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Effect of Egyptian propolis on cryptosporidiosis in immunosuppressed rats with special emphasis on oocysts shedding, leukogram, protein profile and ileum histopathology

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ABSTRACT

Objectives: To investigate the activity of Egyptian propolis extracts (ethanol and water) on cryptosporidiosis in experimentally infected dexamethasone-immunosuppressed rats.
Methods: A total of 180 male rats (190–220) g BWt were randomly divided into 9 equal groups (G1–G9). Groups of rats were kept as (G1): normal control, (G2–G9): immunosuppressed with dexamethasone and (G3-G9): infected with *Cryptosporidium* oocysts. Rats from (G4–G9) were given orally ethanol and water extract of propolis (at a dose of 50 mg/kg BWt) and nitazoxanide (standard anti-cryptosporidial drug at a dose of 100 mg/kg BWt) to infected rats with different regimes. Faecal pellets were collected from all groups to monitor oocysts shedding from the 2nd to the 15th day post infection. At the end of the experiment, blood was collected from all groups for determination of leukogram and serum proteins. Ileum specimens were also examined histopathologically.
Results: The highest reduction of oocysts shedding in faecal samples was 88% in rats prophylactically treated with propolis ethanol extract at the 4th dpi, and in rats prophylactically treated with water extract of propolis, was 91% at the 6th dpi. There was a marked increase in neutrophils count and α_2- and β-globulins levels in infected rats treated with both extracts, while a significant decrease was detected in lymphocytes compared to the infected non treated group. β-Globulin level markedly increased in the rats administered nitazoxanide. Histopathological changes were observed in the ileum of rats infected with *Cryptosporidium*.
Conclusions: Egyptian propolis extracts have an activity on cryptosporidiosis in rats. Moreover, propolis modulated the immunity in dexamethasone-immunosuppressed rats.

1. Introduction

The protozoan organisms of the genus *Cryptosporidium* are obligate, intracellular parasites that infect the epithelial cells lining the luminal surfaces of the digestive and respiratory tracts of a wide range of hosts [1,2]. Many outbreaks of human cryptosporidiosis have been attributed to contaminated water and food [3]. In immunodeficient individuals as people with acquired immune deficiency syndrome, chronic diarrhea may be resulted from cryptosporidiosis [4].

Effective therapy for cryptosporidiosis is limited [5], so the main mode of action remains preventive hygiene management. Despite the effectiveness of more than 200 drugs have been tested for their anti-cryptosporidium effects *in vitro* and *in vivo* [6,7], there is no specific chemotherapeutic intervention for cryptosporidiosis.

Propolis (bee glue) is a sticky dark colored material that honeybees collect from living plants, mix with wax, and use in construction and adaptation of their nests. It possesses anti-cryptosporidial [8], anti-protozoal [9], antiparasitic [10], antibacterial [11], antiviral [12], anti-inflammatory [13], antitumor [14], immunostimulant [15,16] and antioxidant [17] properties. The anti-cryptosporidial activity of different

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propolis extracts were previously studied *in vitro* [8]. There is no published scientific data are available on the anti-cryptosporidium effect of propolis *in vivo*.

This study was performed to evaluate the efficacy of propolis extracts ethanol (EEP) and water (WEP) in comparison with nitazoxanide as a commercial drug against cryptosporidiosis in immunosuppressed rat model based on oocysts shedding, leukogram, serum proteins, and pathological aspects.

2. Materials and methods

This study was carried out according to the guidelines for animal experimentation, and approved by the Institutional Animal Care and Use Committee, National Research Centre, Dokki, Giza, Egypt.

2.1. Egyptian propolis

Egyptian propolis sample was collected from beehives located in Dakahlia governorate, Egypt. The sample was kept in the dark and stored at −20 °C until ethanol and water extracts are processed.

Propolis extracts (ethanol & water) were prepared according to the method of Abd El-Aziz *et al.* [17]. Briefly, 50 g of crude propolis were grounded and extracted with 500 mL ethanol 80% (1:10 w/v) by shaking incubator (150 rpm) in the absence of light at room temperature for a week. The extract was then filtered and the supernatant was evaporated to dryness in a rotary evaporator (Heidolph 2000, Germany) under reduced pressure at 40 °C and stored at 4 °C. To prepare WEP, 45 g of crude propolis were finely grounded and extracted with 5 volumes of distilled water with shaking in the shaking incubator at 30 °C for 3 days. The extract was then filtered and the obtained supernatant was concentrated by freeze-dryer (Labconco Lyophilizer, USA). The obtained dried ethanolic and water extracts of propolis were re-suspended in phosphate buffered saline (pH 7.2). Both propolis extracts were administered orally at a dose of 50 mg/kg BWt.

2.2. Dexamethasone phosphate

It is a glucocorticoid synthetic material, used to induce immunosuppression in the rats to establish *Cryptosporidium* infection. After determination of the daily water consumption rate for rats, the dexamethasone phosphate (Fortecortin, Merck, Germany) was added in the drinking water at a dose of 0.25 mg/kg/day [18]. Throughout the experimental period except the normal control group (G1), rats were maintained on dexamethasone treatment.

2.3. Nitazoxanide

Nitazoxanide is a synthetic nitrothiazolyl–salicylamide derivative. It is used as a commercial standard drug for treatment of cryptosporidiosis. Nitazoxanide was suspended in 1% (w/v) dimethyl sulphoxide (DMSO) solution in water at a dose of 100 mg/kg BWt, once daily for five successive days. Nitazoxanide was supplied by Romark Laboratories, Tampa, Florida, USA.

2.4. Parasite

Oocysts of *Cryptosporidium* spp. were obtained from neonatal Holstein–Friesian calves (age; 3–15 days) suffering from diarrhea at a farm in Abu Rawash, Giza governorate, Egypt. Purification of oocysts from faecal samples using discontinuous sucrose gradients; the pelleted faecal solids were re-suspended in 2.5% potassium dichromate (w/v) then preserved at 4 °C. Preserved stool with potassium dichromate was passed through steel sieve to remove the large debris. After that, the sieved stool was applied to discontinuous sucrose gradients [19]. Sheather's solution was prepared, diluted with 0.025 M phosphate-buffered saline to produce two dilutions (1:2 and 1:4 solutions that had a specific gravity of 1.103 and 1.064, respectively) and supplemented with 1% Tween 80. Gradients were prepared in 50 mL polypropylene centrifuge tubes (Falcon) by layering by layering 10 mL of solution (1:4) over 10 mL of solution (1:2). Five ml aliquot of the sieved faeces in potassium dichromate was layered carefully over 1:4 solution and centrifuged at 4500 rpm for half an hour at 4 °C. The oocysts-enriched layer occupying the interface between the sucrose layers was transferred to new 50 mL polypropylene tubes. The oocysts-enriched layer was diluted with 20 mL (0.9% saline) and centrifuged. The pellets were pooled and washed with saline and centrifuged (at 4500 rpm, 10 min at 4 °C) then re-suspended in potassium dichromate (2.5%) then preserved at 4 °C.

Goat kids for maintenance and amplification of *Cryptosporidium* oocysts: At birth, two female Baldi goat kids (age; 2 days) were separated from their dams. During the first 24 h, kids were fed colostrum from their dam. Then the kids were fed a milk replacement twice daily *ad libitum*. At the 4th day of age, each kid was inoculated orally with 10^6 oocysts in 10 mL of water just before the kids drank milk. Freshly faecal samples were collected from the 1st dpi until no oocysts were detected in faeces to monitor the development of the parasite. Faecal smears were stained by a modified Ziehl–Neelsen technique and oocysts counts per 50 fields under oil immersion at 100× magnifications were scored. Following the onset of oocysts shedding, faeces were collected daily in screw-top containers, mixed with an equal volume of potassium dichromate (2.5%) and stored at 4 °C. These oocysts were purified by discontinuous sucrose gradients [19], counted using a hemacytometer under microscope (Model CX41, Olympus, Japan) and stored in potassium dichromate (2.5%) at 4 °C.

2.5. Animals used

A total of 180 adult male Sprague–Dawley rats (190–220) g BWt were used. Before the start of the experiment, faecal samples from all rats were examined by floatation concentration technique using saturated salt solution to insure that all rats are parasitic free. Other faecal smears were stained with modified Ziehl–Neelsen stain to insure the absence of *Cryptosporidium* infection. Rats were housed in plastic cages of 10 rats each, wire mesh tops, and wood shavings for bedding in well ventilated animal room under standardized conditions (20 ± 3) °C; relative humidity 50% ± 5% and 12 h light/dark cycle). All nutrients including water were supplied *ad libitum* to meet the requirements of the NRC [20]. Rats were acclimatized for 15 days before the start of the experiment.

2.6. Experimental plan

This experiment was performed at the Experimental rat Unit of Lab Animal House, National Research Centre, Dokki, Giza,

Egypt. One hundred and eighty male rats were randomly divided into 9 groups, each of 20 rats (10 rats/cage). Dexamethasone was administered in drinking water for 10 days in all groups of rats except rats in (G1). Groups of rats were kept as (G1): normal control, (G2): immunosuppressed and (G3): infected control with 4×10^6 *Cryptosporidium* spp. purified oocysts. Rats of (G4), (G5) and (G6) were infected with 4×10^6 *Cryptosporidium* spp. purified oocysts and were administered orally on 5th day-post infection (dpi) with nitazoxanide at a dose of 100 mg/kg BWt and 50 mg/kg BWt of EEP and WEP for 7 successive days, respectively. Groups; (G7), (G8) and (G9), infected rats were administered orally with nitazoxanide at a dose of 100 mg/kg BWt and 50 mg/kg BWt of EEP and WEP for three days before infection and then administered again the same dose on the 5th dpi for 7 successive days, respectively. The experimental plan was shown in Figure 1. Daily observations were performed for all groups to record the clinical signs and mortality rate.

2.7. Sampling

2.7.1. Faecal samples

Fresh faecal pellets were collected from rats from the 2nd to the 15th dpi to monitor oocysts shedding. Faecal smears were stained with a modified Ziehl–Neelsen stain [21]. Oocyst counts were scored per 50 fields of a faecal smear under oil immersion at ×100 magnification. The efficacies of propolis extracts and nitazoxanide were assessed by comparing the median numbers of oocysts present in treated versus infected control rats. Moreover, the reduction percentages in the median number of oocysts in treated versus infected control rats were calculated.

2.7.2. Collection and processing of blood samples

At the 15th dpi, two blood samples (0.5 mL each) were collected from retro-orbital plexus of veins of each rat. The first blood sample was collected in vacutainer tubes with ethylene diamine tetra acetic acid tripotassium (K3EDTA) and used for evaluation of the leukogram (Total and differential leukocytic counts) according to the method of Feldman *et al.* [22]. The second blood sample was placed in a plain centrifuge tube for serum separation. Sera were stored at −20 °C until further proteins analysis.

2.8. Analytical techniques

Total serum proteins concentrations were determined according to the method of Henary *et al.* [23]. Test kit was supplied by BioMérieux, France. Helena serum protein electrophoresis procedure was used for the separation and quantitation of serum proteins using cellulose acetate electrophoresis (Helena, France) [24].

2.9. Histopathological examinations

At the 15th dpi, rats were euthanized by over dose of chloroform. From each rat, ileum was rapidly removed and fixed in 10% formol saline for detection of histopathological alterations and *Cryptosporidium* developmental forms using haematoxylin and eosin stain [25].

2.10. Statistical analysis

Differences between infected control and treated groups were analyzed by Wilcoxon Mann–Whitney *U* test in the oocysts shedding. Statistical significance was tested at $\alpha = 0.05$ and 0.1 level [26]. Data of leukogram and serum proteins were statistical analysis using a one-way analysis of variance followed by Duncan's multiple range test. Differences were considered significant at $P < 0.05$ level [27] using SPSS version 16.0 computer program.

3. Results

3.1. Clinical signs and mortality rate

Dexamethasone caused a progressive loss of body weight and decreased in the activity of rats, while these symptoms improved in groups of rats treated by EEP and WEP. Faeces of infected rats were soft (loose) in consistency, but did not develop diarrhea.

The mortality rate among all treated groups of rats was lowered than infected non treated rats (45%) except for that observed in nitazoxanide treated group (55%). In rats treated with WEP (G6 & G9), the mortality rate was the lowest among all treated groups (30%).

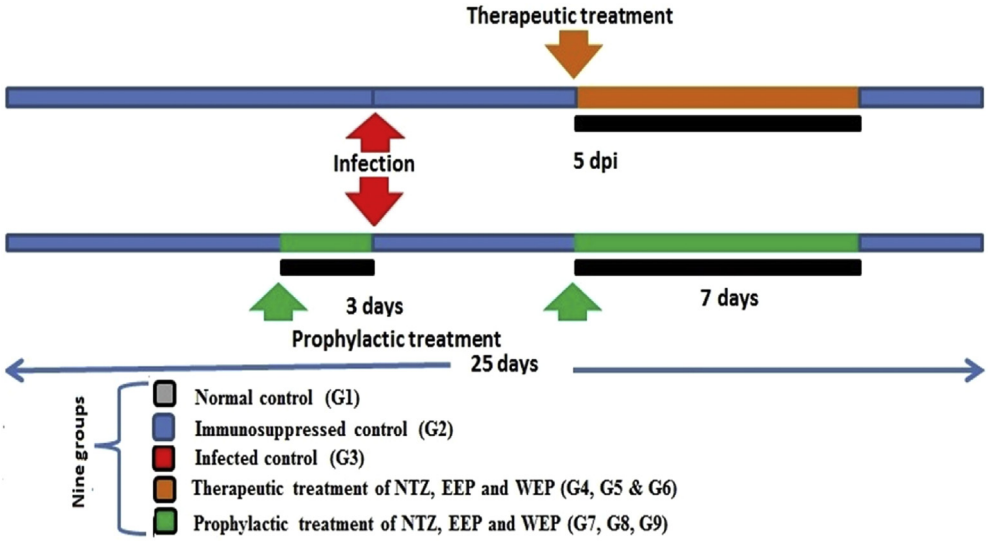

Figure 1. Experimental plan of therapeutic and prophylactic treatment with Egyptian propolis extracts [ethanol (EEP) and water (WEP)] and nitazoxanide (NTZ) in dexamethasone-immunosuppressed rats infected with *Cryptosporidium* spp.

3.2. Effects of propolis extracts (EEP & WEP) and nitazoxanide on Cryptosporidium oocysts shedding counts and reduction%

The oocysts shedding in the infected (G3) increased progressively from the 2nd to 8th dpi and peaked at the 6th dpi. From the 9th to 15th dpi, there was no statistical difference in median oocysts count between any groups of rats except for that achieved in EEP-treated rats (G5) at the 13th and 15th dpi. So, the median oocysts count of all treated groups was compared to infected group only at the 2nd, 4th, 6th, 7th and 8th dpi (Figure 2 and 3). In rats therapeutically treated with EEP (G5) and WEP (G6), a significant reduction in oocysts count was observed at the 2nd, 6th, 7th and 8th dpi. The maximal reduction reached 89% and 75% at the 7th dpi, respectively (Figure 2). There was marked reduction in oocysts at the 4th, 6th and 7th dpi in both groups of rats (G8 & G9) prophylactically treated with EEP and WEP. The highest reduction of oocysts shedding reached to 88% in group of rats (G8) treated prophylactically with EEP at the 4th dpi and it reached to 91% in rats prophylactically treated with WEP (G9) at the 6th dpi (Figure 3).

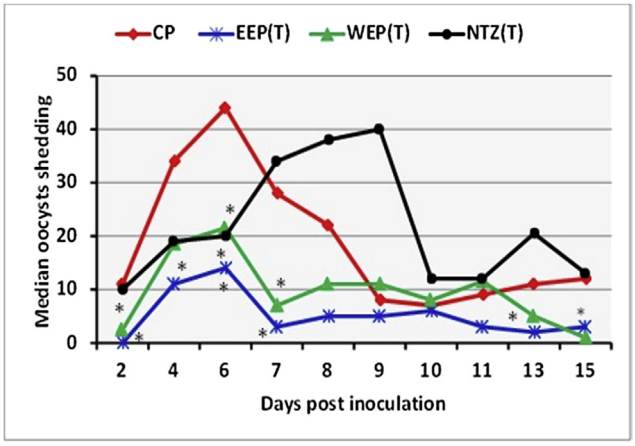

Figure 2. Effect of propolis extracts [ethanol (EEP) and water (WEP)] and nitazoxanide (NTZ) – as a therapeutic treatment – on oocysts shedding in dexamethasone-immunosuppressed rats infected with *Cryptosporidium* spp. compared to infected non treated rats (control positive: CP). *$P < 0.05$ compared with infected non treated.

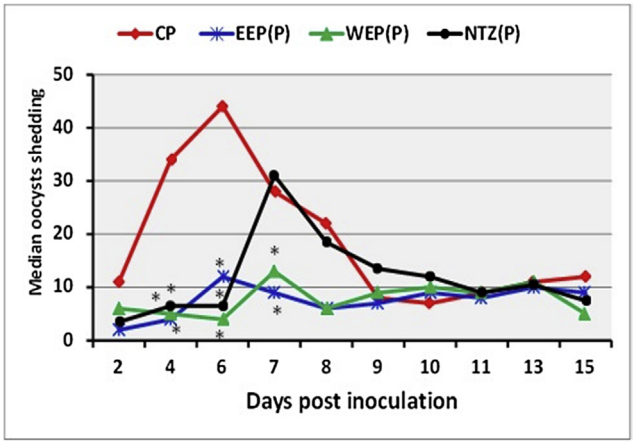

Figure 3. Effect of propolis extracts [ethanol (EEP) and water (WEP)] and nitazoxanide (NTZ) – as a prophylactic treatment – on oocysts shedding in dexamethasone-immunosuppressed rats infected with *Cryptosporidium* spp. compared to infected non treated rats (control positive, CP). *$P < 0.05$ compared with infected non treated.

In rats therapeutically treated with nitazoxanide (G4), there was no statistical difference in the reduction of median oocysts count except for that at the 6th dpi (55%) compared to the infected rats (G3) (Figure 2). In contrast, rats prophylactically treated with nitazoxanide (G7) shed oocysts at significantly lower levels (81% and 85%) than infected rats (G3) only at the 4th and 6th dpi, respectively (Figure 3).

3.3. Leukogram

Results of Leukogram are shown in Table 1.

Rats of G2 administrated dexamethasone showed marked decrease in the total leukocytic count (TLC) and lymphocytes and eosinophils counts, while the neutrophils count was significantly increased compared to normal control rats (G1). The infected group (G3) showed a significant decrease in absolute neutrophils and basophils counts while absolute lymphocytes and monocytes counts markedly increased in comparison with dexamethasone treated rats (G2). Rats treated with nitazoxanide (G4 & G7) showed a significant increase in absolute neutrophils count and a significant decrease in absolute lymphocytes and monocytes. There was a trend of increase in TLC in all groups treated with propolis. This increase was significant in rats treated prophylactically with EEP (G8) and WEP (G9) compared to the infected group (G3). In all groups treated with propolis, a marked increase in absolute neutrophils counts associated with significant decrease in absolute lymphocytes count and no significant differences in absolute eosinophils, basophils, and monocytes counts were recorded in comparison with the infected group (G3).

3.4. Serum proteins profile

Results of serum proteins are shown in Table 2.

Serum total proteins, albumin, and globulins concentrations significantly decreased, while no significant difference was observed in (A/G) ratio in dexamethasone treated rats (G2) than the normal group (G1). In infected rats (3), total proteins, albumin, and globulins markedly increased with no statistical changes in A/G ratio in comparison with dexamethasone treated rats (G2). Group of rats administered dexamethasone (G2) showed a significant decrease in all globulin fractions compared to the normal control (G1) except for that noticed in α_2-globulin level. Infected rats (G3) showed an increase in the level of α_1-, α_2- and β-globulins compared to dexamethasone group (G2), while γ-globulin level was decreased. These increases were statistically significant only in the values of α_1-globulin. Rats administered nitazoxanide (G7) showed marked increase in globulins (α_2-, β- and γ-) level and a marked decrease in albumin concentration and A/G ratio compared to infected group (G3). On the other hand, β-globulin significantly elevated in nitazoxanide treated rats (G4). In all groups of rats treated with propolis (G5, G6, G8 & G9), the total proteins level increased than the infected control (G3). This increase was not statistically significant in group (G8). Although, a significant decrease was recorded in albumin level of rats treated with EEP (G5 & G8), the albumin level showed no significant changes in WEP-treated rats (G6 & G9). Moreover, in all groups of rats treated with propolis, there was a significant decrease in A/G ratio while globulins level increased than infected control (G3). All globulin fractions significantly increased in rats therapeutically treated with EEP (G5). But, α_2- and β-globulin levels significantly elevated in

Table 1

Total and differential leukocytic counts in different experimental groups of dexamethasone-immunosuppressed rats treated with nitazoxanide, ethanol extract of propolis and water extract of propolis therapeutic and prophylactic treatment of *Cryptosporidium* spp.

Groups	Total leukocytic counts	Differential leukocytic counts				
		Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
G1	11.26 ± 0.89^{b}	3.05 ± 0.28^{a}	0.56 ± 0.07^{c}	0.04 ± 0.04^{ab}	7.12 ± 0.59^{e}	0.48 ± 0.14^{bc}
G2	7.63 ± 0.65^{a}	5.97 ± 0.60^{c}	0.12 ± 0.02^{b}	0.07 ± 0.03^{b}	1.20 ± 0.09^{ab}	0.29 ± 0.09^{ab}
G3	8.82 ± 0.24^{a}	4.39 ± 0.28^{b}	0.04 ± 0.02^{ab}	0.00 ± 0.00^{a}	3.68 ± 0.13^{d}	0.71 ± 0.04^{cd}
G4	8.11 ± 0.14^{a}	7.46 ± 0.14^{d}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.61 ± 0.06^{a}	0.04 ± 0.03^{a}
G5	8.88 ± 0.18^{a}	7.31 ± 0.10^{cd}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.45 ± 0.15^{b}	0.12 ± 0.09^{a}
G6	9.31 ± 0.63^{a}	6.14 ± 0.48^{c}	0.04 ± 0.02^{ab}	0.00 ± 0.00^{a}	2.76 ± 0.12^{c}	0.38 ± 0.15^{abc}
G7	7.99 ± 0.59^{a}	6.08 ± 0.54^{c}	0.04 ± 0.03^{ab}	0.04 ± 0.02^{ab}	1.56 ± 0.11^{b}	0.26 ± 0.07^{ab}
G8	11.03 ± 0.66^{b}	9.50 ± 0.35^{e}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.98 ± 0.12^{ab}	0.55 ± 0.21^{bcd}
G9	12.67 ± 0.69^{b}	11.18 ± 0.73^{f}	0.01 ± 0.01^{a}	0.00 ± 0.00^{a}	0.61 ± 0.03^{a}	0.85 ± 0.06^{d}

Numbers are counted as $\times 10^3/\mu L$. Means with different superscripts in the same column are significantly different at $P < 0.05$. Groups of rats (G1–G9); G1: normal control, (G2–G9): immunosuppressed with dexamethasone, (G3–G9): infected with *Cryptosporidium* oocysts, (G4, G5 and G6): were given orally therapeutic treatment with nitazoxanide (at a dose of 100 mg/kg BWt), ethanol and water extracts of propolis (at a dose of 50 mg/kg BWt), respectively. (G7, G8 and G9): were given orally prophylactic treatment with nitazoxanide, ethanol and water extracts of propolis as a same dose in therapeutic treatment, respectively.

Table 2

Serum proteins profile in different experimental groups of dexamethasone-immunosuppressed rats treated with nitazoxanide, ethanol extract of propolis and water extract of propolis therapeutic and prophylactic treatment of *Cryptosporidium* spp. (g/dl).

Groups	Total proteins	Albumin	Total globulins	Albumin/Globulin ratio	Globulin fractions			
					α_1-	α_2-	β-	γ-
G1	8.18 ± 0.08^{b}	4.10 ± 0.12^{b}	4.06 ± 0.10^{bc}	1.01 ± 0.06^{c}	0.92 ± 0.07^{b}	0.60 ± 0.06^{a}	1.62 ± 0.04^{bc}	0.92 ± 0.06^{e}
G2	6.48 ± 0.19^{a}	3.42 ± 0.10^{a}	3.02 ± 0.14^{a}	1.14 ± 0.06^{c}	0.36 ± 0.02^{a}	0.70 ± 0.07^{ab}	1.40 ± 0.05^{a}	0.56 ± 0.02^{bcd}
G3	8.12 ± 0.21^{b}	4.36 ± 0.13^{bc}	3.76 ± 0.20^{b}	1.17 ± 0.07^{c}	1.14 ± 0.09^{bcd}	0.68 ± 0.06^{a}	1.52 ± 0.08^{ab}	0.42 ± 0.05^{ab}
G4	8.29 ± 0.11^{bc}	4.45 ± 0.21^{b}	3.84 ± 0.15^{b}	1.19 ± 0.11^{c}	1.04 ± 0.05^{bc}	0.68 ± 0.07^{a}	1.70 ± 0.12^{cd}	0.40 ± 0.03^{a}
G5	8.94 ± 0.10^{d}	3.36 ± 0.10^{a}	5.58 ± 0.09^{e}	0.60 ± 0.02^{a}	1.56 ± 0.07^{e}	1.62 ± 0.04^{e}	1.84 ± 0.02^{def}	0.56 ± 0.04^{bcd}
G6	8.74 ± 0.20^{cd}	4.40 ± 0.16^{b}	4.34 ± 0.05^{c}	1.01 ± 0.03^{c}	0.96 ± 0.04^{b}	0.88 ± 0.07^{b}	2.04 ± 0.06^{f}	0.46 ± 0.04^{abc}
G7	8.45 ± 0.16^{bc}	3.19 ± 0.10^{a}	5.26 ± 0.15^{de}	0.61 ± 0.02^{a}	1.24 ± 0.07^{cd}	1.60 ± 0.10^{e}	1.80 ± 0.08^{cde}	0.60 ± 0.06^{cd}
G8	8.60 ± 0.15^{bcd}	3.48 ± 0.22^{a}	5.08 ± 0.07^{d}	0.69 ± 0.05^{ab}	1.32 ± 0.12^{d}	1.40 ± 0.03^{d}	1.84 ± 0.05^{def}	0.52 ± 0.04^{abcd}
G9	9.00 ± 0.17^{d}	4.06 ± 0.16^{b}	4.94 ± 0.14^{d}	0.82 ± 0.05^{b}	1.22 ± 0.09^{cd}	1.10 ± 0.07^{c}	1.96 ± 0.04^{ef}	0.66 ± 0.04^{d}

Means with different superscripts in the same column are significantly different at $P < 0.05$. Groups of rats (G1–G9); G1: normal control, (G2–G9): immunosuppressed with dexamethasone, (G3–G9): infected with *Cryptosporidium* oocysts, (G4, G5 and G6): were given orally therapeutic treatment with nitazoxanide (at a dose of 100 mg/kg BWt), ethanol and water extracts of propolis (at a dose of 50 mg/kg BWt), respectively. (G7, G8 and G9): were given orally prophylactic treatment with nitazoxanide, ethanol and water extracts of propolis as the same dose in therapeutic treatment, respectively.

groups (G8 and G9). The highest increase in γ-globulin level was achieved in rats treated with WEP (G9).

3.5. Histopathological findings in ileum

Histopathological findings in the ileum tissue of rats from different experimental groups are illustrated in Figures 4–6.

In the normal control rats (G1), the histology of the ileum tissues was within the normal limit (villi, submucosa, inner and outer muscularis layers and serosa) (Figure 4a). In dexamethasone treated rats (G2), the ileum showed sub-epithelial cell edema, atrophy and sloughing of the upper tips of some villi with infiltration of inflammatory cells (Figure 4b). In rats infected with *Cryptosporidium* spp. (G3), the ileum showed thickening and flat of the villi, degeneration, atrophy, and necrosis with sloughing of the upper tips of the villi, inflammatory cellular infiltration in the lamina properia and submucosa with edema in the inner circular and the outer longitudinal muscle (Figure 4c). Different developmental stages of *Cryptosporidium* spp. were detected at the brush border of the epithelial cells and crypt of Lieberkühn which showed degenerative changes (Figure 4d).

In rats treated with nitazoxanide (G4), the ileum showed a shortage of the villi, hyperplasia of goblet cells (Figure 5a) and few *Cryptosporidium* spp. in different developmental stages at the brush border of epithelial cells of the villi (Figure 5b). The ileum of rats treated with EEP (G5) showed necrosis and sloughing of the upper portion of the villi, dilatation of the blood capillary, edema and inflammatory cellular infiltration in the submucosa (Figure 5c), in addition to presence of faint staining very few *Cryptosporidium* spp. at the brush border of epithelial cells of the villi (Figure 5d). The ileum of rats treated with WEP (G6) showed shortage and flat of the villi with sub-epithelial inflammatory cellular infiltration with edema in the upper tip of the villi with very few *Cryptosporidium* spp. at the brush border of epithelial cells of the villi (Figure 6a). In rats treated with nitazoxanide (G7), inflammatory cellular infiltration and some epithelial edema were seen in the ileum (Figure 6b). The ileum of rats treated with EEP (G8) showed shortage and thickening of the villi, hyperplasia of goblet cells, dilatation of the blood capillary, edema and inflammatory cellular infiltration in the lamina properia and submucosa with edema in inner circular and outer longitudinal muscle (Figure 6c). Few *Cryptosporidium* spp. were detected at the brush border of the epithelial cells of the villi. The ileum of rats treated with WEP (G9) showed shortage, thickening and sloughing of the villi, with sub-epithelial inflammatory cells and edema in the upper tip of the villi and edema in the inner circular and outer longitudinal

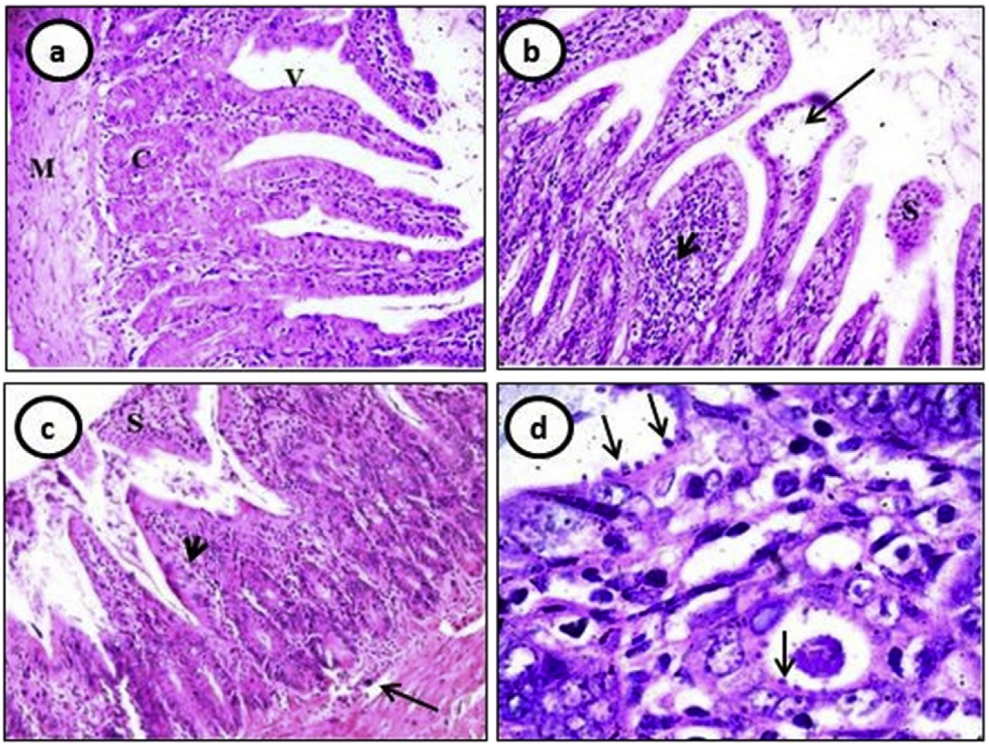

Figure 4. Ileum histopathological changes in different experimental rat groups.
(a): Group 1 (normal control) showing normal histological structure villi (V), crypt of Lieberkühn (C) and muscular layers (M) (H&E ×200), (b): Group 2 (dexamethasone) showing sub-epithelial cell edema (arrow), atrophy and sloughing of the upper tips of some villi (S) with infiltration of inflammatory cells (arrowhead) (H&E ×200), (c): Group 3 (infected) showing thickening and flat of the villi, degeneration, atrophy, necrosis with sloughing of the upper tips of the villi (S), inflammatory cells infiltration in submucosa and lamina properia (arrowhead) and edema in inner circular and outer longitudinal muscle (arrow) (H&E ×200), (d): Group 3 showing different developmental stages of *Cryptosporidium* spp. at the brush border of the epithelial cells of villi and crypt of Lieberkühn (arrow) (H&E ×1 000).

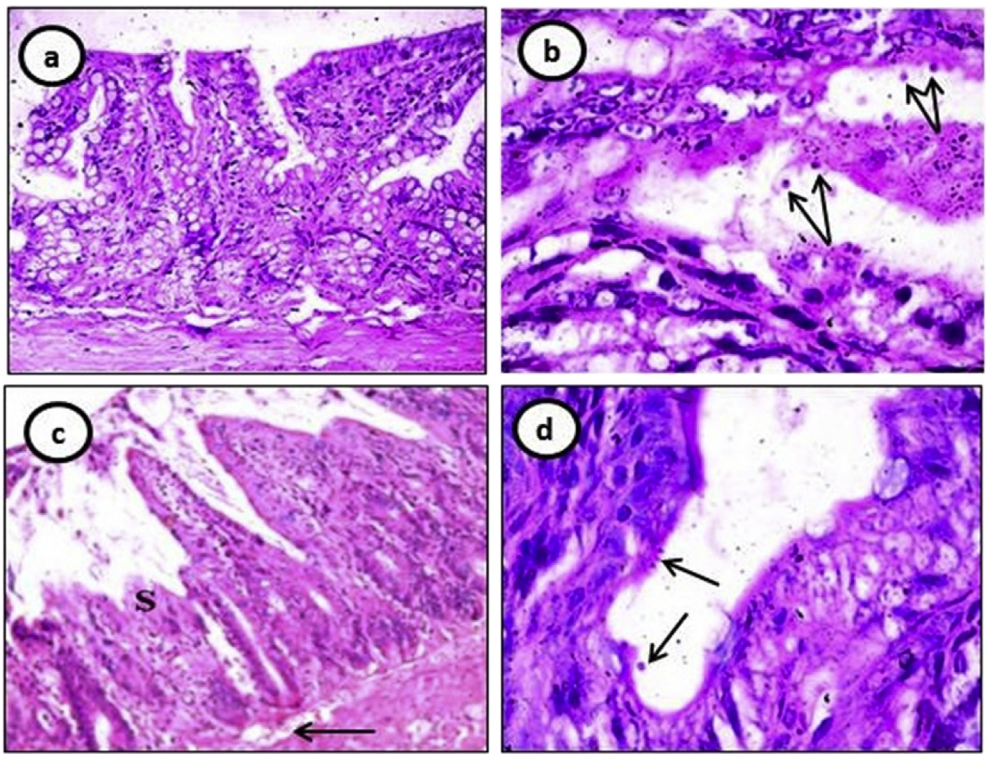

Figure 5. Ileum histopathological changes in different experimental rat groups.
(a): Group 4 (nitazoxanide therapeutic) showing shortage of the villi and hyperplasia of goblet cells (arrow) (H&E ×200), (b): Group 4 showing presence of few parasites of *Cryptosporidium* spp. in different developmental stages at the brush border of epithelial cells of the villi (arrow) (H&E ×1 000), (c): Group 5 (propolis ethanol extract therapeutic) showing necrosis and sloughing of the upper portion of the villi (S), dilatation of the blood capillary, edema (arrow) and inflammatory cells infiltration in the submucosa (arrowhead) (H&E ×200), (d) Group 5 showing presence of faint staining very few parasites of *Cryptosporidium* spp. at the brush border of epithelial cells of the villi (arrow) (H&E ×1 000).

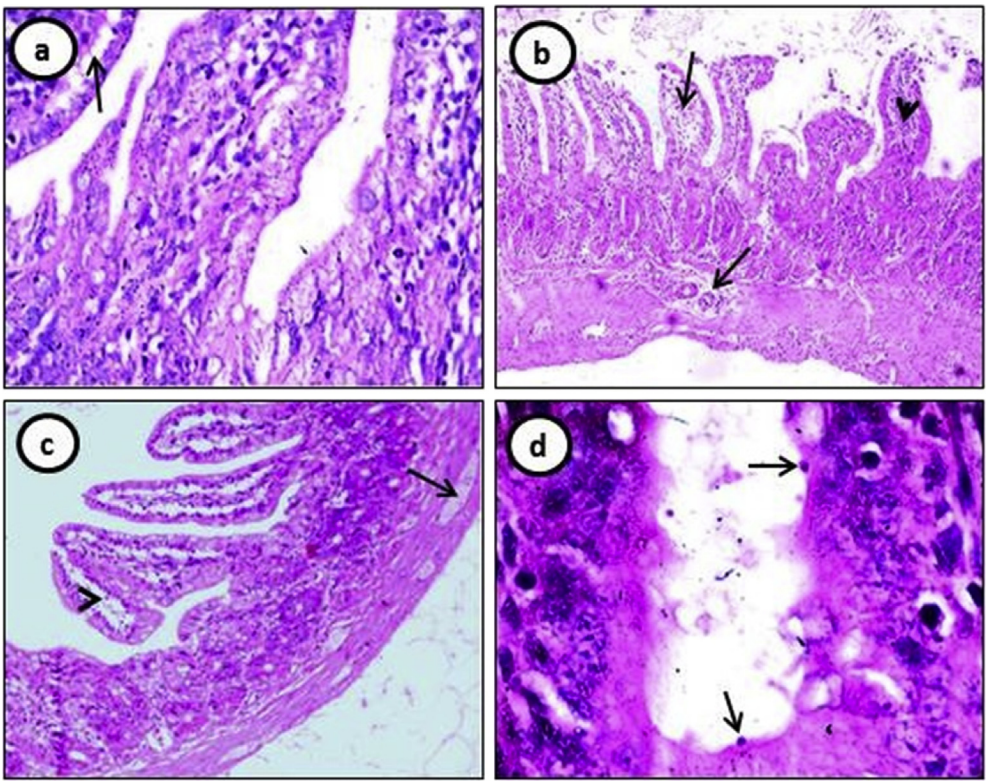

Figure 6. Ileum histopathological changes in different experimental rat groups.
(a): Group 6 (propolis water extract therapeutic) showing edema (arrow) and inflammatory cells infiltration in the submucosa (arrowhead) (H&E ×400), (b): Group 7 (nitazoxanide prophylactic) showing inflammatory cells infiltration (arrowhead) and edema (arrow) (H&E ×100), (c): Group 8 (propolis ethanol extract prophylactic) showing shortage and thickening of the villi, hyperplasia of goblet cells, dilatation of the blood capillary, edema (arrow) and inflammatory cells infiltration in submucosa and lamina properia (arrowhead) and edema in inner circular and outer longitudinal muscle (H&E ×200), (d): Group 9 (Propolis water extract prophylactic) showing few *Cryptosporidium* spp. at the brush border of the epithelial cells of the villi (arrow) (H&E ×1 000).

muscle. Very few *Cryptosporidium* spp. of different developmental stages were detected at the brush border of epithelial cells of intestinal villi (Figure 6d).

4. Discussion

This study showed that the prophylactic and therapeutic administration of Egyptian propolis (EEP & WEP) at a dose of 50 mg/kg BWt to immunosuppressed rats infected with *Cryptosporidium* spp. altered the oocysts shedding pattern compared to the infected control. The highest reduction of oocysts shedding reached to 88% in group of rats (G8) treated prophylactically with EEP at the 4th dpi and it reached to 91% in rats prophylactically treated with WEP (G9) at the 6th dpi. Although both propolis extracts markedly reduced the oocysts shedding, they failed to inhibit it completely. Moreover, EEP and WEP exerted more efficacies in reducing oocysts excretion when given 3 days before the infection and then for 7 successive days. The activity of Egyptian propolis against *Cryptosporidium* may be due to their rich content of flavonoids, caffeate esters and triterpenoids including lupeol and alpha-amyrin, aliphatic acids, aromatic acids and their esters [28] and possibly the presence of amyrins [29]. The antiparasitic effect of EEP and WEP may be due to its phenolic compounds, which have been reported to be responsible for its anti-protozoal effect through enhancement of oxidative defense mechanisms [30]. Phenolic compounds from different plant origins are the major antioxidants of propolis [31]. Since, the phytochemical constituents of some plants which are the main sources of propolis have been proved to have anticryptosporidial effects [32–34] and reduction of fecal oocysts shedding [35]. Therefore, the anticryptosporidial effects of propolis could be attributed to the phytochemical constituents' particularly phenolic compounds. Furthermore, propolis can stimulate the immune system of rabbits [15] and rats [12] leading to increase the antibody titers which positively correlated with the reduction of oocysts shedding [36].

On the other hand, the results revealed that nitazoxanide administered therapeutically or prophylactically at 100 mg/kg BWt did not achieve any efficacies except for that recorded at the 4th and 6th dpi. The same results reported by Theodos *et al.* [6] who mentioned that there was no statistical differences in the log of oocysts shedding between groups of mice treated with nitazoxanide for 10 days at a dose of 100 or 200 mg/kg BWt and placebo control group. In contrast, different formulations of nitazoxanide at a dose of 100 mg/kg BWt for six days caused reduction in oocysts excretion between 26% and 42% in neonatal mouse [37]. Similarly, a significant reduction in oocysts excretion was obtained in immunosuppressed rat that administered nitazoxanide for seven days at doses from 50 to 200 mg/kg BWt [38] and in immunosuppressed gerbil at a dose of 200 mg/kg BWt for 12 days [39]. Nitazoxanide decreased oocysts shedding and reduced the severity of cryptosporidiosis infection in the experimentally challenged with goat kids [40]. Moreover, Ollivett *et al.* [41] revealed that by the end of the observation period, 85% of the nitazoxanide treated calves stopped oocysts shedding, whereas only 15% of control group stopped shedding. These discrepancies may be at least partially explained by different drug formulations, different animal models used and doses and regime of administration.

The mortality rate in rats treated with WEP was relatively low (30%). This record was correlated with Dimov *et al.* [42] who found that administration of water-soluble derivative of propolis to mice at a dose 50 mg/kg BWt prevented the effects of immunosuppressive drug (cyclophosphamide) and the survival rate of the animals was enhanced. No reduction in mortality rates was detected in nitazoxanide treated rats. Such findings were also observed in goat neonates treated with nitazoxanide at doses of 100 and 200 mg/kg BWt [40].

The present study showed that leukopenia, lymphopenia, and neutrophilia were observed after dexamethasone administration. This data was in accordance with the finding of Vishwas *et al.* [43]. The suppression of TLC recorded in rats resulting from the exogenous corticosteroid treatment which alters the distribution and trapped white blood cells in the bone marrow [22]. It also could be assumed that dexamethasone induced apoptosis in lymphocytes [44]. The dramatic increase in neutrophils count following dexamethasone treatment suggested that dexamethasone decreases the permeability of the vascular endothelium lead to accumulate the neutrophils in the circulation [45]. In rats, infections are associated with neutrophilia and lymphopenia [22,45] which confirmed with the current study. The present findings showed that administration of propolis caused an elevation in TLC values due to immunostimulant effect of propolis [15].

The present data showed that albumin level significant dropped in dexamethasone treated rats compared to normal control ones (G1). Indeed, the total proteins level decreased as a consequence of albumin reduction which constitutes the most homogenous fraction, comprising 35%–50% of total serum proteins in rodents [46]. Although glucocorticoid exerted a catabolic effect on peripheral tissues, an increase of hepatic proteins synthesis in response to short-term treatment of glucocorticoids was recorded [47]. In contrary, after long-term administration with glucocorticoid, the rate of hepatic proteins synthesis was gradually suppressed [48].

The present study showed that the total proteins, albumin and total globulins levels markedly increased in rats infected with *Cryptosporidium* spp. The increase in α_1-globulin may be due to the increase level of one or more of their main individual proteins such as α_1-acid glycoprotein, serum amyloid A and serum amyloid P which were confirmed to be elevated during infection [49] and inflammation [50]. The level of γ-globulin was significantly reduced while the level of β-fraction of globulin was a non-significant increase. This finding may have been associated with the presence of high concentration of IgG in β_2-globulin fraction [51]. In general, it could be stated that all globulin fractions were enhanced after administration of EEP and WEP either therapeutically or prophylactically which may be due to stimulation of cytokines production (Interleukin-1β and Tumor necrosis factor-α) by peritoneal macrophages [52].

Histopathological examination of the ileum revealed that dexamethasone treated alone induced a moderate increase in the number of goblet cell and atrophy and thickening of the mucosa. These findings are consistent with earlier studies [53]. Moreover, the developmental stages of the parasite and histopathological changes were detected in ileum tissue of untreated infected rats (G3). Shortage, atrophy, fusion and flatting of the villi with sub-epithelial inflammatory cells and edema in the upper tip of the villi were previously observed in the ileum tissues of infected rats [54]. These pathological changes were attributed to *Cryptosporidium* displacing brush borders causing asymmetrical loss of epithelial cells resulting in shortening and fusing of the villi. Besides, atrophy of the villi observed during cryptosporidiosis may be due to toxins of *Cryptosporidium* that damage the epithelial cells [55]. During *Cryptosporidium* infection, T-lymphocyte, macrophages and polymorphonuclear cells migrate to lamina properia as a part of the host defense mechanisms against this parasite [56]. These alterations accompanied with inflammation of the mucus membrane lining the intestine and malabsorption induces a change in the transmission of water and electrolytes in the intestine [57]. After propolis extracts (ethanol & water) treatments, there was a marked decrease in the number of *Cryptosporidium* oocysts on the brush border of epithelial cells. Both propolis extracts failed to restore the symmetrical architecture of ileal villi and mucosa. The increased number of goblet cells after propolis and nitazoxanide treatment indicates that the immunity increased in the mucosa of intestine. These cells play an important role in the anti-microbial antibodies production [58].

In rats infected with *Cryptosporidium* oocysts, different developmental stages of *Cryptosporidium* spp. were found at the brush border of the epithelial cells. Histopathological alterations of *Cryptosporidium*-infected small intestine included atrophy of the villi, crypt hyperplasia, inflammatory cells infiltration in the lamina properia, glucose malabsorption, chloride secretion, and a reduced barrier function due to increase paracellular permeability [59].

Egyptian propolis extracts have moderate effect on cryptosporidiosis in dexamethasone-immunosuppressed rat model as evidenced by reduced oocysts shedding. Propolis water extract exerted more efficacy than propolis ethanol extract. Both propolis extracts modulated the leukogram, serum proteins and histopathological changes in ileum resulted from the side effects of both dexamethasone administration and *Cryptosporidium* infection.

Conflict of interest statement

There is no conflict of interests as declared by the authors.

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