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ASSESSMENT OF AMELIORATIVE EFFECTS OF VITAMIN C AND E SUPPLEMENTATION ON HEMATOLOGY INDICES, OXIDATIVE STRESS AND ANTIOXIDANT ENZYME STATUS OF RENAL TISSUE IN FLONICAMID INTOXICATED MALE RABBIT

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Abstract

Vitamins are used as nutrient supplement, having antioxidant properties and could be applied as medical intervention in some disease conditions. Flonicamid is a novel insecticide effective against a range of pests, however its extensive use donating toxicants in the environment, causing various pathological changes when exposed to mammals. Therefore, current study was planned to explore the possible protective effects of vitamin C and E on flonicamid induced toxic effects in male albino rabbits. Twenty five healthy rabbits were sorted in to five groups where GI served as control received only vehicle (corn oil). GII, GIII, GIV and GV were orally administered with 1/10th of LD_{50} (18mg/kg body weight) of flonicamid for 30 days. Vitamin C and E each at 100mg concentration were fed to rabbits of GIII and GIV 30min prior to flonicamid administration separately, whereas in GV both vitamins were given together. Various hematology parameters, serum urea and creatinine content, MDA level, SOD and CAT activity were determined. Following exposure for stipulated time, significant decrease was observed in animal hematology indices including RBCs, platelet count and hemoglobin content. Remarkable induction of nephrotoxicity was observed as evidenced by significant increase in serum urea, creatinine content and renal tissue MDA level whereas SOD and CAT activity were declined. Vitamin C and/or E pretreatment supplementation showed significant protection against toxic effects induced by flonicamid on studied parameters. In conclusion, concomitant use of vitamin C and E as antioxidants exhibited strong therapeutic properties that could be able to ameliorate flonicamid induced biochemical and nephrotoxic effects in male rabbits

Key words: *Flonicamid, rabbit, nephrotoxicity, vitamin C, vitamin E, malondialdehyde*

Introduction

Pesticides (insecticides, nematicides, herbicides, fungicides etc) are commonly used as an important management tool to enhance crop yield and reduce post-harvest losses (1). The excessive use of these synthetic pesticides is appeared as serious environmental problem which are posing hazardous effects on both terrestrial and aquatic ecosystems, on beneficial arthropods, as well as on human health (2,3). It causes acute illness like headache, skin irritation, ocular irritation, vomiting, diarrhea to severe chronic diseases such as cancer in human beings (2, 4, 5). The worldwide utilization of pesticides is approximately 5.6 billion pounds per year and their consumption is increasing unexpectedly day by day (2). From the South Asian countries, Pakistan ranks second in the overall consumption of pesticides and major use of these pesticides is in agriculture sector (6, 7). Unfortunately, due to lack of awareness of farmers about toxic effects of pesticides, huge amount of pesticides are applied in agriculture area of Punjab with no significant effect on crop yield. Currently, more than 30 types of fungicides, 5 types of acaricides, 39 types of weedicides, 6 different types of rodenticides, and 108 types of insecticides are being used in Pakistan. Among these pesticides the major use is of insecticide (8).

Flonicamid is a novel systemic insecticide, having IUPAC name as N-cyanomethyl-4-trifluoromethylnicotinamide. It is a pyridine carboxamide manifesting structural formula as shown in Figure 1. It was developed by Ishihara Sangyo Kaisha, Ltd., and registered in Japan in 2006 under the trade name of Ulala DF. In January 2014, this novel insecticide was registered in forty-one countries of America, Europe, Asia and Africa (9).

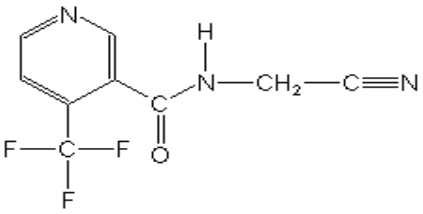

Figure 1. Structural formula of flonicamid

It is commonly applied through foliar spraying and metabolized in to various compounds like 4-trifluromethylnicotinic acid (TFNA), N-(4-trifluromethylnicotinoyl) glycine (TFNG), 4-trifluromethylnicotinamide (TFNA-AM), N-(4-trifluromethylnicotinoyl) glycineamide (TFNG-AM) etc. (10-12). It is very particular towards its effect to control the adult and nymph stages of aphids, white flies (hemipterous pests), thysanopterous pests, mosquitoes, do not harm the beneficial arthropods and exhibit low toxicity against bees, birds, fish and mammal's e.g. male and female rat oral LD50 of flonicamid is 884 mg/kg and 1785 mg/kg, respectively (13). It shows aphid anti-feeding behavior by blocking A- type potassium channels, causing toxicity by inhibiting inward movement of potassium ions through these (Kir) channels, disrupting the salivation and sap feeding ability of insects leading towards the mortality through starvation (11-14). Insecticide Resistance Action Committee (IRAC) placed the flonicamid in group 29 due to its different mode of action as it disrupt the function of chordotonal organs without binding the Nan-lav TRPV channel complex unlike the insecticides of group 9 such as Pymetrozine and Pyrifluquinazon (15.16). Due to its novel

properties, flonicamid has become an integral part of insecticide pest management (IPM) programme and frequently used to control pests on a range of crops like cotton, wheat, apple, peach, cabbage, potatotes and variety of other vegetables that use in our day to day life (12,18,). However, inspite of low toxicity ever- escalating use of flonicamid contribute the decreased level of residue in the environment as compared with neonicotinoids and organophosphates. Earlier studies reported flonicamid toxicity against predators of insect pest such as Chrysoperla and Coccinellid, predators of pomegranate aphids (13). Flonicamid residues have been noticed in various harvested food commodities such as cucumbers, bell peppers, cabbages, apple, peach, tea leaves and paddy rice as well as in several crunches around the Great Lakes Basin in United States (19-22). Flonicamid and its metabolites (TFNA, TFNG, TFNG-AM, TFNA-AM) were detected in orange groves and dried hops after the application of insecticide during field studies (19,21). Due to persistence of pesticide residue on food ultimately enter in to the body of consumer, making adverse effects after bioaccumulation. Flonicamid residues were also detected in human serum and urine samples (23). Prior experimental proofs manifested severe DNA damage in mice after flonicamid exposure (24).

It is the need of the day to explore natural antioxidants that protect the body from oxidative stress induced by toxic residues of environmental pollutants. Vitamin C is the most important water soluble antioxidant in extracellular fluids and involves in various metabolic processes in the human body, including those that are important for the optimal function of the oxygen energy system (25). Vitamin E act as an essential fat soluble antioxidant. It is naturally present in cell membranes, inhibits the reactive oxygen species (ROS) induced generation of lipid peroxyl radicals and protecting the cells from peroxidation of poly unsaturated fatty acids (PUFA) of membrane phospholipids, from oxidative damage of cellular proteins, plasma low-density lipoproteins, DNA and from membrane degeneration (26). They are claimed as powerful antioxidant compounds providing resistance against acute (inflammation) to chronic diseases (cancer). Oxidative stress generated due to free radicals production after exposure to environmental pollutants is the main leading mechanism behind the cause of disease (26-28). Furthermore, vitamin C assists the vitamin E to reappear to its active state in the cell membrane and facilitate the better antioxidant protection in many diseases related with enhanced oxidative stress (29, 30). Under this scenario, in present study vitamin C and E are selected as antioxidant supplements to reduce the effects of flonicamid induced toxicity in male rabbits. Rabbits are considered as good experimental model which are closer to primates in phylogenetic tree as compared to other rodents and represent a better clinico- anatomical morphological anomalies associated with human diseases (31).

To best of our information this is first scientific study to explore antioxidant potential of vitamin C and E to ameliorate the hematological, biochemical and renal toxicity induced by flonicamid exposure in domestic male albino rabbits.

Material and Methods

Chemicals

Flonicamid (purity 98.6%) was obtained from Shandong United Pesticide Industry Co., Ltd. China, (Batch#2020072098510). Vitamin E (purity 99.2%) from Zhejiang Medicine Co., Ltd. China (Batch#201201906205). Non fortified corn oil was obtained from local market. All other chemicals including ascorbic acid (purity, 99%), Thiobarbituric acid (TBA), bovine serum albumin were analytical grade and purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA).

Animal maintenance

Male albino rabbits (*Oryctolagus cuniculus domesticus*) of age between 20-24 weeks and weight between 1.0 to 2.0 kg were purchased from University of Animal and Veterinary Sciences, Lahore. These animals were acclimatized for one week before the start of experiment in the animal house of PCSIR under optimal conditions (temperature 22 ± 3 °C, relative humidity between 30 to 70%, 12h light dark cycle) with free access to commercial diet along with fodder and fresh drinking water *ad labiatum*. All experimental procedures and protocols conducted on animals during current study were strictly followed according to OECD guidelines 423 and same were approved from bioethical committee of Advance Studies and Research Board (AS & RB dated 05/10/2020) of University of the Punjab, Lahore, Pakistan.

Experimental design

Acute oral median lethal dose (LD_{50}) of flonicamid was assessed in male albino rabbits by following the method of Weil (32) through a series of experiments conducted in initial phase of current study (data is not presented). Results revealed that acute oral LD_{50} of flonicamid was 180mg/kg body weight (bw). Further investigations were based on the selection of safe dose i.e. $1/10^{th}$ of LD_{50} of flonicamid (18mg/kg bw/day) given orally through feeding gavage for 30 days to induce toxicity in rabbits. Flonicamid and vitamin E were prepared in non-fortified corn oil whereas aqueous solution of vitamin C prepared at appropriate concentrations to meet the requirement of daily dose of specified groups. Male albino rabbits were randomly divided in to five groups on the basis of various treatments.

Group I- served as control received only vehicle i.e. non-fortified corn oil.

Group II – received only $1/10^{th}$ dose of LD_{50} of flonicamid per kg bw.

Group III – received 100mg of vitamin C and $1/10^{th}$ dose of LD_{50} of flonicamid per kg bw.

Group IV – received 100mg of vitamin E and $1/10^{th}$ dose of LD_{50} of flonicamid per kg bw.

Group V – received 100mg of vitamin C & vitamin E each and $1/10^{th}$ dose of LD_{50} of flonicamid per kg bw.

Antioxidant solutions were given orally through feeding gavage 30 minutes prior to flonicamid oral administration. Body weight changes were noted at zero day to every 10^{th} day of whole study period (30 days) to adjust the treatment dose accordingly. At the end of stipulated time, animals were anesthetized, sacrificed and selected organ i.e. kidney were removed quickly, washed with cold normal saline (0.9%), blot dried on filter paper and weighed.

Blood and tissue sampling

Blood was collected from the ear vein of each rabbit through 3cc syringe after placing the animal in restrain box. Blood was taken at the start (zero day) of experiment and then after every 15th day of experimental period which was further stored in two types of specialized test tubes. One containing EDTA (ethylene diamine tetra acetic acid) for complete blood count and other is plain vacutainer to obtain serum after centrifugation at 4000 rpm for 15min at 4°C. Kidney tissue (1:10 w/v) homogenate was prepared by taking one gram kidney tissues and homogenized it with 100mM potassium chloride buffer (pH7.0) containing 0.3mM EDTA through mechanical driven Teflon glass homogenizer (Polytron, Heidolph RZR I, Germany). The homogenate was cold centrifuged at 3000rpm for 60min at 4°C (eppendorf, Centrifuge 5415R Germany). The resultant clear supernatant was stored at -80°C and will be used for further investigation regarding oxidative status and antioxidant enzymes (33,34).

Evaluation of hematology and renal function markers

Automated Hematology Analyzer (Sysmex KX-21N, USA) was used to analyze various hematology indices such as red blood cells (RBC), white blood cells (WBC), hemoglobin and platelets. Serum renal markers including urea and creatinine were measured using standard commercially available Sigma Aldrich diagnostic kits (Saint Louis, USA), based on couple enzyme reaction producing colored product taking absorbance at 570nm through spectrophotometer (Shimadzu UV-1900).

Assessment of renal lipid peroxidation and antioxidant enzymes

Estimation of malondialdehyde level

Malondialdehyde (MDA) level is correlated with tissues fatty acid peroxidation, was measured through thiobarbituric acid (TBA) method according to Okhawa et al. (35). This assay is based on reaction of MDA with TBA-reacting substances in acidic condition. Pink colored chromophore was measured spectrophotometrically (Shimadzu UV-1900) at 530nm and calculated by using molar extinction co-efficient (1.56×10^{-5} /mol/cm). The values will be expressed as nmol/g of protein/min.

Protein estimation

Protein content of kidney tissues (1.0 % homogenate in 0.25M ice cold sucrose solution) was estimated according to method of Lowry et al. (36) via alkaline copper solution and folin –phenol reagent. Kidney tissue protein content were estimated by using bovine serum albumin (BSA) protein as standard.

Superoxide dismutase activity:

Activity of superoxide dismutase (SOD) of renal tissue homogenate was measured according to procedure described by Beauchamp and Fridovich (37). Briefly, the reaction mixture comprises of tissue homogenate along with 0.1mL of nitroblue tetrazolium (NBT), 0.2mL of methionine and 0.1mL of riboflavin. Tube containing reaction mixture was placed under UV light for certain time. The percent inhibition in the rate of NBT reduction

according to specific time was recorded at 560nm through spectrophotometer (Shimadzu UV-1900). One unit of SOD activity correspond to the enzyme required to inhibit 50% absorbance in contrast to control and was expressed as IU/mg of protein

Catalase (CAT) activity:

Catalase activity of kidney homogenate was evaluated by colorimetric assay described by Sinha (38). This assay was based on the formation of green colored chromic acetate. The catalase content of enzyme preparation were expressed in terms of micromoles of H_2O_2 consumed per mg of protein per min.

Data analysis

The data was subjected to statistical analysis using GraphPad Prism 8.0.2 by means of Two-way Analysis of variance (ANOVA) followed by Dunnetts' Multiple Comparision test, comparing mean of each group with mean of control. Data was expressed as mean of five replicates ± SE. A p value < 0.05 was considered as significant.

Results

In current toxicity study, a non-significant increase (8.26%) of white blood cells (WBC) was observed in only flonicamid exposed rabbits of GII as compared to control GI. WBC raise level was reduced in vitamin supplemented groups but still higher than control (Figure 2A). Red blood cells in control group was 5.67E12±0.35/L while in GII, GIII, GIV and GV were 4.60E12±0.28, 5.81E12±0.29, 4.96E12±0.19 and 5.83E12±0.27, respectively. Current results from two way ANOVA following Dunnetts multiple range test confirmed significant decrease p = 0.002 of RBCs in only flonicamid exposed group while in GV where both vitamin C and E co-administered with flonicamid non-significant reduction was observed as compared to control GI (Figure 2B). Similar reductions in case of hemoglobin (p = 0.033) and platelets (p = 0.033) were perceived in GII equated to GI as presented in Figure 2C and 2D, respectively.

Figure 3 manifested the flonicamid induced renal impairments, as represented by disturbed levels of kidney biomarkers, flonicamid intoxicated rabbits exhibited increased serum urea (25.23%) and creatinine level (17.09%) when compared to control group (p = 0.002, p = 0.002). Oral supplementation of vitamin C and E reduced the plasma urea and creatinine values near to normal (control group) when compared with only flonicamid fed group (GII).

Renal lipid peroxidation level was increased due to flonicamid intoxication in all the test periods when compared to control. Renal MDA levels were decreased significantly in vitamin C and/or E supplemented groups (Figure 4A). Renal SOD and CAT activity were significantly reduced 12.41% (p = 0.002) and 14.89% (p =0.002) in GII as compared to GI, respectively. However, remaining groups showed non-significant decrease of above said antioxidant enzymes which could be related to protective effects of vitamins supplementation (Figure 4B, 4C).

Discussion

Pesticide contamination is a worldwide environmental and public health issue

effecting all kind of living creatures including plants, animals and human beings due to their carcinogenic and highly toxic nature. Flonicamid is a novel systemic insecticide, which is due to low toxicity to mammals and effective killing property of selective insects has become an essential part of IPM (integrated pest management). But frequent use of this insecticide donate toxic effects on human health and ecosystem. It is harmful to human after acute oral administration and its target organs are hematopoietic tissues, liver and kidney (39, 40, 41). Both vitamin C and E are powerful antioxidants and several studies had been conducted revealing their ameliorative effects in pesticides induced toxicity (27, 33). However, there are currently no reports to assess the antioxidant potential of vitamin C and/or E in healing the damages induced by flonicamid exposure in male rabbits. Present study revealed that flonicamid exposure alter the hematology indices in blood tissue of exposed rabbits including WBCs, RBCs, hemoglobin content and platelets count. A dose of 18mg/kg bw of flonicamid in rabbits displayed a high count of WBCS indicating that insecticide exposure donate some toxicants in blood and in response body immune system tried to overcome the toxicants, resulted in increase of WBCs. Riaz and Yousef (42) had reported similar observations of increase in WBCS (11.56%) after malathion exposure in rabbits at 20mg/kg bw concentration for 30days. Shakoori et al. (43) had described 46% increase of WBCs in rabbits fed with danitol. In current study, flonicamid exposure to rabbits caused reduction of RBCs, platelets and hemoglobin content in blood tissue. These findings are in corroboration to Riaz and Yousef (42), demonstrated that malathion exposure to male rabbits at 20mg/kg bw concentration reduced the RBCs count 9.15% in comparison to control group. This reduced value may be associated with less production of RBCs due to damaging effect of pesticide on erytropoeitic tissues or could be increased rate of destruction of RBCs due to toxicants. Similarly, decreased hemoglobin could be related to decline in RBCs count. Previous findings of Kumari and Banergi (44) demonstrated that dose and time related exposure of pesticide as well as age of animal were important factors playing crucial role in reduction of RBCs and hemoglobin concentration in blood tissue of exposed animal. The decrease in haemoglobin concentration was also observed in blood of rabbit treated with danitol (43). In present study, decreased platelets count in comparison to control could be associated with destruction of platelets due to flonicamid toxicants or impairment in formation of platelets from bone marrow (42). Okolie et al. (45) stated that rabbit's exposure to phostoxin insecticide cause the reduction in the platelet count. Variations in hematology indices in flonicamid exposed rabbits were reduced near to control group after vitamin C and/or E supplementation through oral route (Figure 4). Abdul- moneum et al. (49) described in previous investigation that male white New Zealand rabbits were exposed with dietary diazinon at 12.5 mg/kg diet showed remarkable hematotoxicty as evidenced by increased WBCs, decreased RBCs and hemoglobin content in blood tissue. However the use of antioxidants vitamin C (at concentrations 500 or 750mg/Kg diet) and vitamin E (at concentration 25 or 50mg/kg diet) were significantly improved the blood hematology indices near to control

group. Significantly ($p < 0.05$) elevated level of serum urea and creatinine in male albino rabbits, noticed in this study is a classical sign that kidney was harmfully affected by flonicamid exposure. One of the sensitive and demarcated indicator of renal injury is serum creatinine concentration in mammals (42). Current results depicted that elevated level of serum urea and creatinine were observed in all flonicamid intoxicated rabbits and these values were reduced to normal in rabbits fed with vitamin C and/or E. These findings are in corborration to Abdul-moneum et al. (49) reported that use of vitamin C and E in diet along with diazinon significantly reduced the elevated level of creatinine in male rabbits. Riaz and Yousafzai (42) reported that rabbits fed with 20mg/kg bw malathion and cypermethrin for 30days cause increase in serum creatinine phosphokinase and urea concentrations. Urea is removed from body through glomerular filtration and excreted out, so any impairment in kidney function causes reduced ability to excrete urea from blood in to urine, resulting in high serum urea concentration (39).Previous literature suggested that oxidative stress was the main leading mechanism behind the lipid peroxidation of cellular membranes of various organs including liver, kidney, brain, testis and fetus after animal's exposure to pesticides (46,47). Vitamin C and E play an important role against pesticide induced toxicity as an antioxidant agent to scavenge free radicals, thereby resulting protection of vital cells (21). High MDA level in kidney tissues is an indicator of tissue lipid peroxidation as found in present study after flonicamid exposure. Concomitant use of vitamin C and E were effectively controlled the MDA level (Figure 6) that could be indication of the reduced renal tissue injury. In a study by Erdemeli et al. (46) on acetamiprid (ACMP) induced toxicity model, 25 mg/kg bw per day was administered to Blb-c male mice for 21 days. There was an increased value of MDA and both SOD and CAT activities were decreased. When the ACMP treated groups were supplemented with vitamin E at 100mg/kg bw/day concentration, MDA level, SOD and CAT activities were measured near to control group.

Conclusion

In conclusion, pyridine carboxamide based novel insecticide flonicamid significantly affected the heamatological indices, oxidative stress and renal functional markers as well as renal antioxidant enzymes SOD and CAT of male albino rabbits after exposure for specific time period. However, synergistic use of vitamin C and E plays crucial role in protecting biological system from induced toxic effects of flonicamid through reduction of lipid peroxidation and elevation of antioxidant enzyme status. Consequently, precautionary measures must be taken by concerned health authorities to avoid excessive use of aforementioned pesticide in both public and agricultural sector and supplementation of vitamins could be helpful to provide protection against induced toxicants.

Declaration of conflicting interests

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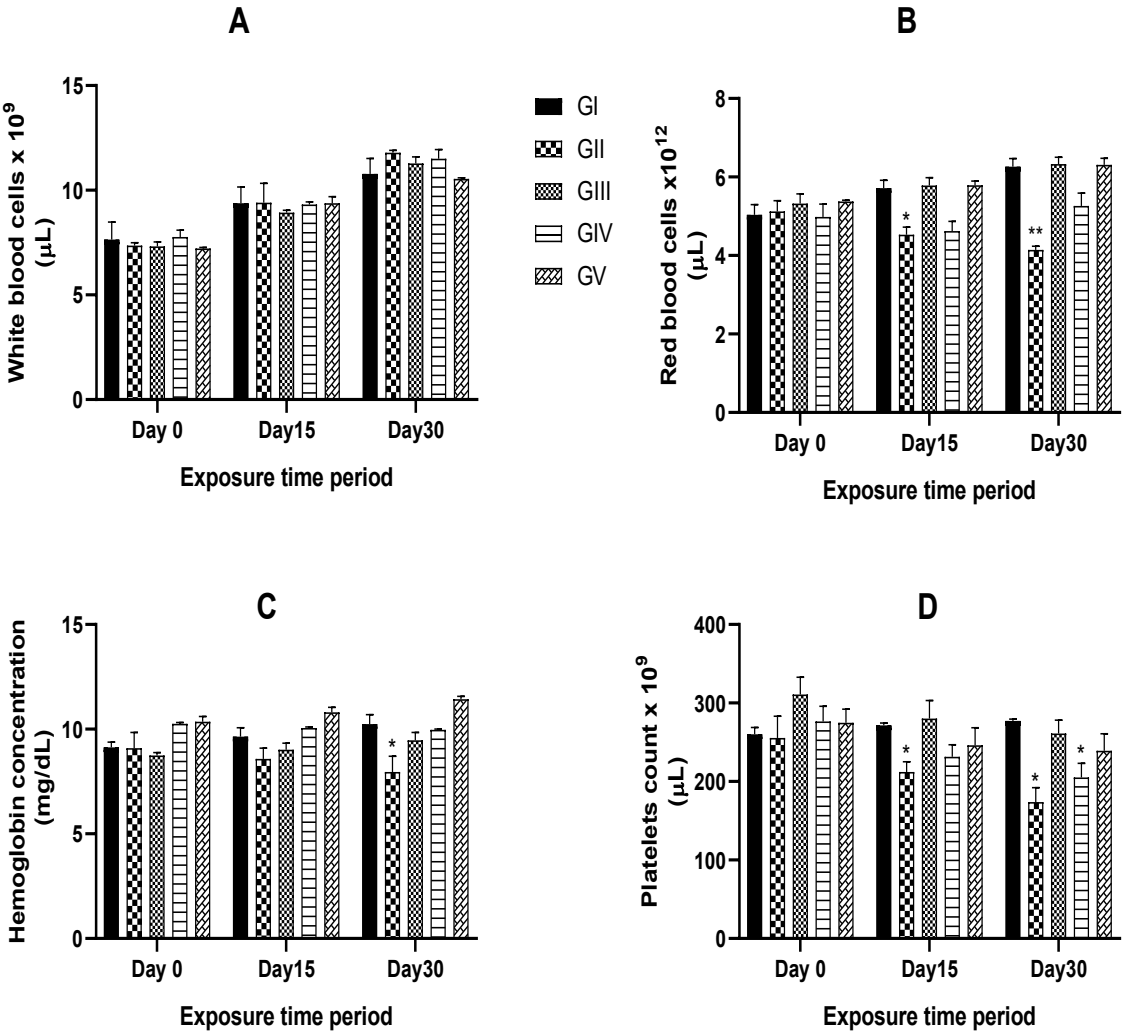

Figure 2. Variations in hematological parameters in treated rabbits: (A) White blood cells count (B) Red blood cell count (C) hemoglobin content (D) Platelet count alterations induced after flonicamid exposure and attenuated effects on co-administration of vitamins supplementation. Values are mean ± SE of five replicates. * Statistically significant from control at $P < 0.05$. ** significant from control at $P < 0.002$. *** significant from control at $P < 0.001$.

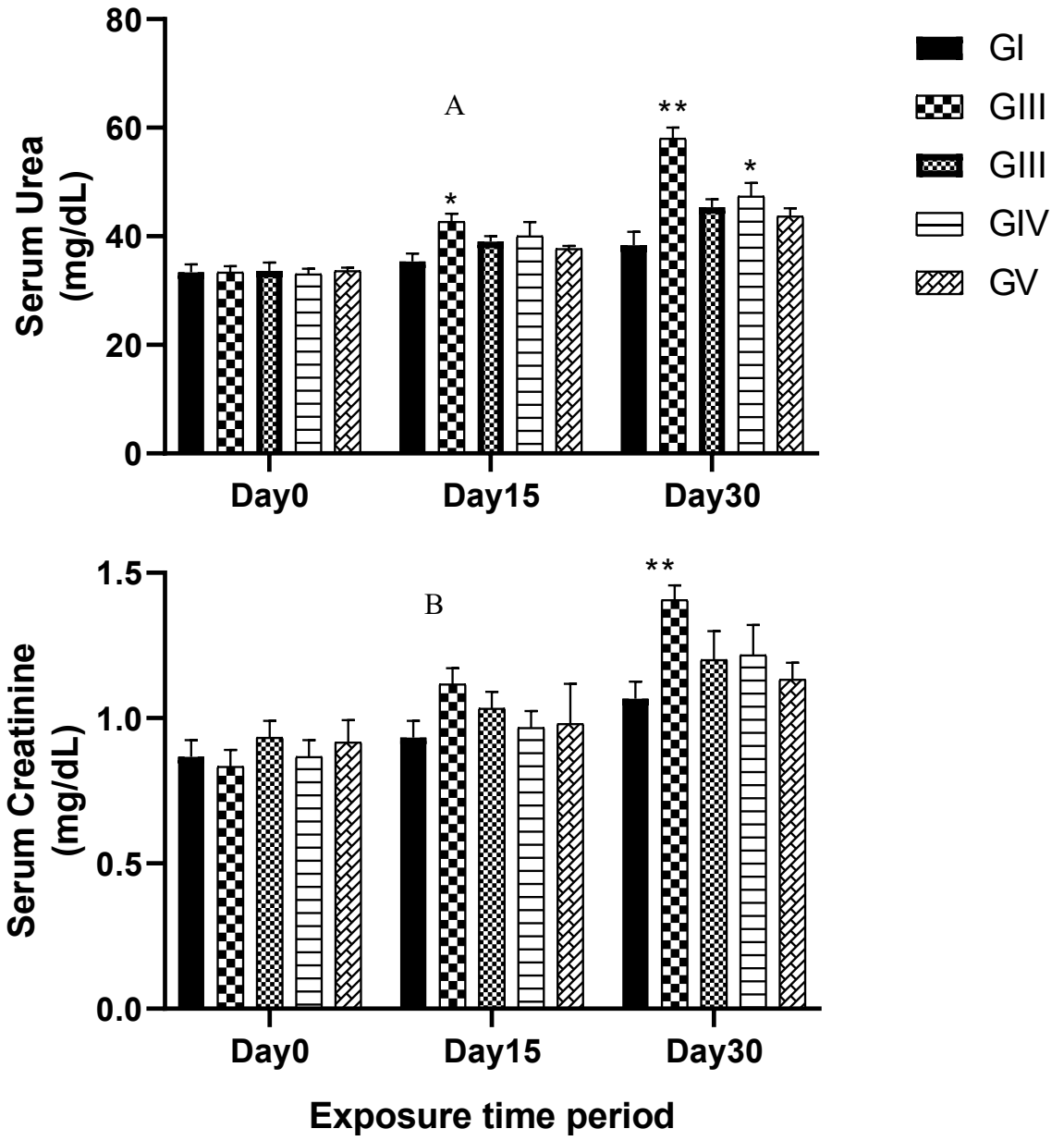

Figure 3. Changes in serum renal function markers in flonicamid exposed rabbits and ameliorative effects of vitamin C and E concomitant supplementation: (A) serum urea (B) Serum creatinine phosphokinase level. Values are mean ± SE of five replicates. * Statistically significant from control at P < 0.05. ** significant from control at P < 0.002. *** significant from control at P < 0.001.

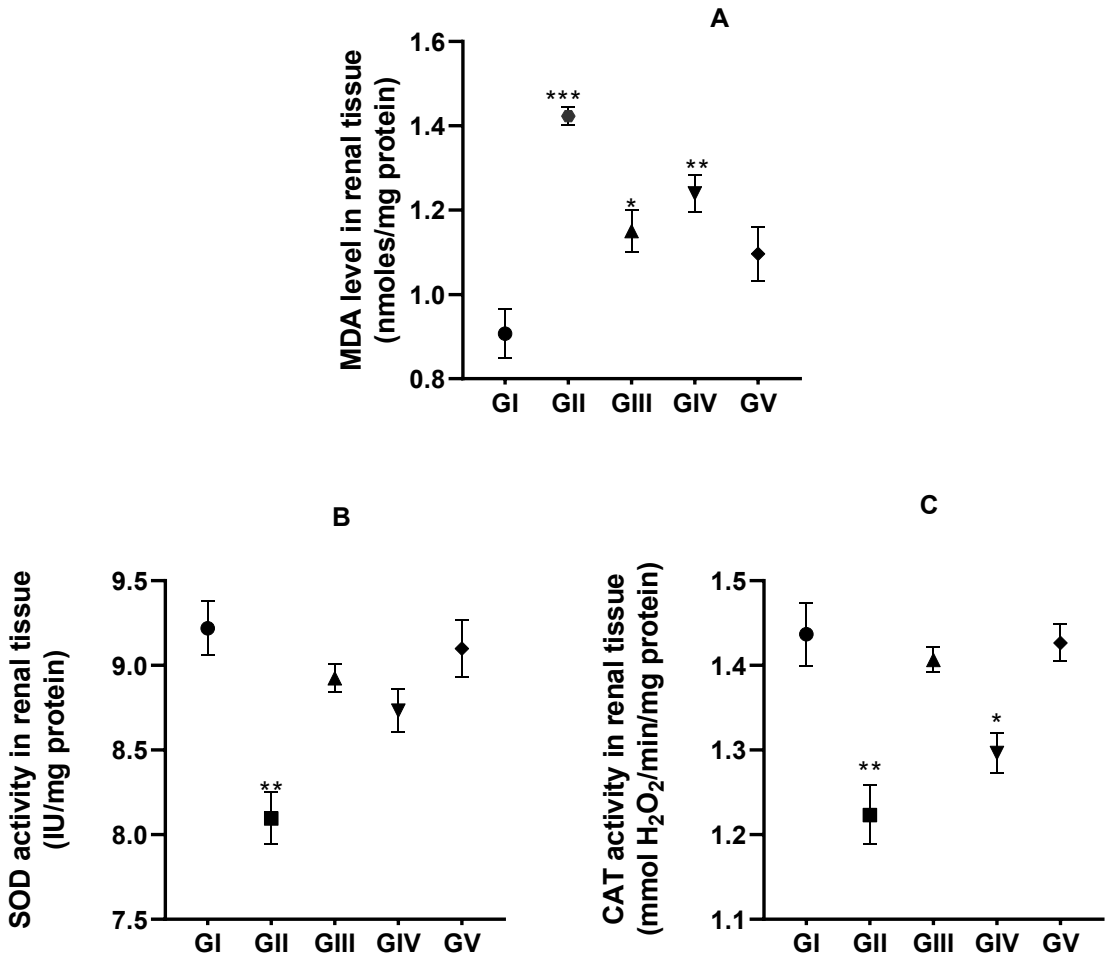

Figure 4. Influence of flonicamid toxicity on kidney tissue oxidative stress marker and antioxidant enzymes activity as well as protective effects of vitamins C and/or E renal tissues: (A) MDA level representing lipid peroxidation of renal tissues (B) SOD activity (C) CAT activity. * Statistically significant from control at $P < 0.05$. ** Significant from control at $P < 0.02$. *** Significant from control at $P < 0.001$.