



CELL MEDIATED AND HUMORAL IMMUNE-RESPONSES OF GROWING RABBITS FED CITRUS LIMON OVERABUNDANT DIET

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ABSTRACT: This study was conducted to assess the effect of Citrus limon powder (CLP) as immune-enhancer phytobiotic of growing New Zealand White rabbits (NZW). Forty eight growing rabbits were randomly allocated to three equal groups in a Randomized Complete Design (RCD). Rabbit groups were fed a basal diet served as control group, while the other two groups were fed basal diet mixed with CLP dosed at 10 and 20g/kg of feed, respectively. After 7 weeks of the experiment rabbits were injected with sheep red blood cells (SRBC's) as immunizing agent then blood samples were taken 7 days later. Serum titers (\log^2) for both total antibody and mercaptoethanol (ME)-sensitive antibody to detect IgG were assayed by microtiter procedures. Sections of the immune-organs were collected from same rabbits for histological investigations. The results of the cell mediated immune-responses showed highly significant difference ($p<0.01$) in the values of total and differential leukocytes, and chemokinetic movement and phagocytic activity as well as, humoral immune-responses (TIg, IgG, IgM and precipitation test) significantly ($p<0.01$) affected by feeding growing rabbits on 10 and 20g CLP compared with those fed control diet. Moreover, histological structure of thymus and spleen of treated rabbits showed normal structure.

In conclusion, 10 and 20g CLP/kg diet enhance growing rabbit immune-responses with normal immune organ structure.

Key words:Rabbit -Cell Mediated Immunity – Humoral Immune-responses - Citrus limon.

INTRODUCTION

Optimal management and nutrition can reduce costs and economize productions to offer higher quality products to consumers (Laudadio et al., 2009). Further, due to concerns of bacterial resistance, the use of antibiotics has been under scrutiny. Different antibiotics may be used simultaneously or intermittently in poultry and animal diet. Ban the use of antibiotics as growth promoters, leading them to find alternatives in animal feeding (Dibaji et al., 2012; Aziz Mousavi et al., 2012). One of these, natural products originating from fruits, vegetables and it's by products has been used as feed supplementations for farm animals long time ago. A lot of experiments studied the effects of herbs, plant extracts and essential oils on broiler performance gave however contradicting results. Some investigators reported affirmative effect of medicinal plants on broiler performance (Cross et al., 2007), while other trials using different additives and essential oils did not affect broilers production (Ocak et al., 2008). Lemon is an important plant of the family Rutaceae. The main bioactive compounds are; Limonene, Terpinene, Pinene and Geranial. These compounds are classified as Terpenoids, which play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic and other pharmaceutical functions (Sayed et al., 2013). Furthermore, Akbarian et al. (2013) showed that fruits of the Citrus family (particularly orange and lemon), are rich in Phenolic compounds, which phenolic compounds exhibit considerable antimicrobial activity. Their antimicrobial ability may modulate the gut ecosystem to affect feed efficacy. Dried Citrus sinensis peel (DCSP) can significantly improve the immune system activities, and this action has been attributed to their antioxidant properties (Chen et al., 2012). Recently, Abbas et al. (2015)

concluded that DCSP feed supplementation ameliorated the gastrointestinal microbiota and immune system traits. Moreover, it has been demonstrated that herbal extracts can increase the antibody production, especially immunoglobulin G (IgG), indirectly improving the immune system activity with their anti-virus and anti-bacteria action (Catala-Gregori et al., 2007). Kadam et al. (2009) reported that addition of lemon juice to drinking water improved immunity of broiler chickens under heat stress condition. Nobakht, (2013) indicated that feeding 5% dried Citrus limon peel (DLP) tended numerically to increase the heterophils/lymphocyte ratio as a stress indicator that may impair immunity. These results imply that feeding high levels of DLP in broiler diets may apply negative impact on humoral immunity of broilers. While, Abbas et al. (2015) speculated that supplementing citrus pulp up to 2% of diet had no impact on humoral immunity of broiler chickens. Rafiee et al. (2016) found that, dietary supplementation of 1.0% lemon verbena increased ($p < 0.05$) average weight gain and feed intake by 5.81% and 3.29%, and reduced feed conversion ratio by 2.59%, respectively compared to control group. The ratio of heterophyl to lymphocyte was reduced ($p < 0.05$) by 20.68% via significant decrease in heterophyl by 15.55% and significant increase in lymphocyte by 4.51% in birds fed lemon verbena at the rate of 1.0% compared to those fed the control diet, and concluded that, lemon verbena and vitamin C improved some performance data and blood metabolites, which somehow suppressed the negative effects of heat stress. Reihan and Majid (2017) concluded that, dietary consumption of DLP particularly at high levels is not recommended because it may compromise the growth performance of broiler chickens. They added graded levels of

dried citrus to broiler diets. Where dietary treatments are control with no additive, as well as, DLP1: 2.5, 5, and 7.5%; DLP2: 5, 7.5, and 10%; and DLP3: 7.5, 10, and 12% in starter, growing, and finisher phases, respectively. DLP elevated the relative weight of intestinal segments and decreased jejunal crypt depth. Chickens fed on DLP had lower antibody titer against IDV than control bird. Moreover, antibody titer against SRBC decreased in broilers fed on DLP. However, there have only been limited studies conducted to investigate the effects of Citrus limon as powder on immune responses in broiler rabbits.

Therefore, the present study was conducted to assess the effects of substitution of Citrus limon powder (CLP) as growing rabbit immune-enhancer.

MATERIALS AND METHODS

Experimental animals

This experiment was carried out at the farm of Animal and Poultry Production Department, Faculty of Agriculture, Minia University in a semi closed housed on galvanized wire cages (40 × 50 × 35 cm) provided with feeders and automatic drinking watery system, a period of 14-16 hours of day light was provided. Feed and water were available all time ad libitum, during the experimental period (8 weeks). All rabbit groups were kept under the same managerial, hygienic and environmental conditions.

Preparation of dry lemon

Lemon was provided from a private commercial market at El-Minya Governorate Egypt. Dried at 40°C until constant weight. The dry lemon was finally milled, sieved (1 mm mesh) and stored in a well tight polyethylene bags at room temperature 25°C. Composite sample of lemon powder was taken in sample plastic bag for nutritional analysis.

Ration and Lemon Supplementation

Three batches of rabbits ration each of 500 kg were formulated to contain; 44%

ground yellow corn, 40.5% wheat bran, 13.5% soybean meal (44% crude protein), 0.5% lime stone, 1% sodium chloride and 0.5% vitamin & mineral premix according to NRC (1977). Citrus limon powder (CLP) was added and thoroughly hand mixed with other feed ingredients of each batch at 0, 10 and 20g CLP/kg diet. Experimental rations were packed in polyethylene bags until feeding.

Animals grouping and treatments

In a feeding experiment lasted 8 weeks, Forty eight growing New Zealand White (NZW) rabbits aged eight weeks weighed in average 1543.33 ± 25 g were randomly blocked by weight into three groups (16 animals each), where the 1st group fed a basal ration free of Citrus limon (control), while the 2nd and 3rd groups were fed on the same basal ration supplemented with 10g and 20g CLP/ kg diet respectively.

Collection of blood samples

At the end of the experiment, 5 ml blood samples were taken at 07:00 – 08:00 am from the marginal ear vein under vacuum in clean tubes with or without heparin before slaughtering time, coagulated blood samples were centrifuged for 15 minutes on 5000 rpm and the clear serum was separated and stored in a deep freezer at -20°C until analysis. Non-coagulated blood was tested shortly after collection for estimating total, deferential white blood cell count, chemokinesis and phagocytic activity testes.

Total leucocytes count (Nx10³)

The white blood cells were counted according the method of (Campbell, 1995). The number of the cells per cubic millimeter of blood was calculated using the following equation:

$$\frac{\text{Number of WBC's count /mm}^3 \text{ of blood} \times (N/64)}{160 \times 120} = N \times 50$$

Where “N” is the number of leucocytes counted in 4 large (64 – small) square.

Differential leucocytes count:

Differential leucocytes counts were determined according to (Phillip et al.,

2009). 10 µl of blood is placed near one end of a slide on a flat level surface; the blood is then placed with the edge of another slide held at about 30-degree angle to the slide being prepared. After air-drying, smears were fixed in absolute methanol and subsequently stained with Giemsa stain. Stained films were examined microscopically using oil immersion; then percentage of each type of leukocytes in relation to the total number of leucocytes counted was calculated.

Cell mediated immunity

Cell Mediated immune-responses were determined using the following techniques, chemokinesis and phagocytosis.

Phagocytosis (%):

The phagocytic activity of macrophages as well as other phagocytic cells in whole blood was examined using the binocular Optika Research LED Fluorescence Microscope (model B-500TIFL) as follows:- The whole blood was kept in cold saline solution (0.9 % NaCl). One drop of blood sample was mixed with a drop of paraffin oil and examined and photographed (Abu El-Maged, 1991).

Microporefilters assay (Chemokinetic Assay)

In an attempt to more precisely quantify the chemotactic response, a modified Borden's chamber assay was used (Boyden, 1962) in which cells migrate from one compartment, through micropore filter, towards a compartment containing chemoattractants (SRBC's). The method employed here after (Gearing and Rimmer, 1985; Abu El-Maged , 1991; and El -Feki, 1994), the two compartments were formed by the wells of two microtitration plates on top of each other with cellulose acetate micropore filters (5µm millipore 67/20 Molsheim , France) partitioning the wells. The lower test wells were filled with chemoattractant (SRBC's) and in case of controls the lower wells was filled with saline (0.9 % NaCl). 5µm filter micropores were then placed over all lower wells. The

upper plate was inverted and placed over the lower plate with smearing petroleum jelly around the perimeter of both plates where they were then clamed tightly together. 0.3 ml of blood was introduced into each chamber through a hole made in the base of each well. The whole plate was then covered with aluminum foil to reduce evaporation of liquid and then placed in humid incubator at 37°C for 16-18 hours. Following incubation, the fluid was removed from the upper wells using syringe and needle, and replace with 0.3 ml of methanol for the fixation of the cells. After 20 minutes the methanol was removed and the two plates were separated. The filter paper was then fixed in 10% formalin for 20 minutes and stained in Delafield's haematoxylin for 5 minutes then washed with distilled water and each one was mounted in Canada balsam on microscopic glass slide with the under surface uppermost. Two counts were made on each of 5 filters. The chemokinetic effect obtained was expressed by a chemokinetic index according to (El-Feki, 1994), calculated as following:

Mean number of cells per h.p. field with chemokinetic stimulus
Chemokinetic index (CI) = -----
Mean number of cells per h.p. field with control stimulus

Humoral immune response:

The humoral response to SRBCs was determined using the following techniques; quantitative determinations of total immunoglobulin (TIg), IgG and IgM by hemagglutination of antibodies and detection of precipitation antibodies.

Hemagglutination

Detection of agglutinating antibodies was assessed after 8 weeks of treatments using passive hemagglutination techniques. In our study the response to immunological challenge was assessed at the same time: a sheep red blood cell (SRBC) hemagglutination test (Wegmann and Smithies, 1966; Aitken and Parry, 1974; Klasing, 1988; Deerenberg et al., 1997). The SRBC hemagglutination assay entails exposing the animal to SRBC and

quantifying the resulting production of SRBC-antibodies. Each animal was injected intraperitoneally with SRBC in Phosphate Buffered Saline (PBS, pH = 7.2, approximately hematocrit ~2%), using a dosage of 0.1 ml/100 g of body mass. One week later, a 0.5-1.0 ml blood sample was taken from each animal. Samples were centrifuged to separate serum, which was stored at under -20°C until analysis.

Hemagglutination Technique

Twenty-five µl of serum from each sample was serially diluted with 25 µl of PBS, using a V-shaped 96-well microtiter plate, resulting in a two-fold dilution series (i.e., 2-1, 2-2, 2-3, 2-4,...). Twenty-five µl of a suspension of 2% SRBC in PBS was then added to each well. The plate was incubated at 37°C for 1 h and examined visually to determine the highest dilution (= lowest concentration) of serum that led to an agglutination of SRBC. Results are expressed as the negative of the base-2 dilution factor (Wegmann and Smithies, 1966; Aitken and Parry, 1974). Mercaptoethanol (ME)-resistant antibody (IgG) was measured as described by Yamamoto and Glick (1982). The titers, both total and IgG, were expressed as the log₂ of the reciprocal of the highest dilution giving visible agglutination. Treatment with Mercaptoethanol inactivates IgM, and as a result, hemagglutination observed after treatment with 2-ME is due mostly to the presence of IgG antibodies. The difference between total antibody and IgG titers was determined the IgM titer.

Precipitation test:

Precipitating antibodies were detected in control as well as treated groups' sera using the immuno-double-diffusion technique. In this technique antigen and antibody were allowed to migrate towards each other in a gel and a line of precipitation was formed where the two reactants meet. As this precipitate is soluble in excess antigen, a sharp line is

produced at equivalence. This technique only identifies antigen and antibodies (Hudson and Hay, 1980).

Procedures:

- 1- Boil 0.5g agar in 100 ml water until it dissolved (15-20 min).
- 2- Allow agar solution to cool to 5°C and the pipette 10 ml onto each glass plate (0.2 ml/cm²) placed on a leveling table.
- 3- Allow the agar to gel and then leave overnight at room temperature (or 4-6h at 40°C) until completely dry. Protect pre-coated plates from moisture.
- 4- Prepare 1% (w/v) agar solution by heating in 100 ml PBS (phosphate buffer saline) in boiling water bath until completely dissolved.
- 5- Allow agar solution to heat to 45°C and carefully pipettes a 5 ml onto each plate. Allow solidifying.
- 6- Wells are then punched in the gel using the cork borer or gel punch in pattern of a central well surrounded by 5-6 wells of varying distances to ensure optimal antigen – antibody ratios during diffusion.
- 7- Remove the plugs of agar from the wells using Pasteur pipette attached to vacuum line. Adjust pressure so that the plugs are quickly and eventually removed.
- 8- Prepare dilutions of antigen and serum in PBS.
- 9- Pipette 20 µl neat serums into the central well of the pattern and pipette the dilutions of antigen (20 µl /well) in order of concentration in the surrounding wells.
- 10- Pipette 20 µl neat antigen into the central well of another set wells bored in the same pattern and pipette the dilution of serum (20 µl/ well) in order to dilution in the surrounding wells.
- 11- Incubate overnight at room temperature in humid chamber.
- 12- Wash the gel in 5 changes of 100 ml of PBS over 48 h.
- 13- Dry by covering with a sheet of filter paper and leaving for 16 h at room temperature or 2-6 h at 40°C

14- Immerse gel in stain for 5-10min until stained bands are visible.

15- Destine in 3-4 changes of destine solution until background are visible.

16- Dry in air at room temperature.

17- Examine plates for immune-precipitation line.

Histopathological examinations

Microscopic changes were observed through random selection of thymus and spleen samples from each test and control animals. Tissues were preserved in 10% formalin followed by dehydration in ascending grades of alcohol. Clearing by xylene and embedding in paraffin wax. Paraffin sections (5 μ m thickness) were stained with hematoxylin and eosin (H & E) for histological examination (Diab et al., 2012).

Statistical analysis:

The study was conducted based on a completely randomized design (CRD) with three treatments and 16 replicates per treatment 1 animal each. Data were analyzed by Statistical Analysis System software (SAS, 2003) using the generalized linear model (GLM) procedure. The statistical model was as follows:

$$X_{ij} = \mu + T_i + e_{ij}$$

Where; X_{ij} = Value observed in each experimental unit, μ = Mean population, T_i = The effect of each treatment, and e_{ij} = The effect of experimental errors.

However, significant differences among treatment means for each trait in experiment was detected using Duncan's multiple rang test (Duncan, 1955).

RESULTS AND DISCUSSION

Total and differential count of white blood cell

From Figure (1) it is clear that, the addition of CLP (10 and 20g /kg diet) to growing rabbit ration enhance ($p < 0.05$) WBC's of rabbits where, it increases the total number of WBC's compared with control group. There was intense increase on lymphocytes % while addition of CLP clearly promoted

it nearly to rabbit normal range. Conversely, monocytes and neutrophils % recorded highest % by feeding rabbits on basal diet plus CLP compared with control group. Contrary, adding CLP to growing rabbit diet failed ($p < 0.05$) in enhancing monocyte %, which increased. There was no significant difference between CLP supplemented groups and control group in eosinophil and basophil values. Neutrophillia that appeared may be due to increase granulopiosis as result of CLP supplementation to the diet. Further it is constitute the first line of defense with efficient chemotactic response, it suggested that rabbits of treated group were better equipped for the non-specific cellular response when invaded by foreign agents viable or innate. On the other hand, significantly ($p < 0.05$) higher level of globulin (data not showed), suggested that rabbits of treated group had potential for better humoral immune status, than those of control group. These results match those observed in earlier studied of Behboudi et al. (2016) who investigated the effects of dietary supplementation with thyme and drinking water contained lemon juice on H, L and H/L ratio of broilers under heat stress. Where they found that, thyme or lemon juice alone did not affect H and L percentage and H/L ratio, whereas, their interaction significantly decreased H percentage ($p \leq 0.05$) and H/L ratio ($p \leq 0.01$) and increased L percentage ($p \leq 0.05$). While, Rafiee et al. (2016) found that, the ratio of heterophyl to lymphocyte of broilers chicks under heat stress was reduced ($p < 0.05$) by 20.68% via significant decrease in heterophyl by 15.55% and significant increase in lymphocyte by 4.51% in birds fed lemon verbena at the rate of 1.0% compared to those fed the control diet.

Cell mediated reactions

The effects of dietary treatments on cell mediated immune responses (Chemokinesis and Phagocytic activity %)

are presented in Figures (3 to 8). Rabbits received CLP diets (CLP10 and CLP20 g/kg diet) had moderate and strong chemokinetic index respectively compared with control group, which recorded the weakest chemokinetic index. Moreover, rabbits fed control diet had lowest phagocytic activity compared with CLP groups. Furthermore, rabbits received, (CLP 20g) had highest phagocytic activity compared with other groups. It is well known that phagocytosis is integral component of the inflammatory response and innate immunity. While, if the response to the substance has increased speed or frequency of migration it is termed chemokinesis. Phagocytosis is the first step of the macrophage response to invading microorganism while CLP elevated the innate immune response in the body by increasing the ability of heterophils and mature monocytes (Figures 6, 7 and 8) to engulfing antigens (phagocytosis). It is known that macrophages produce some Reactive Oxygen Species (ROS) during phagocytic process. Accordingly, oxygen radical production indicates the actual killing capacity of the macrophages. It is suggested that hydrogen peroxide (H₂O₂) and Nitric Oxide (NO) by macrophages may contribute to inflammation and tissue injury (Loskin and Laskin, 1996). Modulation of immune response to alleviate diseases has since long been of great interest to researchers (Spelman et al., 2006; Ozek et al., 2011; Mahima et al., 2012). Stimulating the non-specific immune modulation, essentially granulocytes, macrophages, natural killer cells and complement functions (Hashemi and Davoodi, 2010; Mirzaei-Aghsaghali, 2012). Several plant extracts, compounds and formulations have also been patented and this include various polysaccharides, lectins, peptides, flavonoids and tannins which are used in various in vitro models to assess their immune responses (Cherng

et al., 2008). In literature, many plants have been listed; having immune modulatory effects and some of them have been proven using modern scientific methodologies (Barnes et al., 2007). Several studies have shown that *Citrus limon* has a strong capability for scavenging superoxide radicals, hydrogen peroxide and nitric oxide from activated macrophages, reducing iron complex and inhibiting lipid peroxidation (Behboudi et al., 2016; and Rafiee, et al., 2016).

Humoral immune response

The effects of dietary treatments on total immunoglobulin, IgG, IgM (mg/dl), and Precipitation test are presented in Figure 2. Data showed that there were no significant differences among CLP levels (CLP10 and CLP 20g/kg diet), moreover, they recorded the highest values of total immunoglobulin, IgG and IgM compared with control group. Moreover, when rabbits were fed on CLP levels there were positive (formed a sharp line, Figure 10 and 11) Precipitation test compared with those fed control diet which had negative precipitation test (absent of sharp line, Figure 9). Lowest antibody titer against SRBC (hemagglutination) was observed in rabbits fed on control diet that was substantially lower than those fed on 10 and 20g CLP/kg diet. Moreover, dietary administration of CLP significantly increased antibody titer against SRBC as compared with the rabbits in control group ($p < 0.05$). Data of humoral immune responses refer to that some can be performed in fluid i.e (total immunoglobulin, IgG, IgM and hemagglutination). However others such as precipitation can be executed in gel. These tests are illustrated in Figures 9, 10 and 11. In these cases, antibodies are called precipitins. They react with dissolved antigens and form large complexes that become visible as a fine precipitate. The physical form has the majority influence of antibodies detection. A precipitation

reaction occurs as a result of the combination of antibodies in solution with soluble substances in which the antibodies react. Generally, our results indicated that rabbits fed on control diet hadn't any precipitation execute as clear in Figure 9. This might be due to the lower amount of total antibody (Figure 2). In contrast to our data, Reihan and Majid (2017) indicated that chickens fed on 10 and 12% dried lemon pulp had lower antibody titer against IDV than control birds. Moreover, antibody titer against SRBC decreased in broilers fed on 5 and 7.5 dried lemon pulp. While, Nobakht (2013) indicated that feeding 5% DLP tended numerically to increase the heterophil to lymphocyte ratio as a stress indicator that may impair immunity. These results imply that feeding high levels of DLP in broiler diets may apply negative impact on humoral immunity of broilers. In contrast, Chen et al. (2012) reported that, dried Citrus sinensis peel (DCSP) can significantly improve the immune system activities, and this action has been attributed to their antioxidant properties. Moreover, it has been demonstrated that herbal extracts can increase the antibody production, especially immunoglobulin G (IgG), indirectly improving the immune system activity with their anti-virus and anti-bacteria action (Catala-Gregori et al., 2007). Abbas et al. (2015) studied the effect of different levels of dried Citrus sinensis peel (DCSP) on broilers immune system, and found that, supplementation of Citrus sinensis had no significant effect on SRBC hemagglutination, IgG and IgM after day 28 ($p>0.05$) of treatment. However, SRB Cheamagglutination on day 42 was significantly different ($p<0.05$). According to the results of this study on day 28, the lowest titer of total anti-SRBC was related to control treatment and the highest rate was related to DCSP-0.25 % treatment. Moreover, on day 42, the lowest titer of total anti-SRBC was related to

control treatment and the highest rate was related to DCSP-0.75% treatment. While, according to this study, on day 42, the lowest titer of IgG was related to control treatment and the highest rate was related to DCSP-0.25 and DCSP-0.75% treatments. On day 42, the lowest titer of IgM was related to DCSP-0.25% treatment and the highest rate was related to DCSP-1% treatment. However, Sayed et al. (2013) working on broiler chicks, investigated the effects of lemon essential oil on gastrointestinal tract, blood parameter and immune responses (0.1 and 0.2 g/kg Lemon essential oil) SRBC response and immunoglobulin G and M were not affected by treatments.

Histological changes of some lymphatic organs

1- Spleen:

The slide sections of control and treated groups were in normal histological structure (Figure12). Thus, it showed that the splenic parenchyma was composed of the white and red pulp. It is surrounded by a capsule of dense connective tissue which sent out trabecular dividing the splenic parenchyma into incomplete compartments. The connective tissue of the capsule and trabeculae contained some smooth muscles cells. The white pulp consisted of lymphatic tissue arranged in sheaths around central arteries, as well as, lymphoid nodules appended to the sheaths (the per-arterial lymphatic sheaths).

These sheaths were formed mainly of dense accumulation of small lymphocytes with deeply stained nuclei. The lymphatic follicles were spherical or ovoid structures. They presented as either primary follicles, which were formed mainly of densely packed small lymphocytes, or secondary follicles, which could be differentiated into an outer dark zone formed of small lymphocytes and inner light one called germinal center containing large and medium sized lymphocytes, dendritic cells and few macrophages. The germinal zone

between the white pulp and red pulp consisted of loose lymphoid tissues, many active phagocytes and large number of red blood cells. The red pulp is a reticular tissue, which differentiated into splenic cords and blood sinusoids. The cords consisted of macrophages, monocytes, lymphocytes, plasma cells, and many blood elements (erythrocytes, platelets and granulocytes).

2-Thymus:

Histological structure of the thymus of control and treated group shows normal architecture. Each lobe consists of lobules, which are partly separated by connective tissue. The lobule consists of an outer dark cortex and inner pale medulla. Islands of reticular cells occur in the medulla; the cells are usually vacuolated rather than laminated unlike those of mammalian corpuscles. Each thymic lobule consisted of a dark staining peripheral zone (the

cortex) and light staining central zone (the medulla). Both the cortical and medullary zones have the same cellular types (Figure13). The cortex consisted mainly of small and packed lymphocytes arranged in a continuous layer, which passed from one lobule to the other. The medulla consisted of large number of reticuloepithelial cells, thus it stained lightly. Fewer numbers of thymic lymphocytes were found in the medulla, but these cells are fully mature and smaller than those found in the cortex.

CONCLUSIONS

Citrus limon powder (CLP) feed supplement to growing rabbits resulted in enhancing the immune responses. Feed supplementation with CLP had significant effect on cellular and humoral immune responses. The results suggest a possible use of CLP as immune-enhancer for growing rabbits.

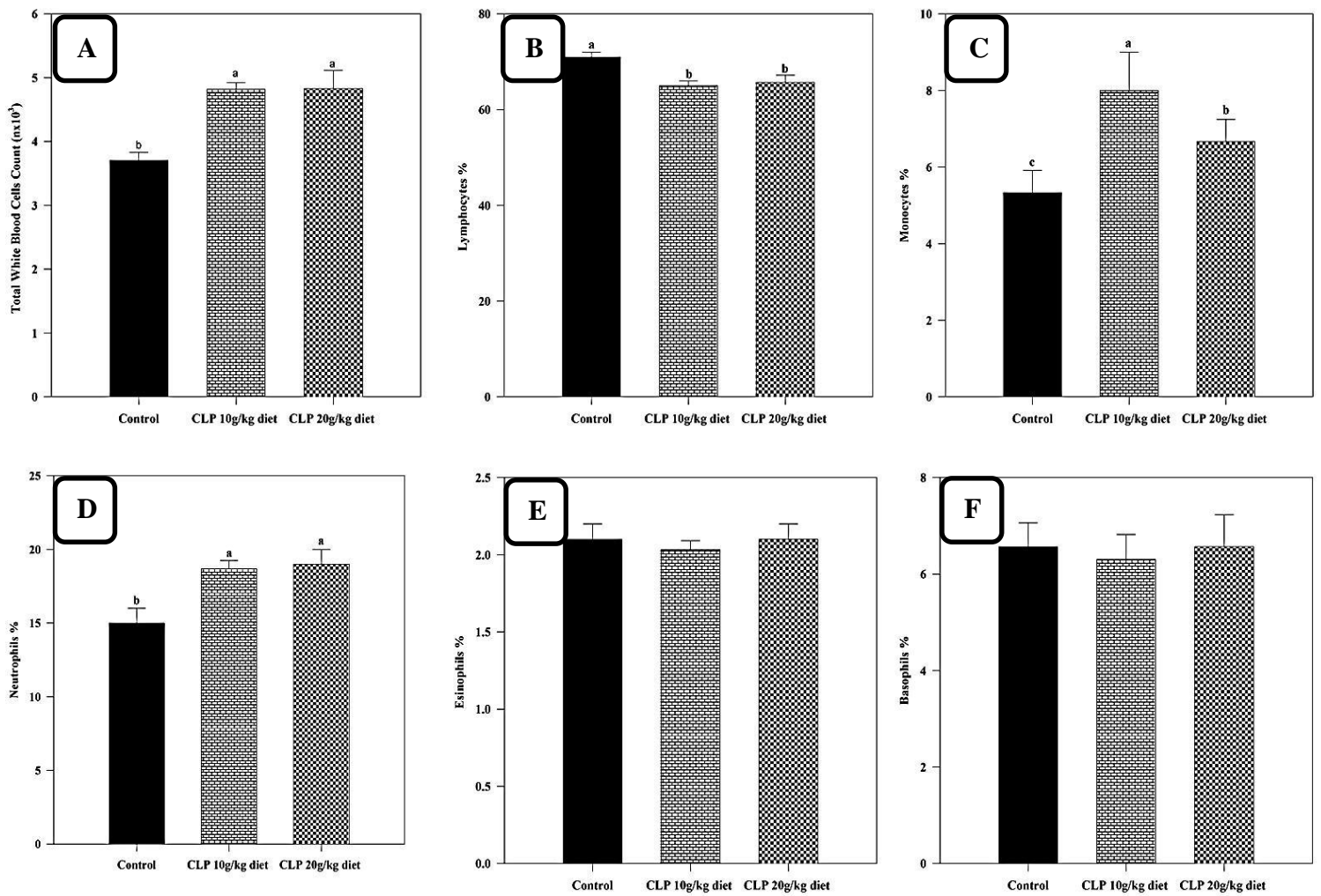


Figure 1. (A) Total white blood cell count, (B) Lymphocytes %, (C) Monocytes %, (D) Neutrophils %, (E) Eosinophil % and (F) Basophils % of New Zealand white rabbits as affected by dietary 0, 10 and 20g Citrus limon powder.

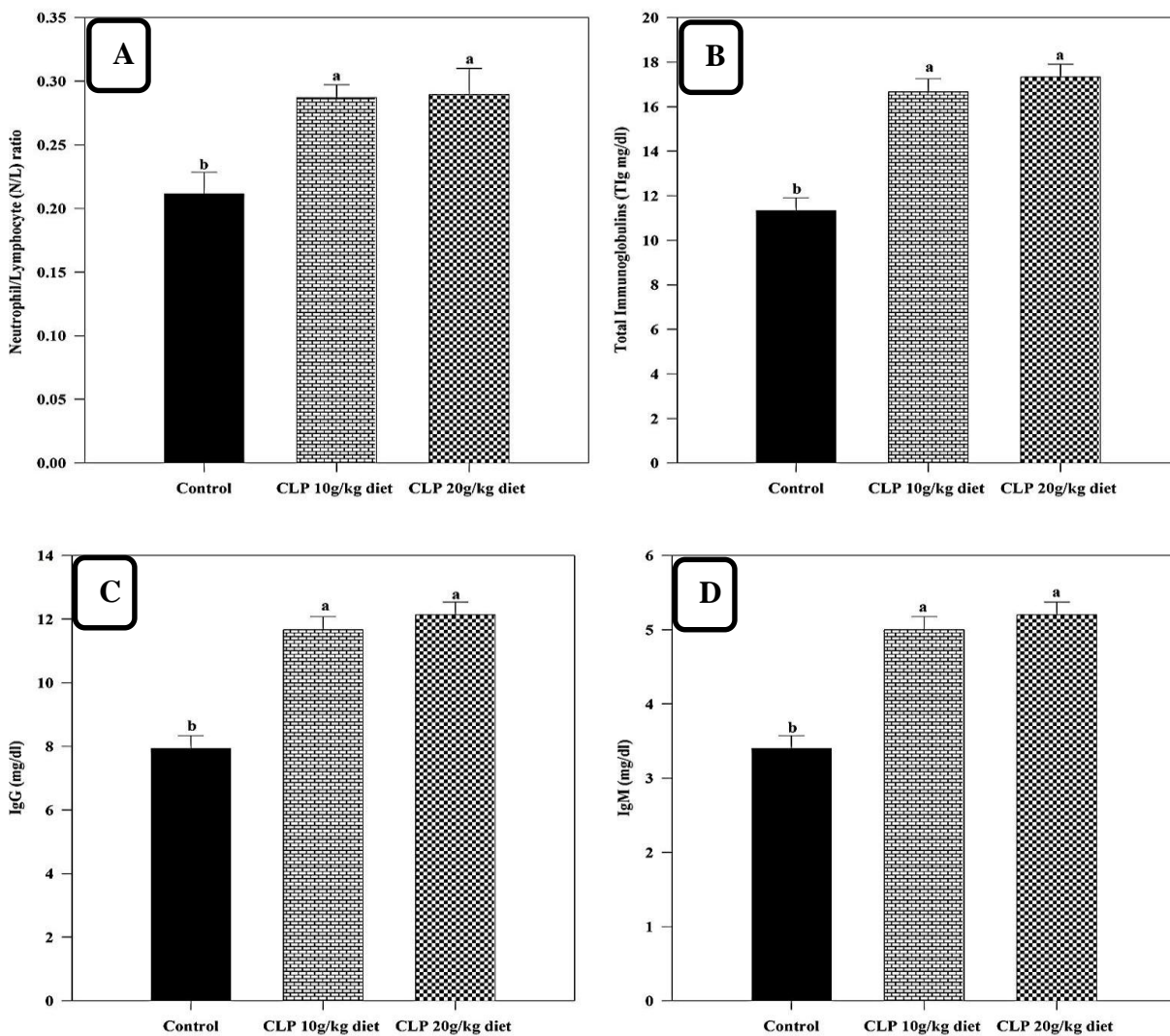


Figure 2. (A) Neutrophil/Lymphocyte (N/L) ratio, (B) Total Immunoglobulins TIg (mg/dl), (C) IgG (mg/dl), and (D) IgM (mg/dl) of New Zealand white rabbits as affected by dietary 0, 10 and 20g Citrus limon powder.

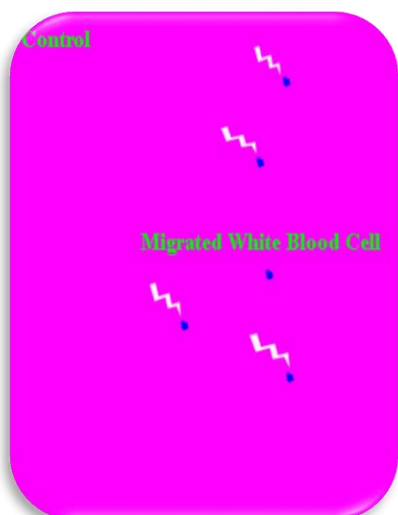


Fig. (3): Photomicrograph of chimokines index of rabbits fed on control diet. Shows weak white blood cells migrations.



Fig. (4): Photomicrograph of chimokines index of rabbits fed on 10g CLP/kg diet. Shows moderate white blood cells migrations. Arrows

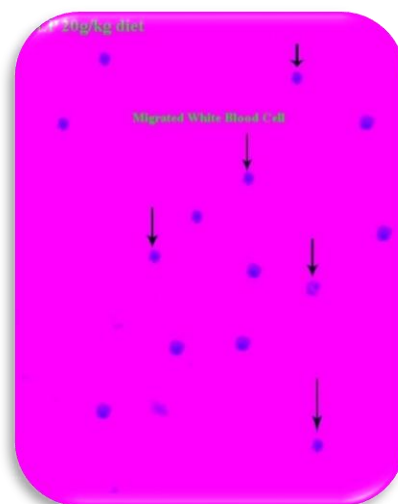


Fig. (5): Photomicrograph of chimokines index of rabbits fed on 20g CLP/kg diet. Shows Highest white blood cells migrations. Arrows

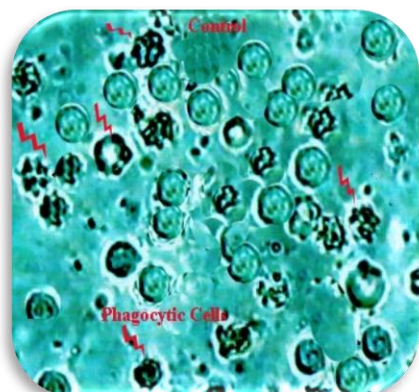


Fig. (6): Photomicrograph of Phagocytic Activity of rabbits fed on control diet. Shows weak phagocytic activity against *Candida albicans*. Zigzag arrows

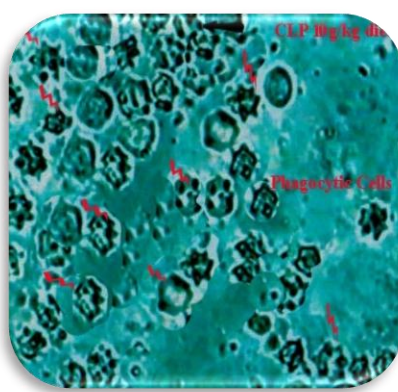


Fig. (7): Photomicrograph of Phagocytic Activity of rabbits fed on 10g CLP/kg diet. Shows moderate phagocytic activity against *Candida albicans*. Zigzag arrows

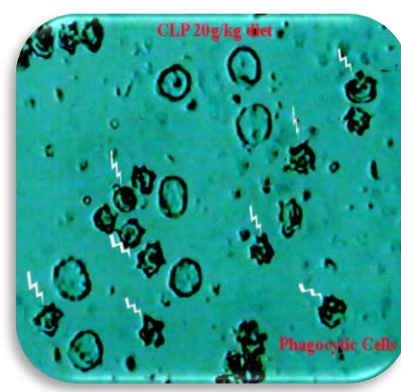


Fig. (8): Photomicrograph of Phagocytic Activity of rabbits fed on 20g CLP/kg diet. Shows highest phagocytic activity against *Candida albicans*. Zigzag arrows



Fig. (9): Precipitation of Antibodies of rabbits fed on control diet. Note. There was no formation of sharp precipitating line (UV 360 nm wave length).



Fig. (10): Precipitation of Antibodies of rabbits fed on 10g CLP/kg diet. Note. The formation of sharp precipitating line (UV360nm wave length).

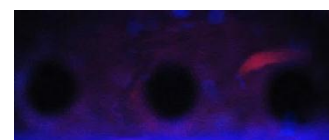


Fig. (11): Precipitation of Antibodies of rabbits fed on 20g CLP/kg diet. Note. The formation of sharp precipitating line (UV 360 nm wave length).

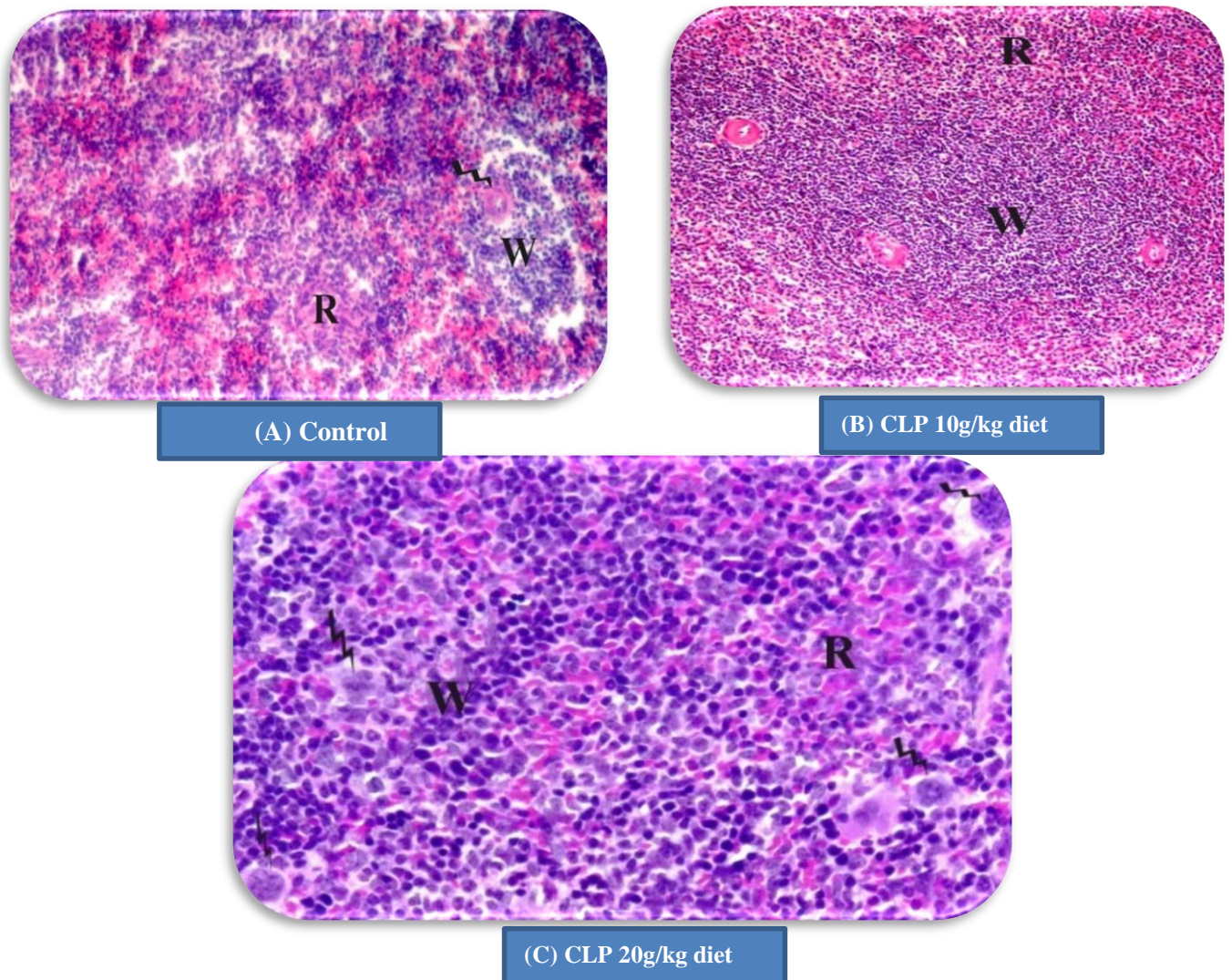


Fig. (12):(A) Photomicrograph of control rabbit's spleen section showing red pulp (R) and white pulp (W), (B and C), Section of spleen of rabbits treated with 10g and 20g CLP/kg diet for 8 weeks showing, improvement in lymphocytic population with capsule (C) as well as infiltration of leucocytes and macrophages (Zigzag arrows). (H&E. x500).

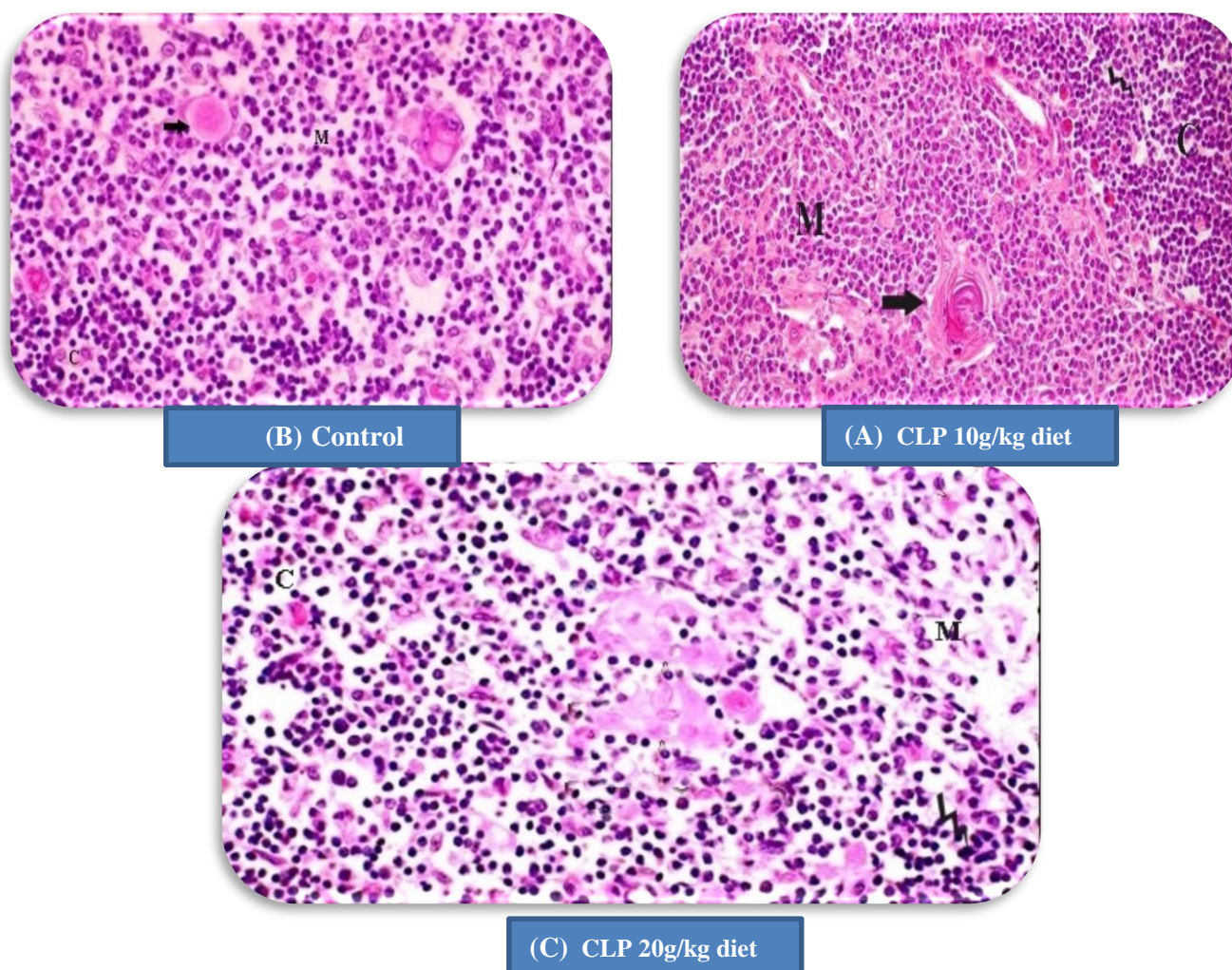


Fig. (13): (A) Photomicrograph of thymus section of control rabbits, showing the cortex (C) and medulla (M). Note, Hassle's corpuscle (thick arrows). (B and C) Section of the thymus of rabbits treated with 10g and 20g CLP/kg diet for 8 weeks showing, cortex (C) and medulla (M) with increasing in lymphocytic population and number of rosettes (Zigzag arrows). (H&E. x500).

REFERENCES

- Abbas, E.; Antonello, S.; Mosè, A.; Zohreh, P.; Nariman, M.; and Alireza, S., (2015).** Effect of dried *Citrus sinensis* peel on gastrointestinal microbiota and immune system traits of broiler chickens, *Ital. J. Anim. Sci.*, 14: 4, 712-717. DOI: 10.4081/ijas.2015.4194
- Abu El-Maged, A. A., (1991).** Encapsulation, chemotactic and Chemokinetic activities of the haemocytes of different stages of *periplaneta Americana*. *Bull. Fac. Sci., Assuit. Univ.*, 20(2-E): 109-122.
- Aitken, I. D.; and Parry, S. H., (1974).** The comparative serological response of the chicken, pheasant and quail to a soluble particulate antigen. *Immunology*, 27: 623-629.
- Akbarian, A.; Golian, A.; Kermanshahi, H.; Farhoosh, R.; Raji, R.; De Smet, S.; and Michiels, J., (2013).** Growth performance and gut health parameters of finishing broilers supplemented with plant extracts and exposed to daily increased temperature. *Span. J. Agri. Res.*, 11(1): 109-119.
- Aziz, Mousavi, S. M. A.; Seidavi, A. R.; Dadashbeiki, M., (2012).** Effect of different levels of synbiotics on carcass characteristics of broiler. *Res. Opin. Anim. Vet. Sci.*, 2: 161-165.
- Barnes, J.; Anderson, L. A.; and Phillipson, J. D., (2007).** *Herbal Medicine*. 3rd Edn., Pharmaceutical Press, London, pp: 1-23.
- Behboudi, H.; Esmaeilipour, O.; Mirmahmoudi, R.; and Mazhari, M., (2016).** The Influence of drinking water containing lemon juice and thyme supplemented diet on performance and some blood parameters of broilers under heat stress. *Iran. J. Appl. Anim. Sci.*, 6(1): 169-174.
- Boyden, S. V., (1962).** The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocyte. *J. Exp. Med.*, 115: 453-466.
- Campbell, T. W., (1995).** *Avian hematology and cytology*(2nd). Iowa state university press, Ames, pp.104.
- Catala-Gregori, V.P., Hernandez, F., Megias, M.D., Madrid, J., (2007).** Effect of formic acid and plant extracts on growth, nutrient digestibility, intestine mucosa morphology, and meat yield of broilers. *J. Appl. Poultry Res.* 16:555-562.
- Chen, Z.T., Chu, H.L., Chyau, C.C., and Duh, P.D., ((2012).** Protective effects of sweet orange (*Citrus sinensis*) peel and their bioactive compounds on oxidative stress. *Food Chem.* 135:2119-2127.
- Cherng, J. M., W. Chiang and L. C. Chiang, (2008).** Immunomodulatory activities of common vegetables and spices of Umbelliferae and its related coumarins and flavonoids. *Food Chem.*, 106: 944-950.
- Cross, D.E., McDevitt, R.M., Hillman, K., and Acamovic, T., (2007).** The effect of herbs and their associated essential oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age. *Brit. Poultry Sci.* 48:496-506.
- Deerenberg, C., Arpanius, V., Daan, S., and Bos, N. (1997).** Reproductive effort decreases antibody responsiveness. *Proc. R. Soc. Lond. B Biol. Sci.* 264: 1021-1029.
- Diab K., Elmakawy A, Abd-Elmoneim O.M. and Sharaf H.A., (2012).** Assessment of genotoxicity and histopathological changes induced by polyethylene glycol (PEG6000) in male mice. *J. Cytology & Histol.*, 3(153): 1-7.
- Dibaji SM, Seidavi A, Asadpour L., (2012).** Effect of dietary inclusion of the symbiotic Biomin IMBO on broilers' some blood metabolites. *Res Opin Anim Vet Sci.*, 2: 10-13.

- Duncan, D.B. (1955).** Multiple range and multiple F tests. *Biometrics*.11, 1- 42.
- El-Feki, M. A., (1994).** The role of chemo attractants in phagocytosis of catfish .*Bull., Fac., Sci., Assuit., Univ., .23 (2-E) 185-200.*
- Gearing, A.; and Rimmer, J. J., (1985).** Amphianlymphokines: leucocyte chemotactic factors produced by amphibian spleen cells following antigenic and mitogenic challenge in vitro. *Develop. Comp. Immunol., 7: 281-290.*
- Hashemi, S. R. and H. Davoodi, (2010).** Phytogenies as new class of feed additive in poultry industry. *J. Anim. Vet. Adv., 9: 2295-2304.*
- Hudson, L.; and Hay, F.C., (1980).** Practical immunology. Hudson, L. and Hay, F.C. (Eds): Blackwell Scientific Publications.
- Kadam, A.S., Lonkar V.D., Patodkar V.R., Kolangath S.M. and Bhosale T.A., (2009).** Comparative efficacy of supplementation of natural (Citruslimon juice), herbal and synthetic vitamin C on the immune response of broiler chicken during summer stress. *Asian J. Poult. Sci.* 3,57-62.
- Klasing, K.C., (1988).** Influence of acute feed deprivation or excess feed intake on immunocompetence of broiler chicks. *Poult. Sci., 67, 626-634.*
- Laudadio, V., Tufarelli V, Dario M, D'Emilio F.P., Vicenti A., (2009).** Growth performance and carcass characteristics of female turkeys as affected by feeding programs. *Poult., Sci., 88, 805-810.*
- Loskin, D. L., and Laskin, J. D., (1996).** Macrophages inflammatory mediators and lung injury. *Methods: A companion to methods in Enzymal., 10:61- 70.*
- Mahima, A. Rahal, R. Deb, S.K. Latheef and H. A. Samad, (2012).** Immunomodulatoryand therapeutic potentials of herbal, traditional indigenous and ethnoveterinarymedicines. *Pak. J. Biol. Sci., 15: 754-774.*
- Mirzaei-Aghsaghali, A., (2012).** Importance of medical herbs in animal feeding: A review. *Ann. Biol. Res., 3: 918-923.*
- Nobakht, A., (2013).** Effects of different levels of dried lemon (*Citrus aurantifolia*) pulp on performance, carcass traits, blood biochemical and immunity parameters of broilers. *Iran J Appl. Anim. Sci., 3:145–151.*
- N.R.C., (1977).** **National Research Council.** Nutrient Requirements of Rabbits, National Academy of Science, Washington, D.C, USA.
- Ocak, N., Erener, G., Burak, A.K.F., Sungu, M., Altop, A., Ozmen, A., (2008).** Performance of broilers fed diets supplemented with dry peppermint (*Menthapiperita L.*) or thyme (*Thymus vulgaris L.*) leaves as growth promoter source. *Czech J. Anim. Sci.* 53:169-178.
- Ozek, K., K.T. Wellmann, B. Ertekm and B. Tanm, (2011).** Effects of dietary herbal essential oil mixture and organic acid preparation on laying traits, gastrointestinal tract characteristics, blood parameters and immune response of laying hens in a hot summer season. *J. Anim. Feed Sci., 20: 575-586.*
- Phillip, C.; Wayne, B., and Shane, R., (2009).** Atlas of clinical avian hematology.1st Ed by John Wiley & Sons United Kingdom. P 16-26.
- Rafiee , F., M. Mazhari, M. Ghoreishi and O. Esmaeilipour, (2016).** Effect of lemon verbena powder and vitamin C on performance and immunity of heat-stressed broilers. *Journal of Animal Physiology and Animal Nutrition.* DOI: 10.1111/jpn.12457
- Reihan B., and Majid T., (2017).** Effect of dietary graded levels of dried lemon (*Citrus aurantifolia*) pulp on performance, intestinal morphology, and humoral immunity in broiler chickens.*Int J Recycl Org Waste*

- Agricult DOI 10.1007/s40093-017-0159-5
- SAS (2003).** Institute. Inc. SAS/STAT user's guide: Statistics (versin6.4th ed.) Vol.2. SAS Insitute. Inc. Cary. NC. 2003.
- Sayed A. H., Mohammad N., Abolfazel Z., Houshang L., Seyyed R. R., and Amir M., (2013).** Effects of lemon essential oil on gastrointestinal tract, blood parameter and immune responses in broilers. Annals of Biological Research, 4 (10):47-51 (<http://scholarsresearchlibrary.com/archi ve.html>)
- Spelman, K., J. Burns, D. Nichols, N. Winters, S. Ottersberg and M. Tenborg, (2006).** Modulation of cytokine expression by traditional medicines: A review of herbal immunomodulators. Altern. Med. Rev., 11: 128-150.
- Wegmann, T. G. and Smithies, O. (1966).** A simple hemagglutination system requiring small amounts of red cells and antibodies. Transfusion, 6, 67-73.
- Yamamoto, Y., and B. Glick, (1982).** A comparison of the immune response between two lines of chickens selected for differences in the weight of the bursa of Fabricius. Poultry Sci. 61:2129 2132.

الملخص العربي

الإستجابات المناعية الخلوية و الإخلاطية للأرانب النامية المغذاه على تركيزات مرتفعة من الليمون المجفف

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أجريت هذه التجربة لدراسة بعض التغيرات المناعية الناتجة عن تغذية الأرانب النامية على تركيزات مرتفعة من الليمون المجفف وكانت مدة التجربة 8 اسابيع. حيث تم إستخدام عدد 48 أرنب (النيوزيلاندى الأبيض) عمر 45 يوم حيث تم تقسيمهم عشوائياً إلى ثلاث مجموعات كل مجموعة 16 أرنب وكان تسكين الأرانب فى بطاريات من السلك المجلفن (35 × 50 × 40سم) فى عنبر مغلق وكان توزيع المجموعات كالتالى:-

1. المجموعة الأولى كمنترول.
 2. المجموعة الثانية تم تغذية الأرانب على العليقة الكمنترول +10 جم ليمون مجفف / كجم عليقة.
 3. المجموعة الثالثة تم تغذية الأرانب على العليقة الكمنترول +20 جم ليمون مجفف / كجم عليقة.
- تم حقن الحيوانات بمحلول من كريات الدم الحمراء للغنم بعد سبعة أسابيع من بداية التجربة ثم جمعت عينات الدم بعد سبعة أيام من الحقن فى نهاية التجربة، لقياس التغيرات فى عدد كريات الدم البيضاء الكلى و النوعى وبعض القياسات المناعية ممثلة فى المناعة الخلوية (قدرة كريات الدم البيضاء على الهجرة ناحية الجسم الغريب و القدرة على البلعمة)، كذلك تم تقدير الأجسام المضادة الكلبية و الـ IgG و الـ IgM و قدرة الأجسام المضادة على ترسيب الجسم الغريب. وتم دراسة القطاعات النسيجية لكلاً من الطحال و الغدة التيموسية بعد صباغتها بالصبغة العامة (H and E).
- وخلصت نتائج التجربة إلى أن إستخدام الليمون المجفف بتركيزات 10 و 20 جم ليمون مجفف / كجم عليقة أثر ($p > 0.05$) معنوياً على عدد كريات الدم البيضاء الكلى و نسبة الكريات اللمفاوية والأحادية والمتعادلة و زاد نشاط معدل هجره كريات الدم البيضاء ناحيه الجسم الغريب و قدرتها على البلعمة ولم تتأثر نسبة الكريات الحامضية و القاعدية بالمعاملة. أما عن الأجسام المضادة فارتفع تركيزها فى السيرم بشكل ملحوظ و كذلك ارتفع كلاً من الـ IgG و الـ IgM. و عن التغيرات النسيجية للطحال والغدة التيموسية فخلى كلاً منهما من أى تغير ضار.
- نستنتج من هذه الدراسة أنه يمكن إستخدام الليمون المجفف حتى 20 جم/ كجم عليقة فى تغذية الأرانب النامية كمنشط مناعى طبيعى.