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# An in vivo appraisal of Punica granatum peel extract's ultrastructural effect on cystic echinococcosis in mice

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## Abstract

In the past decade, interest has significantly increased regarding the medicinal and nutritional benefits of pomegranate (Punica granatum) peel. This study examined the effects of using pomegranate peel extract (PGE) alone and in combination with albendazole (ABZ) on ultrastructural and immunological changes in cystic echinococcosis in laboratory-infected mice. Results revealed that the smallest hydatid cyst size and weight (0.48 ± 0.47mm, 0.17 ± 0.18 gm) with the highest drug efficacy (56.2%) was detected in the PGE + ABZ group, which also exhibited marked histopathological improvement. Ultrastructural changes recorded by transmission electron microscopy including fragmentation of the nucleus, glycogen depletion, and multiple lysosomes in vacuolated cytoplasm were more often observed in PGE + ABZ group. IFN-γ levels were significantly increased in the group treated with ABZ, with a notable reduction following PGE treatment, whether administered alone or in combination with ABZ. Thus, PGE enhanced the therapeutic efficiency of ABZ, with improvement in histopathological and ultrastructural changes.

## Introduction

Cystic echinococcosis (CE) is a widely distributed parasitic disease resulting from the hydatid cyst caused by *Echinococcus granulosus*. It is also known as hydatid disease or hydatidosis and is classified as a zoonotic illness (Kern *et al.* 2017). Echinococcosis has been considered among the 20 neglected tropical diseases targeted by the WHO for disease eradication or control (Abdelbaset *et al.* 2021; WHO 2022).

More than one million people contract echinococcosis globally each year. The cost of treating patients with echinococcosis and damage to the livestock industry is about three billion dollars (Mahmoudi *et al.* 2019). Humans become infected by ingestion of ova accidentally through direct contact with dogs or indirect contact with contaminated food, soil, or water (Jelowdar *et al.* 2017). Although various organs of CE patients may be invaded by the oncosphere, approximately 70% of patients are affected in the liver and approximately 20% are affected in the lungs (Wen *et al.* 2019). The location, size, and number of cysts, affected organs, oxidative stress, and the immune responses of infected individuals play important roles in the pathogenesis and clinical signs of echinococcosis (Heidarpour *et al.* 2012).

Although surgery is the primary treatment method, leakage of cystic fluid is the primary cause of infection recurrence. Therefore, it is mandatory to kill the protoscoleces within the cyst during the surgical procedure (Shi *et al.* 2016). Benzimidazoles such as albendazole (ABZ) are common medications to treat human infections, but such drugs are known to have parasitostatic rather than parasiticidal effects. Moreover, the low water solubility and low absorption of benzimidazoles may be responsible for ineffective treatment (Siles-Lucas *et al.* 2018). Therefore, researchers have made several attempts to increase these compounds' solubility, absorption, and bioavailability by finding new effective compounds for the treatment of cystic echinococcosis. Plant-based anthelmintics have been used for the treatment of many parasitic diseases, including echinococcosis (Atayi *et al.* 2018). Pomegranate (*Punica granatum*), a well-known edible fruit, is a member of the family Punicaceae and is widely known for the tremendous medicinal properties conferred by its seeds, juice, and peels (Singh *et al.* 2018). Various phytochemicals, such as alkaloids, tannins, and volatile oils have been found in different parts of pomegranate, which exhibit a wide spectrum of bioactivities, including antiparasitic, antimicrobial, and antioxidant properties (Ge *et al.* 2021). Pomegranate peel extract (PGE) has a wide range of therapeutic benefits, including the ability to treat and prevent cancer (Dikmen *et al.* 2011), cardiovascular disease (Jurenka, 2008), and diabetes (Middha *et al.* 2012), and improve wound healing

(Hayouni *et al.* 2011). Other potential applications include infant brain ischemia, Alzheimer's disease (Middha *et al.* 2012), male infertility, obesity, arthritis (Kanatt *et al.* 2010), anthelmintic and anti-protozoan activities (Calzada *et al.* 2006; El-Sherbini *et al.* 2009), and antihydatic effects (Labsi *et al.* 2016). However, the ultrastructural effect of PGE against cystic echinococcosis has yet to be determined. This study was conducted to determine the in vivo effect of PGE on cystic echinococcosis through parasitological, transmission electron microscopy (TEM), and histopathological studies. Also, serological assessment of serum IFN-γ in mice was measured.

## Materials and methods

### Parasite

Hydatid cysts were collected from the El-Warrak slaughterhouse in Cairo, Egypt. The protoscoleces (PSCs) were prepared according to Amri *et al.* (2007). Fertile cattle pulmonary cysts were aspirated under aseptic conditions to obtain the hydatid fluid. The fluid was centrifuged, and the sediment containing PSCs was washed with sterile phosphate buffer saline supplemented with 30 mg/mL gentamicin. After being stained with 0.1% eosin, the movement of the PSCs was observed by an inverted microscope to determine their viability. The unstained and moving PSCs were counted as viable. A percentage of viable PSCs of more than 95% was considered suitable for further experimental infection. Each mouse's infective inoculum was adjusted to contain 2000 viable PSCs suspended in 500 µl of sterile phosphate buffer saline for intraperitoneal inoculation (Urrea-París *et al.* 2002).

### Experimental animals

The study was carried out on 85 pathogen-free laboratory-bred female albino mice, six to eight weeks old and weighing 24 ± 2 g. They were purchased from the Biology Supply Center at Theodor Bilharz Research Institute (PO Box 30 Imbaba, Giza, Egypt). The mice were housed in standard individual mouse cages (5 mice per cage) in a controlled room temperature (22 ± 1°C) and humidity, with controlled 12h light/dark cycles and free access to a standard pellet animal diet and tap water. Following the approval of the institutional ethical committee of the National Liver Institute (00507/2023), all animal care and procedures were conducted per international ethical standards.

### Tested drugs

ABZ suspension (ABZ 400 mg/10 mL; Pharma Cure Pharmaceuticals Co., Cairo, Egypt) was dissolved in 0.2 mL chromophore L through mechanical shaking and administered orally via a gastric tube at a dose of 200 mg/kg/d for 5 consecutive days per week for 8 weeks starting at 3 months post-infection (Küster *et al.* 2014). The ABZ suspension was shaken vigorously prior to its intragastric delivery to mice. For more clinical efficacy, ABZ formulations were freshly made every 3 days and refrigerated at 3–5°C.

Fresh PGE was prepared by aqueous extraction and then filtered using Whatman filter paper. Mice were treated by daily intragastric administration of 500 µl of PGE. The PGE dose was adjusted to be 0.65 g/kg dissolved in 0.2 ml chromophore L from the second day post-infection (Labsi *et al.* 2016).

### Experimental design

In the current study, the experimental animals were divided into the following five groups: Group I (5 mice): uninfected and untreated group (normal control); Group II (20 mice): infected untreated group (positive control); Group III (20 mice): infected and treated with ABZ, with treatment started 3 months post-infection; Group IV (20 mice): infected and treated with PGE, with treatment started 3 months post-infection; Group V (20 mice): infected and treated with ABZ and PGE, with treatment started 3 months post-infection. All treatment regimens were continued for 8 weeks, and 1 week after administration of the last dose of the tested drugs, the experiment was terminated. At the end of the study, each mouse was anaesthetized with ether, and blood sampling via a cardiac puncture and sacrifice by cervical dislocation were performed. Blood samples were centrifuged, and sera were separated and stored at -80°C for further serological and biochemical analysis. Each mouse was subjected to parasitological assessment of hydatid cyst size and weight followed by histopathological and TEM examination of the liver tissue and then measurement of serum IFN-γ in all studied groups.

### Parasitological assessment

Each mouse's peritoneal cavity was thoroughly opened during necropsy, and different organs were examined. Digital still photography was used to capture the hydatid cysts, and Adobe Photoshop CS3 (San Francisco, CA, USA) was used to calculate their sizes using the scaled ruler. The cysts were carefully removed, and an analytical balance was used to calculate the weight of the cysts. The following formula was used to calculate the efficacy rate of treatments (Pensel *et al.* 2015). Treatment efficacy= C-T / C X 100%, where C is the mean cyst weight or size in the infected non-treated control group, and T is the mean cyst weight or size in the treated group.

### Histopathological examination

Liver tissue samples of each mouse were fixed in 10% formalin, embedded in paraffin, and processed into blocks. Serial sections of 5 µm in thickness were cut and then stained with hematoxylin and eosin (Harries 1989). The sections were observed by a light microscope (Olympus BX41, Olympus Corporation, Tokyo, Japan).

### Transmission electron microscopy (TEM)

The obtained hydatid cysts were processed as previously reported for ultrastructure examination (Elissondo *et al.* 2006). The samples were photographed by using a JEOLJEM 1230 transmission electron microscope (JEOL, Tokyo, Japan)

### Measurement of IFN-γ in mice serum samples

The sandwich ELISA (Quantikine R&D Systems, Inc., Minneapolis, MN, USA) was used to detect IFN-γ in mouse serum samples. Serum samples were examined according to the manufacturer's recommendations, and IFN-γ concentrations were expressed as pg/mL.

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### Statistical analysis

Data were tabulated and analysed using SPSS Version 20 (SPSS, IBM, Armonk, USA). The descriptive data were examined by the Chi-square test and Fisher's exact test. Numerical data were expressed as mean ± standard deviation (SD). The Kruskal-Wallis test was applied and followed by post hoc tests to determine significant differences between groups. Results with $P < 0.05$ were considered significant.

## Results

### Parasitological assessment results

In the current study, the highest mortality rate (30%) was observed in the infected control group (GII), but the mortality rate of all treated groups was 15%. The hydatid cyst size and weight declined significantly ($P < 0.001$) across all treated groups. The smallest hydatid cyst size and weight (0.48 ± 0.47mm, 0.17 ± 0.18 gm) with the highest drug efficacy of 56.2 % was detected in GV, whereas the largest cyst size and weight (1.25 ± 0.85mm, 0.43 ± 0.29 gm) was observed in GIII, with a drug efficacy of 22.9% across treated groups (Table 1).

### Histopathological results

In the present study, regarding fibrosis and inflammatory infiltration of liver tissue, there were significant differences between treated groups (GIII, GIV, and GV) and the infected control group (GII) ($P < 0.05$) (Table 2). Severe fibrosis and inflammatory infiltration of lymphocytes and macrophages in both portal areas and hepatic parenchyma were found in the infected control group (GII) (Figures 1A, B). Improvements were observed in the ABZ (GIII) (Figure 2C) and PGE (GIV) groups, but marked improvements were noticed in the PGE + ABZ group (GV). Evaluation of liver cell necrosis and steatosis (a solitary, sizable lipid vacuole characterises the cytoplasm of hepatocytes) revealed a significant difference between the studied groups ($P < 0.05$). Therefore, the ABZ group (GII) (Figure 2c) showed the highest values of liver cell necrosis and steatosis. Improvements were reported in PGE + ABZ group (GV) (Figures 2F, G, H). However, the best results were detected in the PGE group (GIV) (Figures 2D, E; Table 2).

### Transmission electron microscopy

The ultrastructure of the hydatid cysts retrieved from GII showed an intact germinal layer with microtriches extending into the laminated layer. Also, there were also undifferentiated cells present with intact nuclear membranes and normal nuclei (Figure 2a). The hydatid cysts from the ABZ group (GIII) showed slightly vacuolated cytoplasm, glycogen depletion, and heterochromatin (pyknosis) in the nuclei (Figure 2b). The hydatid cysts obtained from the PGE (Figure 2c) and PGE+ABZ (Figure 2d) groups that showed disturbance of hydatid layers and lipid droplets. The cytoplasm showed fragmentation of the nuclei, glycogen depletion, and multiple lysosomes in vacuolated cytoplasm. All these changes were more obvious in PGE + ABZ group (Figure 2e).

### Serum IFN-γ detection results

In the current study, the mean serum concentration of IFN-γ was extremely low (60.28 ± 1.14 pg/ml) in GI. The IFN-γ level was augmented starting in all infected and treated groups. There was a significant difference ($P < 0.05$) between the studied groups. The highest significant rise was in the ABZ group when compared with GI and GII. Also, there was a significant decrease in serum IFN-γ with PGE treatment either alone (GIV) or combined with ABZ (GV) compared with the infected control (GII) and ABZ (GIII) groups. Combined treatment with PGE + ABZ (GV) showed the most significant reduction in IFN-γ production compared with all the studied groups ($P < 0.05$) (Figure 3).

## Discussion

Finding novel compounds for the medical treatment of cystic echinococcosis is crucial due to the drawbacks and restrictions of employing benzimidazoles. Punica granatum has garnered significant attention from the public and the academic community due to its potential medicinal properties and relevance to the food industry. The pomegranate fruit exhibits a diverse array of potential therapeutic properties (Shaygannia *et al.* 2016), and it was recently challenged for its immunomodulatory effect against SARS-CoV-2 (Alexova *et al.* 2023).

In the current study, the combination of PGE with ABZ gave better results than PGE alone, denoting a synergitic antihydatic effect between these drugs. Similar results regarding PGE were observed by Labsi *et al.* (2016) who found that the diameter and the weight of hydatid cysts were decreased significantly in the PGE group. Thus, they considered it an alternative natural anthelmintic and antimicrobial drug with less toxic effects as the extract is a rich source of phytochemicals that can produce less toxic effects at higher doses or under long-term administration (Moazeni and Roozitalab 2012).

**Table 1.** Comparisons between studied groups regarding hydatid cyst size and weight

| | GII (Positive control) (n = 14) | GIII (ABZ) (n = 17) | GIV (PGE) (n = 17) | GV (PGE + ABZ) (n = 17) | Kruskal- Wallistest | *P* value | Significantpost-hoc |
|---|---|---|---|---|---|---|---|
| Hydatid cyst size (mm): Mean ± SD. | 5.14 ± 1.75 | 1.25 ± 0.85 | 0.89 ± 0.54 | 0.48 ± 0.47 | | | P1 = 0.0001* P2 = 0.0001* P3 = 0.0001* P4 = 0.04* |
| Median (Minimum–Maximum) | 6 (3–7) | 1.70 (0–2) | 1 (0–2) | 0.80 (0–1) | 52.324 | 0.0001 | |
| Hydatid cyst weight (gm): Mean ± SD. | 0.78 ± 0.08 | 0.43 ± 0.29 | 0.2 ± 0.15 | 0.17 ± 0.18 | 46.325 | 0.001 | P1 = 0.003* P2 = 0.0001* P3 = 0.0001* P4 = 0.008* |

* Highly significant results: P1 = GIII (ABZ) *vs.* GII (positive control); P2 = GIV (PGE) *vs.* GII (positive control); P3 = GV (PGE + ABZ) *vs.* GII (positive control); P4 = GV (PGE + ABZ) *vs.* GIII (ABZ).

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**Table 2.** Comparisons between studied groups regarding histopathological changes in hepatic echinococcosis lesions

| | GII (Positive control) (n = 14) | GIII (ABZ) (n = 17) | GIV (PGE) (n = 17) | GV (PGE + ABZ) (n = 17) | Fisher's exact test | Chi square test | *P* value |
|---|---|---|---|---|---|---|---|
| Inflammatory infiltration | | | | | | | |
| Mild: n (%) | 0 (0.0%) | 4 (23.5%) | 10 (58.8%) | 15 (88.2%) | | 58.267 | 0.001* |
| Moderate: n (%) | 6 (42.9%) | 9 (52.9%) | 5 (29.4%) | 2 (11.8%) | | | |
| Severe: n (%) | 8 (57.1%) | 4 (23.5%) | 2 (11.8%) | 0 (0.0%) | | | |
| Liver fibrosis | | | | | | | |
| Mild: n (%) | 0 (0.0%) | 5(29.4%) | 7 (41.2%) | 17 (100%) | 95.562 | | 0.0001* |
| Moderate: n (%) | 4 (28.6%) | 10 (58.8%) | 10 (58.8%) | 0 (0.0%) | | | |
| Severe: n (%) | 10 (71.4%) | 2 (11.8%) | 0 (0.0%) | 0 (0.0%) | | | |
| Liver cell necrosis | | | | | | | |
| Absent n (%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 407.353 | | 0.0001* |
| Mild: n (%) | 6 (42.9%) | 2 (11.8%) | 17 (100%) | 8 (47.1%) | | | |
| Moderate: n (%) | 5 (35.7%) | 10 (58.8%) | 0 (0.0%) | 9 (52.9%) | | | |
| Severe: n (%) | 3 (21.4%) | 5 (29.4%) | 0 (0.0%) | 0 (0.0%) | | | |
| Steatosis | | | | | | | |
| Absent: n (%) | 5 (35.7%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 54.786 | | 0.0001* |
| Mild: n (%) | 5 (35.7%) | 8 (47.1%) | 17(100%) | 11 (64.7%) | | | |
| Moderate: n (%) | 4 (28.6%) | 9 (52.9%) | 0 (0.0%) | 6 (35.3%) | | | |

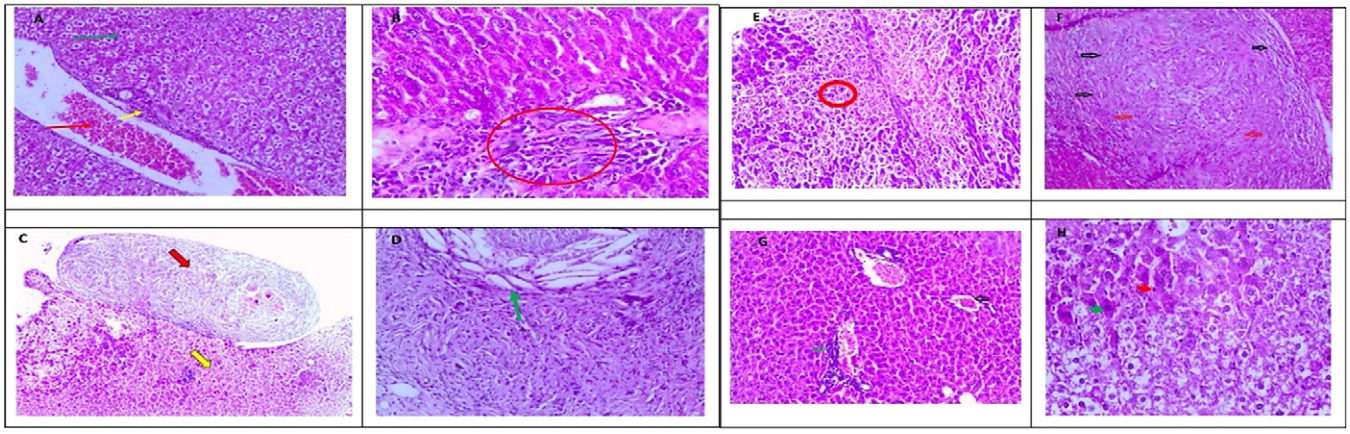


**Figure 1.** Liver sections from *E. granulosus*-infected groups stained by H & E. GII showing congested central vein (red arrow), inflammatory infiltrate in the form of lymphocytic collection (yellow arrow), and the surrounding hepatocytes showing moderate steatosis (green arrow) (x100) (A) and showing the portal tract with moderate infiltration by lymphocytes (red circle) (x200) (B); GIII exhibiting severe degeneration of liver parenchyma, focal necrosis (yellow arrow), and granulomatous reaction (red arrow) (x40) (C); GIV showing wall of granulomatous reaction with dense fibrosis and scattered cholesterol crystal deposition (green arrow) (x100) (D) and exhibiting severe degeneration of liver parenchyma and frequent cellular apoptosis (red circle) (x100) (E), GV showing granulomatous reaction in the form of collection of lymphocytes, histocytes, and proliferating fibroblasts (black arrow) and fibrosis (red arrow) (x100) (F), congested central vein (black arrow) and moderate infiltration of portal tract by lymphocytes (green arrow) (x 100) (G), scattered degenerated necrotic cells with pyknotic nuclei (red arrow) and scattered apoptotic cells (green arrow) (x200) (H).

The present pathology results denote that PGE treatment associated with ABZ improves the ABZ effect and seems to be more efficient, as reduction of side effects particularly in liver were in agreement with Labsi *et al.* (2019). In the current study we tried to highlight the ultrastructural alterations induced by PGE in the treatment of cystic echinococcosis, which were in line with findings reported by the use of alternative drugs (Zhang *et al.* 2017; Yin *et al.* 2018). This corroborates the suppressive impact of PGE on *E. granulosus* cysts. PGE administration resulted in notable alterations in the ultrastructure, specifically disrupting the hydatid layers and lipid droplets. According to Corfield (2015), the formation of the loose mucus-rich glycogen barriers in the laminated layer was attributed to mucins with a specific type of glycosylation, namely mucin O-type glycosylation (Corfield 2015). The lack of glycogen storage vesicles could be attributed to the influence of combined therapies that actively diminish glucose absorption and storage in hydatid cysts, as elucidated by Lacey's (1990) investigation into the impact of the medicinal intervention on the cellular

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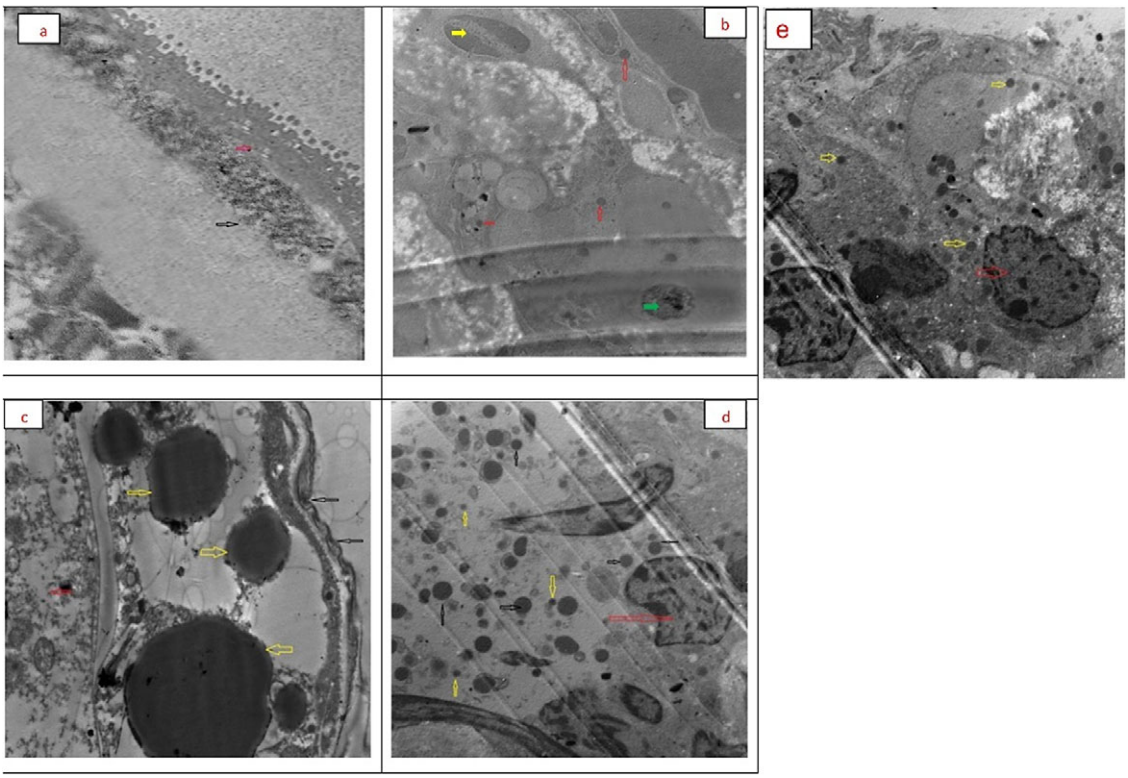


**Figure 2.** Transmission electron micrograph (TEM) of a hydatid cyst (a) from an infected non-treated mouse (GII) showing intact germinal layer (black arrow) and intact laminated layer (red arrow) (×10000); (b) TEM micrograph of hydatid cysts from ABZ treated mouse (GIII) showing auto-phagolysosomes (green arrow), glycogen (red arrow), and cracked nucleus (yellow arrow) (×10000); (c) from PGE treated mouse (GIV) showing the ultrastructure of hydatid cyst with slight disturbance of hydatid layer (black arrow), disturbed cytoplasm (red arrow), and lipid droplets (L) (yellow arrow) (×12000). TEM micrograph of hydatid cysts from PGE + ABZ treated mouse (GV) showing scattered multiple lysosomes (black arrow), scanty glycogen in disturbed vacuolated cytoplasm (yellow arrow), distortion and fragmentation of the nucleus (red arrow) (×12000) (d) (×10000) (e).

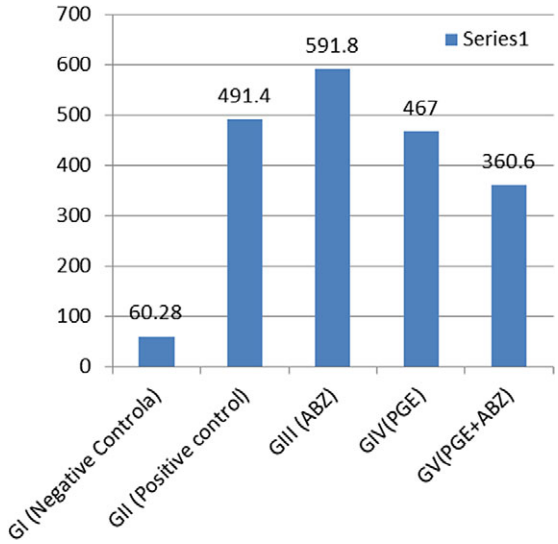


**Figure 3.** Mean values of IFN-γ among the studied groups of experimental animals.

microtubular structures of the metacestode (Lacey 1990). Observing enhanced cellular vacuolisation, swelling of mitochondria, lipid droplets in the germinal layer, and lamellar bodies in the laminated layer may indicate a widespread tissue stress response in reaction to the parasite, potentially leading to apoptosis or necrosis. The process of vacuolisation has the potential to result in significant cytoplasmic content leakage (Kim *et al.* 2011). The observation of lipid droplets in the syncytium of the germinal layer may suggest a disturbance in the metabolic processes of the cyst due to therapeutic intervention (Verma *et al.* 2013).

The assessment of treatment response to a chemotherapeutic agent in cystic echinococcosis is contingent upon multiple factors, one of which is an evaluation of the host immune system response. The immune system's response to echinococcosis is facilitated by T cells, with Th1 cells capable of generating IFN-γ, IL-2, and lymphotoxin and Th2 cells producing IL-4, IL-6, IL-5, and IL-10. Th1-type responses are deemed pivotal in causing damage to the parasite while augmenting Th2-type responses has a contrary effect (Díaz 2017). The cytokine cascade against parasites has been found to involve IFN-γ, according to earlier studies (Xu *et al.* 2017, Zhang *et al.* 2018). Consistent with these findings, the present study observed a significant elevation in serum IFN-γ levels across all groups relative to the normal control group's level. The ABZ group exhibited the highest value, followed by the PGE group, with significant reductions observed in serum IFN-γ levels. Earlier studies in experimentally infected mice support these findings; such studies have assumed that an increase in levels of IFN-γ was responsible for damage to formed cysts and included protoscoleces (Rogan 1998). These findings were also in line with those of Riganò *et al.* (2004) who recoded predominant production of IFN-γ in patients with inactive hydatid cysts. Conversely, patients with cysts active or transitional state displayed an exclusive expression of Th2 cytokines, the authors referred their observation to dependence of immune response on clinical manifestations and the therapeutic intervention's efficacy (Riganò *et al.* 2004). Shirgholami *et al.*

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(2021) also reported a significant increase in levels of IFN-γ concentrations when compared to the control; however, their ABZ group showed the lowest concentration of IFN- γ (Shirgholami *et al.* 2021). We attribute the difference reported in ABZ-treated mice to the duration of infection, administered dose of the drug, and drug regimen.

A significant decrease in the weight of hydatid cysts was observed across all infected treated groups, accompanied by an increase in IFN-γ levels. The protective function of IFN-γ was ascribed to its ability to stimulate peritoneal macrophages and local antibodies that can detect protoscoleces antigens and activate the complement system, removing approximately 90% of the parasite inoculum (Mourglia-Ettlin *et al.* 2011).

Overall, results showed that P. granatum in combination with albendazole had a stronger effect in reducing the hydatid cyst size and weight. The combination also had higher efficacy than either drug alone. Combining ABZ with PGE was most effective in reducing histopathological and ultrastructural changes, with notable reduction in IFN-γ levels. Further studies are recommended to assess immunoregulatory cytokines such as Il-10 and to calculate the ratio of IFN gamma/Il10.

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**Competing interest.** None.

**Ethical standard.** The study took place under the rules set forth by the Ethical Committee for the handling and using laboratory animals from the National Liver Institute No. 00507/2023.

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