Effect of propolis in diet supplementation on the histopathological changes in some organs and challenge tests against Newcastle disease in broiler chicks.

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Abstract

The present study was conducted to evaluate the effect of dietary supplementation with propolis on histopathological changes of some tissue and challenge tests against Newcastle disease virus at 21 and 42 day old chicks.

One hundred and eighty Ross broiler chicks were used in day-old they were divided randomly to six groups (30 birds/group), feed and drinking water offered ad libitum and experimental groups were treated as follows:

The first four groups received (0.5, 1, 2, 3) g propolis/kg in feed respectively, the five group served as positive control received basal diet without propolis but vaccinated and group six served as negative control received basal diet without propolis and non-vaccinated.

Histopathological changes in bursa of Fabricius showed normal histology with mild condense proliferation of lymphocyte in the cortex at the G4 as compared with other groups which did not show any pathological lesions. Histopathological changes in the liver of G 4 showed mononuclear cell aggregation in liver parenchyma and promenance of kappafer's cells as compared with other groups (G1,G2,G3,G5 and G6) which showed normal tissue. The microscopical examination of the spleen, kidney and thymus showed no histopathological alteration in all the groups. The challenge test showed that the total mortality was zero in group 4 and 3 and low in groups 1 and 2 as compared with groups 5 and 6.

Introduction

Propolis or bee glue, as it is commonly named, is a natural resinous mixture produced by honeybees (Apis mellifera) from substances collected from parts of plants, buds and exudates (Ghisalberti, 1979). This resin is masticated, salivary
enzymes are added, and then it is mixed with beeswax and probably with other compounds of bee metabolism (Burdock, 1998).

Propolis, a resinous substance produced by honey bees from exudates collected from different parts of plants (Fischer and Vidor, 2008), presents several biological activities (Fischer et al., 2007a, b; Vatansever et al., 2010; Gregoris and Stevanato, 2010), even though many of its action mechanisms are unknown. Propolis pharmacological activity against several viral infections has been evaluated in studies with influenza virus (Serkedjieva et al., 1992), adenovirus (Amoros et al., 1992), and herpes simplex viruses (Schnitzler et al., 2010; Nolkemper et al., 2010). The wide spectrum of propolis biological activities together with the need for new virucidal substances, renew the interest for this bee product regarding its antimicrobial potential (Nolkemper et al., 2010).

Within the several last year's using the prebiotics, probiotics and natural products is going to substitute for antibiotics in order to improve immune system and fight against pathogens in human and animal life. In contrast to antibiotics these products do not have side effects and are very useful in food chain (Aghdam et al., 2011).

**Materials and Methods**

**Experimental Animals:**

The chicks (Rose, Syria Origin), were brought from Hatchery Association of wade AL-Rafidian – Bagdad /Abu grab. The 180 one day old chicks were divided randomly into 6 groups) (all biosecurity protocols were applied). Organ samples were taken from 5 chicks randomly from each group for assessment of histological section, take same of organs (liver, kidney, spleen, thymus and bursa of Fabricius) at 21 and 42 day. The chicks in G1,G2,G3,G4 and G5 were vaccinated with ND (La Sota) via drinking water at day 15 followed by booster dose of Newcastle virus vaccine (La Sota) at day 25 and with IBD Gumbo L strain (Ceva-Hungary) at day (19) and G6 left without any vaccine as control negative.

**Preparation of Poultry House**

The experiment was done in poultry farm of animal resources department at the College of Agriculture / Baghdad University, Poultry house, after cleaning and disinfection (by Formaline and Sodium Hypochlorite and left for 2 days, finally all doors and windows were opened and ventilator were switched on to assure complete removal of residual toxic gases before chicks admittance, the experiment began at 19/12/2011 to 3/2/2012.
Diet Composition and Contents: The basal diet was formulated for broilers in which yellow corn and wheat were the major sources of energy, whereas the soybean and plant protein were the major sources of protein in this diet. This diet was fed to all groups. Other ingredients were same as in the groups (table 1). Nutritional requirements were adjusted according to the Nutritional Requirements Council (NRC, 1994).

a. **Starter:** The chicks fed on starter diet from 1 to 20 days at the beginning from experiment.

b. **Final:** The chicks used from 21 days until the end of the experiment (day 47). It was composed of the followings:

Table (1) showing the Composition of experiment's diets prepared in this study.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Percentages of ingredients in Starter</th>
<th>Percentages of ingredients in Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>plant Protein (5% protein)</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Soybean meal (45% protein)</td>
<td>31.5%</td>
<td>22%</td>
</tr>
<tr>
<td>Yellow corn</td>
<td>47%</td>
<td>47%</td>
</tr>
<tr>
<td>Wheat</td>
<td>12%</td>
<td>20%</td>
</tr>
<tr>
<td>fat</td>
<td>2.5%</td>
<td>4%</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.5%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Vitamins</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Ca co3</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>0.3%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Histopathological Examination:

The Specimens where taken at 21 and 42 day old with dimensions 1 cubic cm were taken from liver, spleen, kidney, thymus and bursa of Fabricius, the tissues were fixed in 10% formalin buffer solution immediately after removal. After 72 hrs of fixation, the specimens were washed with tap water and routinely processing with a series of upgrading alcoholic concentrations started from 70% to absolute 100% for 2 hrs in each concentration to remove water from the tissues, then clearance was done by xylol. Specimens were infiltrated with semi-liquid paraffin wax at 58°C on two stages. blocks of specimens were made with paraffin wax and sectioned by rotary


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microtome at 5μm thickness for all tissues. Section were stained with Hematoxylin and Eosin (H & E), stained and examined using light microscope (Luna, 1968).

**Haemagglutination tests**

a) Twenty five microlitre of PBS was dispensed into each well of plastic U-shape bottomed 96 wells microtitre plate.
b) Twenty five microlitre of virus suspension (i.e. infective allantoic fluid) was placed in the first well.
c) Two fold dilution of 0.025 ml volumes of the virus suspension were made across the plate.
d) Twenty five microlitre of 1% (v/v) washed chicken RBCs was dispended to each well.
e) The suspension was mixed by tapping the plate gently the RBCs were allowed to settle for about 30 minutes at incubator. When control RBCs were settled to a distinct button, HA was determined by tilting the plate and observing the presence or absence of tear–shaped streaming of the RBCs. The titration was read to the highest dilution giving complete HA (No streaming); this represented 1 HA unit (HAU) and was calculated accurately from the initial range of dilutions (OIE, 2004).

**Source of the strain:**

A velogenic field strain was received from Dr. Abdul Ameer Zahid.

**Egg Inoculation:**

Nine- to ten- days old embryonated chicken eggs were used for propagation of the virulent NDV. The eggs were candled before inoculation and all non-viable eggs were discarded. The air cell were marked and sterilized with 70% alcohol and were allowed to dry.

The seed virus suspension was diluted in sterile PBS and 0.25 ml of the dilution 10⁻³ was inoculated into the embryonated eggs by intra-allantoic route. The inoculated eggs were incubated at 37 °C and examined after 24 hour, dead embryos, if any, were discarded and the remaining embryos were reincubated further, and candled every six hour.

Embryos which died subsequently were stored at +4 °C. After 48 hour of incubation, the eggs were removed from the incubator and chilled for at least 24 hrs, (Young *et al.*, 2002).
Harvesting:

The sterilizing solution were allowed to dry, the tops of the eggs were removed and the allantoic fluids aseptically aspirated after depression of the embryo. The inclusion of any yolk material and albumin were avoided. Allantoic fluids from these embryos were collected with aseptic precaution and tested for haemagglutination activity by spot test using 10 percentage pooled and washed chicken erythrocyte. The harvested fluid was centrifuged for 20 minutes at 1500 rpm.

The supernatant was used as the source of virus. As the harvest progressed, the bottles were removed, closed, numbered and placed immediately at +4 °C.

A small pool was also made from the harvested Allantoic fluids and titrated in embryonated eggs for virus content (Palya, 1991). have haemagglutinins on their envelops that will attach to special sites (receptors) on the surface of red blood cells, causing them to clump together “haemagglutination”, therefore HA-positive allantoic fluid was tested by haemagglutination Inhibition test with Reference hyper immune serum to NDV to ascertain that an isolate was NDV (Thayer and Beard, 1998)

Titration of Virulent Virus

The HA test gives an indication of the amount of virus in the sample, but does not tell if the virus is viable “live”. To determine if the virus is viable, the infectivity of the virus was measured by titration in embryonated eggs. Nine- days old embryonated chicken eggs were used for titration of the virus. Series of 10-fold dilution (10⁻¹ to 10⁻¹⁰) were prepared in antibiotic mix Phosphate Buffer Solution (PBS), pH 7.0-7.4 and 0.25 ml of the dilution 10⁻⁵ to 10⁻¹⁰ was inoculated into each of 10 embryos per dilution by the allantoic route, sterile PBS was used for the control group. The eggs were examined twice daily for embryopathy, mortality of embryos was recorded up to 72 hours after inoculation. the allantoic fluid was collected from the chilled dead embryos and tested for HA activity by Rapid plate test for confirmation of the presence of ND virus(Allan et al.,1975). Embryo lethal dose (ELD₅₀) of field isolate was determined following the method of (Reed and Muench 1938) and found to contain ELD₅₀ 10⁻⁷.₅/ml.

Statistical Analysis:
Data were analyzed by using complete randomize design (CRD) in factorial experimental by the falling model.
The Data were analyzed by using SPSS (2008). To calculate the difference between means was used Duncan-multiple test (Duncan, 1955).

Results and Discussion

Histopathological study
Bursa of Fabricius (BF)

Histological section of BF showed normal histology with mild condense proliferation of lymphocyte in the cortex of BF at the G4. Although, bursa of Fabricius is a major organ in antibody production in chickens (fig. 1).

![Histological section in the bursa of chicken at 42 day old post administration 3g propolis showed mild proliferation and lymphocytes in the cortex of group 4. (H&E stain 400X).](image)

The microscopical examination of bursa in groups received 1and 2g propolis showed no clear histopathological lesion and bursa showed normal structure similar to the control negative (fig.2).

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The present findings reveals the stimulating effect of propolis on B-cells which is leading to increase the humoral immunity. The result of this study of histological section of BF is in agreement with Ziaran et al. (2005) who reported that the number of proliferating cells in bursa of Fabricius increased at highest level of propolis in the diet. The germinal center increased noticeably as compared with the controls positive and negative at this level. Also these finding are in agreement with José et al.,(2011) who mentiand that the evaluating the stimulatory effect of ethanolic extract of propolis on the Bursa of Fabricio in F1 male chicks Rhode Island Red X Rhode Island White, It was show statistical significant differences between the treatments (P<0.05).and who found that the ethanolic propolis extract has an stimulatory effect on the Bursa of Fabricio in F1 male chicks Rhode Island Red X Rhode Island White compared to the control groups.

**liver**

Section of liver from G4 (42 day old) showed mono nuclear cell aggregation in liver parenchyma as well as proliferation of kupffer's cells (fig.3), which was not noticed in both groups 5,6 this can be attributed to absence of propolis. Moreover G1 and G2, did not show any pathological lesion in liver.
The results of histological sections of liver in G4 is in agreement with Ziaran et al. (2005), who reported that there was an increase in the thickness of blood vessel and the number of lymphoid cell in per portal area of liver was at highest level of propolis in the diet of chickens. The present data was also in agreement with Attalla and Ayman (2008), who mentioned that the honeybee propolis ameliorated the recovery of hepatotoxicity in mice, where it acts as an antioxidant scavenges free radicals and could restore the normal liver functions and normal histology.

**Histological examination in Spleen, Thymus and Kidney:**
The microscopical examination in all groups did not show any histopathological alteration in spleen, kidney and thymus tissue.

**Challenge tests:**

**Virus used for challenge:**
A velogenic field strain was received from Dr. Abdul Ameer Zahid The velogenic strain was titrated before use as described by Reed and Muench (1938) and found to contain ELD$_{50}$ $10^{7.5}$/ml.
Table (2). Explain the result of ELD$_{50}$.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Total No. of embryos</th>
<th>No. of dead embryos</th>
<th>No. of live embryos</th>
<th>Ratio of dead</th>
<th>Percentage of dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$</td>
<td>10</td>
<td>9</td>
<td>_</td>
<td>9/10</td>
<td>90%</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>10</td>
<td>9</td>
<td>_</td>
<td>9/10</td>
<td>90%</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>4/10</td>
<td>60%</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5/10</td>
<td>50%</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>10</td>
<td>2</td>
<td>8</td>
<td>0/10</td>
<td>20%</td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>10</td>
<td>_</td>
<td>10</td>
<td>0/10</td>
<td>0%</td>
</tr>
</tbody>
</table>

Results of the challenge tests:

In the challenge experiment at 42 days old with virulent velogenic NDV showed high mortality of served as positive and negative control where she was (2, 3) from 5 chicken respectively also gave less mortality Amounting to (2, 1) from 5 chicken in G1 and G2 alternatively and there were noticed same clinical signs in G1 and G2 such as ruffled feathers, loss of appetite, depression, as well as swelling of the head and eyes. However results from G3 and G4 that did not register any mortality and there were did not showed any clinical signs on chickens and This is proof that the positive role of propolis.

Table (3). Challenge test and protected percentage.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No. of chicks</th>
<th>Live chicks</th>
<th>Dead chicks</th>
<th>Protected percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>60%</td>
</tr>
<tr>
<td>G2</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80%</td>
</tr>
<tr>
<td>G3</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>G4</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>Control positive</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>60%</td>
</tr>
<tr>
<td>Control negative</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>40%</td>
</tr>
</tbody>
</table>

The results of this study was in agreement with the finding Hegazi et al. (1995) who studied the effect of some bee products on immune response of chicken.
infected with virulent NDV. They found that, the mortality rate reduced in groups infected with virulent NDV and subsequently treated either with propolis or honey if compared with the infected groups only.

The present finding agreement with Shalmany and Shivazad (2006), who mentioned that average weight gain and feed consumption, feed efficiency were significantly higher for propolis fed birds and inclusion of Propolis also reduced mortality rate in comparison to the control diet. Also these finding are agreement with Giurgea et al (1981) There was daily administration of propolis extract to chickens changed the blood concentration of cholesterol, total proteins and amino acid, as well as stimulated the immune system which resulted in decreased mortality as compared to the control.

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