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### Original Paper

## Alleviation of imidacloprid-induced oxidative stress and immune damage by *Spirulina platensis* in broiler chickens

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### ABSTRACT

Imidacloprid (IM) is a neonicotinoid insecticide being used extensively for crop protection and pet flea control programs. Nevertheless, IM is a strong environmental toxicant for both animals and humans. As a result, the present study aimed to assess the ameliorative efficacy of *Spirulina platensis* (SP) against IM-induced oxidative stress and immune damage in broiler chickens. Chicks (n=102) were divided randomly into six equal groups (n= 17 each). Group I administered filtered water orally for 4 weeks. Group II administered IM 10 mg/kg b. wt. orally daily for 4 weeks. Group III given SP 5 g/kg of diet for 4 weeks. Group IV received IM plus SP for 4 weeks. Group V received IM for 2 weeks, followed by SP for another 2 weeks. Group VI received SP for 2 weeks, followed by IM for another 2 weeks. Results revealed that IM significantly decreased body weight while increased feed conversion rate, total leukocytic count and lymphocyte percent. There was a decrease in serum Haemagglutination inhibition antibody titer against Newcastle disease vaccine. Antioxidant enzymes such as reduced glutathione and catalase significantly decreased; while malondialdehyde increased in bursa of Fabricius tissue accompanied with histopathological changes. Administration of SP in combination, before and after treatment with IM significantly ameliorated the IM-induced oxidative stress and immune damage due to its antioxidant properties.

### 1. INTRODUCTION

Insecticides are commonly used in agriculture, veterinary and medicine. Indiscriminate use of pesticides to combat insects by the farmers causes pesticide entrance into the food chain, leading to residue-related exposure to humans and animals (Mondal et al., 2009).

Imidacloprid (IM) was the first neonicotinoid insecticide to be approved for use and it is now the most valuable marketable product due to its high efficacy against insects (Hussein and Singh, 2016).

Pesticide residues can induce immunosuppression in animals, either directly or indirectly, through the intervention with stress mechanisms and the neuroendocrine system (Gawade et al., 2013). Despite some studies proved the immunotoxic effects of IM in rats, no attempts have been made to testify the immunological implications of IM in chickens (Gatne et al., 2006).

Recent literature suggests that imidacloprid-induced toxic manifestations is correlated with an increase in the production of reactive oxygen species (ROS), which may explain the different forms of toxic responses (Yu et al., 2008). As the synthesis of ROS exceeds the antioxidant capability of the target cell, damage to macromolecules such as nucleic acids, lipids, and proteins occurs, causing changes in the target cell function and eventually cell death (EL-Gendy et al., 2010).

Enhanced production of free radicals by IM can hinder immune function in chickens, resulting in vaccine failure and increased susceptibility to diseases. The immunotoxic effects of IM may include impairment of immune tissues and organs (bursa of Fabricius, spleen, thymus, bone marrow, and lymph nodes) and reduction of Haemagglutination inhibition (HI) antibody titer against vaccines (humeral immunity).

Antioxidants can enhance immune responses through lowering the level of free radicals released by insecticides (Kammon et al., 2012). Protective effects of natural antioxidants against pesticide-induced oxidative damage have recently received an interest, especially when the production of free radicals is involved.

*Spirulina platensis* (SP) is a fresh-water blue-green algae with a high nutrient value and a wide variety of therapeutic applications. It contains highly effective antioxidants and free radical scavengers (Basha et al., 2008). Active ingredient of *spirulina*, C-phycoerythrin has anti-inflammatory, hepatoprotective, neuroprotective, immunomodulatory, antitumor and anticancer activities in addition to free radical scavenging and antioxidant function (Basha et al., 2008).

Therefore, the aim of this study was to assess the effects of SP as natural antioxidant against immunotoxicity, and oxidative damage induced by imidacloprid in broiler chickens.

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## 2. MATERIAL AND METHODS

### 2.1. Chemicals

Imidacloprid (Avenue 70% WG) was purchased from STARCHEM company as commercial product for agricultural use.

*Spirulina platensis* (SP) powder was obtained from National Research Center (Cairo, Egypt).

Diagnostic kits of MDA, GSH, CAT were supplied from Biodiagnostic company, Cairo, Egypt.

### 2.2. Experimental design

A total number of 102 healthy one-day-old broiler chicks (Cobb 500) supplied from El-Dakahlia company with an average body weight (40-50 g). Chicks were housed on deep litter system under proper environmental conditions of temperature, humidity, light and had free access to feed and water. Experiment was conducted according to the guide for care of laboratory animals approved by the ethical animal committee of Benha university (Approval no. BUFVTM 07-02-21). Before the experiment, the chicks were acclimatized to the environment for seven days. On Day 7, chicks of all groups were weighed (average b. wt. 140-150 g) and vaccinated against Newcastle disease (ND) and Infectious Bronchitis Virus (IBV) (Combivac C) by ocular route and were randomly segregated into six groups (17 chicks per each group) as following:

Group I served as vehicle control group received filtered water orally daily for 4 weeks.

Group II received imidacloprid (IM) at a dose of 10 mg/kg b. wt. (1/10 LD50) (Gupta and Lather, 2016) orally daily for 4 weeks.

Group III received *spirulina platensis* (SP) 5 g per kg of diet (Bonos et al., 2016) for 4 weeks.

Group IV (Co-treated group) received SP 5 g per kg of given diet and IM 10 mg/kg b. wt. orally daily for 4 weeks.

Group V received IM 10 mg/kg b. wt. for 2 weeks orally, followed by SP 5 g per kg of diet for another 2 weeks.

Group VI received SP 5 g per kg of diet for 2 weeks, followed by IM 10 mg/kg b. wt. orally for another 2 weeks.

Chicks received booster vaccination of NDV at 21<sup>st</sup> day. After 4 weeks of experiment (35<sup>th</sup> day old) chicks were individually weighed, euthanized, then blood samples and bursa of Fabricius tissues were collected.

### 2.3. Body weight and feed conversion ratio

Body weights and feed intake of experimental groups were checked weekly food conversion rate (FCR) of each group was calculated weekly by dividing the amount of feed consumed (g) during the week by gain in weight (g) during the same week.

### 2.4. Blood sampling

Blood samples were collected from 7 randomly chosen chicks from each group at Day 21, and at end of experiment (35<sup>th</sup> day) from wing vein, then allowed to clot at room temp. Sera were then separated and preserved at -20 °C till used for evaluation of HI test. Other blood samples collected at the end of experiment (35<sup>th</sup> day) and received on disodium EDTA 10% solution (20 µL/ml blood) were used for leukogram parameters.

### 2.5. Immunological examinations

The possible effect of IM on the humoral immunity in broiler chicks was assessed by measuring serum antibody

titer against ND vaccine strain by Haemagglutination inhibition (HI) test. The results were expressed as log<sup>2</sup> (Ndifon, 2011).

### 2.6. Tissue preparation and evaluation of oxidative stress markers in bursa of Fabricius tissue

Bursa of Fabricius tissue were dissected and washed with a phosphate buffered saline (PBS) solution, pH 7.4 containing 0.16 mg/ml heparin to remove any red blood cells and clots. One gram of each tissue was homogenized in 5 ml of cold buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA) per gram tissue, using sonicator homogenizer. Tissue homogenates was centrifuged by cooling centrifuge 4000 rpm for 20 min. then stored at -80 °C (Ohkawa et al., 1979) for analysis of malondialdehyde (MDA), reduced glutathione (GSH) and catalase (CAT) according to the manufacturer's instruction of the kits.

### 2.7. Histopathological studies

Bursa of Fabricius tissue samples were fixed in 10% formalin. Thereafter, paraffin-embedded sections of these tissues were cut (4 µm thickness) and stained by H&E (Bancroft and Layton, 2019).

### 2.8. Statistical analysis

The results were presented as mean ± SE using one-way ANOVA variance test by using SPSS software. A significant difference was used at ≤ 0.05 probability level.

## 3. RESULTS

### 3.1. Effect of imidacloprid and/or *spirulina platensis* on body weight and feed conversion rate of broiler chicks:

Our results showed significant decrease in b. wt. of group II from 1<sup>st</sup> to 4<sup>th</sup> weeks, group V in 1<sup>st</sup> and 2<sup>nd</sup> weeks and group VI in 3<sup>rd</sup> and 4<sup>th</sup> weeks compared to group I and III, while significant increase of b. wt. of group IV from 1<sup>st</sup> to 4<sup>th</sup> weeks, group V in 3<sup>rd</sup> and 4<sup>th</sup> weeks and group VI in 1<sup>st</sup> and 2<sup>nd</sup> weeks compared to group II (Table 1).

As presented in table (2), significant increase in FCR of group II from 1<sup>st</sup> to 4<sup>th</sup> weeks, group V in 1<sup>st</sup> and 2<sup>nd</sup> weeks and group VI in 3<sup>rd</sup> and 4<sup>th</sup> weeks compared to group I and III, while significant decrease of FCR of group IV from 1<sup>st</sup> to 4<sup>th</sup> weeks, group V in 3<sup>rd</sup> and 4<sup>th</sup> weeks and group VI in 1<sup>st</sup> and 2<sup>nd</sup> weeks compared to group II.

### 3.2. Effect of imidacloprid and/or *spirulina platensis* on leukogram of broiler chicks:

As illustrated in table (3), there was a significant increase in total leukocytic count (TLC), and lymphocyte % in group II compared to group I and III. In contrast, these parameters were significantly decreased in group IV, V and VI compared to group II. While no significant difference in basophils, monocytes, and eosinophils % of all treated groups from those of group I (vehicle control) and group III (SP).

### 3.3. Effect of imidacloprid and/or *spirulina platensis* on humoral immunity (HI antibody titer) in broiler chicks:

As shown in table (4), IM produced significant decrease in the titer of antibodies against ND vaccine in group II and V compared to group I, III and VI, while significant increase in group IV compared to group II in day 21 (after 2 weeks of experiment). Also, significant decrease of antibody titer in group II compared to group I and III while significant increase in group IV, V and VI compared to group II in day 35 (after 4 weeks of experiment).

3.4. Effect of imidacloprid and/or *spirulina platensis* on oxidative status of bursa of Fabricius tissue in broiler chicks:

As illustrated in figure 1, results showed a significant decrease in CAT and GSH, while significant increase in MDA in group II compared to group I and III. A significant increase in CAT and GSH and significant decrease in MDA in group IV, V and VI compared to group II.

3.5. Effect of imidacloprid and/or *spirulina platensis* on histopathological changes of bursa of Fabricius in broiler chicks:

As shown in figure 2, bursa of Fabricius sections of group I and III revealed normal histoarchitecture structure with un-depleted lymphoid follicles. In contrast, severe lymphoid depletion in the cortex and medulla of lymphoid follicles were demonstrated in group II. Other three groups IV, V and VI notably showed recovery of the histopathological appearance with mild lymphoid depletion.

Table 1 Effect of imidacloprid and/or *spirulina platensis* on weekly body weight of broiler chicks.

Time	Body weight of experimental groups					
	Group I	Group II	Group III	Group IV	Group V	Group VI
1 <sup>st</sup> week	409.19 ± 11.58 <sup>a</sup>	312.47 ± 8.15 <sup>c</sup>	415.26 ± 8.32 <sup>a</sup>	375.75 ± 11.83 <sup>b</sup>	317.94 ± 7.51 <sup>c</sup>	411.06 ± 9.06 <sup>a</sup>
2 <sup>nd</sup> week	792.75 ± 13.65 <sup>a</sup>	627.53 ± 16.48 <sup>c</sup>	802.65 ± 17.58 <sup>a</sup>	680.38 ± 18.11 <sup>b</sup>	632.41 ± 17.48 <sup>c</sup>	799.65 ± 10.57 <sup>a</sup>
3 <sup>rd</sup> week	1112.50 ± 30.38 <sup>b</sup>	858.82 ± 23.34 <sup>d</sup>	1180.59 ± 28.08 <sup>a</sup>	983.44 ± 17.11 <sup>c</sup>	1004.29 ± 20.06 <sup>c</sup>	986.01 ± 19.92 <sup>c</sup>
4 <sup>th</sup> week	1475.75 ± 27.81 <sup>b</sup>	1068.00 ± 30.18 <sup>d</sup>	1667.31 ± 36.82 <sup>a</sup>	1256.44 ± 18.12 <sup>c</sup>	1266.82 ± 41.24 <sup>c</sup>	1245.79 ± 22.54 <sup>c</sup>

The data are presented as means ± S.E. (n = 17). Different superscript letters within the same row denotes significantly different mean values (p ≤ 0.05).

Table 2 Effect of imidacloprid and/or *spirulina platensis* on weekly Feed Conversion Rate (FCR) of broiler chicks.

Time	FCR of experimental groups					
	Group I	Group II	Group III	Group IV	Group V	Group VI
1 <sup>st</sup> week	1.29 ± 0.06 <sup>c</sup>	2.04 ± 0.17 <sup>a</sup>	1.26 ± 0.09 <sup>c</sup>	1.66 ± 0.11 <sup>b</sup>	2.06 ± 0.17 <sup>a</sup>	1.29 ± 0.09 <sup>c</sup>
2 <sup>nd</sup> week	1.38 ± 0.08 <sup>c</sup>	2.19 ± 0.13 <sup>a</sup>	1.40 ± 0.14 <sup>c</sup>	1.80 ± 0.16 <sup>b</sup>	2.21 ± 0.13 <sup>a</sup>	1.42 ± 0.14 <sup>c</sup>
3 <sup>rd</sup> week	1.40 ± 0.04 <sup>c</sup>	2.24 ± 0.18 <sup>a</sup>	1.42 ± 0.11 <sup>c</sup>	1.83 ± 0.11 <sup>b</sup>	1.86 ± 0.13 <sup>b</sup>	1.81 ± 0.09 <sup>b</sup>
4 <sup>th</sup> week	1.50 ± 0.07 <sup>c</sup>	2.22 ± 0.10 <sup>a</sup>	1.52 ± 0.07 <sup>c</sup>	1.88 ± 0.12 <sup>b</sup>	1.83 ± 0.12 <sup>b</sup>	1.89 ± 0.11 <sup>b</sup>

The data are presented as means ± S.E. (n = 17). Different superscript letters within the same row denotes significantly different mean values (p ≤ 0.05).

Table 3 Effect of imidacloprid and/or *spirulina platensis* on leukogram of broiler chicks

Haematological parameters	Experimental groups					
	Group I	Group II	Group III	Group IV	Group V	Group VI
TLC (×10 <sup>3</sup> /mm <sup>3</sup> )	23.43 ± 1.00 <sup>c</sup>	42.53 ± 2.58 <sup>a</sup>	22.86 ± 1.12 <sup>c</sup>	30.43 ± 0.95 <sup>b</sup>	29.84 ± 2.38 <sup>b</sup>	29.57 ± 3.05 <sup>b</sup>
Lymphocytes %	48.72 ± 2.07 <sup>c</sup>	71.96 ± 1.25 <sup>a</sup>	50.47 ± 1.58 <sup>c</sup>	58.70 ± 1.59 <sup>b</sup>	61.66 ± 1.51 <sup>b</sup>	61.11 ± 1.71 <sup>b</sup>
Monocyte %	4.79 ± 0.18 <sup>a</sup>	4.61 ± 0.21 <sup>a</sup>	4.44 ± 0.26 <sup>a</sup>	5.12 ± 0.21 <sup>a</sup>	4.94 ± 0.20 <sup>a</sup>	4.69 ± 0.19 <sup>a</sup>
Basophils %	2.01 ± 0.12 <sup>a</sup>	2.17 ± 0.11 <sup>a</sup>	2.11 ± 0.11 <sup>a</sup>	1.99 ± 0.13 <sup>a</sup>	2.14 ± 0.15 <sup>a</sup>	2.25 ± 0.14 <sup>a</sup>
Eosinophils %	2.79 ± 0.09 <sup>a</sup>	2.61 ± 0.13 <sup>a</sup>	2.69 ± 0.08 <sup>a</sup>	2.89 ± 0.13 <sup>a</sup>	2.84 ± 0.16 <sup>a</sup>	2.86 ± 0.11 <sup>a</sup>

The data are presented as means ± S.E. (n = 7). Different superscript letters within the same row denotes significantly different mean values (p ≤ 0.05).

Table 4 Effect of imidacloprid and/or *spirulina platensis* on humoral immunity (HI antibody titer) in broiler chicks.

Experimental groups	HI antibody titer (log <sup>2</sup> )	
	Day 21	Day 35
Group I	7.32 ± 0.24 <sup>a</sup>	8.40 ± 1.50 <sup>a</sup>
Group II	4.89 ± 0.28 <sup>c</sup>	6.60 ± 0.05 <sup>c</sup>
Group III	7.52 ± 0.33 <sup>a</sup>	8.70 ± 0.05 <sup>a</sup>
Group IV	6.26 ± 0.17 <sup>b</sup>	7.50 ± 0.21 <sup>b</sup>
Group V	4.75 ± 0.25 <sup>c</sup>	7.20 ± 0.13 <sup>b</sup>
Group VI	7.48 ± 0.29 <sup>a</sup>	7.60 ± 0.26 <sup>b</sup>

The data are presented as means ± S.E. (n = 7). Different superscript letters denotes significantly different mean values (p ≤ 0.05).

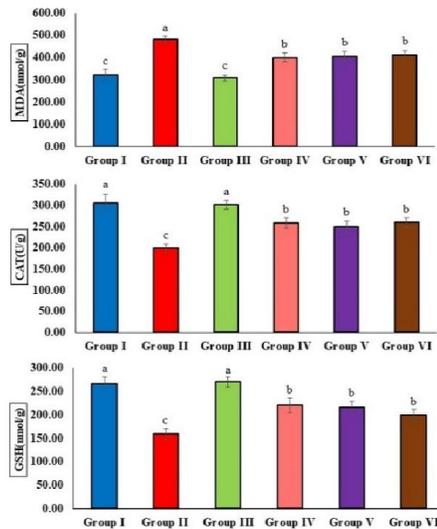


Figure 1 Lipid peroxidation and antioxidant parameters of bursa of Fabricius tissue in control and treated groups of broiler chicks. The data are presented as means ± S.E. (n = 7). MDA: Malondialdehyde, GSH: Reduced Glutathione, and CAT: Catalase. Different letters indicate significantly different mean values (p ≤ 0.05).

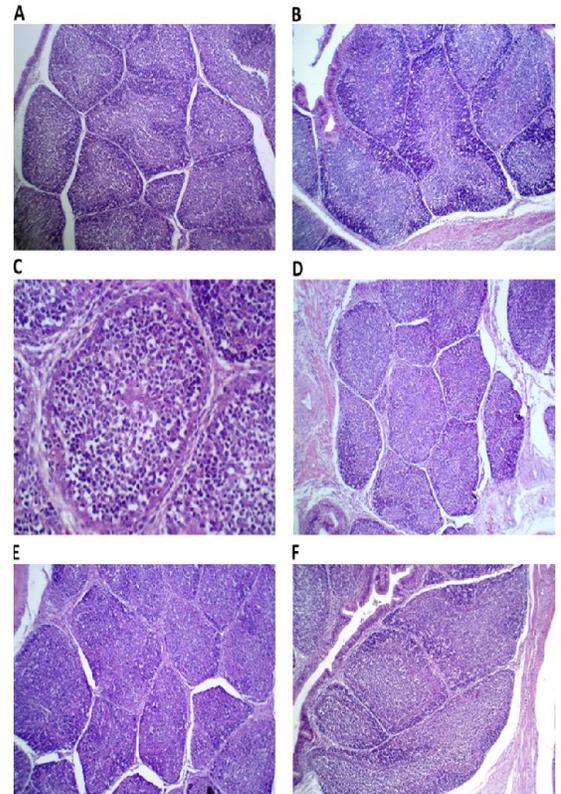


Figure 2 Effect of imidacloprid and/or *spirulina platensis* on histopathology of bursa of Fabricius tissue in broiler chicks. A and B: Bursa sections from group I and III, respectively, showing normal lymphoid follicles and bursal mucosa (H&E ×200). C: group II showed severe lymphoid depletion in the lymphoid follicle (H&E ×400). D, E, F: group IV, V and VI, respectively, showing nearly normal histological structure (H&E ×200).

#### 4. DISCUSSION

Neonicotinoids have recently received a lot of attention because of their adverse impacts on non-target species and ecosystems. Because of its widespread use in agriculture, imidacloprid exposure is of a great concern among rural populations (Tao et al., 2019).

Pesticides toxicity causes adverse effects on gross performance parameters in broilers. Concerning body weights of imidacloprid-treated chicks that decreased significantly, our results were in consistent with the previous studies reported by Sasidhar et al. (2014). This might be attributed to decreased food consumption as a result to the adverse effects of pesticides, which caused the endangered animal to consume less food and water (Loss of appetite), resulting in body weight loss (Li et al., 2007). Feed conversion rate (FCR) was significantly increased weekly as a result of decreased weight gain in IM group. In contrast, groups treated with *Spirulina platensis* showed improvement in body weight gain and feed conversion, this might be due to improving the feed utilization efficiency (Kaoud, 2012).

In the present study, significant increase in number of TLC in IM- treated group was observed which is in accordance with that recorded by (Badgujar et al., 2013; Mohany et al., 2012; Soujanya et al., 2013). This could be due to the activation of leucopoiesis by imidacloprid, which might act as an immunosuppressive agents at this dose as reported by Ravikanth et al. (2018) and due to increased percent of lymphocytes.

The activation of defense mechanism and humeral immunity in chicks was assessed through Haemagglutination inhibition (HI) test. IM resulted in a significant decrease in antibody titer against ND vaccine. This result have confirmed by the adverse damaging effect on lymphoid organs such as spleen, bursa of Fabricius and liver. Bursa of Fabricius tissue was focused in the recent study as it well-known as the main lymphoid organ of chicken and a major pathway by which environmental antigens stimulate the immune system (Ifrah et al., 2017). Imidacloprid causes oxidative stress by increasing free radical production (Sauer et al., 2014). Oxidative stress is a biochemical disruption that occurs when the production of free radicals, such as reactive oxygen species (ROS), exceeds the capacity of endogenous antioxidant agents, leading to membrane lipid peroxidation and cellular organelle injury (Kapoor et al., 2010). This explain the immunotoxic effect of IM specially the damage of bursa of Fabricius tissue. Broilers administered imidacloprid showed substantial increase of bursal MDA level as a result of lipid peroxidation by ROS while significant reduction in CAT and GSH levels as compared to control group, this may be due to the increased intake of GSH and CAT to compensate the extra free radical output (Duzguner and Erdogan, 2010; Kapoor et al., 2011). This confirmed by histopathological picture of bursal tissue that showed lymphoid depletion in the cortex and medulla of lymphoid follicles similar to results reported by Kammon, (2012).

*Spirulina platensis* supplementation resulted in significant improvements in immune function, oxidative stress and pathology of bursa of Fabricius in the current research. *Spirulina* has been shown to enhance immune function, and growth parameters (Khan et al., 2005). This might be due to limiting the amount of free radicals generated in cells by its antioxidant scavenging activity (Abdel-Daim, 2014). In accordance with our findings, the antioxidant properties of *Spirulina* contributed to its preventive function against IM toxicity.

#### 5. CONCLUSION

It is concluded that imidacloprid has immunological detrimental effects and oxidative impact in chickens with *spirulina platensis* supplements alleviating the negative effects of imidacloprid.

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