Egyptian propolis (EEP); Experimental evaluation of immunomodulatory effects on swiss albino mice

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ABSTRACT

Propolis is a natural substance has antibacterial, antioxidant, anti-inflammatory and immunomodulatory activities. The present study aimed to investigate the immunostimmulant effect of Egyptian propolis on mice. Ninety Swiss albino mice apparently healthy of both sexes were divided into 3 groups of 30 each. Group 1 (G1) was kept as a control; Group 2 (G2) was supplemented with 100 mg of ethanolic extract of propolis (EEP) in water intake; Group 3 (G3) was supplemented with 200 mg of EEP in water intake. Mice in all groups were kept under the same environmental, managerial and hygienic conditions. The result revealed that administration of 200 mg Propolis/kg had significantly hyper responder compared to the control group in all examined parameters. The phagocytic percentage was 74.00 ± 0.57 % and 67.66 ± 0.88% in G3 and G2 respectively compared to 61.00 ± 0.52 %, in G1 after 21 days of supplementation. The phagocytic index was 1.91 ± 0.05 and 1.43 ± 0.03 in G3 and G2 respectively compared to 0.84± 0.01 in G1. The level of lysozyme showed significant increase, and it was 334.5 ± 23.01 and 273.2 ± 34.8 in G3 and G2 respectively compared to 197.02± 15.85 in G1 after 21 days of supplementation. While tumor necrosis factor (TNF-α) showed significant decrease and it was 52.4 ± 0.18 and 57.59 ± 0.30 in G3 and G2 respectively compared to 59.30 ± 0.60 in G1 after 14 days of supplementation. The level of IgG showed significant increase and it was 891.00± 2.30 and 759.66± 1.45 in G3 and G2 respectively compared to 702.66 ± 3.71 in G1. The level of IgM showed significant increase and it was 202.33± 3.17 and 177.00± 2.51 in G3 and G2 respectively compared to 156.33 ± 1.20 in G1.

Key words: Ethanolic extract of propolis (EEP), Phagocytosis, Immunostimulant.

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1. INTRODUCTION

Propolis (bee glue) is a natural sticky resinous mixture produced by honey bees *Apis mellifera* collected from various part of plant sources (Banskota *et al.,* 2001; Wagh and Borkar, 2012). Propolis has more than 300 phytocompounds. These compounds are collected from different leguminous plants and collectively termed as propolises which are valuable sources of new and biologically active molecules possessing antimicrobial property and these phytochemicals have inhibitory effects on all types of microorganisms (Wagh and Borkar, 2012). Propolis has been used in folk medicines and complementary therapies since 3.000 B.C in Egypt and in different nations (Hegazi, 1998) and has become one of the most popular functional foods all around the world (Wang *et al.,* 2013). Propolis showed a broad spectrum of bioactivities, such as antioxidant (Sulaiman *et al.,* 2011), antibacterial (Mascheroni *et al.,* 2010), immunomodulatory (Sforcin, 2007), antiviral (Nolkemper *et al.,* 2010), cardioprotective (Zhu *et al.,* 2011), antiproliferative effects and anti-inflammatory effects (Paulino *et al.,* 2006). Egyptian propolis becomes a subject of research by biologists and chemists (Kujumgiev *et al.,* 1999; Hegazi *et al.,* 2000a, 2000b and Hegazi and Abd El-Hady, 2002). Little was known about the immunomodulatory action of propolis until the 1990s, but in the last decade new and interesting articles were published, providing an important contribution to this research field (Sforcin, 2007). Several studies have previously reported immunostimulatory and anti-inflammatory effects of propolis in mammals (Zhang *et al.,* 2009 and Talas and Gulhan 2009). In fishes, propolis has been reported to have an

important effect on aquatic environment (Christybapita *et al.,* 2007) and to enhance non-specific immune responses and disease resistance of Nile tilapia through dietary supplementation (Abd-el-Rhaman, 2009). The effect of propolis on the immune system has also been investigated by some authors, who showed its ability to activate macrophages and stimulate antibody production by sheep red blood cells (SRBC) - immunized mice (Scheller et al., 1989). Tatli Seven & Seven (2008) reported that propolis stimulated immune system and decreased mortality rate by improving immunity in broilers. They remarked that propolis supplementation in poultry diets as alternative to antibiotics may be recommended in broilers in heat stress conditions.

Therefore, the present study was performed to evaluate the use of propolis as an immunostimulant in mice. So the present study aimed to investigate the immunostimmulant effect of EEP on mice.

2. MATERIALS AND METHODS

2.1. Ethanolic extract of propolis (EEP)

Crude (Egyptian) propolis samples from *Apis mellifera* were collected kindly by beekeeper from private farm in Zagazig, El-Sharkia government, Egypt. Propolis was hardened in freezer (-40 ºC) to kept dry and cold until used. Hundred grams of frozen propolis is grained in a handy grinder, then dissolved in 300 ml of 96% ethanol. This mixture was kept in the incubator at 30 ºC for two weeks in a dark bottle closed tightly. After incubation procedure, supernatant was filtered twice with Whatman No. 4 then with Whatman No. 1 and filter paper (Uzel et al., 2005). Ethanolic extracts of propolis (EEP) were freshly prepared and administered to animal with different doses.

2.2. Animals

This study was carried out on 90 Swiss albino mice apparently healthy of both sexes. The mice aged (4-6 weeks) weighed about 25-30 gram, were obtained from the Animal Health Research Institute (Dokki, Giza, Egypt). The mice were kept in polypropylene cages under proper environmental conditions at room temperature 22-24ºC and 12h light/dark cycle and fed with a commercial pellet diet (Wadi El Kabda Co., Cairo, Egypt). The animals were acclimatized to laboratory conditions for two weeks before beginning the experiment. The experiment continued for 3 weeks on which constant weight of diet was given for each mouse. All the experiment were designed and conducted according to the ethical norms approved by the Ethical Committee of Animal Health Research Institute. Mice were randomly grouped into 3 groups (30 mice in each group) G1 (control group), G2 (mice received an oral daily dose of propolis extract (100 mg/kg) (El-Khayat, *et al.,* 2009) on water intake and group G3 mice received an oral daily dose of EEP extract (200 mg/kg) (Missima & Sforcin, 2008 and El-Mahalaway *et al.,* 2015) for 2 weeks on water intake. There were no pregnant females were used in this study.

2.3. Blood sample collection (serum preparation)

For the collection of autologous serum (AS), 300 µl of blood was collected by retro-orbital puncture using heparinized blood capillary tubes under mild ether anesthesia. After collection, the blood samples were incubated at room temperature for 10 minutes and left to clot for 2 hrs at 4°C, the clotted blood was centrifuged; the serum was removed from the clot. The serum was centrifuged again for 30 min at 500xg at 4°C to remove any remaining insoluble material. It was stored in clean stopper plastic vial at 4°C for a week at maximum or stored at -20ºC until the analysis of serum parameters.

2.4. Phagocytic activity

The phagocytic activity was assessed according to Zhang et al. (2010) with some modification. Phagocytosis assay was done at 3, 7, 14 and 21 days after EEP supplementation. The results are expressed as phagocytic percentage and phagocytic index.

2.5. lysozyme assay

Lysozyme activity was measured by agarose by gel lysis assay according to Schultz (1987).

2.6. TNF-α determination

The content of The TNF-α was evaluated in serum as described by Ogata et al., (1993) using a commercially available enzyme-linked immunosorbent assay (ELISA) kit supplied by Biosource, USA, according to the manufacturer's protocol. TNF-α activity were measured at day 3, 7 and 14 after supplementation of EEP.

2.6. Immunoglubulin assay

IgG and IgM were measured in serum, using a commercially available enzyme-linked immunosorbent assay (ELISA) kit supplied by GenWay Biotech, Inc. USA, according manufacturer's instructions.

2.7. Effect of EEP on growth performance

Body weight after supplementation of mice with EEP was individually recorded at 3, 7 and 14 days to evaluate the effect of EEP on growth performance.

2.8. Statistical Analysis

All data were analyzed for mean and standard error of mean and were statistically analyzed by conducting analysis of variance (ANOVA) test for least significant Difference (LSD) for determination of the significance between means at $P > 0.05$ according to Petrie and Watson (1999).

3. RESULTS

The obtained results revealed that, administration of mice with EEP in dose of 200 µg/mouse/day showed improvement in cellular and humeral immunity than 100 µg/mouse/day of EEP in comparison with control group.

3.1. Phagocytic activity

Peritoneal macrophage phagocytic activity/index was determined in mice supplemented with EEP at dose of 100 mg/kg (G2), EEP at dose of 200 mg/kg (G3) and untreated group (G1). After 3 days of supplementation the phagocytic percentage showed no significant increase in any of groups and the phagocytic index was nearly similar in all groups Figure (1). The phagocytic percentage showed significant increase in G3 and G2 respectively in comparison with G1 after 7, 14 and 21 days of supplementation (Table.1). After 7, 14 and 21 days of supplementation the phagocytic index reveled significant increase in G3 and G2 respectively in comparison with G1 (Table.2 and Figure1).

3.2. Lysozyme activity

The highest serum lysozyme activity was seen in group G3 and G2 respectively compared to G1 after 21 days of EEP supplementation. G2 showed no significant difference with control group ($p>0.05$) after 3,7 and 14 days of supplementation (Table.3)

3.3. TNF-α activity

After 3 days of supplementation with EEP TNF-α level showed no significant decrease in any of treated groups compared to control group (G1). While TNFα level revealed significant decrease at 7 days, 14 days in G3 compared to G1. The level of TNF-α in G3 was 55.12 ± 0.36 and 52.4 ± 0.18 after 7 days and 14 days respectively compared to 59.36± 0.54 and 59.30 ± 0.60 in control after 7 days and 14 days respectively. While G2 showed significant decrease at 14 days only compared to G1. It was 57.59 ± 0.30 compared to 59.30 ± 0.60 in G2 compared to G1 respectively Table (4).

3.4. Immunoglobulins assay

The effect of EEP supplementation on serum IgG and IgM are shown in Table (5). Supplementation of EEP revealed significant increase in IgG level in EEP treated groups in comparison with control. Serum IgG level was 891.00± 2.30 and 759.66± 1.45 in G3 and G2 respectively compared to 702.66 ± 3.71 in G1 (Table.5). Supplementation of EEP showed significant increase in IgM level in EEP treated groups in comparison with control group. Serum IgM level was 202.33± 3.17, and 177.00± 2.51 in G3 and G2 respectively compared to 156.33 ± 1.20 in G1 (Table.5).

3.5. *Growth performance*

The supplementation of EEP revealed no significant increase in body weight after 3 days in treated groups, while it showed significant increase in G3 and G2 in comparison with G1 after 7 days of supplementation. After 14 days of supplementation of EEP the body weight showed significant increase in G3 in comparison with G1 and it was 31.00 ± 0.20 g in G3 compared to 29.87 ± 0.21 g in G1 Table (6).

Table (1): Phagocytic percentage in mice supplemented with EEP

Group	phagocytic percentage %			
	3 days	7 days	14 days	21 days
G1	61.00 ± 0.49^{a}	61.00 ± 0.57^{c}	60.33 ± 0.88^{c}	61.00 ± 0.52^{c}
G2	60.66 ± 0.30^{a}	64.33 ± 0.32^{b}	66.33 ± 0.64^{b}	67.66 ± 0.88^{b}
G3	63.33 ± 0.33^{a}	67.33 ± 0.88^{a}	71.00 ± 0.53^{a}	74.00 ± 0.57^{a}

The mean difference within the same column and bearing different superscripts are significantly different at $p > 0.05$ levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (2): Phagocytic index in mice supplemented with EEP

Group	phagocytic index			
	3 days	7 days	14 days	21 days
G1	0.87 ± 0.008[a]	0.84± 0.022[b]	0.85 ± 0.02[c]	0.84± 0.01[c]
G2	1.02± 0.02[a]	1.33 ± 0.03[a]	1.42± 0.02[b]	1.43 ± 0.03[b]
G3	1.18± 0.06[a]	1.36 ± 0.05[a]	1.80± 0.04[a]	1.91 ± 0.05[a]

The mean difference within the same column and bearing different superscripts are significantly different at p > 0.05 levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (3): Serum Lysozyme activity (µg/ml) in mice supplemented with EEP

Group	Serum Lysozyme (µg/ml)			
	3 days	7 days	14 days	21 days
G1	210.02±16.95[a]	182.41± 31.5[b]	213.9± 14.56[b]	197.02± 15.85[b]
G2	233.09± 19.13[a]	252.2 ± 18.21[b]	252.2 ± 19.11[b]	273.2 ± 34.8[a]
G3	292.4± 21.07[a]	357.3 ± 22.21[a]	357.3 ± 22.8[a]	334.5 ± 23.01[a]

The mean difference within the same column and bearing different superscripts are significantly different at p > 0.05 levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (4): The effect of EEP supplementation on TNFα (pg/ml) in mice

Group	TNFα (pg/ml)		
	3 days	7 days	14 days
G1	59.66 ± 0.86[a]	59.36± 0.54[a]	59.30 ± 0.60[a]
G2	59.21± 0.17[a]	58.91 ± 0.32[a]	57.59 ± 0.30[b]
G3	56.3 ± 0.03[a]	55.12 ± 0.36[c]	52.4 ± 0.18[c]

The mean difference within the same column and bearing different superscripts are significantly different at p > 0.05 levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (5): The effect of oral supplementation of EEP on IgG and IgM in mice

Group	IgG	IgM
G1	702.66 ± 3.71[c]	156.33 ± 1.20[c]
G2	759.66± 1.45[b]	177.00± 2.51[b]
G3	891.00± 2.30[a]	202.33± 3.17[a]

The mean difference within the same column and bearing different superscripts are significantly different at p > 0.05 levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (6): The effect of supplementation of EEP on body weight

Group	Body weight (g)		
	3 days	7 days	14 days
G1	28.62 ± 0.23[a]	29.00 ± 0.40[b]	29.87 ± 0.21[b]
G2	28.60± 0.35[a]	29.62± 0.23[a]	30.00± 0.20[b]
G3	28.97 ± 0.43[a]	30.50 ± 0.20[a]	31.00 ± 0.20[a]

The mean difference within the same column and bearing different superscripts are significantly different at p > 0.05 levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

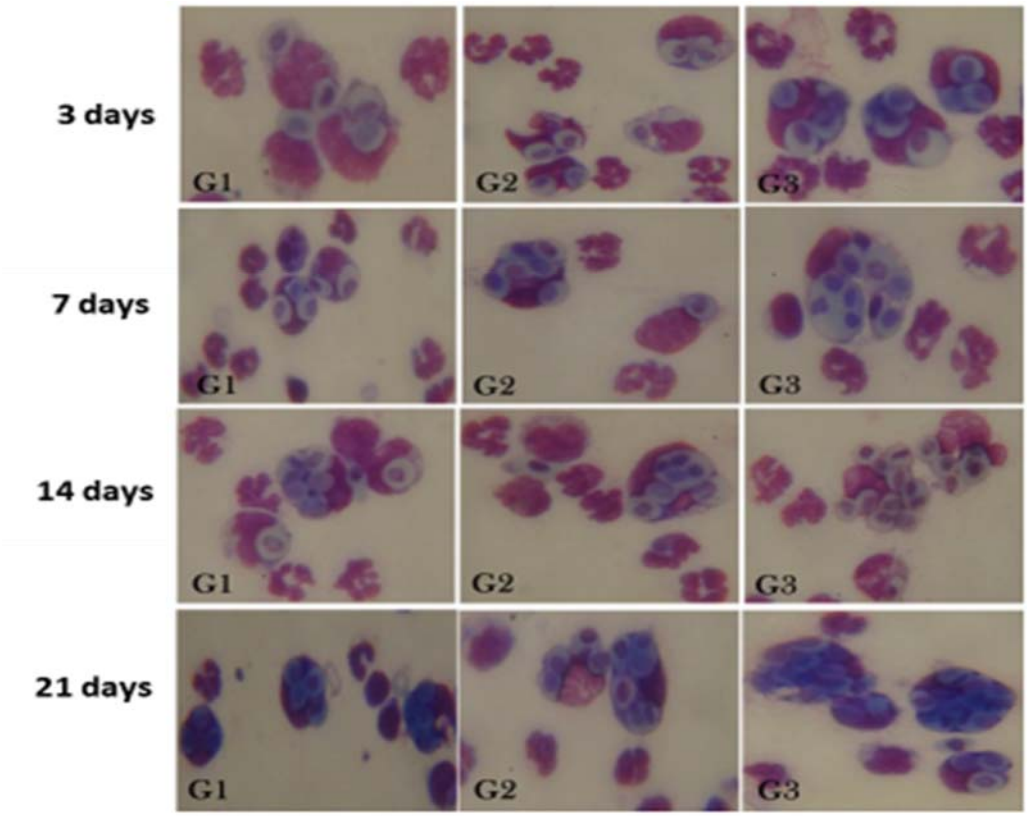

Figure (1): Effect of chitosan supplementation on mice peritoneal macrophage phagocytic activity engulfed chicken red blood cells. The phagocytic activity was examined under light microscopy at a total magnification power of 100x.

4. DISCUSSION

This study has been conducted to assess immuostimulant effect of Egyptian propolis. Mice were supplemented with two doses of EEP (200 mg/kg and 100 mg/kg). The highest dose of EEP revealed more effect on cellular and humeral immunity than the low dose, which means that the effect of EEP is a dose dependent.

The supplementation of EEP showed significant increase in phagocytic percentageand phagocytic index of macrophage than the control group. This result concur with the reports of previous workers Dimov et al., (1992); Ivanovska et al., (1995); Murad et al., (2002) and Cuesta et al., (2005) who described the increments in the intensity, mobility and activities of leukocytes, and in the production of IL-1, TNF and activating factors of mammalian leucocytes after in vitro or in vivo treatment with propolis. Probably, the increase in the leucocytic count might have resulted in the enhancement of the nonspecific defense, because leucocytes are the key elements in the immune system and are the major affecter and effector cells on which propolis exerts its activities. The result of present study is in agreement with Cuesta et al., (2005) who reported that water and ethanolic-extracts of propolis increased the phagocytic percentage in gilthead seabream. Besides, Talas and Gulhan (2009) reported many changes in count of differential leucocyte following administration of toxic concentration of propolis via immersion route in rainbow trout. The results of enhancing phagocytic activity after supplementation with EEP are in accordance with Tatefuji et al., (1996); Murad et al., (2002) and Orsoli and Basic (2003) who recorded that propolis enhanced the macrophage functions and lymphocyte proliferation in several mammalian species.

The lysozyme activity is an important indicator of the immune defence in both invertebrates and vertebrates (Ellis, 1990). Lysozyme are proteins of low molecular weight found in polymorphonuclear leukocyte and mononuclear cells, lysozyme are considered as a member of innate humoral factors that elaborate from the body and showed dramatic increase in concentration in response to infection or tissue injury (Weir, 1983). Lysozyme has an antibiotic ability and is released by leukocytes, it damage bacterial cell walls especially of Gram-positive and some Gram-negative bacteria (Grinde, 1989) by hydrolysis glycoside link between N-

acetylmuramic and N-acetylglucosamine in peptidoglycan layer of bacterial cell wall and activate the complement system and phagocytes by acting as an opsonin (Magnado, 2006).

The present study showed that the dietary supplementation of EEP increase lysozyme activity compared to control especially in G3. Where, the higher levels of serum lysozyme activity in the treated groups could have contributed to the noticeable enhancement of the non-spesific defense mechanisms (Engstad et al., 1992). The results of this study is concordance with Alishahi and nejad (2013) who reported that food supplemented with 0.5% and 1% PEE showed significant increase in serum lysozyme activity and stimulated the immune response in B. barbulus.

The production of pro-inflammatory cytokines such as TNF-α is increased in acute inflammatory responses associated with infection, injury, trauma or stress (Avitsur et al., 2006). The present study demonstrated that the dietary supplementation of EEP showed significant decrease in the level of TNF-α as compared to control group. Daily administration of 200 mg/kg EEP for two weeks in mice (G3) showed the more significant decrease of TNF-α as compared to the control group. Propolis contains a number of natural active constituents that have been shown to exert a variety of medical properties including anti-inflammatory activity (Khayyal, et al., 1993; Ozturk, et al., 2000). the results of this study is in agreement with previous work by Khayyal et al., (2003) who mentioned that daily administration of aqueous extract of propolis for two months to asthma patients decreased pro-inflammatory cytokines production suggesting the anti-inflammatory effect of propolis. in the other study by Seven et al., (2012) who reported that administration of propolis in rats increased the osteoprotegrin and decreased TNF-α, and nuclear factor-kappa B ligand which inhibited the osteoclastogenesis. These results are in contrast to previous study by El-Mahalaway et al., (2015) who reported that administration of propolis at dose of 200 mg/kg induced the level of TNF-α in rat.

Immunoglobulin is a protein produced by plasma B-cells that has the ability to recognize and neutralize foreign objects (Solem and Stenvik, 2006). Specific immunoglobulins in combination with lymphokines could indicate collectively the status of humoral immunity playing essential role in protecting against bacterial as well as viral infections (McKee et al., 2007). The results of this study showed that daily administration of EEP for two weeks (G3 and G2) showed significant increase in IgG and IgM compared to G1. In concordance with this result, Gunathilaka et al., (2015) who observed that serum lysozyme activity and plasma Ig significantly higher in fish fed 1% propolis in powder form and 0.5% propolis in liquid form. Also, Yonar et al., (2011) reported that after oral administration of propolis the plasma Ig level of rainbow trout increased significantly. Similar results have been reported by Sforcin et al., 2005 and Sforcin, 2007) who reported that propolis stimulate Ig production in rats regardless of season and origin and an ethanolic extract of propolis was shown to increase Ig production in mice.

After treatment of mice with different concentrations of EEP the body weight of mice was measured. EEP administration at concentration of 200 mg/kg (G3) showed significant increase in comparison with G1 control group. This results is in concordance with Hegazi et al., (1996) who studied the effect of Egyptian Propolis on chicken body weight and lymphoid organs. Body weight showed significant increase after one week post injection and increase thymus weight after 14 days post injection up to the end of the experiment. On other hand, Abd- El-Rhman (2009) reported significant increase in average weight-gain (AWG), specific growth rate (SGR) and feed conversion ratio (FCR) in tilapia fed with propolis enriched diet. In contrast to this result Galal et al., (2008) reported that there was no significant difference among treated groups with EEP for body weight in hens. Another finding by Alishahi and nejad (2013) who revealed that supplementation of food by EEP did not induce any specific change in all growth indices including: AWG, SGR, FCR and FER in fish. On the other hand Cuesta et al., (2005) have not observed either mortality or growth rate alteration after daily intake of propolis in the diet during 6 weeks in gilthead seabream. They used propolis with the origin of southern region of Iran, whereas propolis used in this experiment was originally from the northern Egypt. The difference between the origins of propolises, which influence its quality, may be one of the main reasons for the incoherence among the different results.

In conclusion, Egyptian propolis has powerful immunostimulant effect. Administration of mice with Egyptian EEP in dose of 200 mg/kg showed improvement in cellular and humeral immunity than 100 mg/kg of EEP in comparison with control group, this mean that the effect of EEP is dose dependent. Propolis administration in high dose improves growth performance.

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