treatment of giardiasis through experimental study on the white laboratory mice. Stool cyst counts ELISA was also used for antigen detection.

2. Materials and methods

2.1. Animals

Fifty male Swiss albino mice (*Mus musculus*), free from any intestinal parasitic infection, aged 3–4 weeks and weighing 25–35 g each, were obtained from the animal facilities of King Saud University, Riyadh, Saudi Arabia. The mice were bred under specified pathogen-free conditions and fed with feed (P 684) of the General Organization for Grain Silos and Flour Mills production in Riyadh, Kingdom of Saudi Arabia. To ensure that mice are free from intestinal parasites, stool samples were examined for three consecutive days before starting the experiment. Smears were directly swabbed after addition of D'Antoni's iodine [40]. The experiments were approved by state authorities and followed Saudi Arabian rules for animal protection.

2.2. Source of the parasite

G. lamblia cysts were obtained from fresh stool samples of outpatients at King Khalid University in Riyadh, Saudi Arabia, where microscopic examination showed that there were cysts per each microscopic field (400×). Informed written consent was obtained from outpatients.

2.3. Infection of mice

Each mouse was orally inoculated with *Giardia* cyst 10^5/mL cyst. Subsequently, fresh faecal pellets were collected every 24 h to be sure they become infected [41]. The collected pellets from each group were weighed, and the bedding was changed to eliminate re-infection.

2.4. Preparation of the pomegranate peel extract

P. granatum peels were obtained from fruit purchased from a local market. According to the method described by Abdel Moneim [28], pomegranate peel extract was prepared with slight modifications. Air-dried powder (100 g) of pomegranate peels was extracted by percolation with 70% methanol at room temperature and then kept at 4 °C for 24 h. Thereafter, the extract was concentrated under reduced pressure (bath temperature 50 °C) and dried in a vacuum evaporator. The residue was dissolved in distilled water and used in this experiment [38].

2.5. Experimental design

Animals were divided into five groups, ten animals in each group. Three groups were assigned as the control groups. Group 1 was infected with 10^5/mL of *Giardia* infestation cysts. Group 2 was fed daily for 7 days before infection and throughout the experimental period by oral gavage with citrus pectin (Sigma) which consists of a complex set of polysaccharides. Group 3 was fed daily starting from 7th day of infection and throughout the experimental period by oral gavage with citrus pectin until the

end of the experiment. The other two groups were assigned as experimental groups. Group 4 was fed daily for 7 days before infection and throughout the experimental period by oral gavage with 100 μL pomegranate peel extract (300 mg/kg). Group 5 was fed daily starting from 7th day of infection. The dose and the route of injection were selected on the basis of the previous studies [37].

2.6. Parasitological studies

2.6.1. Determination of the infection

The stool samples were microscopically examined on daily basis starting from the first day of the infection up to the time-point when the *Giardia* cysts were detected and the mice were infected.

2.6.2. Determination of the infection intensity

After confirmation of the infection, the intensity of infection on Days 10, 15 and 20 was determined as described by Roberts-Thomson *et al.* [42]. Detection of *Giardia* parasite antigen in the animals stool in the Days 5, 7, 14, 21 and 28 after infection was performed by ELISA using the monoclonal antibodies (ProSpect *Giardia* Microplate Assay, Remel).

2.7. Statistical analysis

The data were statistically processed using One-way ANOVA, and the statistical comparisons among the groups were performed with Duncan's multiple range test using a Microsoft Excel 2010. A probability level of $P < 0.05$ was chosen to indicate significant differences among the means. Means connected with stars are considered significantly different from each other.

3. Results

In the present investigation, the effect of pomegranate peel extract on *G. lamblia* infections was investigated as an anti-*Giardia* material. It was observed that 10 days following infection by *G. lamblia*, the production of cysts differed between pomegranate-treated and un-treated mice. In the former, the rates of excreted cysts reached 5.70 ± 0.67 and 7.10 ± 0.73 in those treated 7 days prior to infection and those starting treatment 7 days post-infection, respectively. Cysts excretion was reduced by about 50% on Day 10 in the experimental group compared with the control group. Furthermore, by Day 20 post-infection, the pomegranate treatment significantly lowered the shedding of cysts to approximately 75.6%, as shown in Table 1. However, the rates of cysts production were significantly different on Days 10, 15 and 20 in control groups. On the other hand, the differences among the experimental groups were significant regarding the number of cyst detected on Days 10, 15 and 20.

Table 1
Rate of detection *Giardia* cysts in the stool among the different studies groups.

Groups		Numbers of cysts in stool		
		Day 10	Day 15	Day 20
Control groups	1	$11.80 \pm 2.15^{*}$	$10.60 \pm 1.17^{*}$	$8.60 \pm 0.69^{*}$
	2	$11.10 \pm 1.66^{b*}$	$9.60 \pm 0.84^{ab*}$	$8.40 \pm 0.96^{a*}$
	3	$11.60 \pm 1.95^{b*}$	$9.70 \pm 0.94^{ab*}$	$8.50 \pm 0.70^{a*}$
Experimental groups	4	$5.70 \pm 0.67^{b**}$	$2.80 \pm 0.63^{a*}$	$1.10 \pm 0.73^{a*}$
	Reduction %	51.69%	73.85%	87.20%
	5	$7.10 \pm 0.73^{b**}$	$3.80 \pm 0.78^{a*}$	$2.10 \pm 0.99^{a*}$
	Reduction %	39.80%	64.10%	75.60%

Data are expressed as mean ± SD. Different letters indicate significant differences in the Tukey test. *P*-value represents the relationships among groups in one row. *: $P \leq 0.05$; **: $P \leq 0.001$.

As shown in Table 2, immunological investigations indicated that the detection rates of *Giardia* antigen in the stool by ELISA test displayed gradual reduction in *Giardia* antigen in the stool of the experimental groups which received pomegranate extract compared with control groups. However, the *Giardia* antigen reduction rate in the experimental groups which were fed with pomegranate extract from 7th days of infection reached 35.1% and 96.8% on Days 5 and 28, respectively. Furthermore, *Giardia* antigen in the experimental groups which were fed with pomegranate extract 7th days after infection didn't show reduction on Day 5, but on Day 28 the highest *Giardia* antigen reduction rate was observed. In contrast, control groups didn't show any improvement. Generally, the dose used in the experimental groups induced noticeable reduction in the number of cysts. Furthermore, using pomegranate peel extract before infection showed higher ability to reduce the infection rate and the number of cyst produced compared with using the extract after infection. This observation was also recorded for *Giardia* antigen detection in the stool till Day 14.

Table 2
Rate of detection *Giardia* antigen in the stool among the different studies groups.

Groups		*Giardia* antigen in stool				
		Day 5	Day 7	Day 14	Day 21	Day 28
Control groups	1	$0.74 \pm 0.09^{**}$	$8.15 \pm 0.68^{**}$	$20.05 \pm 0.92^{**}$	$18.40 \pm 1.03^{**}$	$17.90 \pm 0.83^{**}$
	2	$0.66 \pm 0.08^{**}$	$8.19 \pm 1.12^{**}$	$20.15 \pm 1.32^{**}$	$18.34 \pm 1.54^{**}$	$16.79 \pm 0.77^{**}$
	3	$0.71 \pm 0.07^{**}$	$8.19 \pm 0.76^{**}$	$19.91 \pm 0.93^{**}$	$18.46 \pm 0.84^{**}$	$17.92 \pm 0.81^{**}$
Experimental groups	4	$0.48 \pm 0.12^{**}$	$4.08 \pm 1.06^{**}$	$3.19 \pm 0.99^{**}$	$1.07 \pm 0.39^{**}$	$0.56 \pm 0.24^{**}$
	Reduction %	35.1%	49.9%	84.1%	94.0%	96.8%
	5	$0.77 \pm 0.09^{**}$	$7.71 \pm 1.13^{**}$	$3.67 \pm 1.20^{**}$	$0.51 \pm 0.11^{*}$	$0.45 \pm 0.09^{**}$
	Reduction %	0.0%	5.3%	81.6%	97.2%	97.4%

Data are expressed as mean ± SD. *P*-value represents the relationships among groups in one row. *: $P \leq 0.05$; **: $P \leq 0.001$.

4. Discussion

Giardia infects both adults and children in developed countries worldwide and is currently responsible for the largest number of water-borne outbreaks of diarrhea [43,44]. Metronidazole drug for the treatment of intestinal infections caused by *G. lamblia* is reported to have unpleasant side effects [25,35]. Development of parasite resistance to treatment besides the negative side effects of conventional drugs might be the main reasons that lead experts to look for safe and effective alternative agents for the *Giardia* treatment. Globally, herbal curatives have been studied under controls and was technologically approved by researchers in many countries [45]. An additional constraint in using anti-*Giardia* medications comes from the consumer and the ever-increasing demand for the drug-free production of foods [46] as they are not only natural products, but may also include new therapeutic molecules to which resistance has not yet developed [38]. Pomegranate peel extract which contains biologically active components may be active against diarrhea [47]. In the present investigation, pomegranate peel extract was examined as an alternative and effective medication for treatment and protection against *G. lamblia*. The extraction procedure is an important factor that affects the efficacy of the plant extract as a treatment against infections. The type of solvent used for the extraction process is also of critical importance and considered a key factor that affects the positive determination of biologically active compounds from plant origin. The most commonly used solvents for investigation of antimicrobial activity from plant materials are organic solvents (methanol and ethanol) and water [48]. In this investigation, the methanol extract of pomegranate peel was tested for anti-*Giardia* activity and it demonstrated positive results. Experimental groups treated with extract of pomegranate peel in this study showed cysts reduction on the 10th day after infection. Moreover, curing rate was highly significant in the experimental group which reached 73.85% on Day 15 post-infection, indicating the effectiveness of pomegranate peel extract as an anti-*Giardia* therapy. These results were in agreement with El-Shennawy *et al.* [49] who noted 72.15% reduction of *Giardia* in a group receiving pomegranate peel extract. Furthermore, antigen detection was significantly reduced in the *P. granatum*-treated mice by the 14th day post infection and was mostly eliminated by the 28th day post infection. These results were supported by Al-Mathal and Alsalem [37]. Many other studies have reported pomegranate peel as an effective anti-protozoan treatment [35,36]. Furthermore, significant improvement in the antioxidant status and protection of the host tissue from injuries induced by pomegranate extract has been well documented by Dkhil [38]. This might be the main reason for the reduction rate of cysts in treated mice with pomegranate peel extract. In addition, El-Shennawy *et al.* [49] recorded a remarkable decrease manifested by high percentage of dead adult worms of *Schistosoma* (72.2%) treated with pomegranate peel extract. Moreover, several studies indicated that peel extracts of *P. granatum* could represent promising bioactive natural agents such as anti-parasites that deserve further investigations [37,45,50]. Mansour *et al.* [51] found higher concentration of phenolic compounds such as gallic acid, ellagic acid, caffeic acid, *p*-coumaric acid, quercetin, and vanillic acid in methanolic pomegranate peels extract than water extract. Concentration of these compounds could be the main reason for the potential giardicidal activity of methanolic pomegranate peel extract investigated in this study.

In conclusion, the results of the present study reinforced the effectiveness of peel extracts of (*P. granatum*) as a safe treatment against giardiasis through experimental study on the white laboratory mice. Peel extracts have proven to be valuable agents in prevention and treatment of *G. lamblia* infection, and will form basis for further investigation of pomegranate efficacy against other parasites. Moreover, it indicates potential discovery of new natural biologically active compounds and the accumulation of traditional knowledge about plants used for the treatment of giardiasis. Further investigations are needed to identify the active ingredients in pomegranate peel and to perform toxicity test to identify safe levels for use.

Conflict of interest statement

We declare that we have no conflict of interest.

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