



RESEARCH ARTICLE

Evaluation of the therapeutic effect of propolis on *Fasciola gigantica* and *Clostridium novyi* type B infections in sheep

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ABSTRACT

The use of natural products for disease control is a promising approach to solving the problem of drug resistance. The aim of the research reported here was to evaluate the fasciolicidal and anti-*Clostridium novyi* type B activities of propolis administered orally to sheep infected with *Fasciola gigantica* and *C. novyi* type B. Sheep infected with both pathogens were divided into two groups: an infected treated group and an infected non-treated group. The treatment was oral administration of 50 mg propolis extract/kg daily for 15 days. The body weight of the sheep, fecal egg counts of *F. gigantica*, serum levels of *F. gigantica* IgG, concentrations of cytokines (IL-2, IL-10, and IL-17), and bacterial counts of *C. novyi* were evaluated. Following treatment, the sheep had increased body weight and a significant decrease in the egg count, which was reduced by 54.54% at 15 days post treatment. The level of anti-*Fasciola* IgG increased, whereas levels of IL-2, IL-10, and IL-17 decreased in propolis-treated sheep. Treatment of sheep with propolis produced a significant reduction in fecal count of *C. novyi*, from 8×10^9 to 3×10^3 colony units per gram at 15 days post treatment. This research highlights the therapeutic potential of Egyptian propolis extract as a treatment against *F. gigantica* and *C. novyi* type B infections, and investigated its mode of action through its effect on some cellular and humoral responses in sheep with both infections.

Keywords: Propolis; *Fasciola gigantica*; *Clostridium novyi*; cytokines.

INTRODUCTION

Fasciola is a trematode parasite with a worldwide distribution. It is responsible for a considerable amount of disease, and production losses which are estimated to amount to more than three billion dollars per year (Keiser & Utzinger, 2009) in farm animals in developing countries. The World Health Organization (2015) considers ruminants to be the main source and reservoir of fasciolosis for humans. The WHO (2017) identified fasciolosis as a re-emerging neglected tropical disease, associated with endemic and epidemic outbreaks in human populations. Fifty million people worldwide have been affected with fasciolosis. *Fasciola* plays an important role in the infection of animals, and is considered to be a stress factor, which facilitates infection with *Clostridium novyi* type B (El Shanawany *et al.*, 2019). Immature *Fasciola* flukes produce anaerobic necrotic lesions in the liver, which provide a suitable environment for the germination of spores of *C. novyi* type B in the liver. During *Fasciola* invasion of the liver, *C. novyi* releases toxins

into the blood stream, causing necrotic hepatitis (INH), which leads to local necrosis and significant damage to the microvascular system, causing subcutaneous bleeding and blackening of the skin, a condition known as black disease (Navarro & Uzal, 2016; Boulianne *et al.*, 2020). *Clostridia* infection progresses rapidly, and has a high mortality rate. Previously, the main treatment of fasciolosis was the oral flukicide Triclabendazole, which is effective and widely used. However, recent studies have confirmed the development of resistance against this drug (Hardi *et al.*, 2019). Smith (2014) showed that *C. novyi* is highly sensitive to penicillin and tetracyclines, but antibiotic treatment is rarely effective, due to the acute course of the disease and the large amount of toxin produced in most infected animals. Therefore, many authors have used alternative medicines, such as plant extracts like *Moringa oleifera*, which is used as an antihelmintic and antibacterial (El-Kholy *et al.*, 2018; El Shanawany *et al.*, 2019), or natural products, such as bee-like propolis has been used in medicine for a long time in many countries (Pasupuleti *et al.*, 2017). Propolis successfully

suppresses the multiplication of intracellular pathogens; it has anti-protozoal efficacy (Asfaram *et al.*, 2020). Propolis also acts as a hepatoprotective (Wagh, 2013) and antioxidant (Kocot *et al.*, 2018). Propolis contains more than 300 synergistic compounds (Hegazi & Abd El Hady, 2001; Anjum *et al.*, 2019) which increase the antibacterial activity of propolis (Przybytek & Karpinski, 2019). It also has anti-inflammatory (Franchin *et al.*, 2017) and anti-viral (Sforcin, 2016) effects, and has potent fasciolicidal activity (Hoste *et al.*, 2006; Hegazi *et al.*, 2007a, 2007b).

An alternative safe and effective drug for fasciolosis and *Clostridia* infection is urgently needed. The objective of the current study was the assessment of the therapeutic effect of propolis on natural *Fasciola gigantica* and *C. novyi* infections in sheep, and to introduce a safe, efficacious natural product as an alternative or complementary medicine.

MATERIALS AND METHODS

Ethical approval

The protocol for this study, and the method for collecting and using the infected blood, were certified by the Ethics Committee as following the institutional guidelines of the National Research Centre's Animal Research under approval protocol No. 16/219.

Propolis extraction

Propolis was collected from an apiary in a farm near El-Mansoura City, Dakahlia, Egypt. It was kept in a refrigerator in a lightproof bag until use. Propolis ethanol extract was prepared according to a procedure published by Trusheva *et al.* (2007). After extraction, each 500 mg of propolis produced 50 mg dry matter extract.

Preparation of crude *F. gigantica* antigen

Adult *F. gigantica* worms were collected from the livers of naturally infected buffaloes, which were slaughtered at El Monib abattoir, Giza Governorate. The antigen was prepared as described by Oldham (1983). The protein content of the prepared antigen was estimated using the method described by Lowery *et al.* (1951). Bovine serum albumin was used as the standard, and 10% sodium carbonate in 0.5 N sodium hydroxide, 0.5 gm copper sulfate in 1% sodium potassium tartrate, and folin-ciocalteau reagent (Fisher scientific, Co., USA) was used as a protein reagent. The optical density of the protein sample was measured at 720 nm.

Treatment protocol

Fifty-five sheep reared in the Nile river basin were used in this research. Their ages ranged from 6 to 12 months. Of the 55 animals, 8 sheep (14.54%) were infected. They were divided into two groups; group 1 contained the control infected but untreated animals, and group 2 was treated with a dose of 50 mg propolis extract/kg (Soufy *et al.*, 2017). The treatment was 0.5 gm raw propolis/kg, which contained 50 mg ethanol extract, administered orally once per day for 15 days. Feces were collected from each animal daily. Each fecal sample was divided into two parts. One part was examined for the *Fasciola* egg count per gram, and the other one was used for the examination of bacteria, to estimate *C. novyi* colony units per gram (CFU/g). Blood samples were collected by jugular venipuncture on days 0, 3, 9, and 15. The sera were separated and stored at -20°C until they were analyzed.

1-Evaluation of therapeutic efficacy and immune responses of propolis

a-Clinical examination of animals

Thorough clinical examination was conducted according to the methods of Radostits *et al.* (2006). Treated and non-treated sheep were weighed at day zero and re-weighed at 15 dpt.

b-Parasitological parameters

Counting of *Fasciola* eggs

For each individual fecal sample, one gram of feces was mixed with 45 ml of water in a graduated cylinder. The mixture was filtered, and then allowed to sediment. The number of *Fasciola* eggs per gram was counted (Happich & Boray, 1969; Mooney *et al.*, 2009). The efficacy of the treatment with propolis was estimated by the reduction in the percentage of eggs, as calculated by: [(Number of eggs at zero day of treatment–number of eggs of treated animals/number of eggs at zero day of treatment) × 100] according to Foreyt (1988).

c-Detection of specific *F. gigantica* IgG antibodies

Indirect ELISA analysis was used to evaluate the IgG antibody response of sheep following treatment with propolis at different intervals, and to compare them to the infected untreated control group, as published by Santiago and Hillyer (1988). The antigen concentrations, serum dilution, and anti-sheep IgG conjugate horseradish peroxidase dilution were estimated using checkerboard titration. The chemical was purchased from Sigma Chem. Co., St. Louis, USA. The OD values were read using an ELx800™ Microplate ELISA Reader (USA) at 450 nm. The cutoff values were measured as mean OD values of negative serum +3 SD (Awad *et al.*, 2009).

d-Quantification of cytokines in sheep sera

The concentrations of cytokines IL-2, IL-10, and IL-17 in the sheep sera were determined using commercial kits purchased from Biovision Co., China, following the manufacturer's instructions.

2-Evaluation of propolis effects on *C. novyi* type B

Bacterial *C. novyi* type B count (CFU/g)

One gram fecal samples from each sheep were collected at days 0, 3, 9, and 15, and homogenized in 9 ml of phosphate-buffered saline at pH 7.2. Ten-fold serial dilutions of the homogenates were cultured in duplicate on neomycin blood agar, using the method of Markey *et al.* (2013). A colony counter was used to count bacterial colonies (Black, 1996). The colony count was confirmed microscopically and biochemically as described in the method of Pires *et al.* (2012).

Statistical analysis

One-way ANOVA tests were used to analyze the data. The results are expressed as mean ± standard deviation (SD), and the results were considered to be significant at values of $p \leq 0.05$, for treated groups and naturally infected non-treated groups (Steel & Torrie, 1980).

RESULTS

Condition and body weight

The animals' appetite gradually increased over the course of treatment, which led to increased energy. There were significant increases in live weight in sheep treated with propolis, compared with their weight before treatment. The infected sheep had a body weight of 35 ± 1 kg before

treatment, and 40 ± 1 kg after treatment. Their wool became shiny, and the animals had recovered completely from the intermittent diarrhea at the end of the experiment.

Therapeutic activity of propolis

1-Anti-parasitic effect of propolis in naturally infected sheep

As depicted in Figure 1, the egg count for infected animals ranged from 10 to 12 eggs per gram, and the mean egg count was 11 ± 1.0 egg/gram. At 3 dpt the egg count began to decrease, and reached 5 eggs per gram at 15 dpt. There was a significant reduction in egg count ($p \leq 0.001$) to 54.54% at 15 dpt (Figure 2). The ovicidal activity of propolis following treatment of infected sheep produced no morphological changes, compared with normal *F. gigantica* eggs.

2-IgG level in sheep naturally infected with *F. gigantica* and treated with propolis

The level of antibodies against *F. gigantica* (IgG) in treated infected sheep was significantly higher at all times post treatment than control infected non-treated sheep, as shown in Figure 3.

3-Efficacy of propolis on the cytokine response in sheep naturally infected

Figure 4 shows a significant reduction in IL-17 level in naturally infected sheep treated with propolis at different days post treatment compared with positive control untreated

animals. The levels of IL-2 and IL-10 were significantly decreased ($P \leq 0.05$) 15 days post treatment with propolis compared with those in untreated infected animals.

4-Evaluation of the effects of propolis on *C. novyi* in sheep naturally infected

C. novyi is a gram-positive rod occurring singly or in short chains. The cells are short, thick, straight, and round-ended. Their spores are elongated, oval, and pear-shaped, wider than the cell. Round colonies of *Clostridium* spp. were identified in blood agar, which was used for subculture. Colonies have edges like wisps of hair, surrounded by a zone of hemolysis. *C. novyi* produces turbidity and gas, with a pinkish discoloration in cooked meat media. All of the isolates fermented dextrose. Lactose, sucrose, and maltose produced acid and gas but did not ferment mannitol. All suspected samples were catalase and oxidase negative, and were Indole, VP and MR negative. These properties of the organisms are typical of *C. novyi*.

The number of *C. novyi* in the control group of untreated sheep naturally infected with *F. gigantica* ranged from 8×10^9 to 9×10^9 CFU/g. In the group treated with propolis, the counts of *C. novyi* were significantly reduced to 3×10^3 at 15 dpt ($p \leq 0.05$), as shown in Figure 5.

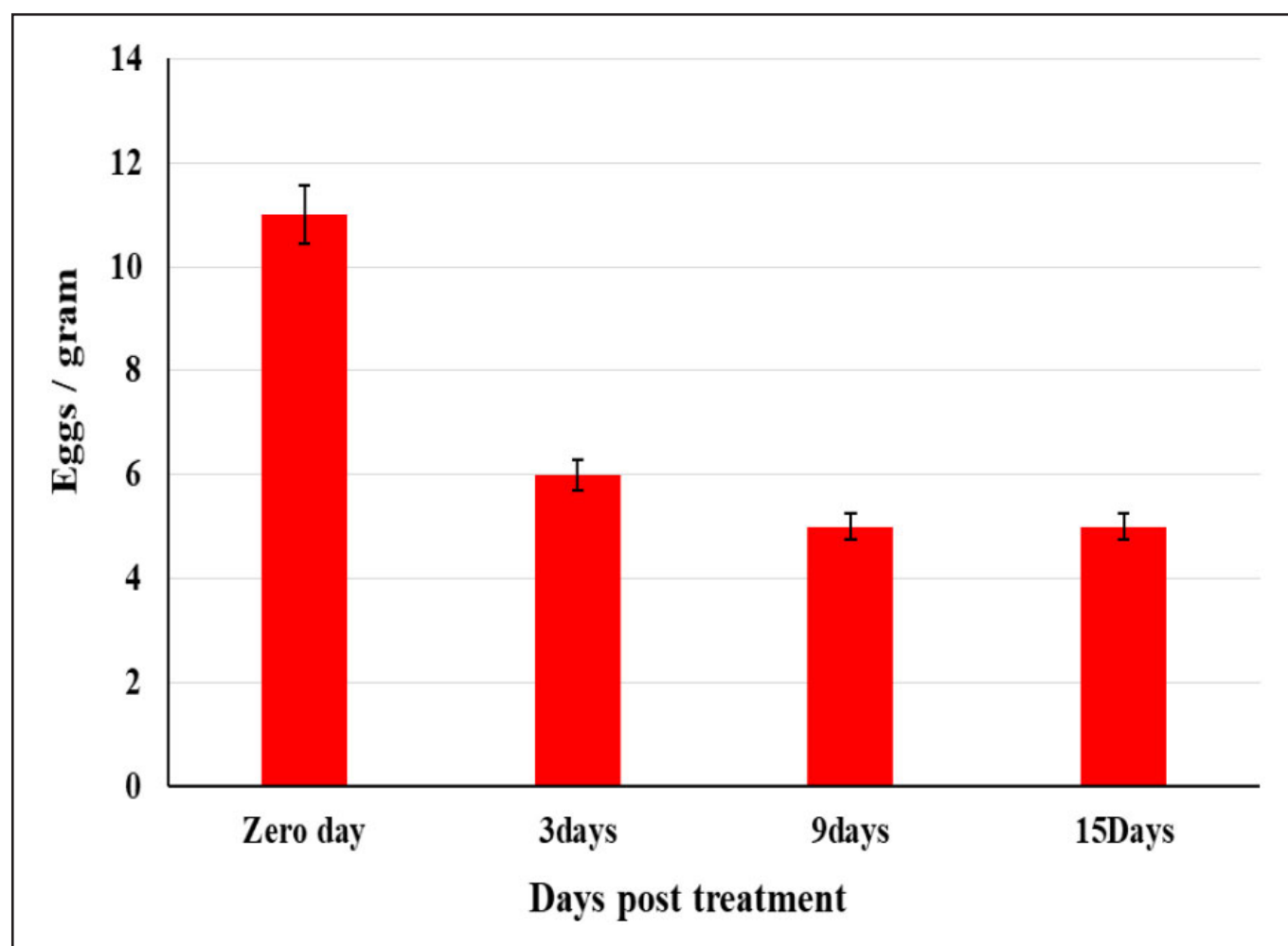


Figure 1. Effect of propolis administration on egg count of naturally infected sheep with *F. gigantica*. Bars represent mean \pm standard deviation (SD) of the *Fasciola* egg count / gram.

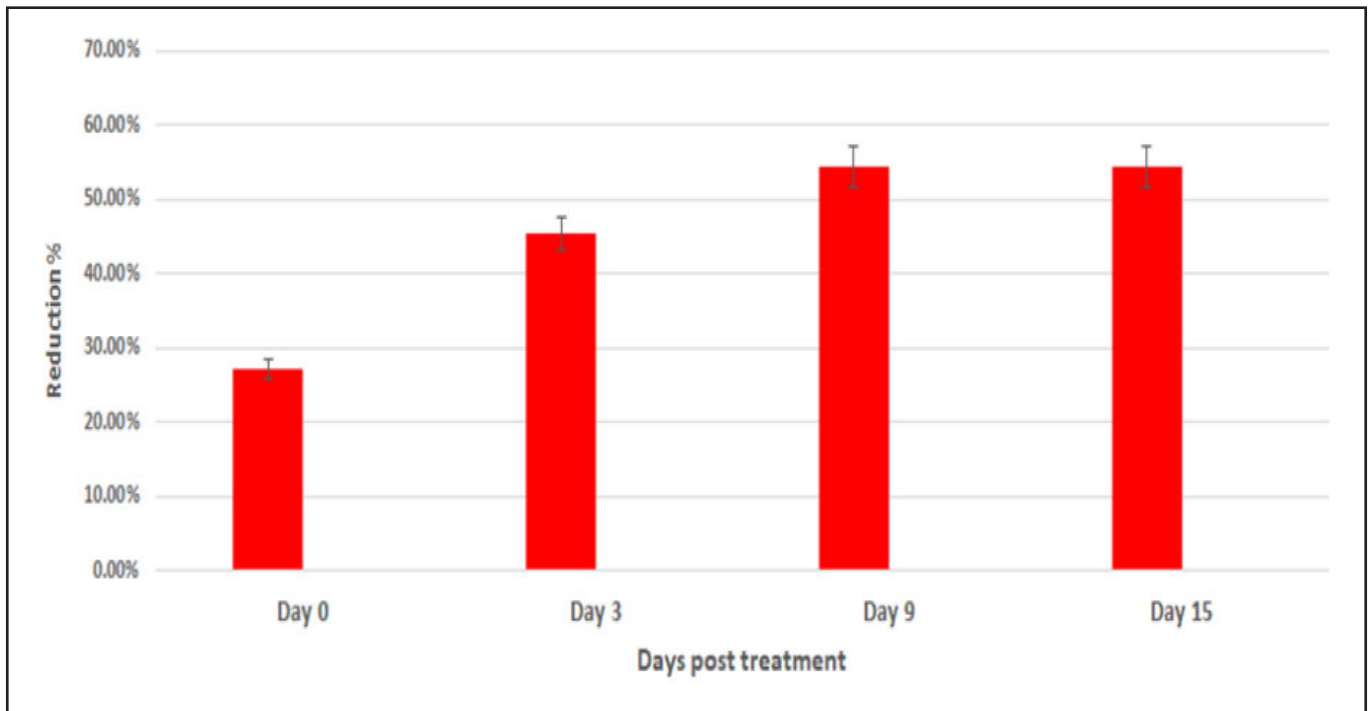


Figure 2. Reduction % in egg counts of naturally infected sheep with *F. gigantica* post treatment with propolis. Bars represent mean \pm standard deviation (SD) of the reduction % in egg counts.

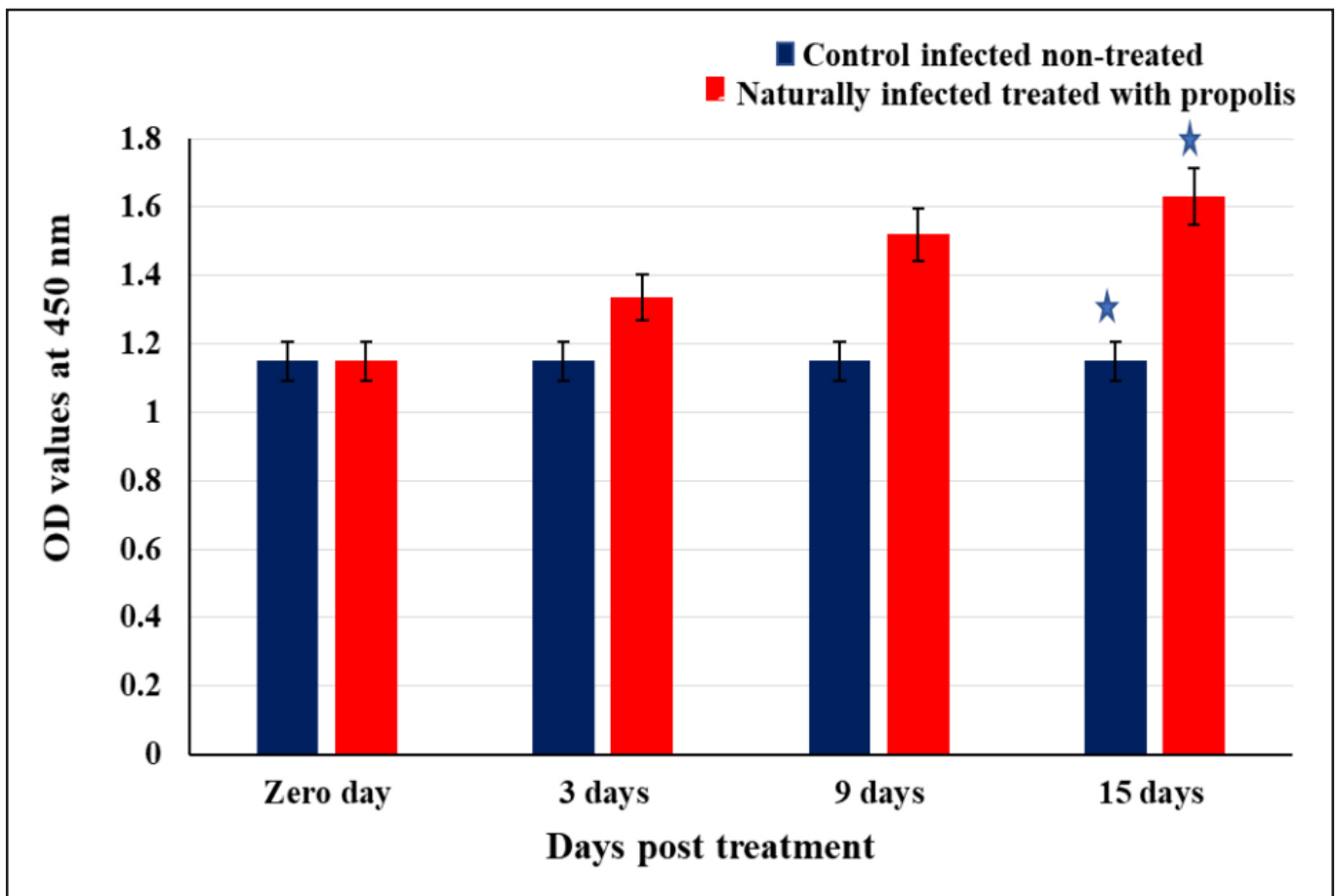


Figure 3. IgG levels of naturally infected sheep treated with propolis at different days post treatment. Bars represent mean \pm standard deviation (SD) of the optical density (OD), *Denotes significance ($p \leq 0.01$) between the control infected non-treated and infected treated group.

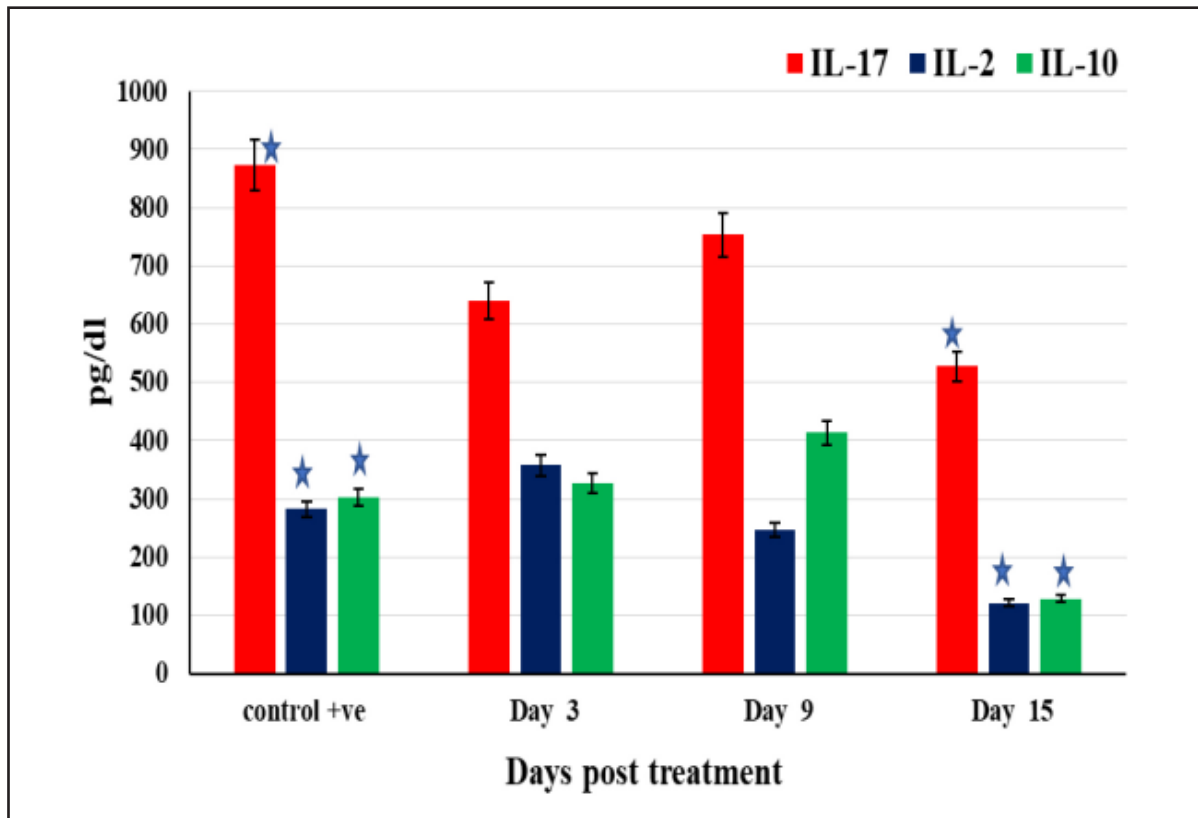


Figure 4. Cytokines level (IL-17, IL2 and IL-10) of naturally infected sheep with *F. gigantica* treated with propolis at different days post treatment, Bars represent mean \pm standard deviation (SD) of the optical density (OD), *Denotes significance ($p \leq 0.05$) between the control infected non-treated and infected treated group.

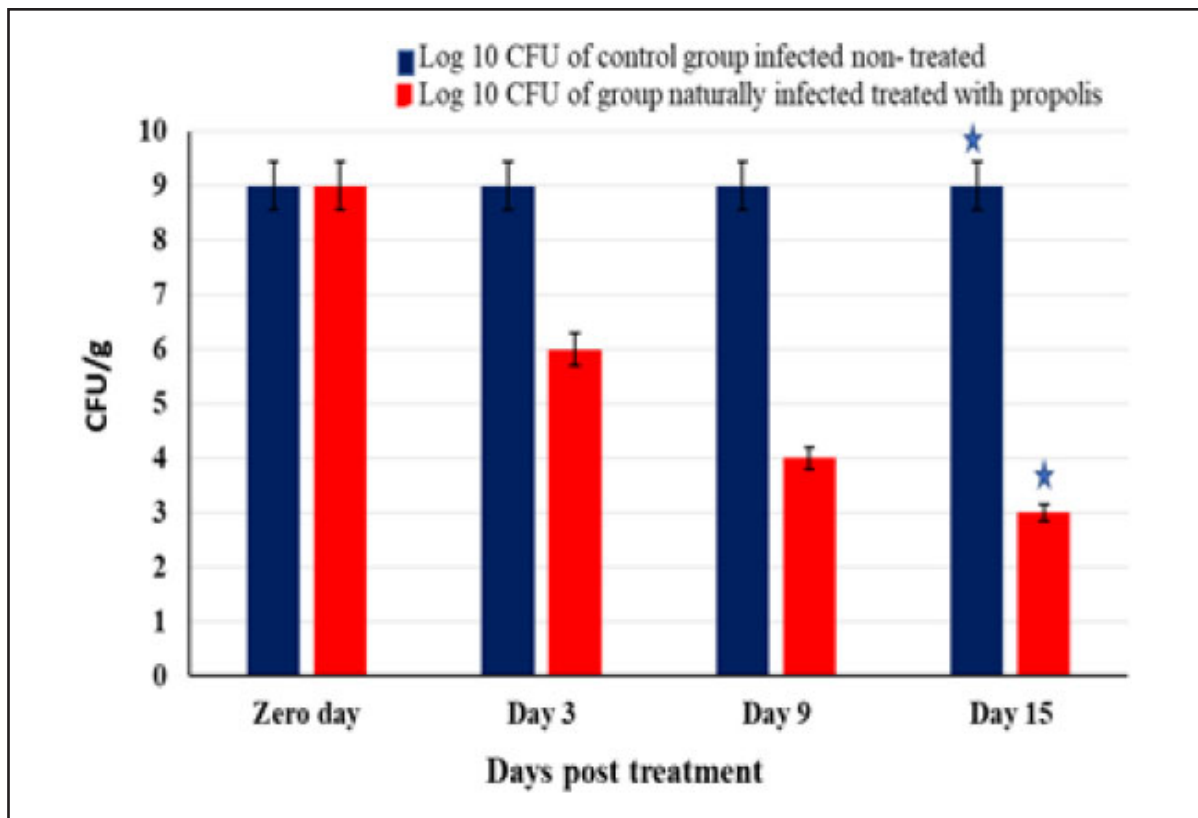


Figure 5. The bacterial counts of *Clostridium novyi* in natural infected *Fasciola gigantica* and treated with propolis, Bars represent mean \pm standard deviation (SD) of of the bacterial counts, *Denotes significance ($p \leq 0.05$) between the control infected non-treated and infected treated group.

DISCUSSION

In the current study, propolis was used as a natural product for the treatment of sheep co-infected with fascioliasis and *C. novyi* type B infection. The study demonstrated the therapeutic potential of Egyptian propolis extract against both infections in sheep. The adoption of natural products in therapy is a good alternative to drugs, which are expensive and less potent, due to host resistance and the risk of residues in host tissues (Dias et al., 2012). Previously, propolis has been shown to be a potent therapeutic agent against infections, and is safe to use (Wagh, 2013).

In the current study, the treated sheep were significantly heavier than the infected untreated group, and the wool of the treated sheep became shiny by the end of the experiment. This result is attributed to propolis gradually increasing the appetite, which led to increased viability and consciousness in treated animals. In the untreated control group we observed short-term diarrhea, which leads to a loss of body weight. These results confirm that propolis supplements improved growth and carcass yield (Seven et al., 2008; Abbas et al., 2012; Kupczyński et al., 2012; Morsy et al., 2013). The increased weights may be due to the anabolic activity of propolis. Yaghoubi et al. (2008) found that flavonoids affected the immune response and improved growth in young calves. Propolis also stimulated the growth of underdeveloped lambs and calves (Hudnall, 2007). Propolis has biological, antibacterial, antimicrobial, and anti-fungal activities that have led to improve health status and consequent increases in body weight (Hegazi et al., 2014a, 2014b; Papachroni et al. 2015, Hegazi et al., 2018a).

This research identified a significant decrease ($P < 0.001$) in fecal egg shedding of naturally infected treated sheep. The egg count decreased gradually after treatment with propolis from 11 eggs per gram and reached 5 eggs per gram at 15 dpt, a reduction of 54.54%, compared to infected non-treated animals. The reduction of egg count may be attributed to the direct toxic effect of propolis against *F. gigantica* worms, which resulted in alterations in worm fecundity and distortion of their eggs, as reported against *H. contortus* by Cabardo and Portugaliza (2017). The current reduction in egg count was probably due to tegmental damage to the adult *Fasciola* worm which resulted in worm death (Hegazi et al., 2007b). Propolis might act as a fasciolicide, due to the presence of flavonoids which destabilized the cell membrane and cuticle collagen of the parasite and led to reductions in egg-laying (D'addabbo et al., 2011). Previously, Hassan et al. (2016) found that Egyptian propolis ethanolic extract caused disintegration of the hypodermis, cuticle damage, and distortion of the excretory pores of *Toxocara vitulorum* adult worm *in vitro* after incubation for 24h with the propolis extract as determined by scanning electron microscopy.

The *Fasciola* eggs detected in sheep feces treated with propolis showed no degeneration and no significant morphological changes. This result supported our contention that propolis extract affected *Fasciola* worms rather than its eggs. The fasciolicidal activity of propolis may be related to tannins, which has been shown to cause paralysis in *Fasciola* worms (Molan et al., 2000; Hoste et al., 2006; Hegazi et al., 2007a).

We explored the reasons behind the anthelmintic effect of propolis extract. There was a significant increase of IgG level in naturally infected sheep compared with the control infected non-treated group. This increase started 3 dpt and continued until the end of the experiment, and was probably

responsible for reducing the severity of infection. Propolis can stimulate IgG, IgM, and IgA to combat invading organisms, leading to a reduction in worm numbers and a consequent reduction in egg shedding (Hegazi et al., 2018b; Sena-Lopes et al., 2018). These results agreed with those of Dantas et al. (2006), who found that Bulgarian propolis at concentrations of 25 and 50 mg/kg decreased parasitemia and prevented hepatic or renal toxic effects in Swiss mice experimentally infected with *Trypanosoma cruzi*. The antibody can also interact with a critical site on the surface membrane of the parasite. Such "neutralizing" antibodies may even interfere with the welfare of adult worms in the liver (El Ridi, 2002). Hegazi et al. (2017) reported that propolis strongly stimulated the production of toxoplasma antibodies compared with a control group from the second week post treatment to the end of their experiment. Hegazi et al. (2018a) showed that Egyptian propolis acts as an immune enhancer. Indonesian propolis extract at 25, 50, and 100 mg/kg increased the level of IgG against surface antigens of *Plasmodium berghei* (Syamsudin et al., 2009).

Significant reduction in the levels of the cytokines IL-2, IL-10, and IL-17 in naturally infected sheep treated with propolis was observed in the current study at different intervals post treatment. This effect of propolis on the level of cytokines had previously been observed. Brazilian green propolis significantly reduced the production of IL-1 α , IL-1 β , IL-6, IL-12p40, IL-4, IL-13, and TNF- α (Szliszka et al., 2013). Treatment with oral propolis extract suppressed the levels of TNF- α , IFN- γ , and IL-2 in the sera of mice when compared to control as shown by Fatahinia et al. (2012). Hegazi et al. (2017) found a significant reduction of TNF α , IL-1 β , and IL-6 levels in rats after infection with *T. gondii* and treatment with propolis.

In the present study, the bacterial count of *C. novyi* ranged from 8×10^9 to 9×10^9 CFU/g in naturally infected sheep with *F. gigantica*. After the treatment with propolis, the count reduced gradually reaching 3×10^3 CFU/g at 15 dpt. This result demonstrated the significant antibacterial activities of propolis, which is probably due to the presence of chrysin and pinocembrin as major flavonoid components (Molnár et al., 2017), and aromatic compounds and caffeic acid (Parolia et al., 2010). The antibacterial action of propolis has been suggested to be due to the interaction between pinocembrin, galangin, pinobanksin, and phenolic compounds (Castaldo & Capasso, 2002; Hegazi & Abd El Hady, 2002; Wagh, 2013). Propolis can prevent the division of bacterial cells, destroy cell walls and bacterial cytoplasm, and stop protein synthesis (Khalil, 2006; Parolia et al., 2010). The antibacterial effect of propolis is not restricted to *C. novyi*, but acts against other strains, as shown by Piotrowski et al. (2018), who demonstrated the bactericidal effect of propolis on *Clostridium difficile* strains. Boyanova et al. (2006) found that Bulgarian propolis was active against most anaerobic strains of *Clostridium*, *Bacteroides*, and *Propionibacterium* species. Hegazi et al. (2007b) found that Siwa propolis, Dakahlia propolis, and Matrouh propolis, all from Egypt, inhibited the growth of *C. novyi* type B associated with fascioliasis. Hegazi et al. (2019) also demonstrated that propolis-ALG NPs exhibited a synergistic antibacterial activity against different bacterial strains. Gao et al. (2014) showed that the artemisinin C (the most important constituent of Brazilian green propolis) activated macrophage phagocytosis. The flavonoids galangin and pinocembrin from purified propolis extracts have been shown to stimulate neutrophil chemotaxis and phagocytic activity (Sampietro et al., 2016).

CONCLUSIONS

The current study provided evidence to support the contention that propolis extract has health benefits, and investigated the biological effects of the extract. The results demonstrated that Egyptian propolis has immunomodulatory, anti-parasitic and antibacterial effects. Propolis can be developed into a potent apitherapeutic agent, with some precautions taken to avoid allergens associated with the use of bee products, and the determination of the appropriate dosage.

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Conflict of Interest

The authors declare no conflicts of interest with regards to this research or the manuscript prepared for publication.

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