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# Egyptian Propolis 12: Influence of Propolis on Cytokines of *Toxoplasma gondii* Infected Rats

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**A B S T R A C T**

This investigation conducted to study the influence of Egyptian propolis on immunological responses with special reference to cytokine levels of infected rats with *Toxoplasma gondii* and treated with 0.1 ml propolis day after day till the end of the experiment (28 days). Immune response of rats was evaluated weekly. The results revealed that the propolis was the highest in the toxoplasma antibodies titer when comparing with control group from the 2nd week to the end of experiment. Serum level of cytokines was consistently higher in the treated and propolis infected rats compared with controls. Serum cytokine levels of rats after infection with *T. gondii* showed significantly reduced levels of tumor necrosis factor-α (TNFα), IL-1β and IL-6 levels if compared to those in infected control rats or propolis treated group.

## Introduction

*Toxoplasma gondii* is extreme zoonotic tissue cyst-forming protozoan. Rats are considered as animal model for *Toxoplasma gondii* infection (Dubey, 1988). The parasite develops adaptive humeral and cell-mediated immune responses following a primary infection in cats, sheep and human (Innes and Vermeulen, 2006), the immunological response against *T. gondii* antigen clearly a strong cytotoxic T cell response (Sayles *et al.*, 2000; Johnson *et al.*, 2004). As *T. gondii* is an obligate intracellular parasite, cellular immunity has been considered the major response to eliminate the parasite within the host, yet humoral immunity also plays an important role in shaping the immune responses (El Fadaly *et al.*, 2012). Suzuki *et al.*, (2011) stated that cytokines secreted in the immune response to *T. gondii* include upregulated factors (IFNγ, IL-2, TNFα, IL-1, IL-7, IL-12, IL-15) and down regulated factors (IL-4, IL-6, IL-10).

Propolis is collected by honeybees from the buds of trees, which used to make the protective shield at the entrance of beehive (Ghisalberti *et al.*, 1978). It has been used since ancient times as a medicine (Hegazi, 1998) due to its biological properties as an antimicrobial (Hegazi *et al.*, 2014a),

antifungal (Hegazi *et al.,* 2000), antiprotozoal (Hegazi *et al.,* 2014b), antiparasitic (Hegazi *et al.,* 2007a,b) and antiviral agent (Hegazi *et al.,* 2012a; Fan *et al.,* 2013), antioxidant (Abd El Hady *et al.,* 2007), hepatoprotective (Gonzales *et al.,* 1995), immunostimulating (Hegazi and Abd El Hady, 1994; Takagi *et al.,* 2005), localized plaque psoriasis (Hegazi *et al.,* 2013) and cytostatic (Banskota *et al.,* 2001). The chemical composition of propolis appeared to be extremely complex and more than 300 compounds have been identified so far (Marcucci, 1995), the most important ones being polyphenols (Hegazi and Abd El Hady, 1997). So, this investigation conducted to study the influence of propolis on immunological responses with special reference to cytokine levels of infected rats and treated with propolis.

## Materials and Methods

### Propolis

### Propolis extraction and sample preparation

The Egyptian propolis sample (25 grams) was collected from apiary farm near El-Mansoura City, Dakahlia Province, Egypt. The resinous materials were kept in dark bag in the refrigerator till being extracted with ethanol.

Propolis was prepared through cut into small pieces and extracted at room temperature with 250 ml of 70% ethanol (1:10 w/v) according to Hegazi *et al.,* (2007). After 24 h, the extracts were filtered and evaporated to dryness under vacuum at 40°C and stored in desiccators as Hegazi *et al.,* (2014). The percentage of extracted matter was 0.8 gm/dry weight. 0.1 gm/dry weight dissolved in 10 ml normal saline was done to treat with 0.1 ml propolis day after day till the end of the experiment (28 days).

### *Toxoplasma gondii* strain

In the present study, the RH strain was maintained and secured in Zoonotic Diseases Department, National Research Center, Egypt, via using regular mice peritoneal passage every 3 days for continuous collection of fresh tachyzoites according to El Fadaly *et al.,* (2012, 2015) The RH strain tachyzoites maintained through successive intra-peritoneal tachyzoites - tachyzoites passages in mice every (72 HPI), the obtained tachyzoites from mice ascetic fluid were used for intra-peritoneal acute infection after counting and dilution as necessary ($10^3$).

### Animals

A total of sixty female Wistar rats weights >110 gm. were obtained from Laboratory Animals House, National Research Center, Egypt. These animals were used as long as the term of the study, housed in standard environmental conditions at temperature (24°C) and relative humidity (50%) with a 12:12 light: Dark cycle. with free access to a standard commercial diet and water. Ten mice were used for harvesting and secure the peritoneal RH tachyzoites with continuous regular flow, by successive intraperitoneal tachyzoites-tachyzoites mice cycle every 72 hours. The obtained tachyzoites from mice ascetic fluid were diluted for adjusting the tachyzoites count at $10^3$ /ml and exposed to infected and infected treated with propolis treated with 0.1 ml propolis day after day till the end of the experiment (28 days). The rats were monitored for mortality daily, and during the experiment rats were weighed at regular intervals (every 7 days). Experiments were performed according to the Guide for the Care and Use of Laboratory Animals and Ethical Approval of animal rights according to Committee, National Research Centre, Egypt. The experimental design was given in table 1.

## Monitoring infections

The rats were monitored for mortality daily, and in some experiments rats were weighed at regular intervals. At the 7, 14 and 28 days, a group of rats were killed and their weight measured.

## Sulfadiazine and pyrimethamine drug

The treatment of choice for toxoplasmosis is a combination of sulfadiazine (Tablet: 500 mg) and pyrimethamine (Tablet: 25 mg) as WHO (2008).

## Laboratory studies

## Measurement of sera IgM and IgG

Blood samples were collected via the tail vein into heparinized capillary tubes and separating the serum portion by centrifugation at 3500 rpm for separation of serum, which then kept in deep freeze at -80ºC until the determination of the IgM and IgG (at 7, 14 and 28 days) by ELISA (Hassan *et al.,* 2016). It tracks any complex including antigen and antibody couples. All control and infected rats were examined for infection by an ELISA kit designed in our laboratory. *Toxoplasma* lysate antigen (TLA) was prepared from tachyzoites of *T. gondii* RH strain (Daryani *et al.,* 2003), Briefly, the RH strain (about $2 \times 10^9$ tachyzoites) harvested in PBS were filtered and centrifuged at 750 *g*, three times for 15 min. The pellet was solubilized by adding distilled water and then the solution was supplemented with protease inhibitor, 5 mm phenylmethylsulphonyl fluoride. The suspension was freeze–thawed five times. The protein content of TLA was determined using Bradford method (Bradford, 1976) and then stored at −20 °C until used.

ELISA was carried out using a procedure described by Voller *et al.,* (1976). The 96-well, flat-bottom microtiter plates were coated overnight at 4 °C with 10 μg $ml^{-1}$ solution of TLA in carbonate buffer, pH 9.6 (100 μl per well). Plates were washed with phosphate buffered saline tween (PBST) (PBS, pH 7.4, containing 0.05% Tween 20) for three periods of 3 min. The ELISA plate was blocked for 1 h using 100 μl of 3% skim milk powder in PBS 0.05% Tween 20 and washed. Sera samples diluted in 3% skim milk in PBS were added at a volume of 100 μl and at a concentration of 1: 100. After washing, plate was incubated with peroxidase-labelled rabbit anti-rat IgG (Sigma-Aldrich Company, St. Louis, MO, USA) diluted 1: 10 000 in PBST plus 3% skim milk and incubated for 1 h at 37 °C. Finally, the enzymatic activity was revealed using the substrate tetramethylbenzidine (Sigma). After 20 min of incubation at room temperature, the reaction was stopped by adding 50 μl of $H_2SO_4$ 1.25 m and optical density (OD) was measured at 450 nm with ELISA reader. A sample was considered positive with the mean OD value of infected rats, which was higher than the mean of control rats plus three standard deviations (cut-off). Titer was defined as the reciprocal of the highest dilution that produced OD readings more than 0.1 OD unit above background. The absorbance was measured at 405 nm, the IgG anti-Toxoplasma <15 UI/ml was reported negative and the level >15 UI/ml was reported positive. In regard to IgM levels lower than 1 UI/ml was reported negative and levels equal or higher than 1 UI/ml was reported positive.

## Cytokine levels in serum samples

Blood samples were obtained from anesthetized animals. Serum samples were stored at -80°C until analyzed. Sera were diluted 1/10 in PBS then TNFα, IL1β and IL6 levels were measured at 7 and 28 days using ELISA (enzyme linked immunosorbent assay) technique as described by Roberts *et al.,* (1995), ELISA reagent kits (produced by

Biosource, Lucerne Chem AG, Lucerne, Switzerland) according to the manufacture's instruction. All measurements were performed in triplicate. Experiments were repeated three times, with 3 animals per group. The concentration of cytokines was determined spectrophotometrically. The absorbance was read at 450 nm. Standard curve was constructed by using cytokine standards. The cytokine concentrations for unknown samples were calculated according to the standard curve. Concentrations were determined from a standard curve and the absorbance readings were converted to pg/ml based upon standard curves obtained with recombinant cytokine in each assay.

## Statistical analysis

The results obtained in the present study were represented as means ± standard error, and were analyzed using analysis of variance (ANOVA). Samples were compared using the unpaired Student t test (two-tailed), with equal variance and unpaired samples, and calculated using Excel (Microsoft, Seattle, WA). The significance of difference between means at $P<0.05$ was calculated using the Duncan Multiple Range Test (Steel, and Torrie, 1980).

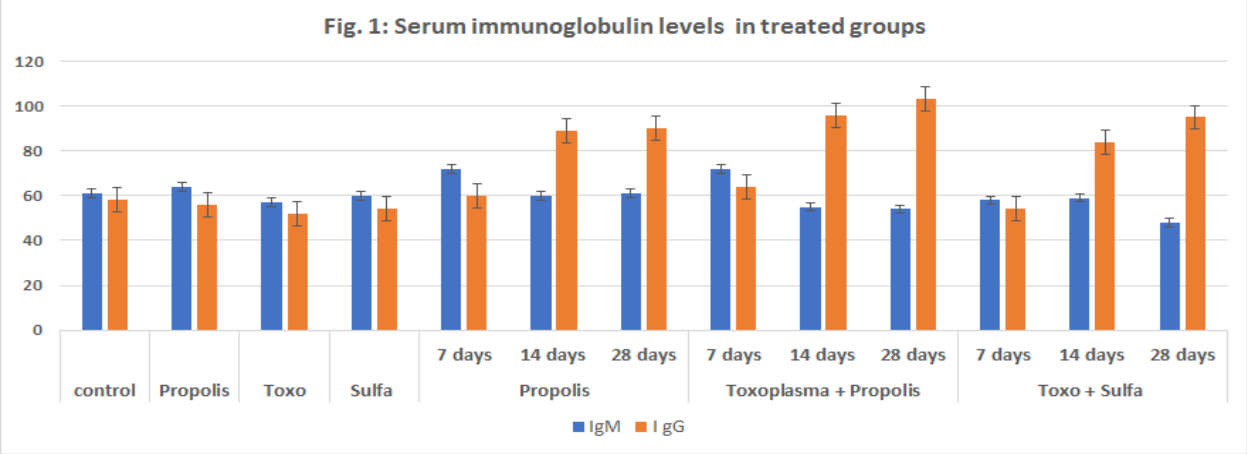
## Results and Discussion

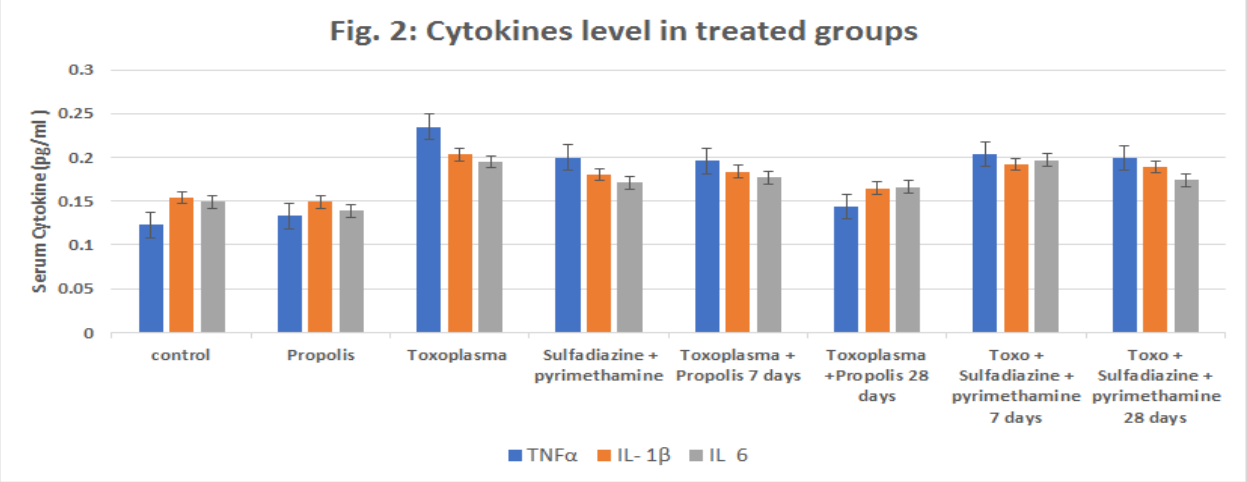
Antibody response at day 7 (IgM) and at 28-day (IgG) post infection with *Toxoplasma gondiii* was detected by the enzyme-linked immunosorbent assay. The antibodies were increased significantly after the intraperitoneal infection with *Toxoplasma gondiii* which reached its maximum level at day 7 (IgM) and at 28-day (IgG) post infection as detected by the enzyme-linked immunosorbent assay (Fig.1). Reciprocal titers were determined in sera from rats infected with *Toxoplasma gondiii,* then treated with propolis (0.1 ml) day after day till the end of the experiment (Fig. 1). Figure 2 illustrated serum cytokine levels detected by ELISA assay in rat infected with *Toxoplasma gondiii* and non-infected as well as infected and treated either with propolis or combination of sulfadiazine and pyrimethamine. Serum cytokine levels of rats after infection with *T. gondii* showed significantly elevated levels of tumor necrosis factor-α (TNFα), IL-1β and IL-6 levels if compared to those in infected control rats. Treatment with propolis (0.1 ml) day after day till the end of the experiment showed significantly reduction in TNFα, IL-1β and IL-6 levels. Values represent the means ± SEM. ($P<0.05$).

The target of this investigation directed to study the immune response of rat infected with *Toxoplasma gondii* whish is an opportunistic intracellular parasite that known to infect approximately one-third of the human population (Joynson, and Wreghitt, 2001). Also, rat was considered as natural resistance species to Toxoplasma infection (Remington and Krahenbuhl. 1982). Antibody response reached its maximum level at day 7 (IgM) and at 28-day (IgG) post infection intraperitoneally with *Toxoplasma gondii* infection as detected by the enzyme-linked immunosorbent assay (Fig. 1). Similar funding was observed elevation of serum antibody titer levels during the course of acute and chronic infection with *Toxoplasma gondiii* in mice (Chang *et al.,* 1990 and Beaman *et al.,* 1991). In this investigation, the specific antibodies (IgM and IgG) were increased significantly when detected in rat infected with group and infected then treated with propolis (0.1 ml propolis day after day till the end of the experiment). In the propolis, groups showed significantly increased their antibodies if compared with control sera of healthy rats.

**Table.1** The experimental design

| Group Name | No. of animals | Treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | Saline | Honey | Toxo plasma | Sulfadiazine + pyrimethamine | Toxoplasma + Honey | Toxo+ Sulfadiazine + pyrimethamine |
| Control | 10 | + | - | - | - | - | - |
| Honey | 10 | - | + | - | - | - | - |
| Toxoplasma | 10 | - | - | + | - | - | - |
| Sulfadiazine + pyrimethamine | 10 | - | - | - | + | - | - |
| Toxoplasma + Honey | 10 | - | - | + | - | + | - |
| Toxoplasma + Sulfadiazine + pyrimethamine | 10 | - | - | + | - | - | + |



Favre *et al.,* (1984) found that a rise in IgM first, followed by the IgA response, also they observed a simultaneous rise in IgM (Turunen *et al.,* 1983; Favre *et al.,* 1984). The elevation in the antibody in the rats treated with propolis than infected with *Toxoplasma gondii* or treated with combination of sulfadiazine and pyrimethamine may be attributed to the propolis contains a variety of biologically active compounds such as flavonoids, vitamins, antioxidants and hydrogen peroxides (Hegazi and Abd El Hady, 2002 and Abd El-Hady *et al.,* 2007). The major components of propolis exerted beneficial effects by polyphenols, caffeic acid phenethyl ester (CAPE), chrysin and other flavonoids (Hegazi *et al.,* 2000, Hegazi and Abd El Hady, 2002 and Hegazi *et al.,* 2007a, b). Propolis has modulatory action on murine peritoneal macrophages, increasing their microbicidal activity and stimulant action on antibody production (Sforcin, 2007). The synergistic effects of wide range of compounds present in propolis are the results of antioxidants activity (Hegazi and Abd Al Hady, 2009) and hepatocytes protection (El-Khatib *et al.,* 2002) and anti-inflammatory (Rashid *et al.,* 2016).

Administration of propolis to rats significantly reduced TNFα, IL-1β and IL-6 levels in infected rats with *Toxoplasma gondiii*. Detection of serum cytokine levels by ELISA assay of rat which was obtained for infected and non-infected as well as infected and treated either with propolis or combination of sulfadiazine and pyrimethamine as shown in Figure 2. Results from this figure showed serum cytokine levels of rats after infection with *T. gondii* showed significantly reduced levels of tumor necrosis factor-α (TNFα), IL-1β and IL-6 levels if compared to those in infected control rats or propolis treated group. Significant reduction in rat's serum TNFα, IL-1β and IL-6 levels after treatment with propolis may be intimately involved in the pathogenesis of *T. gondii* infection or associated with an acute phase response (Beaman *et al.,* 1994 and Hunter *et al.,* 1994). The serum cytokines of rat infected with *Toxoplasma gondii* were increased significantly when detected. Rat's infected and treated with propolis showed reduction in the cytokines level. This due to infection with *T. gondii* is characterized by the development of acute hyperinflammatory and lethal ileitis (Gaddi and Yap, 2007). The primary function of the innate immune system is the detection of pathogens and the rapid activation of host defense mechanisms (Iwasaki and Medzhitov, 2004 and Yarovinsky and Sher, 2006). Propolis contains natural flavonoid, chrysin (5,7-dihydroxyflavone), it has beneficial effects including anti-tumor and anti-oxidant activities. The mode of action of chrysin is to decrease gene expression of pro-inflammatory cytokines such as, tumor necrosis factor-α, IL (interleukin)-1β, IL-4, and IL-6 in mast cells (Bae *et al.,* 2011). Chrysin also significantly reduced the serum levels of pro-inflammatory cytokines, interleukin-1 beta (IL-1β) and IL-6 (Fitzpatrick *et al.,* 2001; Ahad *et al.,* 2014). Ansorge *et al.,* (2003) demonstrate that propolis has a direct regulatory effect on basic functional properties of immune cells where cytokines produced by monocytes/ macrophages (IL-1β, IL-12), by Th1 type (IL-2) as well as Th2 type (IL-4) lymphocytes were found to be also suppressed. Also, Mossalayi *et al.,* (2014) found that propolis decreased both monokines and interferon γ (IFNγ) production and induces potent anti-inflammatory activity due to their complementary immune cell modulation. Propolis inhibitory effects on lympho proliferation may be associated to its anti-inflammatory property. In immunological assays, the best results were observed when propolis was administered over a short-term to animals (Sforcin, 2007).

Masek and Hunter (2013) stated that macrophages can be infected by *T. gondii*, they are able to limit parasite replication and produce cytokines that contribute to resistance, making them important regulatory and effector cells during toxoplasmosis. Neutrophils influence the T-cell response by enhancing the functions of dendritic cells (van Gisbergen *et al.*, 2005) or inflammatory monocytes (Soehnlein *et al.*, 2008). Infection of mice by intraperitoneal (i.p.) inoculation with low numbers of a highly virulent strain of *T. gondii* or with a high inoculum of low-virulence strains results in recruitment of neutrophils to the peritoneal cavity (Mordue *et al.*, 2001).

We therefore conclude that the treatment with propolis through the experiment showed raised antibody titer and decreased the serum cytokines (IFN-γ, IL-1 and IL-6) level in infected with *Toxoplasma gondii*.

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## Competing interests

The authors declare that they have no competing interests.

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