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The *in vivo* preventive effect of some medicinal plant extracts on the development of hydatid cyst infection

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Abstract

This study aimed to explore the preventive effect of the methanolic extracts of the seeds of *Citrullus colocynthis*, and the aerial parts of *Ruta graveolens* and *Peganum harmala* on the development of secondary infection of *Echinococcus granulosus in vivo.* The viable protoscolices were subjected to 40 mg/mL of methanolic extracts of the three plants for 4 hours of exposure times *in vitro*, then injected into male Albino-Wistar rats for 8 months. Compared to the negative control group, *R. graveolens* methanolic extract showed high significant ($p< 0.05$) protoscolicidal and preventive effects on the secondary hydatid cysts growth similar to that recorded in albendazole (ABZ) drug. However, *P. harmala* and *C. colocynthis* methanolic extracts showed low preventive effects on the secondary hydatid cysts' growth. The preventive effects were represented by the reduction in the infection rate, cysts number, size, and weight. In addition to the high significant changes ($p< 0.05$) in values of hematological and biochemical parameters, elevation of gamma interferon (IFN-γ) levels and decline of interleukin-10 (IL-10) and IL-4 cytokines compared to the negative control group.

Keywords: *E. granulosus*, Hydatid cysts, Medicinal plants, Secondary infection

1. Introduction

Cystic Echinococcosis (CE) is a serious zoonotic disease caused by *Echinococcus granulosus'* larval stage. The parasite's life cycle requires canines (dogs) to act as definitive hosts and herbivores to act as intermediate hosts. Humans contract hydatidosis by ingesting the parasite's eggs, just as herbivores do [1]. The close contact between dogs, sheep, and human beings is responsible for the trans-infections in certain occupations such as shepherds and farmers [2]. Thousands of animals are slaughtered during the Eid-ul-Adha, marriage, birth, and other occasions. Infected offals of some of these animals provide a good meal for both the domestic and stray dogs which can be seen around abattoirs, farms, and close to people's homes, which accordingly make complete life cycle for the parasite causing CE [3].

Hydatid cysts of *E. granulosus* have two layers derived from the parasite: an inner nucleated germinal layer and an outer acellular laminated layer, both of which are surrounded by a fibrous capsule produced by the host [4]. These cysts develop in internal organs (mainly the liver and lungs) of humans and intermediate hosts as unilocular fluid-filled bladders, protoscolices branching asexually from the germinal layer and growing from the brood capsules' inner wall [5].

Human echinococcosis is complicated to treat, and sometimes requires extensive surgery, which is the best choice of treatment [6]. The spillage of cyst contents during surgery inside the body is the main cause of secondary parasitic infections [7]. A significant cellular inflammatory response may be associated with secondary infections that may cause pathological changes such as increased leukocytosis, mainly of eosinophils, lymphocytes, neutrophils, and macrophages, 3 to 5 days after infection. As early as week one post-infection, elevated levels of tumor necrosis factor-alpha (TNF), gamma interferon (IFN-γ), interleukin-10 (IL-10), interleukin-4 (IL-4), and interleukin-5 (IL-5) can be detected [6].

Neutralization of the hydatid cyst content is strongly recommended before opening or removing the cyst using effective scolicidal agents [7]. Currently, several scolicidal agents such as benzimidazole carbamate groups (mebendazole, albendazole (ABZ), and thiabendazole), are used for inactivation or neutralization of the content of the cyst during and after surgery. Unfortunately, hydatid cyst treatment methods are faced with limitations; these agents are effective and can destroy parasites in the cases where cysts are small and few, however, they are usually not responsive in some advanced cases [8], and their use can result in some complications such as leukopenia, neutropenia, the elevation of liver transaminase, sclerosing cholangitis, liver necrosis, alopecia and liver dysfunction [9]. Due to the increasing demand for safe and effective active compounds, the fact that vaccinations are ineffective in the majority of cases [10], and the fact that parasites have occasionally developed resistance to available synthetic therapeutics [11], it is critical to look for alternative sources of anti-parasitic drugs. The medical properties of medicinal plants and their active compounds have played a key role in the worldwide maintenance of health [12]. These plants produce a wide variety of secondary metabolites including alkaloids, flavonoids, phenolics, curcumin, saponins, and terpenes that have been used to cure many diseases and aches for ages [13].

The current study aimed to do more screening work for novel, safe, and effective natural compounds that could be used as anti-parasites with fewer adverse effects. To achieve this goal, the aerial parts or seeds of Some wild medicinal plants (*Citrullus colocynthis* (*Cucurbitaceae*), *Peganum harmala* (*Zygophyllaceae*), and *Ruta graveolens* (*Rutaceae*)) (Figure 1). were collected from various regions in Jordan, extracted using absolute methanol, and used for further investigations.

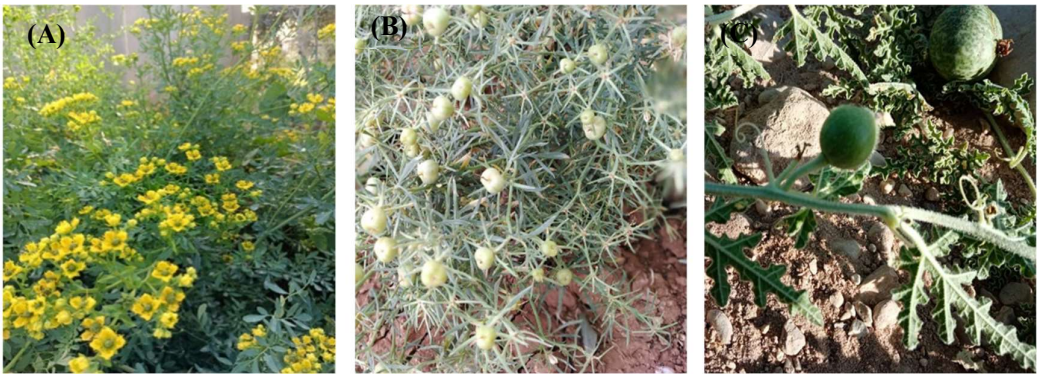

Figure 1 Plants used in the study; (A) *R. graveolens;* (B) *P. harmala;* and (C) *C. colocynthis*.

2. Materials and methods

2.1 Collection and preparing the extracts of the plant specimens

In June 2020, *R. graveolens* and *P. harmala* aerial parts, including stems and leaves, were taken from various locations throughout Jordan, while *C. colocynthis* seeds were taken in July 2020. Prof. Sawsan Oran, a plant taxonomist at the University of Jordan's Department of Biological Sciences in Amman, Jordan, verified the authenticity of these species. The voucher specimen's numbers for *C. colocynthis*, *P. harmala*, and *R. graveolens* were deposited in the Department of Biology, Faculty of Science, Mutah University, Jordan (MU2021-21, MU2021-20, and MU2021-19, respectively). The seeds of *C. colocynthis* and the aerial parts of *P. harmala* and *R. graveolens* were thoroughly washed, dried, and ground using a blender. For each plant, 100 grams of the powder were soaked in absolute methanol (10:1 v/w ratio) for 72 h at room temperature with continuous shaking. After that, the suspension was filtered through a Whatman paper No.1, and the crude methanol extract was concentrated under reduced pressure using a rotary evaporator at 45°C. The resulted extract was kept in the freezer at -20°C in airtight containers [14].

2.2 Collection of parasite larvae from infected organs with hydatid cysts

This study was carried out in AL-Karak province in Jordan, slaughtering animals is under the control of the veterinary section for meat inspection. Regular visits (periodically) to the slaughterhouse were performed to inspect for the presence of hydatid cysts. The hydatid cyst-infected organs of the slaughtered animals were identified by veterinarian descriptions. The infected organs were placed in plastic bags, in an icebox, then transported within 1 h to the Parasitology research lab at the Department of Biological Sciences, Mutah University. The samples were examined to determine the site of infection particularly, the liver and lungs.

The hydatid fluid was transferred into a test tube under a sterile condition, allowed to sit for 15 min, and washed with phosphate buffer saline (PBS). Extracted protoscolices were kept at 37°C in a sterile Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum. To prevent bacterial contamination, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate were added to the medium [4].

Fertility tests were conducted on several cysts found in slain animals at random. A needle (Gauge 18) was used to puncture the cyst wall, which was then opened up with a scalpel and scissors. The contents were transferred to a sterile container and inspected under a compound microscope (Olympus, Japan) for the presence of protoscolices. The presence of free protoscolices in the cystic fluid was assessed by microscopic inspection of a wet mount drop to indicate cyst fertility. Cysts without protoscolices, as well as heavily suppurative or calcified cysts, were considered infertile. The vitality of protoscolices was determined using the motility of flame cells and staining with 0.1% aqueous eosin solution. Unlike dead protoscolices, living protoscolices do not absorb the stain [3].

2.3 Animals and experimental design

The effect of methanolic plant extracts on protoscolices viability was evaluated using healthy adult male albino rats of the Wistar strain. The animals were obtained from the animal house of Mutah University and they aged 50 days and weighed between 150 to 200 g. Ethical approval with reference number 49-2021 was obtained for the study. The animals were allowed to adapt to the standard environmental laboratory conditions for 2 weeks before conducting the experiments in the air-controlled room with a cycle of 12 h of lightness and 12 h of darkness in the animal house of Mutah University, food pellets and water were provided *ad libitum.*

2.4 Calculation of the median lethal dose (LD_{50})

The oral median lethal dose (LD_{50}) of plant extracts and their range was calculated according to the proposed new methods. Overnight-fasted rats were used in this experiment. The oral treatment methods were divided into 3 stages; in each stage, the animals were divided into different groups, as shown in (Table 1). Groups 1 to 4 received 1mL of plant extract for each animal orally, while the control group 5 (G5) received physiological saline with 1mL for each animal by the same route. For predicting the median LD_{50}, the treatment process started with the lowest concentration of the plant extract 100 mg/kg body weight (BW) until reaching the highest concentration of 5000 mg/kg BW (the high degree of safety) through three stages, as shown in (Table 1). In stage one, the tested groups were given increasing concentrations of extracts starting from 100, 200, 400, and 800 mg/kg BW. General symptoms of toxicity and mortality in each group were observed within 24 h. If there was no deadly effect shown by the highest concentration in stage one, the stage two started from 1000, 1500, and 2000 mg/kg BW, any toxicity or mortality in each group was observed within 24 h, and if the mortality rate was zero, stage three was started from 3000, 4000 and finally 5000 mg/kg BW. If one rat was dead at any concentration in any stage, confirmation was done by duplicating the animals' number in the target stage, if half or more of the animals were dead in this stage by the successive concentration of extract, the LD_{50} was calculated according to the following equation as mentioned by Chinedu et al [15]; $LD_{50} = ((M_0 + M_1) / 2)$, Where M_0: is the highest dose of test substance that gave no mortality and M_1: is the lowest dose of test substance that caused mortality [15].

Table 1 Recommended doses for the proposed (new) LD_{50} method.

Stage	Dose of body weight (mg/kg)				
	Group 1	Group 2	Group 3	Group 4	Group 5
1	100	200	400	800	Saline
2	1000	1500	2000	-	-
3	3000	4000	5000	-	-

2.5 Preventive experiments

To investigate the preventive effect of plant extracts on the progression and transformation of protoscolices into secondary hydatid cysts *in vivo*, the animals (36 rats) were divided into 6 groups, 6 rats in each. Protoscolices

were pre-treated with 40 mg/mL of the plant extracts for 4 h. Stock solutions of methanolic plant extracts were prepared in 30% dimethyl sulfoxide (DMSO) at a concentration of 1g/mL.

The rats in groups 1-3 were injected intra-peritoneally (IP) with protoscolices (2×10^3 protoscolices/rat) which were pre-treated with plant extracts (40 mg/mL), the treatment was continued orally using approximately 1/10 of LD_{50} of each plant extract for 30 days; whereas the rats in group 4 (G4) were injected with protoscolices pre-treated with 20 mg/mL albendazole, and the treatment was continued orally using albendazole with 150 mg/kg body weight daily for 20 days. The rats in the negative control group (G5) were injected with protoscolices pre-treated *in vitro* with 2.4% DMSO, which is the concentration of DMSO received by every plant extract-treated animal, prepared in normal saline. The treatment was continued orally with 2.4% DMSO prepared in normal saline for 30 days. And Finally, the healthy group 6 (G6) did not receive any protoscolices, and the rats were treated orally with 2.4% DMSO prepared in normal saline for 30 days [3]. This was based on the results of the *in vitro* screening test that has been done by our work team and published before [16]. All animals used in the experiment were anesthetized using cotton wool soaked in chloroform 8 months post-treatment starting, and the abdominal cavity was opened until the sternum using medical scissors. The blood samples were directly drawn from the heart using a 5 mL sterile syringe into heparinized sample tubes containing ethylenediamine tetraacetic acid (EDTA) for hematological analysis within 24 hours of collection. The hematological parameters included red blood cells count (RBCs), white blood cells count (WBCs), and differential WBCs count. In addition, the hemoglobin (Hb) level and hematocrit (HCT) were also evaluated. For the biochemical tests, blood samples were introduced into clean dry non-heparinized centrifuge tubes and allowed to stand for 10 min at room temperature, then centrifuged at 3000 rpm for 15 min using a laboratory centrifuge to obtain sera. Sera were carefully removed and analyzed for total protein, albumin, cholesterol, triglycerides, and glucose levels. The activity of liver enzymes, including Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), as well as Creatinine and Urea were measured. Serum cytokines concentration of IL-4, IL-10, and Interferon gamma (IFN-γ) were determined by enzyme-linked immunosorbent assay (ELISA) commercial kits (mouse IL-4, IL-10, and IFN- γ high-sensitivity ELISA kits, MY Biosource), according to the manufacturer's instructions. To evaluate the preventive effect of plant extract on the presence of secondary hydatid cysts, at necropsy, the peritoneal cavity was opened, different visceral organs were inspected and sliced carefully, and the hydatid cysts were carefully separated from each other and photographed by a Phone Digital Camera. The numbers and sizes of the cysts were determined based on the digital caliper, ACCUD Code: 111-006-12. The numbers, sizes, weights, or any evidence of degeneration and calcification in treatment groups were compared to that of the negative and the positive controls [17].

2.6 Statistical analysis

Data were presented and analyzed using Statistical Package for the Social Sciences (SPSS.V24) software. The results were expressed by means ± Standard Deviation (SD). The statistical significance of differences between groups was assessed by the one-sample t-test. $p< 0.05$ was considered significant.

3. Results

3.1. The median lethal dose (LD_{50})

Oral administration of tested plants methanolic extract to rats in single different doses started from the dose of 100 mg up to 5000 mg/kg BW. The *R. graveolens* extract had the highest LD_{50} of 4500 mg/kg BW, while the LD_{50} for the *P. harmala* obtained was 2500 mg/kg BW and *C. colocynthis* showed a low LD_{50} of 1750 mg/kg BW. In an *in vivo* study, abdominal writhing, body tremors, a slight decrease in locomotor activity, accelerated breathing, pulse, and clonic muscular spasms. Some toxicity symptoms were revealed when *P. harmala* and *C. colocynthis* were used at a dose of 2500 mg/kg BW and 1750 mg/kg BW, respectively as shown in (Table 2).

Table 2 Oral toxicity of the tested plants extracts on rats.

Plant extract	LD_{50} (mg/kg BW) (Orally)
R. graveolens	4500
P. harmala	2500
C. colocynthis	1750

3.2. In vivo preventive effects of methanolic plant extracts on the development of secondary infection of E. granulosus

In the current study, the protoscolices pre-treated with the plant extracts *in vitro* were injected into the peritoneum of the laboratory rats to check the preventive effect of these extracts on the growth of secondary

hydatid cysts *in vivo.* The rats that were injected with pre-treated protoscolices were further treated with the plant extracts for 30 days. After 8 months the rats were sacrificed and dissected, and the secondary hydatid cysts were investigated in the peritoneum, lungs, kidneys, liver, and other organs of the body. We found that the hydatid cysts in the abdominal cavity of the negative control group were adhesive to the surrounding tissues, had a larger size, rounded in shape, white or transparent in color, contained clear fluid, and had an elastic cystic wall. Through observation and comparison, we found that in group 1 (G1), which was treated with *R. graveolens* extract, and G4, which was treated with ABZ, no cysts were found in the abdominal cavity. However, the hydatid cysts were visible in most rats treated with *P. harmala* group 2 (G2) and *C. colocynthis* extracts group 3 (G3) (Figure 2). Oral treatment with albendazole (150 mg/kg BW/day) showed a preventive effect on the growth of secondary hydatid cysts in laboratory rats, the infection rate was (0.0%) as shown in (Table 3). Similarly, remarkable preventive effects have been also shown when *R. graveolens* extract (400 mg/kg BW/day) was used for 30 days orally. The results presented in (Table 4), showed that the infection rate was (0.0%), and no hydatid cyst development occurred in rats treated with *R. graveolens* and ABZ.

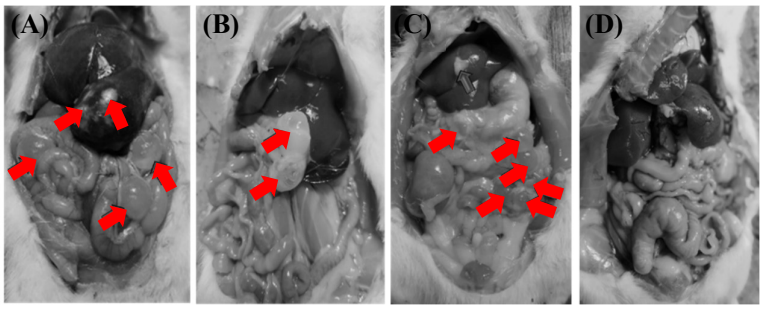

Figure 2 Infected rats with hydatid cysts after 8 months post-infection. (A) treated with normal saline, (B) treated with *P. harmala*, (C) treated with *C. colocynthis*, (D) treated with *R. graveolens.*

The oral treatment with 200 mg/kg BW/day of *P. harmala* (G2) and 150 mg/kg BW/day of *C. colocynthis* (G3) methanolic extracts showed varying preventive effects on the growth of secondary hydatid cysts. The infection rate was 66.7 and 83.30% in methanolic extracts of *P. harmala* and *C. colocynthis*, respectively. In the negative control group, the infection rate was (100%), and the cysts were located in the liver, peritoneum, kidney, and mesentery. The number, size, and weight of hydatid cysts in all treatment groups were measured and compared with the negative control group (G5) and the positive control group (G4) as shown in Table 3. The maximum number, size, and weight of hydatid cysts were observed in the infected rats of the negative control group, the largest mean size was 6.18 ± 1.9 mm, and the total weight of all cysts (20 cysts) was 3.57 g. Treatment with *P. harmala* and *C. colocynthis* extracts caused a significant reduction ($p< 0.05$) in the number and the total weight of hydatid cysts as compared to the negative control. The best results were seen in the group treated with *R. graveolens* and ABZ (G1 and G4, respectively), they showed no hydatid cyst growth at all.

Table 3 Effect of the methanolic plant extracts on prevention of hydatid cyst formation compared to Albendazole as positive control and normal saline as a negative control. (n =6)**.**

Group ID	No. of infected rats	Infection (%)	Infected organs	Total no. of cysts	Size of hydatid cyst (mm)	Total weight of hydatid cysts (g)
G1	0	0.00	ND*	0	0	0
G2	4	66.7	Liver, kidney, and mesentery	7	3.53 ± 0.9**	0.52
G3	5	83.3	Liver, peritoneum cavity and mesentery	11	4.04 ± 0.7**	0.74
G4	0	0.00	ND	0	0	0
G5	6	100	Liver, peritoneum cavity, kidney and mesentery.	20	6.18 ± 1.9	3.57
G6	0	0.00	ND	0	0	0

*ND: not detected. ** Significant at $p < 0.05$, compared to the negative control.

The blood parameters of the negative control (G5) were compared to the healthy group (G6) to confirm the infection. The blood parameters of other groups *R. graveolens* (G1), *P. harmala* (G2), and *C. colocynthis* (G3) were compared to the negative group as well as the healthy control, and the results are shown in (Table 4). The results of RBCs, Hb, and HCT levels revealed a significant increase in the groups treated by *R. graveolens* and ABZ as compared with negative controls ($p< 0.05$), while the mean number of WBCs revealed a significant

decrease ($p< 0.05$). However, in groups treated with *P. harmala* and *C. colocynthis*, the RBCs, Hb and HCT levels did not show any significant differences when compared to the negative control group, whereas a significant decrease was observed in the mean number of WBC ($p < 0.05$).

Table 4 Hematological parameters of rats from the preventive experiment. Data are expressed by mean ± SD, where n = 6.

Blood Parameters	G1	G2	G3	G4	G5	G6
RBC (×l0^6 cell/µL)	8.2±0.51**	6.8±0.35	6.4±0.38	8.08±0.77**	6.6±0.33*	7.8±0.64
WBC (×l0^{3}cell/ µL)	9.9±0.34**	9.8±1.2**	10.0±0.8**	8.4±0.54**	13.6±0.64*	8.2±0.42
Hb (g/dL)	13.6±0.5**	12.0±0.61	11.2±0.38	13.4±1.32**	11.9±0.8*	13.5±0.87
HCT (%)	43.0±0.04**	38.7±0.02	37.5±0.01	42±0.03**	38.0±0.03*	44.0±0.23

*Significant at $p< 0.05$ compared to the healthy control. **Significant at $p< 0.05$ compared to the negative control.

The results of differential WBCs count in the negative control showed significant changes in all types of WBCs as compared to the healthy group ($p< 0.05$). The mean number of lymphocytes and eosinophils showed a significant decrease in G1 (treated with *R. graveolens*) and G4 (treated with ABZ) ($p< 0.05$) as listed in (Table 5). Whereas in G2 and G3 (treated with *P. harmala* and *C. colocynthis*, respectively) the results showed a significant decrease in the mean number of eosinophils, however, there were no significant differences in the mean number of lymphocytes as compared to the negative control group. All blood indices and differential WBCs results in the *R. graveolens* group remained within the normal range compared to the positive control ABZ group, so this value didn't reach statistical significance (Table 5).

Table 5 Differential count of WBCs of rats from the preventive experiment compared to healthy and negative controls. Data are expressed by mean ± SD, where n = 6.

White blood cells (%)	G1	G2	G3	G4	G5	G6
Neutrophils	33.75±2.1**	15.1±0.4	12.42±0.96	28.8±2.1**	11.0±0.61*	28±1.2
Lymphocytes	61.5±4.1**	76.6±4.6	78.2 ±3.8	66.8±3.2**	78.3±3.9*	68.2±2.4
Monocytes	2.0±0.59	1.66±0.27	2.15±0.46	1.3±0.59	1.5±0.45*	2.2±0.46
Eosinophils	2.8±0.9**	6.17±0.98**	7.0±1.1**	2.9±0.47**	9.10±1.5*	1.2±0.41
Basophils	0.35±0.14	0.55±0.12	0.3±0.09	0.2±0.13	0.32±0.82*	0.83±0.75

*Significant at $p< 0.05$ compared to the healthy control, **Significant at $p< 0.05$ compared to the negative control.

The biochemical parameters of the treated groups were also investigated and compared to the negative control. The results of the negative control group (G5) showed a significant increase in the levels of AST, ALT ALP, and total proteins and a significant decline in the level of albumin, serum urea, and glucose as compared to the healthy control (G6) ($p< 0.05$). In the groups treated with *R. graveolens* (G1), ABZ (G4), and *P. harmala* (G2), a significant decrease in the concentration of AST, ALT, ALP, and total protein was observed as compared to the negative control ($p< 0.05$). Moreover, a significant increase in the concentration of albumin, serum urea, and glucose was also seen in the groups treated with *R. graveolens* and ABZ as compared to the negative control ($p< 0.05$) (Table 6). No significant changes were found in the levels of creatinine, cholesterol, and triglycerides throughout the treated groups as compared to the negative control (Table 6). Notably, these values remained within the normal ranges as in the positive control.

Table 6 Biochemical parameters in the serum of rats from the preventive experiment. Data are expressed by mean ± SD, where n = 6.

Biochemical parameters	G1	G2	G3	G4	G5	G6
AST (U/L)	117.80±46.2**	133.50±24**	168.20±36.6	112.80±30**	153.10±21^6*	123.93±16.2
ALT (U/L)	35.10±10.4**	52.77±6**	55.16±9.3	32.90±11.2**	65±4.1*	42.45±5
ALP (U/L)	109.20±21**	154±26.1**	168±42.3	111.70±16.1**	159.30±32.7*	103.22±22.6
Total protein (g/dL)	7.14±0.33**	9.90±0.61**	10.29±0.55	7.03±0.3**	9.06±0.4*	7.53±0.15
Albumin (g/dL)	4.82±0.49**	3.65±0.31**	3.72±0.35	5.73±0.33**	3.80±0.06*	5.40±0.1
Creatinine (mg/dL)	0.32±0.1	0.29±0.1	0.33±0.09	0.33±0.08	0.32±0.02*	0.36±0.04
Urea (mg/dL)	16.33±4.2**	11±3.7**	10.90±2.5	18.35±2.5**	11.40±0.78*	19.28±0.87
Glucose (mg/dL)	112.50±7.8**	96.0±8.3**	90.47± 3.7	119± 5.8**	79.80±7.6*	100.80± 5.78
Cholesterol (mg/dL)	68.90±3.66	70.30±3.3	66.33±15.2	67.83±13.2	68.30±3.5*	76.8±5.3
Triglycerides (mg/dL)	82.40±7.8	83.30±10.9	86.30±10.9	84.97±38.5	85.17±9.1*	96.40±11.5

*Significant at $p< 0.05$ compared to the healthy control. **Significant at $p< 0.05$ compared to the negative control.

Serum cytokines levels of T-helper cell 1 (Th1) IFN-γ and T-helper cell 2 (Th2) (IL-4, IL-10) were measured in the sera of the treated groups. On one hand, The IFN-γ, IL-4, and IL-10 cytokines were significantly increased in the negative control group (G5) as compared to the healthy control group (G6) ($p< 0.05$). On the other hand, IL-4 and IL-10 cytokines were significantly decreased in all treated groups as compared to the negative control group ($p< 0.05$) (Table 7). However, *R. graveolens* and ABZ treatment groups (G1 and G4, respectively) show a higher decrement in the levels of IL-4 and IL-10 compared to the *P. harmala* and *C. colocynthis* treatment groups (G2 and G3, respectively). Moreover, IFN-γ levels in *P. harmala* and *C. colocynthis* treatment groups (G2 and G3, respectively) were lower than those found in *R. graveolens* and ABZ treatment groups (Table 7). Interestingly, our results showed no significant differences between *R. graveolens* and ABZ treatment groups.

Table 7 Serum cytokines levels in the treated and control groups. Data are expressed as mean ± SD, where n = 6.

Cytokines (pg/mL)	G1	G2	G3	G4	G5	G6
IL-4	193.5±13.0**	288.5±8.7**	295.0±15.3**	198.9±12.2**	317.1±13.5*	117±12
IL-10	119.0±8.5**	293.6±19.6**	335.4±51.8**	141.9±15.6**	398.5±33.3*	106±13
IFN- γ	224.3±8.3**	191.3±25.4	156.2±18.1	216.4±15.1**	167.3±22.4*	95±10

*Significant at $p< 0.05$ compared to the healthy control. **Significant at $p< 0.05$ compared to the negative control.

4. Discussion

The current study showed that *R. graveolens* extract and ABZ have higher preventive effects on the development of secondary hydatid cysts growth than *P. harmala* and *C. colocynthis* extracts. The *R. graveolens* extract has been found to have a high noticeable preventive effect which is similar to that of ABZ. *R. graveolens* has a clear destructive effect on the pretreated protoscolices that were injected into the experimental rats intraperitoneally, so they cannot grow and develop into a secondary infection. These results are in agreement with the results of Moazeni et al [18] who reported that the aromatic water of *Zataria multiflora* has preventive effects on the hydatid cyst formation in laboratory mice. Moreover, those findings are well correlated with the results of our previous *in vitro* experiment [16], which showed that *in vitro* exposure of hydatid cysts to *R. graveolens* extract leads to profound protoscolices alterations such as loss of hooks and microtriches, paralysis, bleb formation in the tegument, contraction of soma region and rostellar disorganization, loss of the cellular structure, and death. These changes may be due to the presence of phenolic compounds, such as tannins and rutin, which cause death in worm's larvae via uncoupling oxidative phosphorylation when binding to free proteins in the host animal's gastrointestinal tract or glycoprotein on the worms' cuticles [19,20]. On the other hand, *P. harmala* extract showed a moderate preventive effect on the hydatid cyst formation, which might be attributed to the high quantity of different flavonoids in the aerial parts of the plant [21]. However, *C. colocynthis* methanolic extract showed a low preventive effect on hydatid cyst formation. These results are also in agreement with the results of our previous *in vitro* experiment [21].

This study has also revealed that the mean values of HCT and Hb, and the mean numbers of RBCs and WBCs were within the normal levels in the groups that revealed preventive effects against the secondary infection. On the contrary, the groups which were treated with *P. harmala* and *C. colocynthis* kept almost the same RBCs, HCT, and Hb levels as the negative control. The decrease in these parameters could be related to liver infection, hepatocyte destruction, and increased hemolysis or a disruption in the hemopoietin system, which slows the process of blood cells synthesis. Labsi et al [22] and Moraitaki et al [23] have also reported a similar decline in those blood parameters. Akhtar et al [24] related these significant decreases in blood parameters to the hydatid cyst fluid enzymes that cause destruction in the blood cells. On the other hand, the increasing trend of WBCs, lymphocytes, and eosinophils which has been seen in the infected groups could be considered as a defense mechanism against the inflammatory processes in the body, especially in the liver, spleen, and kidneys, where the inflammation induces the bone marrow to produce more WBCs. These findings are also in agreement with the results of Moraitaki et al [23], Khalaf [25], and Younus et al [26] who found a similar increasing trend of WBCs.

Plasma protein determination is usually used as predictive and diagnostic for liver disorders. The current study revealed a significant increase in serum total protein in the negative control, *C. colocynthis*, and *P. harmala* treatment groups. This could be attributed to the enhanced release of tissue-specific enzymes and other intracellular proteins as a result of parasite-induced cell membrane rupture. Similar results were recorded by Orhue et al [27], Akhtar et al [28], and Radfar et al [29]. Akhtar et al [28] attributed the elevation of protein concentration to the production of antibodies (globulin proteins) against antigens present in hydatid cyst fluid. Moreover, our study revealed a significant decrease in albumin levels all over infected groups. Albumin deficiency has been reported previously to appear in the late stages of liver disease, whereas it is not apparent in the early stages [30].

Concerning glucose levels, the drop in glucose level in the negative control group might be due to the effect of the parasitic infection on the liver which plays an important role in the glucose metabolism in the body. Khalaf [25] and Muhsin et al [31] also reported low levels of glucose in the intermediate hosts of *E. granulosus*.

The measurement of liver enzymes (ALP, ALT, and AST) is usually used to assess liver function. The results of this study showed a significant decrease among all treated groups compared to the negative control group,

except for *C. colocynthis* treatment group. The elevation in ALP, ALT and AST levels in the negative control explained that the hydatid liver infection led to hepatocyte destruction and enzyme release [32,33].

Kidney functions are usually assessed by measuring blood urea and creatinine. Decreased serum urea concentration in the negative control may be related to liver failure and reduced hepatic synthesis of urea from ammonia [25]. The creatinine levels were not affected by the infection and remained within the normal range. Therefore, it can be concluded that there is no adverse effect of the infection on kidney functions. Similarly, the blood lipids (cholesterol and triglycerides) were not affected by the infection and remained within the normal range. These results are not following the findings of Sura [34] who reported a significant increase in serum triglycerides and cholesterol levels in patients with hydatid cysts at the late stages of the disease.

Echinococcosis is known to promote two different cytokine secretion patterns: Th1 and Th2, and they usually down-regulate each other. In the early stage of infection, Th1 produces IL-2, IFN-γ, and lymphotoxin which are associated with the susceptibility to the disease, whereas, in the late and chronic infections, Th2 expresses IL-4, IL-5, IL-6, and IL-10 which are related to the protective immunity. IL-10 has an anti-inflammatory effect, so it suppresses cytokines production by Th1 [35]. Our results showed a significant increase in the cytokines profiles (IL-10, IL-4, and IFN-γ) of the negative control compared to the healthy control group. Rad et al [36], Bayraktar et al [37], and Zhang and McManus [6] reported a similar considerable increase in both Th1 and Th2 cytokines in hydatid cysts patients during the active stage of the disease. These findings could mean an enhanced immunological response when protoscolices grow and develop into a secondary infection, which probably increased cyst antigen stimulation and in turn cause more activation for the immune system. *R. graveolens* and ABZ treatment groups, which showed a good preventive effect, revealed a significant decrease in the Th2 cytokines (IL-4 and IL-10) compared to the negative control, whereas IFN-γ level was significantly increased. Those results are in agreement with Bayraktar et al [37] who reported that the patients who responded to chemotherapy had high levels of the Th1 cytokine IFN-γ whereas the Th2 cytokines (IL-4 and IL-10) were at low levels. This response may play an important role in the process of cyst degeneration [38]. On the other hand, *C. colocynthis* and *P. harmala* extract showed weak preventive effects against protoscolices development and this was reflected in the IL-4, IL-10, and IFN-γ cytokine levels, where a significant increase in IL-4 and IL-10 cytokine levels appeared, while the IFN-γ levels have no significant differences compared to the negative control. These results showed a clear association between IL-4, IL-10, and IFN-γ cytokine levels in the infected groups and the outcome of plant extracts treatment, as well supported by previous investigations.

5. Conclusion

This study shed light on the effects of *R. graveolens* extract as a protoscolicidal agent for the first time. These findings are encouraging the use of *R. graveolens* as a choice for developing novel anti-parasitic medicines in the future. The side effects of chemical constituents of *R. graveolens* on animals should be further investigated before being used as a medicine in humans. However, the *P. harmala* and *C. colocynthis* extracts showed low preventive effect against protoscolices development compared to that of *R. graveolens* extract.

6. Ethical approval

The animals were obtained from the animal house of Mutah University, and they aged 50 days and weighed between 150 to 200g. Ethical approval with reference number 49-2021 was obtained for the study.

7. References

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