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Protective effect of amino acid, Glycine in broilers fed on Imidacloprid treated rations

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Abstract

Broiler chicks were segregated into three groups. Group 1 fed on a basal diet and served as a control, group 2 was given Imidacloprid (IM) at a dose of 50 mg/kg diet and group 3 was dietary supplemented with 0.5 % Glycine (GLY) and IM (50 mg/kg diet) for 4 weeks. Blood and tissue samples were collected on the 7th, 14th, 21st, and 28th days. The obtained findings revealed that IM residues were beyond the maximum residue limit (MRL) (0.02 ppm) and were significantly higher in muscles ranging from 0.042±0.0039 to 0.073±0.0026 ppm and in liver tissues ranging from 0.466±0.033 to 0.790±0.017 ppm) throughout the experimental time interval in IM- treated chicks. IM induced a significant decline in the total erythrocytic count (TEC), packed cell volume (PCV), mean corpuscular volume (MCV), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leucocytic counts (TLC) and lymphocyte percent. IM- treated chicks exhibited a significant reduction in the levels of serum acetylcholinesterase (AchE). Besides, the hepatic antioxidants activities [(superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH)] were significantly decreased. A significant increase in the levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), uric acid, creatinine, hepatic lipid peroxide malondialdehyde (MDA), heterophils and monocyte percent were observed in IM-treated chicks. Furthermore, IM induced a significant histopathological lesions in the liver, kidney and muscles. Conclusively, GLY supplementation inhibited IM accumulation. Therefore, IM residues were not detected in muscles, while ranging from (0.250±0.029 to 0.553±0.023) ppm in liver tissues of GLY and IM- treated chicks. Dietary GLY improved liver and kidney function and ameliorated hematological alterations, neurotoxicity, oxidative stress and histopathological lesions induced by IM.

Keywords: broiler chickens, imidacloprid, glycine, toxicity, histopathology.

1. Introduction

Neonicotinoids represent high selective toxicity to insect over vertebrate nicotinic acetylcholine receptors (nAChRs) (Matsuda Ihara, and Sattelle, 2020). Birds are potentially exposed to neonicotinoid insecticides by ingestion of coated seeds during crop planting (Bean *et al*., 2019). Neonicotinoids have direct and indirect effects on birds through the food chain. Imidacloprid acts in the same systemic manner as other neonicotinoids. Using imidacloprid as seed treatments induces oxidative stress and poses risks to small birds (Gibbons *et al*., 2015). Imidacloprid caused sub-lethal effects such as altered biochemical parameters in birds fed insecticide-treated wheat, at the recommended dose (Lopez-Antia *et al.*, 2013).

The histopathology of the liver of imidacloprid intoxicated mice revealed mild focal necrosis with swollen cellular nuclei, hypertrophied blood vessels, and cytoplasmic lesions in hepatocytes, and in kidneys showed degeneration of the tubules and glomerulus (Arfat *et al*., 2014). Imidacloprid administration at different doses in female albino mice revealed leucocytic infiltration, congestion, and dilated sinusoids, distorted central vein filled with blood in the liver (Ajay *et al*., 2014).

The benefits of Gly supplementation during the grower period are limited as other protein related nutrients become limiting at 17.7% curd protein. Supplementation of 0.713% Gly in the grower period increased body weight gain and reduced feed conversion ratio (Hilliar *et al*., 2020). Low protein diets are typically glycine deficient and produce poor performance. Supplementing the diet with Gly or precursors of Gly can overcome this deficiency (Hilliar et al., 2019). Among the non-essential amino acids, the only amino acid glycine could potentially improve inferior performance in broilers. Glycine is important in amino acid metabolism of growing chicks and significantly affect serum uric acid (Awad et al., 2017). Glycine with other amino acids is involved in several crucial metabolic functions such as synthesis of creatine, haem, glutathione, and the essential synthesis of uric acid for excretion of any excess nitrogen. Glycine is a component of uric acid molecule and is directly involved in the synthesis of uric acid by providing two carbons and one nitrogen atom (Corzo, 2012).

The present study was designed to evaluate the possible effects of glycine supplementation on hematological, biochemical, and histopathological alterations associated with oxidative stress and hepato-renal disorder induced by imidacloprid toxicity in broiler chicks.

2. Materials and Methods

2.1. Imidacloprid

Imidacloprid (99.5% w/w) was manufactured by Payer Crop Science AG. R&D SIM - RT- Analytics, Frankfurt, Germany. Its molecular formula is $C_9H_{10}ClN_5O_2$, and its molecular weight is 255.7 g/M. It was diluted in distilled water to obtain the desired concentrations and mixed with diet.

2.2. Glycine

Glycine is a white, sweet-tasting crystalline solid. It is one of the simplest proteinogenic amino acids. It was obtained from El Nasr Pharmaceuticalchemicals Co. Adwic, Pure lab. Chemicals. The molecular formula of glycine is NH_2-CH_2-COOH. It has no D- or L-configuration because a single hydrogen atom is attached to the α-C-atom where a side chain is attached for most other amino acids.

2.3. Experimental design

Broiler ration obtained from AL-Aman Foundation, Abou- Kabeer, El-Sharkya Governorate **(Table 1)**. Diet formulation is based on nutrient requirements by Natural Resources Conversion Service (NRCS, 2003). Sixty unsexed Ross broiler chick aged one day old obtained from a local hatchery were randomly segregated into three groups (20 chicks/ pen). One day old chicks were vaccinated against Newcastle Disease (ND) and Infectious Bronchitis (IB). The chicks were reared under strict hygienic conditions in accordance with the guidelines for the care and use of experimental animals. The birds were provided with standard feed and clean water *ad libitum* and were acclimatized for 10 days prior to the experiment. The ambient temperature was 25 °C, and relative humidity was 45–55 percent, with 12 h each of dark and light cycles. The temperature of the animal house was maintained between 21-31 °C throughout the experiment.

2.4. Diets

Group1: fed on the basal diet **(Table 1)** and served as the control group;

Group 2: fed on the basal diet + imidacloprid at a dose of 50 mg/kg diet daily according to Ravikanth, *et al*. (2018).

Group 3: fed on the basal diet + 0.5 % glycine diet according to Hofmann *et al*. (2010) + imidacloprid at a dose of 50 mg/kg diet.

The experiment was carried out for 4 weeks. The birds were monitored for clinical signs, if any.

Table 1. Composition of the experimental basal diets

Starter- diet			Finisher grower diet		
Ingredients	Chemical composition	%	Ingredients	Chemical composition	%
Yellow corn 54.00	Protein	> 21%	Yellow corn 54.00	Protein	>19%
Soybean meal(44% Cp)	Fat	> 3.92%	Soybean meal (46%Cp)	Fat	>6.22%
Yellow corn gluten (60%)	Fibers	< 3.26%	Yellow corn gluten (60%)	Fibers	< 3.06%
Soybean oil	Energy	> 2000 K.K.	Soybean oil	Energy	> 2100 K.K.
Dicalcium Phosphate			Dicalcium Phosphate		
Limestone			Limestone		
NaCI			NaCI		
Vit+Min mix (1826)			Vit+ Min mix (1826)		
Sodium bicarbonate			Sodium bicarbonate		
DL-methionine			DL-methionine		
L-Lysine hydrochloride			L-Lysine hydrochloride		

2.5. Sampling

Serum and tissue samples were collected from birds in each group at the end of the 7^{th}, 14^{th}, 21^{st}, and 28^{th} day. Before sacrificing and drawing blood samples, birds were fasted for 2 h. Blood samples were collected from wing vein into heparinized and non-heparinized tubes. Heparinized blood was used for complete blood count. Non-heparinized blood samples were incubated at 37 ^{0}C until the blood clotted then the samples were centrifuged at 3000 r. p. m. for 15 minutes and the clear supernatant serum was separated carefully and stored at -20 ^{0}C for biochemical analysis. Birds were sacrificed by cervical dislocation and samples of liver, kidneys, and muscle tissue samples were collected.

2.6. HPLC analysis

The ration was analyzed prior to treatments. Muscle, liver, and kidney samples were collected from birds of all groups. The cleaned and acidified extracts were transferred into auto-sampler vials and used for HPLC analysis as described in section 2.6.2. bellow.

2.6.1. Extraction /Partitioning

10 g of the comminuted homogenous and frozen muscles and liver samples were weighed into a 50 mL centrifuge tube, 10 mL acetonitrile and the potential internal standard (ISTD) solution (e.g. 100 µL of an ISTD) were added and the tube was closed and shaken vigorously by hand for 1 minute. After that, a mixture of 4 g magnesium sulfate anhydrous ($MgSO_4$),1g sodium

chloride (NaCl), 1g Disodium hydrogencitrate sesquihydrate (Na_3 H Citrate sesquihydrate) (e.g. Aldrich 359084 or Fluka 71635) was added. The tube was closed and shaken vigorously by hand for 1 minute and centrifuged for 5 minutes at 3000 U/min.

2.6.2. Dispersive solid-phase extraction (SPE)

An aliquot of the extract is transferred into a PP-single use-centrifuge tube which contains 25 mg primary-secondary amine (PSA) and 150 mg $MgSO_4$ per mL extract (e.g.: for 8 mL extract 200 mg PSA and 1.2 g $MgSO_4$ was needed). The tube is shaken for 30 s and centrifuged (e.g. for 5 min 3000 U/min. After centrifugation, the cleaned extract is transferred into a screw cap vial, and pH is quickly adjusted to ca. 5 by adding a 5% formic acid solution in acetonitrile (v/v) (pro mL extract ca.10 µL). The cleaned and acidified extracts are transferred into auto-sampler vials and used for IM determination by the HPLC technique. HPLC analysis was performed with an Agilent 1100 HPLC system (USA), with a quaternary pump, the manual injector (Rheodyne), thermostat compartment for the column, and photodiode array detector according to (Anastassiades *et al*., 2003). The chromatographic column was C18 Zorbax XDE (250 mm x 4.6 mm, 5 µm). The column was kept at room temperature. The flow rate of the mobile phase (acetonitrile/water = 80/20. v/v) was 0.8 mL/min., and the injection volume was 20 µL. The detection wavelength was set at 270 nm. The retention time was about 4.064 min. Residues were estimated by comparison of peak area of standards with that of the unknown or spiked samples run under identical conditions**.**

2.7. Evaluation of hematological parameters

Heparinized blood was used to determine total erythrocytic count (TEC). Packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leucocytic counts (TLC), differential count of leukocytes such as lymphocyte (%), heterophil (%), monocyte (%), basophil% and eosinophil (%) were estimated according to Feldman *et al*. (2000). Hemoglobin (Hb) concentration analysis was performed as described by Fairbanks and Klee (1987).

2.8. Serum Biochemical assay

Serum samples were used for biochemical analysis by UV/VIS Spectrophotometer Jasco Model 7800 using Biodignostic Kits, Cairo, Egypt. Alanine aminotransferase (ALT) aspartate aminotransferase (AST) assay is based on measuring the keto acids pyruvate or oxaloacetate formed in its derivative form, 2,4- dinitrophenylhydrazone according to Sahoo et al. (2014). Alkaline phosphatase (ALP) assay is based on estimating the liberated phenol colorimetrically in the presence of 4- aminophenazone and potassium ferricyanide according to Sahoo et al. (2014). Serum creatinine forms a colored complex with picrate in an alkaline medium and was determined according to Bogin and Keller (1987), and serum uric acid assay is based on reactions catalyzed by Uricase and Peroxidase and formation of Colored quinoneimine according to Fosati et al. (1980). Serum acetylcholinesterase (AchE) activity was measured via spectrophotometer according to Ellman et al. (1961). This method can be accomplished by using acetylthiocholine iodide as substrate (1 mM final concentration of acetylthiocholine iodide) for measuring cholinesterase activities.

2.9. Hepatic antioxidants and lipid peroxides assay

Birds were sacrificed and the liver was rapidly removed and stored at -20 ºC for estimation of antioxidants concentration separately weighed, cut into small pieces, and homogenized in an ice-cold isotonic physiological saline solution at a concentration of 0.1g/mL. The homogenates were centrifuged at 3500 rpm for 10 min at -4 ºC and the supernatant was obtained and used for estimation of enzymatic and non-enzymatic antioxidant activities and lipid peroxidation by spectrophotometric methods. The assay of superoxide dismutase (purple color SOD) activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on a substrate for SOD according to (Bannister and Calabrese, 1987). The antioxidative enzyme catalase was evaluated by the reaction with a known quantity of H2O2. In the presence of peroxidase (HRP), remaining H2O2 reacts with 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample (Aebi,1984), reduced glutathione (GSH) was determined in tissue supernatant by a colorimetric method described by Lin Hu *et al*. (1988). Lipid peroxide formation was determined as malondialdehyde (MDA) that react with thiobarbituric acid (TBA) in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product the absorbance of the resultant pink product was measured at 534 nm according to Jentzsch, *et al*. (1996) using kits of Biodiagnostic Cairo, Egypt.

2.10. Histopathological Studies

Tissue slices of liver, kidneys, and muscles from all groups were taken on 14th and 28th days post-treatment (PT) and fixed in 10 % neutral buffered formalin (NBF) solution then dehydrated, cleared and embedded in paraffin wax. Tissue sections of 4-5 micron thickness were prepared and stained with Hematoxyline and Eosin stain (H&E) and examined microscopically (Survarna *et al.,* 2013).

2.11. Statistical analysis

Data were statistically analyzed using analyses of variance (F-test) followed by Duncan's multiple range test. A probability at a level of 0.05 or less was considered significant. Standard errors were also estimated using international busines machine statistical package for social sciences (IBM SPSS) statistics program version 20.

3. Results

3.1. Pesticide residue

Starter diet and grower diet were tested before the experiment and the diets were imidacloprid free. IM residues were not detected in muscles and liver from the control group as well as in muscles from the third group treated with imidacloprid and glycine throughout all time intervals. Imidacloprid residues were above the MRL in the liver from second and third groups and muscles of broiler chicks from second group. Imidacloprid residue was significantly higher in muscles and liver tissues of chicks from the second group treated with imidacloprid only in comparison to the control group. However, glycine in combination with imidacloprid provoked a significant decline in IM accumulation in liver and muscle tissues (**Table 2**).

Table 2: Imidacloprid residue in tissues in mg/kg (ppm) of broilers received dietary Imidacloprid (50 mg/kg) with or without glycine (0.5%).

Samples		Groups / Days	Control	Imidacloprid	Imidacloprid + Glycine	MRLs (Codex, 2003)
Residues (mg/kg)	Muscles	7th	ND a	0.042±0.0039 b	ND a*	0.02 ppm
		14th	ND a	0.049±0.0010 b	ND a	
		21st	ND a	0.055±0.0029 b	ND a	
		28 th	ND a	0.073±0.0026 b	ND a	
	Liver	7th	ND a	0.466±0.033 b	0.250±0.029 c	0.02 ppm
		14th	ND a	0.643±0.024 b	0.293±0.017 c	
		21st	ND a	0.757±0.020 b	0.482±0.011c	
		28 th	ND a	0.790±0.017 b	0.553±0.023 c	

Data were represented as means of samples±SE. Means in the same row with different superscripts [a, b , c] are significantly different (Duncan multiple range test $P < 0.05$). ***** ND=Not Detected

3.2. Hematological study

Hematological observations revealed a significant decrease ($p<0.05$) in mean values of TEC, Hb, PCV, MCV, MCH, MCHC, TLC, and lymphocytes %. On the other hand, IM induced a significant increase ($p<0.05$) in heterophils % throughout all time intervals and in monocytes % on the 28th day of the experiment in the IM – treated group in comparison to the control group. These effects were significantly suppressed by glycine supplementation in the third group (**Table 3**).

3.3. Neurotoxicity and hepatorenal toxicity

Biochemical assays revealed that IM induced a significant ($p<0.05$) inhibition in serum AChE in the second group which was relieved by glycine in the third group on the 7th, 14th, and 28th days of treatments. However, imidacloprid induced significant elevations in serum ALT, AST, uric acid, and creatinine ($p<0.05$) in broilers received imidacloprid alone in the second group and which were significantly attenuated by glycine supplementation in the third group at all time intervals. In addition, IM evoked a significant increase in the levels of serum ALP on the 21th and 28th days of treatment which was significantly ameliorated by glycine on the 28th day of treatment (**Table 4**).

3.4. Oxidative stress

IM induced a significant reduction in the levels of hepatic CAT and GSH concentrations on the 7th, 21st, and 28th days of treatment that was significantly ameliorated on the 21st and 28th days in the third group. Nevertheless, imidacloprid induced a significant decrease in SOD activity ($P< 0.05$), while evoked a significant increase ($P < 0.05$) in lipid peroxide (MDA) at all time intervals that was significantly mitigated by glycine supplementation in the third group. and SOD **(Table 5)**.

3.5. Clinical Signs

Chicks given dietary imidacloprid showed clinical signs as depression, decreased appetite, reduced feed intake, watery diarrhea, muscle tremors, ataxia and sitting on hocks.

3.6. Histopathology

Liver sections of chicks treated with IM (50 mg/kg ration) for two weeks revealed degeneration of hepatocytes with mild fatty change (Fig.A), marked dilation and congestion of blood vessels (Fig.B). Kidney showed hydropic degeneration with infiltration of inflammatory cells (Fig.C), and muscles did not reveal pathological changes after two weeks.

After four weeks of administration, the liver showed coagulative necrosis of hepatocytes accompanied by mild infiltration of inflammatory cells (Fig.D). The kidney slices revealed degeneration of tubular epithelial cells and congestion of renal blood vessels (Fig.E). Muscle tissue sections revealed inflammatory cells. (Fig.F).

Chicks were treated with imidacloprid 50 mg/kg ration and glycine 0.5% of ration after two weeks, the liver showed dilated, congested blood vessels and was surrounded by inflammatory cells infiltration (Fig.G), kidney showed hydropic degeneration in addition to inflammatory cells infiltration (Fig.H), muscles did not reveal pathological changes after two weeks, while after four weeks of administration liver showed hydropic dege neration with infiltration of few inflammatory cells (Fig.I), kidney showed mild vacuolar degeneration of tubular epithelium and few congested blood capillaries (Fig.J), and muscles showed few infiltrations of inflammatory cells around blood vessels (Fig.K).

Table 3: Effect of Imidacloprid (50 mg/kg diet) with or without glycine (0.5%) on some hematological indices of broiler chicks on 7^{th}, 14^{th}, 21^{st}, and 28^{th} days of treatments.

Parameters \ Groups	Time	Control	Imidacloprid	Imidacloprid + Glycine
TEC (10^6/μL)	7^{th} day	2.26± 0.01a	1.66±0.06 b	2.21±0.02 a
	14^{th} day	2.72± 0.06 a	1.69± 0.05 b	2.56± 0.09 a
	21^{st} day	2.80± 0.02 a	1.76± 0.06 a	2.58± 0.09 a
	28^{th} day	2.85± 0.04 a	1.86± 0.02 b	2.77± 0.003 a
Hb (g/dL)	7^{th} day	9.67±0.09 a	7.50±0.12 b	9.73± 0.15 a
	14^{th} day	10.73±0.12 a	9.87±0.09b	10.23±0.18c
	21^{st} day	11.07±0.07a	8.27±0.22b	10.50±0.21a
	28^{th} day	11.40±0.12 a	9.53±0.03 b	10. 73±0.17c
PCV (%)	7^{th} day	30.47±0.26 a	22.80±0.10 b	25.79±0.15 c
	14^{th} day	31.04±0.09 a	26.60±0.30 b	27.83±0.17 c
	21^{st} day	31.47±0.09 a	27.37±0.27 b	28.33±0.38 c
	28^{th} day	32.30±0.46 a	27.40±0.78 b	31.46±0.10 a
MCV (fL)	7^{th} day	120.90± 0.45 a	111.12 ±0.89 b	112.33± 0.33 b
	14^{th} day	121.53± 0.29 a	111.57±0.72b	112.93± 0.23b
	21^{st} day	121.03± 0.03 a	111.83±0.81 b	113.40± 0.06 b
	28^{th} day	122.17± 0.91 a	111.70±0.81 b	114.80± 0.56 c
MCH (pg)	7^{th} day	40.37±0.23 a	38.87± 0.09 b	38.89± 0.07b
	14^{th} day	41.29±0.29 a	39.02± 0.04 b	39.83± 0.16c
	21^{st} day	41.67±0.28 a	39.47± 0.12 b	39.93± 0.17 b
	28^{th} day	43.17±0.49 a	32.87± 0.26 b	40.30± 0.05 c
MCHC (g/dL)	7^{th} day	32.57± 0.30 a	31.53± 0.29 b	33.63±0.20 c
	14^{th} day	34.70± 0.12 a	32.63± 0.09 b	34.67± 0.17a
	21^{st} day	35.33± 0.15 a	32.93± 0.02 b	34.97± 0.12 a
	28^{th} day	37.57± 0.44 a	34.63± 0.38 b	35.83± 0.28 c
TLC (10^3 / μL)	7^{th} day	23.23± 0.62 a	20.57± 0.27 b	25.10±0.61a
	14^{th} day	36.47± 0.83 a	21.87± 0.45 b	26.47±0.74 c
	21^{st} day	37.09± 0.11a	21.77± 0.54 b	27.90±0.55 c
	28^{th} day	37.60± 0.21a	23.47± 0.49 b	35.40±0.46c
Heterophils (%)	7^{th} day	34.00± 0.58 a	99.67± 0.33 b	35.47±0.86 a
	14^{th} day	32.83± 0.17a	99.33± 0.30 b	32.67±0.33 a
	21^{st} day	32.17± 0. 34 a	99.33± 0.67b	33.00±0.13 a
	28^{th} day	24.27± 0.40a	89.77± 0.60 b	32.10±0.31c
Lymphocytes (%)	7^{th} day	66.00± 0.58 a	0.33± 0.02 b	64.53±0.29 a
	14^{th} day	67.17± 0.17a	0..66± 0.03 b	67.33±0.23 a
	21^{st} day	75.83± 0.28a	0.67± 0.17b	67.00±0.68c
	28^{th} day	75.40± 0.57a	0.93± 0.10 b	66.30±0.91 c
Monocytes (%)	7^{th} day	0± 0 a	0± 0 a	0± 0 a
	14^{th} day	0± 0 a	0± 0 a	0± 0 a
	21^{st} day	0± 0 a	0± 0 a	0± 0 a
	28^{th} day	0.1± 0.06 a	9.00± 1.69 b	0.37± 0.09a
Eosinophils (%)	7^{th} day	0± 0 a	0± 0 a	0± 0 a
	14^{th} day	0± 0 a	0.33± 0.09 a	0± 0 a
	21^{st} day	0± 0 a	0± 0 a	0± 0 a
	28^{th} day	0.20± 0.13 a	0.28± 0.14 a	0± 0 a
Basophils (%)	7^{th} day	0± 0 a	0± 0 a	0± 0 a
	14^{th} day	0± 0 a	0± 0 a	0± 0 a
	21^{st} day	0± 0 a	0± 0 a	0± 0 a
	28^{th} day	0± 0 a	0± 0 a	0± 0 a

Data were represented as means of samples±SE. Means in the same row with different superscripts [a,b,c] are significantly different (Duncan multiple range test $P < 0.05$).

Table (4): Effect of Imidacloprid (50 mg/kg diet) with or without glycine (0.5%) on some biochemical parameters in the serum of broiler chicks on 7th, 14th, 21st, and 28th days of treatments.

Parameters	Groups / Days	Control	Imidacloprid	Imidacloprid + Glycine
Ach E(U/L)	7th day	1451.27±21.86 a	1311.04±6.35 b	1406.67±3.33a
	14th day	1556.27±0.02a	1455.00±18.01b	1534.73±17.41a
	21st day	1696.96±34.19a	1556.07±2.49 ab	1620.80±17.79ab
	28th day	1832.50±33.80a	1588.50±17.70 b	1707.50±31.70 c
ALT(IU/L)	7th day	10.45±0.25 a	17.92±0.13 b	13.93±0.46 c
	14th day	13.38±0.36 a	19.90±0.49 b	14.85±0.20 c
	21st day	15.81±0.38 a	22.35±0.20 b	16.75±0.19 a
	28th day	17.12±0.48 a	24.16±0.32 b	18.78±0.21 c
AST (U/L)	7th day	38.97±0.58 a	108.08±0.65 b	89.33±0.67 c
	14th day	48.60±0.70 a	118.26±0.32 b	92.08±0.54 c
	21st day	76.83±0.60 a	123.50±0.29 b	105.93±0.12 c
	28th day	78.67±0.88 a	122.72±0.43 b	107.13±0.30 c
ALP (mmol/L)	7th day	1.58±0.06 a	1.57±0.03 a	1.52±0.06 a
	14th day	1.62±0.06 a	1.73±0.03 a	1.54±0.09 a
	21st day	1.67±0.04 a	1.82±0.04 ab	1.72±0.02 ab
	28th day	1.75±0.02 a	1.89±0.05 b	1.76±0.01 a
Uric acid (mg/dL)	7th day	2.53±0.19 a	5.50±0.28 b	4.48±0.28 c
	14th day	3.79±0.15 a	6.80±0.10 b	5.47±0.26 c
	21st day	4.33±0.24 a	7.80±0.10 b	5.98±0.07 c
	28th day	5.34±0.28 a	9.12±0.13 b	6.18±0.12 c
Creatinine (mg/dL)	7th day	0.21±0.01 a	0.88±0.04 b	0.37±0.03 c
	14th day	0.23±0.01 a	0.79±0.15 b	0.93±0.04 a
	21st day	0.25±0.01 a	1.08±0.16 b	0.52±0.09 a
	28th day	0.27±0.01 a	1.43±0.06 b	0.51±0.05 c

Data were represented as means of samples±SE. Means in the same row with different superscripts [a, b, c] are significantly different (Duncan multiple range test $P < 0.05$).

Table 5: Effect of Imidacloprid (50 mg/kg diet) with or without glycine (0.5%) on hepatic antioxidants activity and lipid peroxidation in broilers on 7th, 14th, 21st, and 28th days of treatments.

Parameter	Groups / Time	Control	Imidacloprid	Imidacloprid + Glycine
SOD (μmol/ g)	7th day	34.93±0.52 a	27.90±0.46 b	41.34±0.43 c
	14th day	36.22±0.40 a	28.23±0.62 b	42.18±0.43 c
	21st day	37.50±0.76 a	29.23±0.39 b	50.11±0.79 c
	28th day	38.06±0.58 a	30.83±0.33 b	60.41±0.83 c
CAT (μmol / g)	7th day	1.67±0.12 a	1.10±0.11 b	1.16±0.12 b
	14th day	1.87±0.09 a	1.56±0.33 a	1.73±0.12 a
	21st day	2.03±0.08 a	1.75±0.07 ab	1.97±0.03 ab
	28th day	2.16±0.04 a	1.83±0.06 b	2.05±0.03 a
GSH (μmol/ g)	7th day	2.20±0.15 a	1.17±0.16 b	1.97±0.03 a
	14th day	3.70±0.11 a	2.80±0.15 ab	3.33±0.33 ab
	21st day	4.57±0.28 a	3.47±0.26 b	4.23±0.23 a
	28th day	4.91±0.05 a	4.04±0.03 b	4.58±0.12 c
MDA (nmol/ g)	7th day	6.47±0.75 a	16.66±0.44 b	12.27±0.92 c
	14th day	7.93±0.52a	18.10±0.55 b	14.61±0.64 c
	21st day	10.67±0.88 a	18.83±0.62 b	13.22±0.61 c
	28th day	12.60±0.31 a	19.79±0.44 b	14.48±0.39 c

Data were represented as means of samples±SE. Means in the same row with different superscripts [a, b, c] are significantly different (Duncan multiple range test $P < 0.05$).

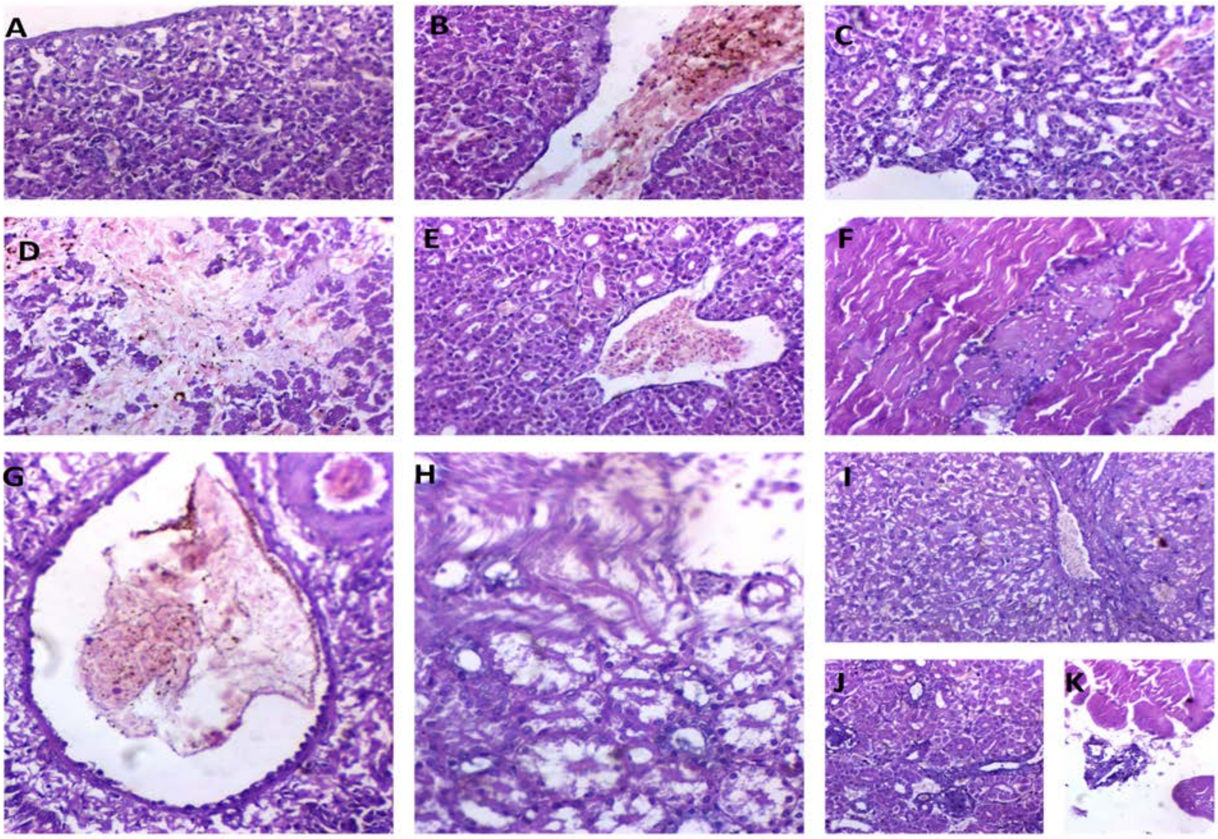

Figure A. Liver sections from second group treated with imidacloprid for two weeks showed degeneration of hepatic cells with mild fatty change (H&EX400).

Figure B. Liver sections from second group treated with imidacloprid for two weeks showed marked dilation and congestion of the central vein (H&EX400).

Figure C. Section of kidney from second group treated with imidacloprid for two weeks showed hydropic degeneration with mild infiltration of inflammatory cells (H&EX400).

Figure D. Section of liver from second group treated with imidacloprid for four weeks showed a focal area of coagulative necrosis of hepatocytes accompanied by mild infiltration of lymphocytes and heterophils. (H&EX400)

Figure E. Section of kidney from second group treated with imidacloprid for four weeks showed degeneration of tubular epithelial cells and congestion of renal blood vessels. (H&EX400)

Figure F. Section of muscle tissue from second group treated with imidacloprid for four weeks showed few infiltrations of inflammatory cells. (H&EX400).

Figure G . Liver section from third group treated with imidacloprid and glycine for two weeks showed dilated, congested blood vessels and mild infiltration of inflammatory cells (H&EX400).

Figure H. Kidney section from third group treated with imidacloprid and glycine for two weeks showed vacuolar degeneration and infiltration of inflammatory cells. (H&EX400)

Figure I. Liver section from third group treated with imidacloprid and glycine for four weeks showed vacuolar degeneration with mild infiltration of inflammatory cells**.** (H&EX200)

Figure J. Section of kidney from third group treated with imidacloprid and glycine for four weeks showed mild vacuolar degeneration of tubular epithelium and few congested blood capillaries. (H&EX400)

Figure K . Section of muscle from third group treated with imidacloprid and glycine for four weeks showed mild infiltration of inflammatory cells around blood vessels. (H&EX400)**.**

4. Discussion

Imidacloprid is categorized as moderately toxic by the EPA, falling under both toxicity neonicotinoid insecticide class II and class III, (US EPA, 2012). In the present study, imidacloprid residues were above the (Codex, 2003) permissible limit (0.02 ppm) in muscles in group 2 and in liver tissue in group 2 and group 3. In accordance with the present study, Ong *et al*. (2017) observed that imidacloprid showed accumulation during the broiler breeding period and the residues at the end of the treatment period showed increment compared to the beginning of treatment period (Ong *et al*., 2017). Imidacloprid metabolites found in the feces include glycine conjugate of methylthionicotinic acid accounted for roughly 80% of the administered doses (Tomlin, 2006). The amino acid, glycine (Gly) could be utilized for attaching to molecules for their excretion (Forman *et al*., 2009).

In the current study, the decline in the mean values of TEC, Hb, PCV, MCV, MCH, and MCHC could be due to the direct toxic action of IM on bone marrow, liver and kidneys which might play a vital role in hemopoiesis and erythropoietin production in respective organs (Ravikanth *et al*., 2017). The significant reduction in total leucocytic count in the present study was in line with the findings of Sasidhar Babu *et al.* (2014) in layer chicks. The lymphocytic depletion observed in the present study might be as a result of hemorrhages in the spleen. Imidacloprid insecticide also has deleterious effects immunologically in the broiler chicks targeting the humoral immune responses (Kammon *et al*., 2012).

Glycine is functional in the biosynthesis of the porphyrin moiety of heme groups (te Braake *et al*., 2008).

This may explain the improvement of the hemoglobin concentration in group 2.

The neurological signs observed in chicks treated with IM could be correlated to the agonistic action of imidacloprid on nicotinic acetylcholine receptors which could induce neuromuscular paralysis (Tomizawa and Casida, 2005).

The significant reduction in the levels of antioxidant indices and the elevation of lipid peroxidation in the liver tissue in group 2 in the present study were in line with Ganguly (2013) and Ravikanth *et al*. (2018). Superoxide radicals undergo dismutation by the action of superoxide dismutase to hydrogen peroxide, while the hydrogen peroxide formed is converted to water and molecular oxygen by catalase to prevent accumulation in the cell (Halliwell, 2015).

The action of glycine is based on its cytoprotective ability from stress injury. Amino acids such as glycine can lower free radical damage by increasing glutathione production (te Braake *et al*., 2008).

Imidacloprid exposure induced hepatotoxic and nephrotoxic damage. In addition, there is an obvious correlation between the lesions and plasma biochemical changes (Kammon *et al.,* 2010). In agreement with the current study, Ravikanth, *et al*. (2017) and Sasidhar Babu *et al*. (2014) observed an increased AST activity signifying muscular damage. Elevated ALP activities may be either primary or secondary to damage in the liver and kidneys. Bataille *et al*. (2011) recorded a decline in the active transepithelial uric acid secretion in the renal proximal tubule which may occur due to cellular stress.

In poultry, uric acid pathways are directly or indirectly dependent on glycine and its precursors (Akinde, 2014). Glycine also plays a critical role in uric acid formation for nitrogen excretion in poultry. Glycine addition decreased serum uric acid concentrations in broilers fed 1.35% Lysine (Powell *et al*., 2009) and increased uric acid excretion (Namroud *et al*. 2010).

Moreover, the hepatic necrosis might be due to oxidative stress induced by imidacloprid that further involved in cellular protein degradation. The dilated sinusoidal spaces were due to the shrinkage and necrosis of hepatic cells. These results were in agreement with Sasidhar Babu *et al.* (2014) and Ganguly (2013). Microscopic changes in the liver revealed large areas of vacuolation, fatty degeneration, large areas of necrosis, and congested sinusoidal spaces. These results were similar to the findings of Eissa (2004) in Japanese quail and Kammon *et al.* (2010) in layer chicks. The vacuolation of hepatocytes might be due to the retention of fluid inside the cell. The cloudy swelling might be due to the reduction of energy necessary for the regulation of ion concentration of the cells (Omiama, 2004). In addition, Kammon *et al* (2010) in layers chicks and Ravikanth (2015) in broilers observed that imidacloprid exposure did not produce any changes in the liver cells except for mild cellular swelling in the hepatocytes and exposure of imidacloprid for 20 and 30 days produced degenerative changes in the hepatocytes and necrosis surrounded by neutrophilic infiltration and explained this lesion by oxidative stress induced by imidacloprid.

In the kidney, the vacuolar degeneration of tubular epithelial cells and hemorrhage between tubules could be due to increased glomerular filtration and capillary permeability. The leakage of proteins causes tubular necrosis (Wankhede *et al* 2017). These findings were in accordance with (Ravikanth *et al*., 2017) and (Gupta and Lather, 2016).

In the present study, the imidacloprid treated chicks (Group 2) showed congestion and hemorrhage in the liver, kidney, and breast muscles. The present results were similar to that obtained by Eissa (2004), Wankhede *et al*., (2017) and Komal (2018) who found that imidacloprid intoxication cause fatty change, congestion, necrosis of hepatocytes and nephritis.

The liver and kidney showed hydropic degeneration and mild vacuolar degenration of tubular epithelium in group3. This improvement in the histopathological lesions may result from the sulphhydryl group of amino acid cysteine, which prevents oxidation of endogenous mitochondrial and microsomal enzymes which participate in the toxicity production. One possible mechanism by which glycine induces these responses is through an increase in the formation of cysteine (Wu, 2013). The obtained findings revealed that chicks in group 3 showed lower degree of lesion compared to group 2.

In the present study, the significant increase in glutathione production induced by glycine supplementation may explain the improvement in the histopathological lesion in group 2 and were in agreement with the findings of Eissa (2004) in Japanese quails who concluded the protective effect of glutathione against pathological changes induced by imidacloprid.

5. Conclusion

Our findings confirmed that glycine supplementation increased imidacloprid excretion and reduced the pesticide accumulation in edible organs and tissue of broiler chicks. Glycine enhanced antioxidants activity and exhibited a preventive effect against oxidative stress and neurotoxicity induced by imidacloprid. Furthermore, glycine reduced hematological alterations and improved hepato-renal function that was supported by regressive pathological lesions in the liver, kidney, and muscles.

References

Aebi H. 1984. Catalase *in vitro*. *Methods Enzymol*., **105**:121-6.

Bean TG, Gross, MS, Karouna-Renier NK, Henry PFP, Schultz SL, Haldik ML, Kuivila KM and Rattner BA. 2019. Toxicokinetics of imidacloprid-coated wheat seeds in Japanese quail (*Coturnix japonica*) and an evaluation of hazard.Environ. Sci. Technol., **53** (7): 3888-3897.

Ajay K, Monika T and Sudhir, KK. 2014. Effect of sub-lethal doses of Imidacloprid on histological and biochemical parameters in female albino mice. IOSR. *Journal of Environmental Science, Toxicology and Food Technology*, **8** (1)**:** 09-15.

Akinde DO. 2014. Amino acid efficiency with dietary glycine supplementation: part 1 and Part 2. *World Poult Sci J.*, **70 (3)**: 461-474.

Anastassiades M, Lehotay SJ, Stajnbaher D and Schenck, FJ. 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residues in produce, *J AOAC Int.*, **86**: 412- 431.

Arfat Y, Nasir M, Muhammad UT, Maryam R, Sameer A, Fan Z, Di-Jie L, Yu-Long S., Lifang H, Chen, Z, Chong Y, Peng S and Ai-Rong Q. 2014. Effect of Imidacloprid on hepatotoxicity and nephrotoxicity in male albino mice. *Toxicology Rep.* **1**: 554-561.

Awad EA, Zulkifli I, Soleimani AF and Aljuobori A. 2017. Effects of feeding male and female broiler chickens on low-protein diets fortified with different dietary glycine levels under the hot and humid tropical climate, *Italian J Animal Sci.* **16**:3, 453-461.

Bannister, JV and Calabrese, L. 1987. Assays for superoxide dismutase, In: **Methods of Biochemical Analysis**, vol. 32, pp. 279–312, John Wiley & Sons, New York, NY, USA.

Bataille AM, Maffeo, CL and Renfro JL. 2011. Avian renal proximal tubule urate secretion is inhibited by cellular stress-induced AMP-activated protein kinase. *Am J Physiol-Renal Physiol.,* **300**: F1327–F1338.

Bogin E and Keller P. 1987. Application of clinical biochemistry to medically relevant animal models and standardization and quality control in animal biochemistry. *J Clin Chem Clin Biochem.,* **25**: 873-878.

Codex Alimentarius Commission. 2003. **Food Standards Programme Codex Alimentarius Commission.** Joint FAO/WHO, The Netherlands .

Corzo A. 2012. Determination of the arginine, tryptophan, and glycine ideal-protein ratios in high-yield broiler chicks. *J Appl Poult Res.* **21**:79-87.

Eissa OS. 2004. Protective effect of vitamin C and glutathione against the histopathological changes induced by imidacloprid in the liver and testis of Japanese quail. *Egyptian J Hospital Med.* **16**:39-54.

Ellman, GL, Courney KD, Andres V, Featherstone, RMA. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. **7**: 88-95.

Fairbanks VF and Klee GG. 1987. **Biochemical Aspect of Haematology, In: Tietz NW, Editor. Fundamentals of Clinical Chemistry**, 3rd ed. Philadelphia*:* WB Saunders, pp. 803–806.

Feldman BF, Zinkl JG and Jain NC. 2000. **Schalm's Veterinary Hematology**, 5th ed., Williams and Wilkins, Philadelphia, 21-100.

Forman HJ, Zhang H and Rinna A. 2009. "Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med.* **30 (1-2)**: 1-12.

Fosati P, Principe L and Berti G. 1980. Use of 3,5-dichloro-2-hydroxybenzene sulfonic acid/4-aminophenazone chromogenic system in the direct enzymatic assay of uric acid in serum and urine. *Clin Chem*. **26**: 227-231.

Ganguly S. 2013. Long term exposure to chemicals, insecticides and heavy metals causing toxicity: A Review. *Int J Pharm res biosci.***2 (3)**: 333-342.

Gibbons D, Morrissey C. and Mineau P. 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. *Environ Sci Pollut Res*. **22**:103-118.

Gupta KRP and Lather D. 2016. Clinico-pathological studies of imidacloprid toxicity in broiler chickens. *Haryana Vet.* **55 (2)**: 163-165.

Halliwell B. 2015. **Free Radicals and Other Reactive Species in Disease**, Encyclopedia of Life Sciences, pp. 1-9.

Hilliar M, Huyen N, Girish CK, Barekatain R, Wu S, Swick RA. 2019. Supplementing glycine, serine, and threonine in low protein diets for meat type chickens. *Poult Sci.* **98(12)**: 6857-6865.

Hilliar M , Hargreave G, Girish CK Barekatain R, Wu SB and Swick RA. 2020. Using crystalline amino acids to supplement broiler chicken requirements in reduced protein diets. *Poultry Sci.* **99**:1551-1563.

Hofmann AF, Hagey LR and Krasowski, MD. 2010. Bile salts of vertebrates: structural variation and possible evolutionary significance. *J Lipid Res.* **51**: 226-246.

Jentzsch AM, Bachmann H, Furst P and Biesalski HK. 1996. Improved analysis of malondialdehyde in human body fluids. *Free Rad Biol Med*. **20 (2)**: 251–256.

Kammon AM, Brar RS, Banga HS, Sodhi, S. 2012. Ameliorating effects of vitamin E and selenium on immunological alterations induced by imidacloprid chronic toxicity in chickens. *J Environ Anal Toxicol*. **4**:7.

Komal (2018). A Review: Pathological studies on imidacloprid toxicity and its amelioration with vitamin C. *The Pharma Innov J.* **7(4)**: 999-1002.

Lin Hu M, Dillard CJ and Tappel AL. 1988. Plasma SH and GSH measurement. *Methods Enzymol*. **233**: 380-382.

Lopez-Antia A, Ortiz-Santaliestra, ME, Francois M and Rafael M. 2013. Experimental exposure of red-legged partridges (*Alectoris rufa*) to seeds coated with imidacloprid, thiram and difenoconzole. *Ecotoxicology,* **2 (1)**: 125-138.

Matsuda, Ihara M and Sattelle DB. Neonicotinoid insecticides: Molecular targets, resistance, and toxicity. *Annu Rev Pharmacol Toxicol*. **60**: 241–255.

Namroud NF, Shivazad M and Zaghari M. 2010. Impact of dietary crude protein and amino acids status on performance and some excreta characteristics of broiler chicks during 10–28 days of age. *J Anim Physio. An N.* **94:** 280-286.

NRCS. 2003. (Natural Resources Conversion Service). **Nutrient Management Technical Note No. 4 Feed and Animal Management for Poultry**. Ecological Sciences Divison October 2003. USDA) United States Department of Agriculture.

Omiama SE. 2004. Protective effect of vitamin C and glutathione against the histopathological changes induced by imidacloprid in the liver and testis of Japanese quail. *Egypt J Hosp Med*. **16**: 39-54.

Ong S, Ab Majid AH and Ahmad H. 2017. Insecticide residues on poultry manures: Field efficacy test on selected insecticides in managing *Musca Domestica* population. *Trop Life Sci Res.* **28(2)**: 45-55.

Powell S, Bidner TD and Southern, LL. 2009. The interactive effects of glycine, total sulfur amino acids, and lysine supplementation to corn-soybean meal diets on growth performance and serum uric acid and urea concentrations in broilers. *Poult Sci*., **88**: 1407-1412.

Ravikanth V. 2015. Mixed toxicity of spinosad (SPD) and imidacloprid (IM) in broilers and its amelioration with vitamin E and silymarin M.V. Sc. Thesis (Veterinary pathology Department) SRI Venkateswara Veterinary University.

Ravikanth VM, Lakshman, D. Madhuri and Kalakumar, B. 2017. Haematological alterations in broilers administered with imidacloprid and spinosad and its amelioration with Vitamin E and Silymarin. *Int J Curr Microbiol App Sci.* **6(4)**: 496-500.

Ravikanth V, Lakshman M, Madhuri D and Kalakumar B. 2018. Effect of spinosad and imidacloprid on Serum Biochemical alterations in male broilers and Its Amelioration with vitamin E and Silymarin. *Int J Curr Microbiol App Sci*. **7(4)**: 2186-2192.

Sahoo D, Rukmini M, and Ray R. 2014. Quantitative analysis of serum levels of alanine and aspartate aminotransferases, γ-Glutamyl transferase and alkaline phospatase as predictor of liver diseases. *Am Inter J Res in Form, Appl and Nat. Scie.* **9**: 51-55.

Sasidhar Babu N, Kumar AA, Reddy AG, Amaravathi P and Hemanth I. 2014. Chronic experimental feeding of imidacloprid induced oxidative stress and amelioration with vitamin C and *Withania somnifera* in layer birds. *Int J Sci. Env ISSN Techno1***. 3(5)**: 1679-1684.

Survarna K, Lyton C and Bancroft JD. 2013. **Bancroft's theory and practice of histological Techniques**, 7th ed. Oxford, Churchil Livingston, Elsevier, England, pp.654.

te Braake FH, Schierbeek K, de Groof A, Vermes M, Longini G and van Goudoever J B. 2008. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr*. **88**: 333-339.

Tomizawa M and Casida J E. 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. *Ann Rev Pharmacol Toxicol*. **45**: 247-268.

Tomlin CDS. 2006. **The Pesticide Manual**. **A world compendium,** 14th ed., British Crop Protection Council. Surrey, England: pp. 598-599.

US EPA. 2012. United States Environmental Protection Agency Ecological Risk Assessment. http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm. Accessed 27Oct2012.

Wankhede V, Hedau M, Ingole RS, Hajare SW and Wade MR. 2017. Histopathological alterations induced by subacute imidacloprid toxicity in Japanese quails and its amelioration by Butea monosperma. *J Pharmacog Phytochem*. **6 (3)**: 252-257.

Wu G. 2013. Discovery and chemistry of amino acids. In: **Amino Acids: Biochemistry and Nutrition.** CRC Press, Boca Raton, USA.