Immunological effect of propolis in *E. coli* infected rats

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A B S T R A C T

This study was undertaken to elucidate the immunostimulant effects of propolis in experimentally induced microbial infection in rats via evaluating leukocytes count and cytokines (TNFα, interleukin (IL)-1β and IL-4), immunoglobulins (IgG and IgM), and leukocyte phagocytic activity. One hundred male Wister rats aging about 5 weeks and weighing about 170-200 gm. rats were divided into five groups as follows: Group 1: control group: consists of 20 male rats served as a control received sterile saline injection. Group 2: propolis: consists of 20 male rats and were given 300 mg/kg body weight propolis extract orally once daily for 16 days. Group *3: E. coli* (Infected): consists of 20 male rats, which were injected intra-peritoneal with *E. coli*. Group 4: Propolis + *E.* coli-infected: consists of 20 male rats, which were given propolis extract orally for 2 weeks then injected intra-peritoneal with *E. coli*. Group *5: E. coli* + propolis: consists of 20 male rats, which were injected intra-peritoneal with *E. coli* then given propolis for 16 days. Leukogram showed that *E.* Coli-Infected group had significant leukocytosis and granulocytosis after 2, 4, 8 and 16 days' post-infection when compared with control, while, there was a significant lymphocytosis after 4 days' post-infection. Propolis + *E. coil* group showed significant decrease in leukocytes and granulocytes when compared with *E. coli* infected group. *E. coli* group showed significant increases in TNF-α and IL-1β, significant decrease in IL-4 after 2 and 16 days when compared with control. On the other hand, Propolis + *E. coli* group induced significant decreases in TNF-α and IL-1β when compared with *E. coli* infected group, While *E. coli* + propolis group induced significant decreases in TNF-α after and IL-lβ after 16 days. *E.* Coli-Infected group showed significant decrease in IgM when compared with control. While, Propolis + *E. coli* group was found to induce significant increase in IgM when compared with *E. coli* infected group. Phagocytic % and phagocytic index showed significant decrease in phagocytic % and phagocytic index in *E. coli* group. On the other hand, Propolis + *E. coli* and *E. coli* + propolis groups showed a significant increase in phagocytic % and phagocytic index when compared to *E. coli* infected group. Therefore, it is concluded that propolis had significant immunostimulant effect in rats infected with *E. coli*.

Keywords: *E. Coli,* propolis, TNF-α, IL-1β, Phagocytic %

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1. INTRODUCTION

Propolis is a resinous hive product collected by honey bees from various plant sources; it is a popular folk medicine possessing a broad spectrum of biological activities *(Banskota et al., 2001)*. The use of propolis goes back to ancient times at least to 300 BC, and it has been used as a medicine in local and popular medicine in many parts of the world, both internally and externally *(Ghisalberg, 1979)*. Propolis has attracted researchers' interest in the last decades because of several biological and pharmacological properties such as immunomodulatory, antitumor, antimicrobial, anti-inflammatory and antioxidant *(Bankova et al., 2000)*. Immunity is the ability of the host system to recognise different arrays of foreign substances (antigens) in the body and react against them. The response of a host to any particular pathogen may involve a complex set of responses; the humoral and/or cellular arms of the specific (adaptive) immune response and the non-specific (innate) responses *(Janeway et al., 2005)*. Macrophages are important in the regulation of immune responses. They are often referred to as scavengers or antigen-presenting cells (APC) because they pick up and ingest foreign materials and present these antigens to other cells of the immune system such as T cells and B cells *(Janeway et aL, 2001)*. On the other hand, after ingestion of the antigens macrophages can destroy themselves through the action of enzymes such as toxic peroxidases present in their cytoplasm, they are known to digest more than 100 bacterial cells before they die *(Ryan and*

Ray., 2004). Endotoxin or lipopolysaccharides (LPSs) produced by Gram-negative bacteria stimulates monocytes and macrophages to release various cytokines including tumor necrosis factor-a (TNF-a) which contributes to organ dysfunction in sepsis *(Nathan et al., 1980). Escherchia coli* is the most common Gram negative bacterium to cause septicemia *(Tunkel and* Scheid., *1993).* It is probably the best-known bacterial species and one of most frequently isolated organisms from clinical specimens *(Mark et al., 2001).* It is one of main species of bacteria that normal inhabitants lower intestines of worm blooded animals including birds and mammals *(Rosario et al., 2004).* LPS is an integral component of the outer membrane of Gram-negative bacteria and plays a major role in the pathogenesis of septic shock *(Morikawa et al., 1996).*

Therefore, this study was undertaken to elucidate the immunostimulant effects of propolis in experimentally induced microbial infection in rats via evaluating leukocytes count and cytokines (TNFα, interleukin (IL)-1β and IL-4), immunoglobulins (IgG and IgM), and leukocyte phagocytic activity.

2. Materials and methods

2.1. Animals:

One hundred male Wister rats aging about 5 weeks and weighing about 170-200 gm were obtained from the Animal House, Faculty of Veterinary Medicine, Benha University, Egypt. All animals were caged and maintained on a standard diet with free access to tape water and were acclimatized for 1 week before starting the experiments.

2.2. Escherichia coli:

Strain of *E. coli* used in this experiment was kindly obtained as a gift from the Department of Bacteriology, Faculty of Veterinary Medicine, Benha University, Egypt. The culture was suspended in nutrient broth at a concentration of 1 X 10^9 CFU/ml and administrated intra-peritoneal at a single dose of 1 ml/Kg body weight following the method of Hegazy et al., *(2010).*

2.3. Propolis:

Propolis was obtained from the Faculty of Agriculture, Benha University, Egypt. Propolis was given as an aqueous extract. propolis samples were mixed with distilled water, heated gently and filtered through Whatman No.1 filter paper. Propolis was freshly prepared and administered to animal by oral gavage *(El-Khayat et al., 2009).* The obtained extract was administered orally at adose of 300 mg/kg b.w *(Basnet et al., 1996a).*

2.4. Chemicals and Reagents:

Ethylenediaminetetraacetic acid, · CLINDIAG Diluent, CLINDIAG Detergent, CLINDIAG Hemolysin (Belgium).

2.5. Experimental design:

In this study, rats were divided into five groups as follows: Group 1: control group: consists of 20 male rats served as a control received sterile saline injection (1 ml/Kg) by IP route. Group 2: propolis: consists of 20 male rats and were given 300 mg/kg body weight propolis extract orally once daily for 16 days. C. Group *3: E. coli* (Infected): consists of 20 male rats, which were injected intra-peritoneal with *E. coli 1* ml of 1 x10^9CFU/m1 single dose. D. Group 4: Propolis + *E.* coli-infected: consists of 20 male rats, which were given 300 mg/kg body weight propolis extract orally for 2 weeks then injected intra-peritoneal with *E. coli* of 1 x10^9CFU/m1 single dose then given propolis 300 mg/kg body weight orally for 16 days. Group *5: E. coli* + propolis: consists of 20 male rats, which were injected intra-peritoneal with *E. coli* of 1 x10^9CFU/m1 then given propolis 300 mg/kg body weight orally for 16 days.

2.6. Blood Samples:

Blood samples were collected by the retro-orbital bleeding method from 5 rats from each group at 2, 4, 8, and 16 days of E. coli injection and blood samples were divided into 2 parts, first part (0.5-1 ml) collected on dipotassium salt of EDTA-tubes for complete blood count and second part (3 ml) collected on plain tubes for serum separation. Blood count was evaluated by using automatic cell counter (H.A.-Vet Clindiag, Belgium).

2.7. Measurement of Immunological parameters:

TNF-α (according to Beyaert and Fiers, (1998), IL-1β (ELISA kit obtained from RayBiotech Inc.), IL-4 (biosource Inc. according to Prokopchuk (2005)), Rat IgG and IgM (my Biosource .com), and phagocytic activity and phagocytic index (according to goddeeris et al. (1986) were measured.

2.8. Statistical Analysis

Statistical analysis was performed using the statistical software package for social science (SPSS) for Windows (Version 16.0; SPSS Inc.,

Chicago, IL). The significance of differences between the experimental groups was evaluated by one-way analysis of variance (ANOVA). If one-way ANOVA indicated a significant difference, then differences between individual groups were estimated using Duncan as a post hoc. Results are expressed as the mean ± standard error of mean. A *P*-value of less than 0.05 was considered significant (Kinnear and Gray 2006).

3. RESULTS:

Leukogram showed that *E.* Coli-Infected group had significant leukocytosis and granulocytosis after 2, 4, 8 and 16 days' post-infection when compared with control, While, there was a significant lymphocytosis after 4 days' post-infection. Propolis + *E. coil* group showed significant decrease in leukocytes after (4, 8 and 16 days) and granulocytes after 2, 4, 8 and 16 days post-infection when compared with *E. coli* infected group. *E. coli* group showed significant increases in TNF-α and IL-1β, significant decrease in IL-4 after 2 and 16 days when compared with control. On the other hand, Propolis + *E. coli* group induced significant decreases in TNF-α and IL-1β after 2 and 16 days when compared with *E. coli* infected group, While *E. coli* + propolis group induced significant decreases in TNF-α after 2 and 16 days but IL-lβ after 16 days. Regarding to antibodies, *E.* Coli-Infected group showed significant decrease in IgM after 2 and 16 days' post-infection when compared with control. While, Propolis + *E. coli* group was found to induce significant increase in IgM after 16 days' post-infection when compared with *E. coli* infected group. Phagocytic % and phagocytic index showed significant decrease in phagocytic % and phagocytic index in *E. coli* group, *E. coli* infection cause impairment of polymorphonuclear leukocytes (PMNLs) function, decreased phagocytic activity.

On the other hand, Propolis + *E. coli* and *E. coli* + propolis groups showed a significant increase in phagocytic % and phagocytic index when compared to *E. coli* infected group.

Table (1): Leukogram after 2 and 4 days after intra-peritoneal injection of *E. coli (lx* 10^9 CFU/ml) and/or treatment with propolis (300 mg/kg body weight)

Groups	WBC, ($x10^3/\mu1$)	Granulocytes ($x10^3/\mu1$)	Lymphocyte ($x10^3/\mu1$)	Monocyte ($x10^3/_{1}11$)
Control D2	11.73 ± 0.38^{d}	5.32 ± 0.45^{c}	6.08 ± 0.18^{a}	0.32 ± 0.01^{b}
Propolis D2	12.55 ± 0.37^{cd}	5.2 ± 0.41^{c}	6.86 ± 0.10^{a}	0.49 ± 0.01^{a}
E. coli-Infected D2	16.05 ± 0.91^{a}	9.41 ± 0.61^{a}	6.50 ± 0.35^{a}	0.14 ± 0.01^{d}
Propolis + E. coli D2	13.79 ± 0.85^{abc}	7.6 ± 0.43^{b}	5.98 ± 0.43^{a}	0.22 ± 0.02^{c}
E. coli + Propolis D2	15.44 ± 0.28^{ab}	8.82 ± 0.13^{ab}	6.38 ± 0.32^{a}	0.23 ± 0.01^{c}
Control D4	12 ± 0.34^{c}	5.49 ± 0.26^{d}	6.16 ± 0.20^{b}	0.35 ± 0.05^{a}
Propolis D4	12.57 ± 0.40^{c}	5.22 ± 0.23^{d}	6.92 ± 0.24^{a}	0.43 ± 0.02^{a}
E. coli-Infected D4	16.49 ± 0.28^{a}	9.84 ± 0.28^{a}	6.51 ± 0.30^{ab}	0.14 ± 0.02^{c}
Propolis + E. coli D4	14.53 ± 0.25^{b}	7.96 ± 0.25^{c}	6.30 ± 0.21^{ab}	0.27 ± 0.01^{b}
E. coli + Propolis D4	15.69 ± 0.19^{a}	8.94 ± 0.22^{b}	6.54 ± 0.14^{ab}	0.21 ± 0.02^{b}

Results are expressed as mean ±S.E.M. Different superscripts (a, b, c, d) at the same check point in the same column indicate significant differences at ($P < 0.05$)

Table (2): Leukogram after 8 and 16 days after intra-peritoneal injection of *E. coli (1x10^9* CFU/ml) and/or treatment with propolis (300 mg/kg body weight)

Groups	WBC, (x10^3/µ1)	Granulocytes (x10^3/µ1)	Lymphocyte (xl 0^{3}4d)	Monocyte (x 10^3/0)
Control D8	11.68 ± 0.28^c	5.37 ± .25^d	6.08 ± 0.18^a	0.23 ± 0.02^b
Propolis D8	12.14 ± 0.37^c	4.86 ± .14^d	6.96 ± 0.40^a	0.32 ± 0.01^a
E. coli-Infected D8	17.37 ± 0.35^a	10.58 ± .36^a	6.63 ± 0.40^a	0.16 ± 0.01^c
Propolis + E. coli 08	15.02 ± 0.37^b	8.25 ± .19^c	6.55 ± 0.22^a	0.23 ± 0.01^b
E. coli + Propolis D8	15.92 ± 0.50^b	9.10 ± .20^b	6.63 ± 0.32^a	0.19 ± 0.01^c
Control D16	11 ± 0.62^{ab}	5 ± 0.49^c	5.73 ± 0.22^a	0.28± 0.08^b
Propolis D16	13.09 ± 0.60'	4.90 ± 0.13^c	7.75 ± 0.66^a	0.43 ± 0.02^a
E. coli-Infected D16	15.09 ± 0.96^a	7.14 ± 0.34^a	7.75 ± 0.69^a	0.21 ± 0.02^b
Propolis + E. coli D16	12.37 ± 0.29^b	5.97 ± 0.23^6	6.17 ± 0.14^a	0.23 ± 0.03^b
E. coli + Propolis D16	13.24 ± 1.16^{ab}	5.95 ± 0.17^b	7.06 ± 1.13^a	0.23 ± 0.04^b

Results are expressed as mean ±S.E.M. Different superscripts (a, b, c, d) at the same check point in the same column indicate significant differences at ($P < 0.05$)

Table (3): TNF-α, IL-1β, IL-4, IgG and IgM after 2 and 16 days of intra-peritoneal injection of *E. coli (1x10^{9}CFU/m1)* and/or treatment with propolis (300 mg/kg body weight):

Groups	TNF-α (pg/ml)	IL-1β (pg/ml)	IL-4 (pg/ml)	IgG (Mg/m1)	IgM (ng/ml)
control D2	25 ± 2.97^c	278.92 ± 28.35^c	46.18 ± 3.43^{ab}	7.78 ± 1.31^{ab}	11.69 + 1.82^a
propolis D2	19.68 ± 2.35^c	245.32 ± 42.09^c	53.47 ± 5.58^a	11.42 ± 1.63^a	13.21 ± 2.38^a
E. coli-Infected D2	67.75 ± 3.91^a	689.98 ± 84.61^a	25.34 ± 4.4^c	3.75 ± 0.42^b	5.4 ± 1.31^b
Propolis + E. coli D2	32.41 ± 3.94^{bc}	470.99 ± 43.61^b	34.95 ± 3.17b^c	6.79 ± 1.42^b	10.15 ± 1.4^{ab}
E. coli + propolis D2	41.37 ± 6.47^b	552.37 ± 25.36^{ab}	33.03 ± 4.64^{bc}	5.97 ± 1.29^b	7.77 ± 1.71^{ab}
control D16	27.34 ± 4.21^{bc}	302.55 ± 46.59^c	50.13 ± 6.9^{ab}	8.62 ± 1.42^{ab}	11.5 ± 1.21^{ab}
propolis D16	20.33 ± 2.82^c	267.07 ± 41.97^c	59.19 ± 5.29^a	11.49 ± 2.22^a	14.68 ± 1.52^a
E. coli-Infected D16	53.19 ± 4.64^a	1246.21 ± 126.25^a	26.14 ± 3.4^c	4.66 ± 0.94^b	5.56 ± 1.10^c
Propolis + E. coli D16	30.56 ± 2.83^{bc}	482.88 ± 46.66^{bc}	37.94 ± 4.1^{bc}	7.36 ± 1.66^{ab}	10.75 ± 1.19^{ab}
E. coli + propolis D16	36.78 ± 3.18^b	605.91 ± 118.03^b	37.64 ± 4.72^{bc}	6.35 ± 1.34^{ab}	8.5 ± 1.35^{bc}

Table (4): Phagocytic % and phagocytic index 16 days after intra-peritoneal injection of *E. coli (1x10^{9}CFU/m1)* and/or treatment with propolis (300 mg/kg body weight)

Groups	Phagocytic %	Phagocytic Index
Control	69.25 ± 1.25^b	6.15 ± 0.13^b
Propolis	79.5 ± 0.65^a	8.48± 0.06^a
E. coli-Infected	54 ± 0.91^d	3.1 ± 0.11^e
Propolis + E. coli	66.75 ± 1.25^b	5.58 ± 0.14^c
E. coli + Propolis	61.75 ± 0.85^c	4.9 ± 0.07^d

Results are expressed as mean ±S.E.M. Different superscripts (a, b, c, d) at the same check point in the same column indicate significant differences at ($P < 0.05$)

4. DISCUSSION

Propolis is an aresinous material collected by bees from bud and exudates of the plants, which is transformed in the presence of bee enzymes *(Burdock, 1998).* Flavonoids, aromatic acids, caffeic acid, terpenes and phenolic constituents appear to be the principal components responsible for the biological and pharmacological activities of propolis *(Bankova et al., 2000; Orsolic et al., 2004).*

Concerning to leukogram, *E.* Coli-Infected group showed significant leukocytosis and granulocytosis after 2, 4, 8 and 16 days' post-infection when compared with control, While, there was a significant lymphocytosis after 4 days' post-infection. Our results agree with *Shin et al., (1998)* who found that Shiga toxin of *E. coli* caused marked increase (seven fold) of granulocytes in the peripheral blood. *Tanaka et al., (2006)* demonstrated that I/P injection of *E. coli* lipopolysaccharide (LPS) stimulate inflammatory response. The response in turn caused release of chemical mediators such as macrophage colony stimulating factor which in turn activates various cell systems as macrophage and neutrophils.

Propolis + *E. coil* group showed significant decrease in leukocytes after (4, 8 and 16 days) and granulocytes after 2, 4, 8 and 16 days' post-infection when compared with *E. coli* infected group. While *E. coli* + propolis group showed significant decrease in leukocytes after 16 days and significant decrease in granulocytes after 4, 8 and 16 days' post-infection when compared with *E. coli* infected group. These results agreed with *Machado et al., (2012)* who demonstrated local and systemic anti-inflammatory action resulting from an immunomodulatory action of propolis. These effects may be due to synergic and/or additive effect(s) of various propolis compounds there by decreasing the inflammation observed. The extracts are also capable of modulating the production of pro-inflammatory and anti-inflammatory cytokines preventing amplification of the inflammatory process.

Regarding to cytokines, *E. coli* group showed significant increases in TNF-α and IL-1β, significant decrease in IL-4 after 2 and 16 days when compared with control. On the other hand, Propolis + *E. coli* group induced significant decreases in TNF-α and IL-1β after 2 and 16 days when compared with *E. coli* infected group, While *E. coli* + propolis group induced significant decreases in TNF-α after 2 and 16 days but IL-lβ after 16 day These results agree partially with *Korish and Arafa., (2011)* who found marked increase in the pro-inflammatory cytokines TNF-α and IL-1β levels and non-significant decrease in IL-4 in animals with lipopolysaccharide endotoxemia reveals picture of systemic inflammatory reaction, On the other hand, propolis treatment with *E. coli* infection was found to cause significant decrease in TNF-α and IL-1β. This may be attribute to the ability of propolis to decrease the infiltration of the liver cells by the inflammatory (neutrophil and monocytes) cells which is suggested to strengthen the defense mechanisms against reactive oxygen species into the liver cells and attenuate the excessive inflammatory injury induced by TNF-α and IL-1β as demonstrated by decreased plasma levels of these cytokines in the treated groups *(Yildiz et al., 2009).* Propolis treatment with *E. coli* infection was found to be associated with a significant increase in IL-4 levels in the treated group. This may be explained by the potential anti-inflammatory effect of propolis which decreases the release of the inflammatory

cytokines from the inflammatory cells and at the same time it stimulates anti-inflammatory cytokines synthesis like IL-4 *(Doughty et al., 2002).*

Regarding to antibodies, *E.* Coli-Infected group showed significant decrease in IgM after 2 and 16 days' post-infection when compared with control. While, Propolis + *E. coli* group was found to induce significant increase in IgM after 16 days' post-infection when compared with *E. coli* infected group. Our results agree with *Giurgea et al., (1983)* who found that rats fed with a standard propolis extract together with *E. coli* antigen, showed an increase in the formation of antibodies compared with rats did not receive propolis. Propolis activates the immune system, increasing macrophage activity *(Dimov et al., 1991)* and increasing interleukin-2, and interleukin-4 levels *(Orsolic and Basic., 2003; Park et aL, 2004).* These cytokines stimulate B lymphocytes and then they are changed to plasma cells, which would be able to produce immunoglobulins *(Diker., 1998).*

Concerning to phagocytic % and phagocytic index, our data showed significant decrease in phagocytic % and phagocytic index in *E. coli* group, *E. coli* infection cause impairment of polymorphonuclear leukocytes (PMNLs) function, decreased phagocytic activity and ineffective opsonization *(Hegazy et al.,* 2010). These results agree with *Abd El-Tawab et al., (2015)* who found significant decrease in phagocytosis and this may be due to exhaustion of immune system by *E. coli* infection. On the other hand, Propolis + *E. coli* and *E. coli* + propolis groups showed a significant increase in phagocytic % and phagocytic index when compared to *E. coli* infected group. These results agree with *Gao et al., (2014)* who found that administration of Brazilian green propolis could promote peritoneal phagocytosis and increased phagocytic index in comparison to the control group. A study conducted by *Fischer et al., (2010)* showed that the polyphenol compounds extracted from Brazilian green propolis could promote specific antibody secretion in mice. Artepillin-C a low-molecular-weight phenolic compound was effective in T helper cells expansion and activation, as well as macrophage activation as demonstrated previously by *Cheung et al., (2011) and Kimoto et al., (1998).* Therefore, we consider a possibility that artepillin-C may be one of the most important ingredients in green propolis in activating macrophage phagocytosis.

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