

The Efficacy of Pomegranate (*Punica granatum*) Peel Extract on Experimentally Infected Rats with *Blastocystis* Spp

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Abstract

Here, the study was conducted to evaluate the effect of pomegranate (*Punica granatum*) peel extract on infected rats with *Blastocystis* spp. Anti-protozoan activities were determined by monitoring *Blastocystis* spp. shedding in stools and histopathological changes of intestine of infected rats. Additionally, we evaluated the antioxidant properties of pomegranate peel extract on different groups through measuring the concentration of Malondialdehyde (MDA).

In this work, *Punica granatum* peel extract-treatment lowered the shedding of cysts very close to nitazoxanide (NTZ) treatment. These data were statistically significant P value ≤ 0.0001 . Pomegranate peel extract was found to have the highest anti-lipid peroxidation effect, assessed by measuring MDA level. The inhibitory effect of pomegranate peel extract on lipid peroxidation was significant when compared to NTZ- treated group (P value ≤ 0.0003). As well, histopathological examination of the intestine showing that *Blastocystis* spp. were often observed in the infected group without treatment either within the luminal material or at the tip of the epithelium compared to the infected treated groups.

Pomegranate peel extract can be used as alternative therapy for blastocystosis and for developing novel anti *Blastocystis* drugs. Additionally, these results show clinical evidence that pomegranate peel extract has components act as powerful antioxidants.

Keywords: *Blastocystis* spp; Rats; Pomegranate (*Punica granatum*); MDA

Introduction

Blastocystis spp. is emerging protozoan parasites that inhabit the intestinal tract of humans and many animals with worldwide distribution [1]. Although the pathogenicity of this parasite is controversial [2], antiprotozoan agents are usually administered to the infected individuals [3]. The first choice of chemotherapeutic agent was metronidazole (MTZ) [4]. MTZ was found to be effective against *Blastocystis* spp. in some studies but not in others [5]. Thus, the susceptibility of *Blastocystis* spp. to standard antimicrobials is not clear [3].

Additionally, Rossignol et al. [6] suggested that *Blastocystis* spp. can be treated effectively with nitazoxanide (NTZ), and this therapy was equally effective in both children and adults. Currently antimicrobial resistance among bacteria, viruses, parasites, and other pathological organisms is a serious threat to the infectious disease management worldwide [7]. Considering the side effects of and resistance to many antibacterial drugs, the plant extracts used in traditional medicine are now used as sources for new treatments [8].

The development of drug resistance against commonly used treatment has necessitated a search for new therapeutic agents from other sources. Recently, there has been considerable interest in the use of plant materials as an alternative method to control pathogenic

microorganisms [9,10]. Plant extracts have shown to be effective against resistant pathogens [11].

Herbal medicine or phytomedicine is now attracting the world's attention as it enhances the health of the body systems without adverse side effects, especially the immune system that protect against pathogens [12].

Punica granatum L, commonly known as pomegranate, is a fruit-bearing deciduous shrub or small tree, native to Asia and belongs to the family Punicaceae [13]. Different parts of the plant such as bark, leaves, immature fruits and fruit rind have medicinal significance [14]. *Punica granatum* is widely employed in various countries as a source of therapeutic agent against a variety of pathogenic microbes [15]. It was utilized as a traditional remedy for thousands of years under the Ayurvedic system of medicine, with extracts from the rind of the fruit and bark of the tree being effective against diarrhea and dysentery [16].

Punica granatum L. is an ancient mystical fruit used in folkloric medicine as a treatment for many diseases such as diarrhea, parasitic worm infections, urinary tract infections, and kidney stones [17,18]. Moreover, many studies indicate that *P. granatum* can slow bacterial growth and inhibit bacterium-induced toxins [19-22]. Furthermore, *P. granatum* peel extract (100 mg/kg) for 10 consecutive days had stimulated immune systems and enhanced cellular immunity in rabbits [23]. Several additional studies have demonstrated the therapeutic effects of *P. granatum* fruit, peel, and juice as powerful antioxidants and anti-inflammatory substances that include polyphenols and tannins [24-31]. *P. granatum* also plays a role in protecting against

cancer diseases [32] and its juice is effective in protecting neuron cells from Alzheimer's disease [33]. The constituents of pomegranate are including high hydrolyzable tannins (punicalins and punicalagins), ellagic acid, a component of ellagitannins, and gallic acid, a component of gallotannins [34]. *P. granatum* has anti-cestodial, anti-nematoidal [35-37], and anti-protozoan activities [8,38,39]. As well, *Punica granatum* peel extract is effective in a murine model of experimental *Cryptosporidium parvum* [40].

Therefore, this study examined the efficacy of aqueous extract of *P. granatum* peel on treatment of *Blastocystis* spp. infection in rats.

Materials and Methods

The study was carried out in the period from January 2016 to May 2016 in the Parasitology Department, Faculty of Medicine, Minia University, Minia, Egypt.

Source of *Blastocystis* isolates

Strains of *Blastocystis* are maintained by repeated subculture with Locke egg (LE) serum medium. The organism could be maintained for more than 3 months [41] in the Parasitology Department, Faculty of Medicine, Minia University Minia, Egypt.

P. granatum materials

Fresh pomegranates (500 gram) were obtained (in order to prepare fresh extraction) from a public market. The peels of pomegranate were separated and oven dried at 33°C for 7 days. The dried peels were powdered in an electric grinder and stored in plastic bags and stored at 4 °C for the next step [42,43].

P. granatum treatments

Therapeutic doses of *P. granatum* were administered to the animals on day 4 post-inoculation (pi), the day cysts appeared in the feces. *P. granatum* doses of 3 g/kg body weight were prepared fresh (3 g/ml *P. granatum* peel in distilled water).

Animals

Six-week-old male-albino rats weighing 80 gm each were obtained from the experimental house, Faculty of Medicine, Minia University. The animals had free access to standard rodent chow and water. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Minia University, Minia, Egypt.

Experimental design

Rats divided into four groups (G1-G4). Each group has six rats: G1: healthy controls, G2: infected/untreated controls G3: infected/ *P. granatum*-treated and G4: infected/ nitazoxanide (NTZ)-treated, from (G2-G4) groups were infected intragastrically with 500 µl LE medium containing 2×10^6 *Blastocystis*. On the fourth day post infection, G3-G4 groups were treated with 500 µl of *P. granatum* doses of 3 g/kg body weight and 500 mg of NTZ [44] respectively administered daily by gastric tubes 1 h before meals for 3 consecutive days [36].

Evaluation of *Blastocystis* spp. Infection in Rats

Detection of cysts shedding in feces

Feces from all rats were examined microscopically at different periods (2,4,6,8, and 10 days) post infection. Quantitative estimation of the infection intensity in the stool samples of *Blastocystis* spp. infected rats was performed according to the method described by [45]. Cysts of *Blastocystis* spp. were counted in at least three fields with estimation of the average number/high power field (HPF) [45].

Histopathological Examination

On the 10th days post infection, all rats from each group were sacrificed. Tissue samples from walls of small intestine, caecum and colon of scarifying animals were collected then fixed in 10% neutral buffered formalin. The organs were routinely processed and sectioned at 4 to 5 µm thickness. The obtained tissue sections were collected on glass slides, deparafinized and stained with Hematoxylin and Eosin stain. The sections were then examined and observed under light microscope at 10, 40 and 100X magnifications [46,47].

Biochemical Determinations

To assess antioxidant property of *P. granatum* extract, pieces of small intestine were aseptically removed, homogenized, as described by El-Shenawy et al. [48]. Intestinal lipid peroxidation was determined as thiobarbituric acid reacting substance and is expressed as equivalents of malondialdehyde (MDA), using 1,1,3,3-tetramethoxy propane as standard [49].

Statistical Analysis

Data were presented as means ± standard deviation (SD) using Statistical SPSS for Windows, issue 15.8. Statistical significance was determined using t-tests (Man Whitney), Chi-square tests, and one-way analysis of variance. P value less than 0.05 was considered significant.

Results

Shedding of cysts in stool

At day 4 post infection (p.t.) rats in the G2, G3 and G4 shed variable cysts in feces. The shedding of cysts varied in the range between 10-11/HPF (Table 1). On the 6th day p.t., the cyst shedding output differed between pomegranate peel and NTZ-treated groups and non-treated group (Table 1). Pomegranate peel and NTZ-treated groups significantly lowered the shedding of cysts. These data were statistically significant (Table 1).

Biochemical determinations

Rats infected with *Blastocystis* exhibited increased levels of MDA production (118.85 ± 0.9 vs. 50.93 ± 0.54 , $P \leq 0.000005$) compared to that of uninfected controls. Treatment of infected rats with *Punica granatum*, and NTZ reduced MDA levels 30.07 ± 5.9 and 61.01 ± 1.1 , respectively, as shown in (Table 2).

Rat group/parasite load Days (post infection)

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
Parasite load in the stool of rats of different studied groups (Mean ± SD)						
Healthy group	0	0	0	0	0	0
Infected untreated	0	0	11.4 ± 0.9	**15.25 ± 0.9	***18.25±0.9	***23.6±1.4
Infected (+ <i>P. granatum</i>)	0	0	11.6±0.5	7.2±0.8	3.4±0.5	1.4±0.5
Infected (+ NTZ)	0	0	10.5±0.5	6.2±0.8	2.4±0.5	0.9±0.4

*: P value: ≤ 0.05, **: P value: ≤ 0.001; ***: P value: ≤ 0.0001.

Table 1: Effect of *P. granatum* peel on the cyst's shedding intensity in the stool of rats of the various studied groups. **: P value between infected untreated rats and infected (+*P. granatum*) and /or infected (+ NTZ) rats.

MDA (NM/g)	Non-infected	Infected	Infected (+ <i>P. granatum</i>)	Infected (+ NTZ)
	50.93±0.54	118.85±0.9	30.07±5.9	61.01±1.1
p. value			0.0003**	0.03**

**: P value between infected untreated rats and infected (+*P. granatum*)and /or infected (+ NTZ) rats; *: P value between non- infected and infected rats.

Table 2: Effect of *P. granatum* peel on the level of malondialdehyde, in the intestine of rats infected with *Blastocystis* spp.

Histopathology

In intestinal H&E histological sections, *Blastocystis* was frequently observed within the luminal material (Figures 1A and 2A) in the infected untreated group. On the other hand, very few organisms were observed within the luminal material of the infected treated groups (Figures 1B and 2B). Associated pathology was in the form of some epithelial damage and mucosal sloughing in the infected untreated group (Figure 1A) compared to the infected treated groups (Figure 1B). Furthermore, the infected untreated group showed also exhibited much goblet cell hyperplasia (Figure 3A) compared to that of the infected treated ones (Figure 3B).

Discussion

In the current study a rat model of *Blastocystosis* was used to determine the efficacy of aqueous *P. granatum* peel extract as a treatment for *Blastocystis* spp infections. Cyst shedding, and histological changes are useful for determining the pathology of *Blastocystis*spp. infections [50]. Therefore, we examined these parameters during the course of *Blastocystis*spp infections with and without *P. granatum* treatment.

In the recent years, the use of plants with preventive and therapeutic effects contributes to health care needs [51]. There are three main reasons for interest in the treating and healing power of plant extracts. First, pharmacological studies have demonstrated that many of plants are known to possess antimicrobial agents; second, people are becoming aware of the side effects associated with the over prescription of traditional antibiotics; third, time to time resistant microorganisms against antibiotics are increasing. Among these plants, *P. granatum* has an important role in folk medicine. Pomegranate is known as a rich source of pharmacological properties which have been evaluated due to antiparasitic, antibacterial, antifungal, antiproliferative, apoptotic and anti-cancer effects as well as protection

against herpes virus, inhibition of LDL oxidation and decrease in plaque formation and reduction of systolic blood pressure [27,34,43].

Blastocystis-infected rats that were treated with *P. granatum* peel in this study showed a complete elimination in fecal cyst shedding by day 10 pi. The reduction and elimination of fecal cyst shedding in response to *P. granatum* treatment seen here may be attributable to the presence of metabolic toxins which have a direct effect on parasite growth in the intestines, the production of the sexual phases, and/or the formation of cysts.

We previously mention that the constituents of pomegranate are including tannins (punicalins and punicalagins), ellagic acid, and gallic acid [34]. It was reported that ellagic acid has anti-microbial activity and punicalagin has anti-food-borne pathogens [52,53].

Furthermore, *P. granatum* peel contains major phenolic compounds, such as organic acids [22,31],that can directly inhibit *Blastocystis*infections. Organic acids have an attenuating effect on the growth of enteropathogenic microbes [54,55]. Specifically, organic acids have inhibitive effects on *Blastocystis* infections [56] and can reduce parasite vitality [57]. Additionally, the hydroxyl group of the phenolic compounds in *P. granatum* can increase toxicity against all organisms [22,58].

Moreover, *P. granatum* decreased MDA production significantly as Sing et al. [59] suggested that Pomegranate ability to suppress hydroxyl radicals is directly related to the prevention of propagation lipid peroxidation thus reducing the rate of chain reaction and the peel extract has the higher antioxidant activity than seeds extract. In addition, it has been reported that, several *P. granatum* metabolites includes polyphenols and tannins which acts as an antioxidant by scavenging reactive oxygen species (ROS), preventing lipid oxidation and production of pro-inflammatory messengers. [24-31]. Also, Sing et al. [59] reported that the Pomegranate ability to suppress hydroxyl radicals seems to be directly related to the prevention of propagation lipid peroxidation thus reducing the rate of chain reaction.

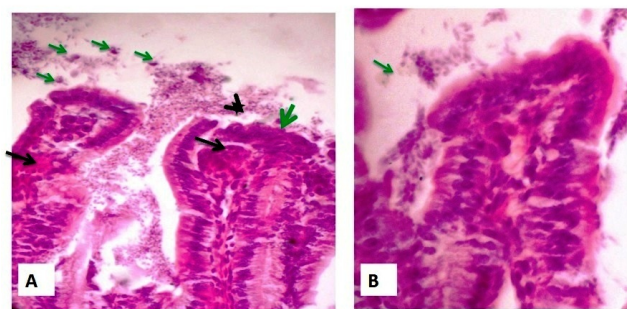


Figure 1 :(A) Photomicrograph of distal ileal section of infected untreated rats shows some preserved villous pattern. The core is infiltrated with few chronic inflammatory cells. The surface is partially ulcerated (40X). (black arrow head). There are areas of edema and congestion of blood vessels (black arrow). The lumen is filled with necrotic debris admixed with mucin and several forms of *Blastocystis*, vacuolar and granular (green arrows) (40X). (B) Photomicrograph of distal ileal section of infected treated rats either with *Punica granatum*, and/or NTZ show intact mucosa of ileocecal area (40X). Very few chronic inflammatory cells are presented in the core. There are very few forms of *Blastocystis* (green arrows) in the lumen.

Based on the observations in the study, it is possible that *P. granatum* is able to activate or desuppress the anti-parasite associated gene(s) in mammals. At the molecular level, *P. granatum* may reduce the level of DNA methylation at promoters of anti-parasite gene(s) and facilitate their expression. As DNA methyltransferases (DNMTs) and some histone methyltransferase (HMTs) have been shown to play a role in the maintenance of DNA methylation globally and loci specifically [60-62] *P. granatum* may cause the promoter hypomethylation via affect the function of DNMTs or HMTs. Therefore, to fully understand how does *P. granatum* eliminate *Blastocystis* spp, it is interesting to investigate whether *P. granatum* plays a role in the regulation of epigenetic modifiers in mammals.

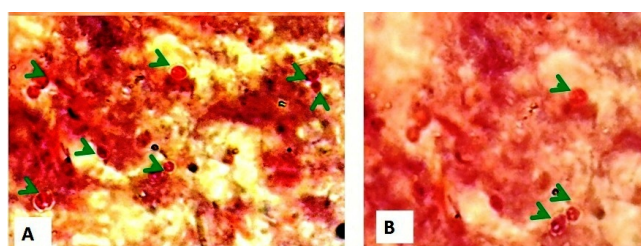


Figure 2: (A) Photomicrograph demonstrates *Blastocystis* in intestinal lumen of infected untreated rats shows several forms of *Blastocystis*, vacuolar and granular (100X) (green arrows head). (B) Photomicrograph of intestinal lumen of infected treated rats either with *Punica granatum*, and/ or NTZ shows very few parasites (100X) (green arrows head).

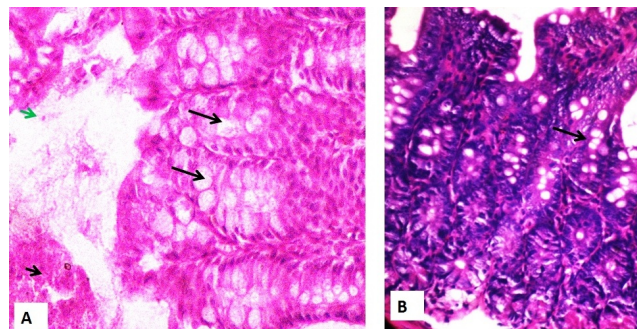


Figure 3: (A) Photomicrograph of cecal section of infected untreated rats shows severe infiltration of the mucosa with chronic inflammatory cells. The mucosal glands are partially destroyed and shows goblet cell hyperplasia (40X) (black arrow). The surface is partially ulcerated. The lumen is filled with necrotic debris mixed with mucin and the organism (green arrows). (B) Photomicrograph of cecal section of infected treated rats shows complete regeneration of the mucosal glands with massive reduction of the inflammatory cell infiltrate. Moderate amount of goblet cells are detected (black arrow). The core is infiltrated with few chronic inflammatory cells. The surface shows no ulceration. The lumen is clear (40X).

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