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Pomegranate (*Punica granatum*) peel is effective in a murine model of experimental *Cryptosporidium parvum*

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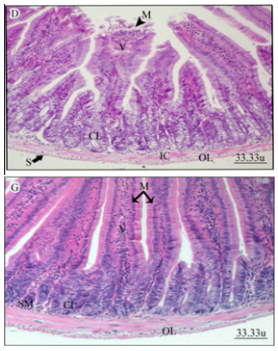
HIGHLIGHTS

- *Cryptosporidium parvum*-infected mice showed weight loss, intestinal morbidity, and mortality.
- Mice treated with *Punica granatum* rapidly recovered from cryptosporidiosis.
- *P. granatum* treatments resulted in no adverse side effects.
- *P. granatum* is a promising efficient and safe treatment for cryptosporidiosis.

GRAPHICAL ABSTRACT

Illium from *Cryptosporidium parvum*-infected mice (D) and Illium from *Punica granatum*-treated, *C. parvum*-infected mice (G). The villi from untreated/infected mice were asymmetrical and showed signs of atrophy and hyperplasia. The villi from infected/*P. granatum*-treated mice showed improved architectural symmetry and decreased atrophy and hyperplasia.



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ABSTRACT

Cryptosporidiosis, a major health issue for neonatal calves, is caused by the parasite *Cryptosporidium parvum*, which is highly resistant to drug treatments. To date, many anti-parasitic drugs have been tested, but only a few have been shown to be partially effective in treating cryptosporidiosis. Previous studies have indicated that pomegranate (*Punica granatum*) possesses anti-plasmodium, anti-cestode, and anti-nematode activities. Therefore, the aim of this study was to evaluate the effect of *P. granatum* peel on suckling mice infected with experimental *C. parvum*. At 4 days of age, 72 neonatal albino mice were randomly divided into five groups: G1: healthy controls, G2: infected/untreated controls, G3: uninfected/distilled water-treated, G4: uninfected/*P. granatum* peel-treated, and G5: infected/*P. granatum* peel-treated. Mice were experimentally-infected by oral administration of 1×10^3 *C. parvum* oocysts per animal. On day 7 post-inoculation (pi), treated mice received an aqueous suspension of *P. granatum* peel orally (3 g/kg body weight). The presence of diarrhea, oocyst shedding, and weight gain/loss, and the histopathology of ileal sections were examined. Infected mice treated with the *P. granatum* peel suspension showed improvement in all parameters examined. Additionally, these mice did not exhibit any clinical symptoms and no deaths occurred. Oocyst shedding was very significantly reduced in the *P. granatum*-treated mice by day 14 pi ($P < .05$), and was completely eliminated by day 28 pi. The mean weight gain of the *P. granatum*-treated mice was significantly higher than that of the infected/untreated controls throughout the study ($P < .01$). Histopathological analysis of ileal sections further supported the clinical and parasitological findings. The histological architecture of villi from the *P. granatum*-treated mice on

Abbreviations: pi, post-innoculation; opg, oocyst per 0.01 gram feces.

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day 14 pi showed visible improvement in comparison with the infected/untreated controls, including renewed brush borders, reduced numbers of *C. parvum* trophozoites, and reduced lymphatic infiltration. On day 28 pi, tissues of the *P. granatum*-treated mice were very similar to those of healthy control mice. These results suggest that *P. granatum* peel is a promising anti-coccidial therapeutic treatment that lacks negative side effects.

1. Introduction

Cryptosporidiosis is a common disease among neonatal calves caused by the protozoan parasite *Cryptosporidium parvum*. Infection by this parasite is one of the most serious health risks for young calves, especially those less than 4 weeks of age. Cryptosporidiosis causes diarrhea and weight loss, and can result in death (Klein, 2008). *C. parvum* is highly-resistant to current drug treatments (Armson et al., 2003). Though the pharmaceutical industry has made several attempts to develop an effective treatment for cryptosporidiosis, this disease still constitutes a major health problem for the livestock industry, calling for the development of a safe and effective treatment (Del Coco et al., 2009).

The United States Food and Drug Administration has approved nitazoxanide, a broad-spectrum anti-parasitic agent that is an effective in vitro drug against cestodes, trematodes, nematodes, and protozoans (Rossignol and Maisonneuve, 1984) in humans. However, nitazoxanide does not show any curative qualities against *C. parvum* infection in calves (Schnyder et al., 2009). Paromomycin is a conventional treatment for cryptosporidiosis (Kayser et al., 2002). However, upon suspension of treatment, fecal oocysts and diarrhea reappeared in calves in field trial (Grinberg et al., 2002). Ingestion of the drug alone may be ineffective, especially against gall bladder and pancreas infections (Fayer, 1997). Halofuginone lactate is an anticoccidial drug that used in the treatment of cryptosporidiosis (Naciri et al., 1993). It seems to reduce the oocyst shedding and decreases the severity of cryptosporidiosis in calves but provides no complete cure (De Waele et al., 2010; Silverlas et al., 2009). Consequently, the available drugs are considered ineffective for the treatment of cryptosporidiosis in calves (Guitard et al., 2006; Massoud et al., 2008; Smith and Corcoran, 2004; Waters et al., 2000; Silverlas et al., 2009). The resistance of *C. parvum* to many antimicrobial drugs may be because it is an intracellular, rather than an extra-cytoplasmic, parasite. Considering the side effects of and resistance to many antibacterial drugs, attention has moved towards plant extracts used in traditional medicine as sources for new treatments (Calzada et al., 2006).

Punica granatum L. (Punicaceae), commonly known as pomegranate, 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 (Navarro et al., 1996; Sudheesh and Vijayalakshmi, 2005). Studies indicate that *P. granatum* can slow bacterial growth and inhibit bacterium-induced toxins (Bialonska et al., 2009; Braga et al., 2005; Choi et al., 2011; Ghosh et al., 2008). Rabbits that received oral doses of aqueous *P. granatum* peel (100 mg/kg) for 10 consecutive days had stimulated immune systems and enhanced cellular immunity (Gracious et al., 2001). 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 (Afaq et al., 2005; Aviram et al., 2000; Aviram et al., 2002; Aviram and Dornfeld, 2001; Cerda et al., 2003; Gasemian et al.,2006; Kim et al., 2002; Suzuki et al., 2004). *P. granatum* also plays a role in protecting against cancer diseases (Syed et al., 2007) and its juice is effective in protecting neuron cells from Alzheimer's disease (Wang et al., 2009).

Despite the many studies conducted to examine the efficacy of *P. granatum* in treating many diseases and microbial infections, much remains unknown about its effects on parasitic infections. However, some studies have indicated that *P. granatum* has anti-cestodial, anti-nematoidal (Akhtar and Riffat, 1985; Fernandes et al., 2004; Korayem et al., 1993; Pradhan et al., 1992; Prakash et al., 1980), and anti-protozoan activities (Calzada et al., 2006; Dell'Agli et al., 2009; El-Sherbini et al., 2009). Additionally, *P. granatum* has been used in traditional medicine to treat diarrhea and dysentery diseases. Therefore, the present study examined the efficacy of aqueous *P. granatum* peel as a treatment for *C. parvum* infections in an experimental murine model of cryptosporidiosis.

2. Materials and methods

2.1. Preparation of oocysts

C. parvum oocysts were collected from naturally-infected calves. Oocysts were concentrated according to Heelan and Ingersoll (2002) and identified by modified Zeihl Neelson (Henriksen and Pohlenz, 1981) and enzyme-linked immunosorbent assays (ELISAs) (*Cryptosporidium* bovine ELISA kit; Cypress Diagnostics, Langdrop, Belgium) as instructed by the manufactures. The identify of purified oocysts was confirmed as *C. parvum* by standard polymerase chain reaction (PCR) for polythreonine gene using *C. parvum* specific primers (cry 44: CTCTTAATCCAATCATTACAAC and cry 39: GAGTETAA TAATAA ACC ACTG) according to Wu et al. (2000) (data not shown).

Sedimented oocysts were collected and stored in a 2.5% potassium dichromate solution at 4 °C. Prior to experimentation, oocysts were concentrated (Heelan and Ingersoll, 2002) and counted in a PBS solution using a hemocytometer.

2.2. Plant materials

P. granatum peels were obtained from fruit purchased from a local market. Samples were authenticated by the Botany Department of the University of Dammam. Peels were cold-dried under ambient conditions, pulverized, and stored at 4 °C.

2.3. Animals

Pregnant, white albino mice (Laurent et al., 1999; Sherwood et al., 1982), no more than 3 months old, were obtained from the animal home of the Arabian Gulf University. Mice were tested for infection over 10 consecutive days, and each litter with the mother was housed in separate cages under hygienic conditions. The mothers remained with their nurslings for the nurslings to feed as needed throughout the course of the experiment. Animal fodder (General Organization of Grain Silos and Flour Mills, Saudi Arabia, Dammam) and water were supplied Ad libitum. Temperature and humidity were maintained at 20–21 °C and 30–40%, respectively. All animal protocols were performed in accordance with the protocols of the Faculty of Medicine, King Faisal University.