Amelioration of oxidative stress-mediated cytotoxicity and genotoxicity induced by copper and flubendiamide *in-vivo* and *in-vitro* by potent antioxidants

Rajesh Mandil (✉ rajesh_mandil@rediffmail.com)

Sardar Vallabhbhai Patel University of Agriculture and Technology

Atul Prakash

U.P. Pt. Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go- Anusandhan Sansthan (DUVASU)

Anu Rahal

Central Institute for Research on Goat (CIRG)

Swati Koli

U.P. Pt. Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go- Anusandhan Sansthan (DUVASU)

Rahul Kumar

U.P. Pt. Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go- Anusandhan Sansthan (DUVASU)

Satish K. Garg

U.P. Pt. Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go- Anusandhan Sansthan (DUVASU)

Research Article

Keywords: Flubendiamide, copper, TUNEL, Micronuclei, Comet, natural antioxidants

Posted Date: December 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1161671/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Present study was designed to assess the toxicity of copper @ 33 mg/kg and flubendiamide @ 200 mg/kg in vivo in male Wistar rats orally once daily for 90 days and protective effect of α -tocopherol, resveratrol, curcumin and catechin and in vitro cyto-genotoxicity in primary cell culture of thymocytes. In vivo study showed significant ($p < 0.05$) increase in AST, total bilirubin and uric acid, creatinine and BUN levels while decrease in total proteins, GSH, SOD and GST levels and increased LPO and GPx with severe degenerative changes were observed in liver and kidney tissues in intoxicated groups. In vitro thymocytes were exposed to 40 μ M concentration of flubendiamide and/or showed significant increase in TUNEL+ve cells, micronuclei, DNA shearing, and comet formation per 100 cells.

Concurrent treatment with α -tocopherol in xenobiotics intoxicated groups showed almost normal values of the biochemical parameters and decreased LPO production and improved antioxidant enzymes activities and histoarchitecture of liver and kidney tissues suggest ameliorative potential of α -tocopherol whereas, resveratrol, curcumin, catechin or α -tocopherol in vitro decreased TUNEL+ve cells, micronuclei induction and comet formation and effect of antioxidants was concentration-dependent and their order of potency on equimolar concentration (10 μ M) basis is: curcumin > resveratrol > catechin = α -tocopherol.

Introduction

Indiscriminate and non-injudicious use of insecticides/pesticides in agricultural practices and waste disposal of heavy-metals and chemical industries in water bodies are entering the food chain unintentionally producing direct impact on human and animal health^{1,2}. Flubendiamide (Pthalmic acid diamide) is a new class of insecticide used extensively as a substitute for organophosphate and organochlorine pesticide. Flubendiamide, a ryanodine class insecticide stimulates ryanodine-sensitive calcium release channels (RyR) of insects^{3,4} and disrupt muscle functions without mammalian ryanodine receptors^{3,5}. Use of flubendiamide in agriculture practices has crossed its threshold and resulting in environmental contamination due to its slow degradation and persistent behaviour and thus adversely affect human and animal health⁶⁻¹⁰.

Though, the United State Environmental Protection Agency (USEPA) has cancelled the registration of all flubendiamide products due to unreasonable adverse effects on the aquatic environment¹¹, however, it is still in use in several countries including India for control of *Lepidopteron sp.* pests in agricultural operations¹². Flubendiamide has been reported to primarily affect liver followed by thyroid and haemopoietic system^{5,13} but does not produces genotoxicity effects on bone marrow cells^{14,15}. Whereas, in our previous studies we have reported flubendiamide induced oxidative stress in spleen and testicular tissues with slightly altered haematological parameters *in vivo* in rats¹⁶⁻¹⁸ and genotoxic behaviour on primary cell culture of rat splenocytes following *in vitro* exposure¹⁸.

Contaminated food and water bodies from industrial effluents contain both essential and non-essential/trace elements that may also produce health hazard. Mn, Fe, Co, Cu and Mo are essential trace metals participate in wide range of redox reactions and their toxicity can be attributed to accumulation of

excess concentration of these metal ions in specific tissues or organs leading to generation of free radicals and consequent oxidative stress is one of the important mechanisms of cell death¹⁹. Copper, although biologically essential, used for control of bacterial and fungal infections as weedicide, fungicide in agricultural practices and as molluscicide in domestic lakes and ponds²⁰ can be highly toxic to biota (plants, animals and human beings) if present in higher concentrations²¹. In India, river, ponds and lakes are commonly used for irrigation and drinking purposes can be a major source of contamination and intoxication where, river water has been reported to have more than 300 µg/ml²² while WHO recommends not more than 2mg/L. Thus, human beings and animals are being continuously exposed to copper through drinking water. Long term chronic exposure to copper causes hepatic and neurotoxic effect in rats^{23,24} and neurodegenerative disorders (Alzheimer's disease and Parkinson's disease) in humans and dogs²⁵⁻²⁷. Copper has also been reported to produce cyto-genotoxic effects²⁸⁻³⁰.

Food-derived components have received great attention in last two decades particularly due to their antioxidant and anti-inflammatory potential³¹⁻³³. Curcumin, α-tocopherol (α-TOH), resveratrol and catechin are well studied biologically active constituents possessing anti-oxidant, anti-inflammatory, anti-apoptotic potential having ability to chelate metal ions and upregulate the antioxidant enzymes have been reported to produce cytoprotective, cardioprotective, antiapoptotic effects in metal- and pesticide-induced oxidative and DNA damage^{33, 34-40}.

Assessment of single chemical toxicity on an individual basis does not reflect actual conditions in environment or in human and animal health where the target is exposed to various chemicals at a same time⁴¹. Thus, the concept of combined exposure to multiple chemicals and assessment of their toxicity has been evolving over decades. Therefore, interaction toxicity studies between two or more toxicants have become important and an emerging area of research in modern toxicology where interaction may lead to synergistic, antagonistic, inhibitory or potentiated toxic effects⁴². Paucity of any information on toxicity of these xenobiotics where flubendiamide is claimed to be safest insecticide and copper as essential micro-mineral prompted us to take up the present study to assess their combined effect and interactive behaviour on biochemical indices, oxidative stress biomarkers, drug metabolizing enzymes and histological changes in liver and kidneys *in vivo* in rats and cyto-genotoxicity assays in primary cell culture of rat thymocytes along with comparative protective efficacy of curcumin, α-tocopherol, resveratrol and catechin against these xenobiotics-induced toxic-insults.

Materials And Methods

Chemicals. Flubendiamide, dexamethasone, resveratrol, catechin, curcumin, α-tocopherol and propidium iodide, ethidium bromide, acridine orange, normal melting agarose (NMA), low melting point agarose, foetal calf serum, Roswell Park Memorial Institute (RPMI-1640), Hank's balance salt solution (HBSS), Paraformaldehyde, Proteinase-K, Triton-X 100, Sodium dodecyl sulfate (SDS) were procured from Sigma-Aldrich (USA). Commercial grade flubendiamide (FAME®; Bayer) was purchased from the local market while copper sulfate pentahydrate (CuSO₄. 5H₂O) from SD Fine Chemical Limited and rest of the other chemicals used were of analytical grade and standard quality. For estimation of different blood biochemical

parameters, commercially available diagnostic kits were procured from Span Diagnostic Ltd. For *in vitro* studies stock solutions of curcumin, α -tocopherol, resveratrol, catechin, flubendiamide, copper sulphate and dexamethasone were prepared in dimethyl sulphoxide (DMSO) and therein DMSO was used as vehicle control in all the experimental protocols to rule out the effect of vehicle, if any.

Experimental animals, design and treatment. Male Wistar rats were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute (IVRI), Izatnagar, India and were maintained under standard managemental conditions in the Departmental Experimental Animal House. Animals had free access to pelleted feed and clean deionized drinking water. Daily light and dark cycle of 12 h was ensured. An acclimatization period of 15 days was allowed before start of the experiment. Study was taken up after the approval of Institutional Animal Ethics Committee of the University (Approval No. 79 IAEC/13 dated 16.07.2013). The study was conducted as per standard guidelines and established procedures for effective compliance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), a statutory Committee set up under Prevention of Cruelty to Animals Act 1960 of Ministry of Environment, Forest and Climate Change, Government of India, 2006.

In vivo study. Fifty-four male Wistar rats weighing 130 to 150g were used in the present study and were randomly divided into nine groups of six animals each. Group I (Healthy control) and group II (vehicle control-corn oil) constituted the control groups and remaining groups were like: group III (copper sulphate), group IV (flubendiamide), group V (copper sulphate+ flubendiamide), group VI (α -tocopherol), group VII (copper sulphate+ α -tocopherol), group VIII (flubendiamide+ α -tocopherol) and group IX (copper sulphate+flubendiamide+ α -tocopherol). Copper sulphate, flubendiamide and α -tocopherol were administered @ 33 mg/kg, 200 mg/kg and 100 mg/kg, respectively, once daily continuously for 90 days by oral gavage. The used doses of flubendiamide and copper sulphate were $1/10^{\text{th}}$ of the LD_{50} values of flubendiamide (>2000 mg/kg) and copper sulphate (>333 mg/kg)^{5,43}. Solutions of copper sulphate and flubendiamide were prepared in deionized water while α -tocopherol was dissolved in corn oil for daily gavaging.

Blood and tissues collection. After the end of experimental period of 90 days, on 91st day blood samples from rats of different treatment groups were collected through retro-orbital plexus using glass capillary in heparinised tubes after overnight fasting as per the method of Sorg and Buckner⁴⁴. Blood samples were centrifuged at 2000 rpm for 15 min and plasma was separated for further biochemical analysis. After collection of blood, rats were humanely sacrificed by cervical dislocation and liver and kidneys were collected and weighed to determine their absolute (g) and relative weights (g/100 g). Pieces of liver and kidneys were preserved in formal saline for histopathological examination while small piece of both organs was stored at -20 °C for estimation of oxidative stress indices. Further, liver tissues stored at -20 °C were washed with ice-cold normal saline and perfused with chilled 1.15% KCl solution containing 0.05 mM EDTA and processed for preparation of microsomes to determine activity of drug metabolizing enzymes.

Biochemical parameters. Total protein, albumin, globulin, bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, uric acid and blood urea nitrogen (BUN) were estimated in

plasma by commercially available diagnostic kits (Span Diagnostic Ltd.) and using semi auto-analyser (Erba, Mannheim).

Oxidative stress biomarkers. Liver and kidney tissues stored at -20°C were thawed and 200 mg of each tissue was weighed and transferred in eppendorf tube containing 2 ml chilled saline. Tissue homogenates were prepared using tissue homogenizer (Heidolph, Germany) under cold condition and centrifuged for 10 min at 3000 rpm. The supernatant obtained was used for estimation of oxidative stress biomarkers. Lipid peroxidation (LPO) was estimated employing the method of Shafiq-U-Rehman⁴⁵. Total protein (TP) estimated by using the method as described by Lowry et al⁴⁶ and reduced glutathione (GSH) by Sedlak and Lindsay⁴⁷. Catalase (CAT) activity was measured in liver and kidney homogenates following the previously described method of Bergmeyer⁴⁸ and superoxide dismutase (SOD) was determined according to Madesh and Balasubramanian⁴⁹. Glutathione-S-transferase (GST) activity was assessed using the protocol described by Habig et al.⁵⁰ and glutathione peroxidase (GPx) by the method of Paglia and Valantine⁵¹.

Drug metabolizing enzymes

Preparation of microsomes. In brief, liver was homogenized in 3 volumes (1:3) of ice-cold (-4°C) homogenization buffer containing 1.15% KCl in 50 mM potassium phosphate buffer solution (pH 7.4). The homogenates were centrifuged at 15000 g for 30 min using ultra centrifuge (Himac CS120GX II, Hitachi, Japan.). The mitochondrial supernatant (8ml) spun at 105000 g for 1 h in ultracentrifuge to sediment the microsomal pellet. Cytosolic fractions were collected from middle separating the lipid layer on top in a separate Eppendorftube. The microsomal pellet was then washed by adding 8 ml of resuspension buffer (homogenization buffer containing 0.05 mM EDTA) and spun again 105000 g for 15 min. The microsomal pellet, so obtained, was resuspended in 4 ml of resuspension buffer and 1 ml aliquots were immediately stored at -80°C until assayed for drug metabolizing enzymes.

Phase I drug metabolizing enzymes. Hepatic microsomal pellets prepared were used for assay of phase I drug metabolizing enzymes, namely-cytochrome P_{450} and CYP_{b5} activities measured as per the protocol described by Omura and Sato⁵². Aminopyrine-*N*-demethylase and aniline *p*-hydroxylase activities were estimated according to the method of La Du et al.⁵³ and hepatic microsomal protein was determined by the method described by Lowry et al.⁴⁶.

Phase II drug metabolizing enzymes. Hepatic microsomal and cytosolic glutathione sulfotransferase were estimated by determining the rate of increase in optical density (OD) at 340 nm at 25°C due to formation of 1-chloro-2, 4-dinitrobenzene (CDNB) conjugate of glutathione as described by Habig et al.⁵⁰ and UDP-glucuronosyltransferase activity was assessed as per the method of Dutton and Storey⁵⁴.

Histopathological studies. Pieces of liver and kidneys preserved in 10% buffered formalin saline were processed employing the standard procedure. Tissue sections of 5-6 μm thickness were cut using microtome (Lieca, Germany) and stained with haematoxylin-eosin as per the method of Luna (1968)⁵⁵. Slides were thoroughly examined under light microscope to observe the histoarchitectural changes, if any.

Interactive Index. Mansour's formula ⁵⁶ was used to determine the type of interaction between the copper and flubendiamide in the groups where used in combination in terms of interaction index.

$$\text{Interaction index (I.I.)} = M + C/A_1 + A_2$$

Where,

M = Mean value of mixture or combination treated group

A₁ & A₂ = Mean values of the individual or alone compound treated group

C = Mean value of control group.

Based on the above formula, the ratings of the interaction indices can be classified as follows:

- a) Positive effect means increase of the concerned biochemical parameters above the control values due to effect of the individual compound. If the values of interaction index obtained is either, I.I. > 1, I.I. = 1 and I.I. < 1, it predicts potentiation, additive effect and antagonism, respectively.
- b) In case of negative effect, i.e. decrease of the concerned biochemical parameters below the control values due to the effect of the individual compound. If the values of interaction index obtained is either, I.I. > 1, I.I. = 1 and I.I. < 1, it predicts antagonism, additive effect and potentiation, respectively.

Finally, I.I. of all parameters were summarized in tabulated form and used to draw the conclusion of type of interaction between compounds when they are present together in body of living beings.

In vitro study. Twenty healthy male Wistar rats weighing 80-100 g were used for *in vitro* study and kept in the Departmental Laboratory Animal House for acclimatization period of one week before the start of the experiment and provided *ad lib* feed and water.

Isolation of thymocytes. Rats were sacrificed by cervical dislocation and whole thymus was collected aseptically. Briefly, thymus was quickly disintegrated into small pieces in chilled phosphate buffered saline (PBS) by scissor and finely minced by vigorous pipetting using glass pipette and suspension was transferred to 15 ml sterile centrifuge tube. Tubes were allowed to stand on ice for 15 min. Top 12 ml of the suspension was collected and pelleted by centrifugation at 1500 rpm for 10 min. The cell pellet was re-suspended in PBS and centrifuged again at 1500 rpm for 10 min. The resultant pellet was then treated with 5 ml of RBC lysis buffer (4.15 g NH₄Cl; 0.5 g NaHCO₃; 0.0186 g Na₂-EDTA; 200 ml DW) and kept for 10 min in ice and centrifuged at 1500 rpm for 10 min. Cells were cleared off the lysis buffer by washings the cells twice with PBS. Further, the cells were re-suspended in 1.0 ml Roswell Park Memorial Institute (RPMI-1640) medium with 10% foetal calf serum (FCS), antibiotic and antimycotic solution. Cell count and their viability was determined employing 0.1% Trypan blue dye exclusion test⁵⁷. Cells with more than 90 % viability were used for this experiment and the cell density was adjusted to obtain 10⁶ cells/ml as per the method standardized previously¹⁸.

Determination of median lethal concentration (LC₅₀) of copper and flubendiamide. LC₅₀ values of copper and flubendiamide in primary cell culture of rat thymocytes were calculated by Probit analysis using Graph Pad Prism8 software as described earlier ¹⁸.

Cyto-genotoxicity studies. LC₅₀ calculated for copper and flubendiamide @ 40 µM were used for exposure to 10⁶ cells/ml of primary culture of rat thymocytes for further *in vitro* studies. Antioxidants viz. curcumin (5 and 10 µM), α-tocopherol (5, 10 and 20 µM), resveratrol (5 and 10 µM) and catechin (10 and 20 µM) were used for treatment to assess their ameliorative potential. For each treatment, samples were run in triplicate.

Induction of apoptosis. To determine the comparative protective efficacy of curcumin, α-tocopherol, resveratrol and catechin against copper and flubendiamide-induced apoptosis in primary cell culture of rat thymocytes, 10⁶ thymocytes/ml per well were taken in 24 well culture plates and intoxicated with 40 µM/ml concentration of copper and/or flubendiamide in the presence of different concentrations of test antioxidants. After 12h of incubation in CO₂ incubator at 37°C percent apoptotic cells were determined as per the protocol described in TUNEL Assay Kit (Invitrogen, USA). Apoptotic cells, which undergo extensive DNA degradation during the late stages of apoptosis, were examined under blue filter of the fluorescent microscope and cells which fluoresced brightly were considered as apoptotic.

Micronuclei-induction assay. Freshly isolated thymocytes (10⁶ cells/ml/well) were seeded in 24-well plate and exposed to both toxicants and treated with all four test antioxidants except control and DMSO exposed group. Micronuclei-induction assay was performed as per the method described by Hayashi et al.⁵⁸ Three hundred cells, both mononuclear and binucleated were examined in each treatment group in blind mode under green filter of the fluorescent microscope to determine the frequency of micronuclei formation.

DNA fragmentation assay. Genomic DNA from rat thymocytes was isolated by using standard phenol-chloroform DNA isolation protocol ⁵⁹. 5x10⁶ thymocytes/well were taken in 24-well plate after viability check by Trypan blue dye exclusion test and exposed to LC₅₀ concentration (40µM) of copper and/or flubendiamide in the presence of different concentrations of curcumin, α-tocopherol resveratrol and catechin, except in control and DMSO group. Concentration and purity of the DNA was determined spectrophotometrically by Biophotometer plus (Eppendorf) at 260 and 280 OD. The integrity of DNA was examined in agarose gel (1.0%) electrophoresis and visualized under UV light in Gel Documentation System (Alpha-Innotech) after staining with ethidium bromide.

Comet assay. Comet formation in thymocytes was determined by employing the protocol described by Dhawan et al.⁶⁰. Individual cell/comets were observed and images were captured at 40X magnification using green filter of fluorescent microscope (Microscan 20 PFM, Nitco) and two slides per treatment were observed and at least 50 cells from each slide were scored for comets formation i.e. total of 100 cells/treatment were scored.

Statistical analysis. Data obtained in *in vivo* studies for blood-biochemical indices, oxidative stress biomarkers, drug metabolizing enzymes and micronuclei formation, were subjected to statistical analysis using One Way analysis of variance (ANOVA) followed by Tukey's B multiple comparison Post-hoc test and

presented as Mean $\pm$ SE of the six observations in each treatment group. The Statistical Package for Social Sciences (SPSS 20.00 Software for Windows) was used for all the statistical analysis. Comparisons were made between the values in control groups and those treated with xenobiotics alone and also between the xenobiotics alone treated and those treated concurrently with α -tocopherol. Median lethal concentrations (LC₅₀) of copper and flubendiamide was determined by Probit analysis method using Graph Pad Prism8 software.

Results

In vivo study

Absolute and relative weights, liver and kidneys functions tests. Observations of the absolute and relative weights of liver and kidneys are presented in Table-1. Perusal of data revealed mild to moderate decrease in absolute weights of liver and kidneys, while relative weight of liver only in copper exposed group of rats. However, flubendiamide alone and in combination with copper and other treatment groups were almost comparable with control and vehicle control group of rats. Similarly, liver function biomarkers were evident of non-significant difference among different treatment groups where non-significant increased serum levels of albumin and decrease in globulin was recorded in combined group instead individual exposure groups compared to control groups. Significant ($p < 0.05$) increase in total and direct bilirubin and enzymatic activities of AST, where copper alone and in combination with flubendiamide have also significantly increased AST activity compared to control and vehicle control groups (Table-2). AST/ALT ratio were recorded to be highest in copper followed by its combination with flubendiamide, α -tocopherol treated groups and flubendiamide alone. Surprisingly, ratio was found to be increased in vehicle treated group also.

Kidney function biomarkers viz. BUN, creatinine and uric acid in copper and flubendiamide combination group were found to be altered with higher values but lower than their alone exposed groups as compared to control and vehicle control, however values obtained were significantly higher in intoxicated groups than control group. α -tocopherol treatment has significantly reduced these markers with exception of creatinine in flubendiamide alone combined exposure groups (Table 3).

Oxidative stress biomarkers in liver and kidneys. Perusal of data on oxidative stress markers of liver revealed non-significant change in the levels of lipid peroxidation while mild to moderate decrease in GSH content in rats exposed to copper and was significant ($p < 0.05$) in flubendiamide alone and combined groups as compared to control and vehicle control groups. The enzymatic activity of SOD was reduced appreciably in copper alone and combined group while it was significantly ($p < 0.05$) in flubendiamide alone exposed group of rats, whereas a significant reduction was recorded in GST activity in combination group than alone exposed groups. Mild to moderate but non-significant increase in CAT and significant increase in GPx activities in copper and flubendiamide alone, respectively compared to control and vehicle groups (Fig. 1 a-g).

Oxidative stress biomarkers data of rat kidneys are presented in Fig. 1 a-g. Significant ($p < 0.05$) increase in lipid peroxidation level and decrease in GSH content were evident in copper and/or flubendiamide exposed

groups compared to control and vehicle control groups, however, levels of LPO and decrease in GSH content was relatively lower in combined than alone exposed groups. Significant ($p < 0.05$) reduction in SOD and GST activities was recorded in copper and flubendiamide alone compared to control and combination groups. Increased GPx activity was comparable among copper and flubendiamide alone exposed groups while combined group was almost comparable with control groups, however activity of CAT remains unaltered in different intoxicated groups. Following simultaneous treatment with α -tocopherol improved the antioxidant status by restoring enzymatic antioxidant activity and GST content in both liver and kidneys.

Drug metabolizing enzymes. The data on hepatic drug metabolizing enzymes are presented in Fig. 2 a-d. CYP₄₅₀, CYP_{b5} and APH, UGT and GST activities were observed to be reduced moderately to significantly in the groups intoxicated with copper alone whereas, increment in the activities of these enzymes were recorded in flubendiamide alone and in combination with copper compared to copper alone but was comparable to both control group of rats. α -tocopherol have also been observed to produce similar effects as copper when used alone, while simultaneous treatment has increased their activities. Activity of ANDM did not show any significant changes across the groups.

Histopathological examination of liver and kidneys. Histopathological examination of liver sections of the rats exposed to copper alone exhibited degenerative changes in hepatocytes, vacuolations in hepatic parenchyma, coagulative necrosis and ruptured hepatic cord (Fig. 3b) and flubendiamide produced congested central vein with rarification of hepatic parenchyma along with degenerative changes (Fig. 3c). Combined exposure group has been also observed to produce congestion of central vein, mild degenerative changes in hepatocytes and vacuoles of different sizes indicating mild fatty changes (Fig. 3d). Treatment with α -tocopherol in copper alone exposed group revealed moderate congestion of central vein, cloudy swelling of hepatocytes and mild changes in liver parenchyma (Fig. 3e), similarly flubendiamide alone and combined groups and simultaneous treatment with α -tocopherol showed milder fatty changes in hepatocytes and cloudy swelling in some areas compared to intoxicated groups (Fig. 3g).

Histopathological findings of kidney tissues in rats exposed to copper revealed severe cellular swelling in renal epithelium along with areas of congestion and desquamation of renal tubular epithelium (Fig. 4b). Flubendiamide exposure leads to degenerated glomerulus along with desquamation of renal tubular epithelium (Fig. 4c) whereas, combined exposure group revealed degenerated renal tubular epithelium, compressed glomeruli with increase in periglomerular space and diffused cloudy swelling in renal tubular epithelium (Fig. 4d). Compared to the alone and combination exposed group of rats along with α -tocopherol has shown to restoration of the histoarchitecture of kidney tissues with mild degenerative changes in renal tubular and mild cellular swelling in renal tubular epithelium, mild renal tubular damage with few areas of congestion along with decreased areas of periglomerular space (Fig. 4e-g). Findings of histopathological examination have clearly suggested ameliorative potential of α -tocopherol against toxic insults produced by copper and flubendiamide.

Interactive index. Based on the findings of biochemical parameters, oxidative stress indices and drug metabolizing enzymes in present *in vivo* study, combined interactive index (I.I.) analysis study was carried out and the data are summarized in Table 4. Out of thirty-one (31) parameters evaluated, seventeen (17)

parameters showed antagonism. Twelve (12) parameters showed potentiation and two (2) parameters were predictive of additive effect. Combined effect of copper and flubendiamide on biochemical parameters viz. total and direct bilirubin, AST, BUN, creatinine and uric acid levels were antagonistic while albumin, globulin and ALT showed potentiation and on total protein it was additive. In liver, LPO, GSH, GPx and total protein showed antagonistic while SOD, GST and CAT showed potentiation. In kidney, LPO, GSH, SOD, GST and GPx showed potentiation while CAT and total protein were additive and antagonistic effect, respectively. CYP₄₅₀, ANDM, APH, microsomal protein content, GST, UGT were observed with antagonist effect while CYP_{b5} showed potentiation.

In vitro study

Median lethal concentrations (LC₅₀) of flubendiamide and copper. Flubendiamide and copper (1.0-80 µM) produced concentration-dependent inhibitory effect on viability of thymocytes as evident from the data summarized in Table 5. Based on Probit analysis of the live-dead thymocytes count data, the median lethal concentration (LC₅₀) values of flubendiamide and copper for thymocytes were found to be 43.45 µM (R²= 0.98) and 40.78 µM (R²= 0.93), respectively. As both these values were almost very close to 40µM, therefore, 40µM was considered as the approximate LC₅₀ of both toxicants for *in vitro* studies in primary cell culture of rats thymocytes.

Comparative anti-apoptotic efficacy of resveratrol, curcumin, catechin and α-tocopherol. Copper and flubendiamide @ 40 µM/ml alone treated wells showed higher number of TUNEL positive thymocytes compared to the control and DMSO-treated thymocytes. TUNEL positive thymocytes in different treatment groups, namely copper and/or flubendiamide along with antioxidants used curcumin, α-tocopherol, resveratrol and catechin have been shown in Fig. (5 and 6). Significantly lesser number of TUNEL positive thymocytes compared to the xenobiotics-alone treated groups and decrease in TUNEL positive cells was concentration-dependent. Based on comparative apparent efficacy of these antioxidants at 10 µM concentration, the order of efficacy was resveratrol>curcumin ≥ catechin ≥α-tocopherol against flubendiamide, whereas to copper it was curcumin >α-tocopherol≥ resveratrol>catechin.

Micronuclei induction. Copper (17.1±1.09), flubendiamide (3.63±0.14) and dexamethasone (4.63±0.20) exposure showed significantly (p<0.05) higher percentage of micronuclei formation compared to 1.10±0.05 % and 1.80±0.05% in negative and DMSO treated cells, respectively (Table 6, Fig. 7 and 8) whereas, simultaneous treatment with antioxidants resulted in significant (p<0.05) decrease in the number of micronuclei formation except in flubendiamide+curcumin (5 µM) treated group (Table 6). The order of efficacy of these natural antioxidants at equimolar concentrations of 10 µM against flubendiamide: α-tocopherol > resveratrol >curcumin=catechin, however decrease in number at 20 µM of catechin and α-tocopherol in flubendiamide treated cells. Similarly, tested antioxidants significantly (p<0.05) reduced the number of micronuclei in copper treatment and the comparative efficacy at 10 µM concentration of each of these against copper was: curcumin > α-tocopherol >catechin ≥ resveratrol, however, α-tocopherol (20 µM) resulted drastic reduction in percent micronuclei formed compared to 10 µM of the same (Table 6; Fig. 8). Further, based on the number of micronuclei formed, copper was found to be more genotoxic than flubendiamide.

DNA fragmentation. Fig.9 and 10 illustrate the DNA fragmentation of rat thymocytes following *in vitro* exposure to copper and/or flubendiamide and simultaneous treatment with test antioxidants for 12 h. DNA from the copper, flubendiamide and dexamethasone exposed thymocytes showed severe shearing of DNA than control groups where shearing was not observed. Simultaneous treatment with curcumin at both concentrations has been observed to be more protective on DNA damage than other test antioxidants.

Comet assay. Observations of per cent comet formation of different treatment groups are summarized in Table 6. Perusal of the data revealed that DMSO did not induce comet formation while dexamethasone conspicuously promoted comet formation as these values were found to be 5% and 29.57%, respectively compared to control (4.06%). Copper and flubendiamide exposure resulted in 28.57 % and 23.52 comets, respectively (Table 6; Fig. 11). Concurrent treatment with antioxidants significantly reduced number of comets formed irrespective of toxicants (Table 6) and the comparative efficacy order of these natural antioxidants at equimolar level (10 μ M) against flubendiamide was curcumin> resveratrol>catechin> α -tocopherol. But at 20 μ M concentration level, catechin was comparatively more effective than α -tocopherol. For copper the order of efficacy at equimolar concentration (10 μ M) was curcumin>resveratrol> α -tocopherol $\geq$ catechin. However, at 20 μ M, α -tocopherol was more effective in decreasing copper-induced comet formation.

Discussion

Copper is an essential trace element required for numerous biological activities responsible for normal development/growth and are building blocks of cuproproteins like ceruloplasmin, tyrosinase and superoxide dismutase and supports the function of numerous cellular enzymes ⁶¹. However, overexposure at sublethal concentration for several weeks may produce detrimental effects in kidney ⁶², spleen and thymus ⁶³ and if the concentration exceeds the body tolerance level, may exert hepatotoxicity ^{62,64,65}. Liver is regarded as the main target organ where hepatocytes are the target cells for copper toxicity ⁶⁵. In present study, significant decrease in absolute weight of liver in rats exposed to copper only. In contrast, no appreciable changes in absolute and relative weights of liver and kidneys in rats exposed to copper and/or flubendiamide were recorded. Elevated serum levels of total and direct bilirubin, AST, AST:ALT ratio in copper and/or flubendiamide group compared to control and vehicle control groups. In harmony with our findings, Manna et al. ⁶⁶, Kumar et al. ⁶⁷ Mohammadyari et al. ⁶⁸, Arafa et al. ⁶⁹, El-Magd et al. ⁷⁰, Abdelazeim et al. ⁷¹ have also demonstrated elevated levels of serum total bilirubin, AST, and AST:ALT ratio in copper oxide nanoparticles after oral administration to rats. Increased AST:ALT ratio in copper intoxicated group indicates liver damage leading to cellular leakage. In our previous study, we have reported marked increase (15.15 % and 27.27%) in hepatic copper concentration in copper alone and in combination with flubendiamide groups ⁶⁴, this could be a reason of suppressing hepatic development. Besides, our results also demonstrated degenerative changes in hepatocytes, vacuolations in hepatic parenchyma, coagulative necrosis, ruptured hepatic cord, and congestion of central vein in the rats exposed to copper (Fig. 6b and 6d). Thus, the liver toxicity is clearly evident with increased serum bilirubin and transaminases ^{65,67}. Simultaneously elevated BUN, creatinine and uric acid suggested kidney dysfunction and is substantiated by degenerative changes, desquamation of renal tubular epithelium, proximal-tubule necrosis and swelling

of tubular epithelium in present study after histopathological examination confirms nephrotoxic potential of copper and flubendiamide. The above manifestations induced by copper were consistent with the oral ingestion of copper sulphate^{72,73}, copper nano-particles^{74,66,75} and slight increase in levels of serum and urinary creatinine^{77,67}. Increased levels of serum albumin and decrease in globulin is an indicator of dehydration¹⁷ and liver/kidney damage with decreased synthesis, nephrotic syndrome where there is renal protein loss, respectively in copper and/or flubendiamide groups. On the other hand, toxicity data of flubendiamide is scanty however reduced serum albumin and significant increase in total and direct bilirubin suggested marked liver damage and loss of function leading to hepatic insufficiency in contrast to the previous studies^{5,13,16} who have reported mild toxic effects on liver, thyroid gland, haemopoietic system and testes.

Long term exposure of copper, their nano-particles and copper salt have been reported to produce hepatotoxicity and nephrotoxicity in rats and mice^{69,77,73,78,65} by inducing oxidative stress which is directly linked to apoptosis and genotoxicity⁷¹. Numerous mechanisms have been proposed to explain cellular toxicity of copper exposure, however most accepted one is stimulation of ROS production which results in oxidative stress⁷⁹. Hosseini et al.⁸⁰ explained that excess of deposited copper increases lipid peroxidation where excess deposition of copper in liver has already been reported in our previous study⁶⁴. Oxidative stress is an important mechanism of toxicity for number of heavy metals and insecticide toxicity. Copper is a redox heavy metal and its redox identity contributes potential toxicity^{81,77} which induces oxidative stress by increased production of reactive oxygen species (ROS) and depletion of cellular antioxidant capacity affecting cellular integrity by causing peroxidative degeneration of polyunsaturated fatty acids in membrane lipids when generation of ROS exceeds antioxidant defence mechanisms^{82,71} and produces histopathological abnormalities in different tissues due to ROS generation and DNA damage⁸³. The results of this study showed increased ROS levels and decreased GSH content and reduction in the activities of SOD and GST while increase in GPx activity. Present findings are in line with the previous toxicity studies of copper and copper nanoparticles^{84,85,67,77,72} and flubendiamide toxicity studies⁸⁶⁻⁸⁸ in liver and kidneys.

Orally administered copper gets absorbed through intestine and reaches liver which is suggested to be the primary storage site, where transition metal copper undergoes redox cycling and promotes ROS production and in presence of GSH and superoxides, Cu^{++} gets converted to Cu^+ which further leads to formation of hydrogen peroxides and hydroxyl radicals^{89,90}. Further, Cu interacts with GSH and forms $\text{Cu(I)-[GSH]}(2)$ complex and gives superoxide and by action of SOD superoxide gets converted to H_2O_2 ^{91,90,92}. Reduced glutathione is very important for survival of cells as it provides protection against free radicals-induced cellular damage. Following exposure of rats to copper or flubendiamide in the present study, significant decrease in GSH levels in liver and kidneys of rats indicates its increased utilization in conjugation with xenobiotics and it is facilitated by glutathione-S-transferase. Thus, there may be detoxification of the xenobiotics which are converted to less reactive chemical moieties or it prevents interaction of xenobiotics with cellular proteins and nucleic acids^{93,94}. GSH also binds with Cu^+ and physiologically regulates intracellular copper levels⁹⁵ and protects against cellular damage induced by GSH-copper complexation and Cu^{++} reduction. Significant decrease in glutathione-S-transferase (GST) activity in liver and kidneys may be

responsible for comparatively better detoxifying ability of this organ and thus comparatively lesser damage to liver compared to kidneys where moderate decrease in GST activity and marked decrease in GSH level was observed in rats of the flubendiamide-treatment group. Our findings are in agreement with the observations of other researchers who have also reported reduction in GST activity following exposure of mice and/or rats to cypermethrin, dimethoate, deltamethrin and fipronil ⁹⁶⁻⁹⁸. This might be the one possible reason for the reduced GSH level of copper-exposed animals. Further, copper exposure significantly raised catalase and GPx activities suggest the activation of defensive mechanisms to protect against copper-associated free radical generation. During oxidative stress conditions increase in SOD, GST, CAT and GPx activities are well documented, however overall comparison of the alterations in the values of different oxidative stress biomarkers observed in liver and kidneys of the rats of groups III, IV and V revealed that kidney is comparatively more vulnerable to oxidative stress-induced insult than liver, may be due to better regenerative and reparative ability of liver⁹⁹. Also, increased GPx activity in liver and kidneys following exposure to copper and flubendiamide is in agreement with the toxicity studies of pesticides and Cu, which it may be due to increased formation of hydroperoxides in kidneys¹⁰⁰ and increase in GPx and catalase activities in liver and/or kidneys of the rats of flubendiamide or copper exposed groups in the present study might be due to adaptive response of the body against the generated free radicals and increase levels of iron in liver of flubendiamide alone and in combination with copper⁶⁴. However, reduced SOD activity in kidney and liver tissues in present study could be due to excessive accumulation of Cu in the liver⁷⁷.

Drug metabolizing enzymes of phase-I and II reactions have been reported to protect liver from dysfunctions caused by xenobiotics. Significant alterations in the activity of different DMEs in the rats exposed to copper and flubendiamide alone and/or in combination groups in the present study revealed decrease in CYPs (CYP₄₅₀ and CYP b₅) activities and impaired function of NADPH-P₄₅₀ reductase in rats exposed to copper could be due to oxidative stress induced generation of reactive oxygen species which inhibit synthesis of heme-prosthetic group^{101,102,103,104} Kim et al. ¹⁰⁵ reported the inhibitory effect of Cu²⁺ on the P₄₅₀-catalyzed reactions due to inability of an efficient electron transfer from NPR to P₄₅₀ and conformational changes in the P₄₅₀. However, inhibition of CYP systems can be effective in protecting liver against xenobiotic toxicity¹⁰⁶.

On the other hand, flubendiamide promotes binding to the lipophilic site of cytochrome P₄₅₀ and induces significant induction in activity of the cytochrome-P₄₅₀ and cytochrome b₅ is in agreement with the findings of Motoba ¹⁰⁷. The increased cytochrome-P₄₅₀ activity might be due to increased *de novo* synthesis of proteins could be due to increased gene transcription and further decrease in rate of degradation of enzymes¹⁰⁸. Simultaneous treatment with α-tocopherol, activities of cytochrome-P₄₅₀ and cytochrome b₅ were almost comparable to control groups suggesting the reparative potential of α-tocopherol. Thus, inhibition of metabolism by α-tocopherol is due to xenobiotics-antioxidant interactions and occurs by two basic mechanisms 1) competition of copper for access to the catalytic site of the relevant drug-metabolizing enzyme and 2) formation of a catalytically inactive, covalently bound complex between a metabolite of the substrate xenobiotics and the P₄₅₀ enzyme¹⁰⁹, because compounds are metabolized by a

variety of different enzymic reactions, and the simultaneous ingestion of two or more compounds may lead to their competition for various enzymic pathways, leading to decreased rates of metabolic transformation.

Aniline-P-hydroxylase (APH) and aminopyre-N-demethylase (ANDM) activities did not alter significantly in any of the treatment groups except in copper-intoxicated group where insignificant decrease in APH activity was observed compared to those in rats of control group, shows that detoxification of copper and flubendiamide through ANDM may not be of considerable biological significance. Aniline-P hydroxylase (APH) activity is an indicator of CYP 2E1 level, while ANDM indicates CYP 1A, 2A 2B, 2D, and 3A activity¹¹⁰. Reduction/inhibition of APH activity might be due to negative regulation of expression of different CYPs as reported by Vakharia et al.¹¹¹ and Noreault et al.¹¹² or non-competitive inhibition of aniline-P hydroxylase system by Cu^{+2} and Fe^{+3} . Several authors have also reported inhibition of APH activity by CuSO_4 , copper and zinc^{113,114}.

Glutathione-S-Transferase and UDP-glucuronosyltransferase (UGT) are generally responsible for the detoxification of xenobiotics. Toxic effects of a given xenobiotic are the net result of a balance between activating and detoxifying pathways and factors modulating the overall biotransformation capacity are of paramount importance. Glutathione-S-transferases (GST) and UDP-glucuronosyltransferase (UGT) activities were found to be significantly decreased in copper alone group and concurrent treatment with α -tocopherol did not significantly improve the GST and UGT activities are in agreement with the findings of Nakahama et al.¹¹⁵ who reported heavy metal-induced inhibition of UGT. UGT prevents the accumulation of potentially toxic compounds and/or their subsequent bioactivation to more toxic intermediates. The suppression in GST and UGT activities in copper-treated group is due to increased lipid peroxidation, generation of super oxide by copper (Fenton and/or Haber–Weiss reactions) which disorganizes the membrane lipid bilayer and modify activation state of UGT. However, in contrast to present findings, Rajpoot et al.¹¹⁶ observed an increase in UGT activity in rats following exposure to xenobiotics and ascorbic acid.

Simultaneous exposure of rats to copper and flubendiamide, the toxic effects were not found to be significantly augmented in the present study; therefore, our observation is contrary to the findings of other researchers who have reported augmented toxic effects of agrochemicals and metals on concurrent exposure¹¹⁶⁻¹¹⁸. Co-exposure of copper and flubendiamide did not have any strong impact on the activities of CYP₄₅₀, ANDM, APH, GST and UGT and further microsomal or cytosolic protein contents in rat liver due to antagonistic effect observed in interactive index study analysis suggesting that the effect produced by flubendiamide was not appreciably affected by copper and vice-versa, and flubendiamide and copper might not have reciprocally and significantly affected the metabolism of each other by altering their activation and/or their detoxication mechanism. Similar results have also been reported following combined exposure to arsenite + parathion¹¹⁹ and malathion+arsenite¹²⁰. However, combined effect of copper and flubendiamide on kidneys tissue oxidative stress biomarker (LPO, GSH, SOD, GST and GPx) was observed potentiating in interactive index analysis while catalase and total protein was shown was additive and antagonistic, respectively, suggest kidneys was more vulnerable to toxic insult than liver following copper and flubendiamide in combination. The results of present study indicated moderate to marked and/or significant alterations in biochemical parameters, oxidative stress biomarkers and histology in liver and

kidneys and drug metabolizing enzymes activity in liver of the rats following exposure to the test xenobiotics suggest that the test xenobiotics induces oxidative stress which is ultimately responsible for hepatic- and renal-insults as evident from the correlation and association data between the oxidative stress markers and liver and kidney function test markers. However, hepatic degenerative changes observed in rats of flubendiamide-treated group were similar to those reported in JMPR following 13 weeks exposure of rats to flubendiamide⁶. Although flubendiamide is a category III compound having low order of toxicity⁵ but hepatotoxic and nephrotoxic effects of flubendiamide, as observed in the present study, seem to be of great concern, therefore, further studies are warranted.

Thymus is a primary while spleen is the secondary lymphatic organ and both play a vital role in building the immune system. T-lymphocytes from thymus mature into functional defence cells like T effector cells, T-killer cells, T helper cells etc. and are responsible for coordinating the innate and the adaptive immune system. In view of the different nature and functions of the thymocytes and splenocytes of thymus and spleen, respectively are likely to have differential susceptibility to toxic effects of xenobiotics. In our previous study, we have reported flubendiamide and copper induced-cytogenotoxic effects in splenocytes¹⁸. Copper-induced activation of both the intrinsic and extrinsic apoptotic pathways and mitochondrial ROS generation in redox cycling are responsible for cytotoxic and DNA strand breaking effects^{121,122}. Similar oxidative stress induced DNA damage have been reported following exposure to pesticides, metals and environmental pollutants^{123,124,125}. Therefore, in this study, we evaluated the vulnerability of thymocytes to cyto-genotoxic effects of both these xenobiotics using TUNEL assay, DNA fragmentation assay and micronuclei induction assay with an endeavour to establish if there is any difference in cyto-genotoxic effects on thymocytes and splenocytes.

TUNEL assay is a gold-standard for cellular self-destruction by apoptosis and significant increase in number of TUNEL₊ve thymocytes following exposure to flubendiamide and copper evidently suggest the involvement of apoptotic signaling cascades in cytotoxic effects. Flubendiamide and copper possibly interact with double-stranded DNA (dsDNA) and induce cellular damage in thymocytic DNA and TdT binds with 3'OH label blunt ends of dsDNA and serve as a marker of apoptosis. Lu et al.¹²⁶ and Tufan et al.¹²⁷ have also reported significant increase in number of TUNEL₊ve fragmented DNA in brain and hippocampus of copper-treated mice and rats respectively. Similar increase in TUNEL₊ve cells has been recently reported in splenocytes as well following exposure to flubendiamide and copper¹⁸.

DNA fragmentation assay in the present study also revealed that the pattern of DNA shearing in copper and flubendiamide-treated thymocytes was similar to that observed in dexamethasone-treated group. DNA damaging effect of flubendiamide and copper in thymocytes in the present study is also substantiated by Comet assay data wherein number of comets formed in thymocytes treated with flubendiamide (23.52%) and copper (28.57 %) were almost similar to those observed in the positive control group (29.57%) treated with dexamethasone in the present study and also reported earlier in our study with splenocytes¹⁸. Thus suggesting that thymocytes and splenocytes are almost equally susceptible to flubendiamide and copper induced genotoxic effects.

Dexamethasone induces apoptosis in thymocytes by causing mitochondrial dysfunction, caspase-3 activation, reactive oxygen species (ROS) production and oxidative damage¹²⁸ whereas ROS generated interact with cellular DNA and produced comet formation inducing single- and double-strand breaks of DNA. Similar increase in DNA fragmentation following exposure to copper has been reported by Vidyashankar and Patki¹²⁹ in HepG2 cells. In the present study, oxidative stress in liver and kidneys could be the reason behind DNA damage and comet formation in thymocytes. Similar results of DNA damage and comet formation has been reported in our previous study conducted on spleen cells¹⁸.

Micronuclei induction assay detects clastogens and aneugens as well as mitotic delay, apoptosis, chromosome breakage and chromosome loss^{130,131} and is used for evaluation of the genotoxic potential of xenobiotics¹³². Compared to 1.1% micronuclei formed in control group and 4.63% in dexamethasone-exposed group, significant increase in number of the micronuclei formation in thymocytes of copper (17.1%) and flubendiamide (3.63%) groups suggest genotoxic potential of copper and flubendiamide, however thymocytes have been found to be more prone to cyto-genotoxic effects. Cyto-genotoxic potential of copper have been reported in mice¹³³ and other tissues¹³⁴ and rat splenocytes¹⁸. However, comparison of the micronuclei data of thymocytes (present study) and of splenocytes reported recently from our laboratory¹⁸ revealed that splenocytes were more susceptible to micronuclei formation following exposure to flubendiamide while thymocytes were more susceptible to copper-induced micronuclei formation. Thus the findings on cell viability, micronuclei and comets formation data obviously suggest differential susceptibilities of different cells to cyto-genotoxic effects of different xenobiotics.

Precise mechanism of cyto-genotoxic effects of flubendiamide is still not clear but possibly the cytotoxic effects on thymocytes due to ROS-induced oxidative stress and apoptosis due to activation of caspases, certain kinases and other cell signaling molecules¹³⁵. Few reports have suggested that several metals and insecticides induces ROS generation which leads to increased cytosolic free Ca²⁺ concentration and further impairs Ca²⁺ clearance system, as a result mitochondrial depolarization and ultimately cell dysfunctions and apoptosis^{136,137}. Our observations on thymocytes in the present study are in agreement with flubendiamide-induced oxidative stress in liver and kidneys of rats and also on acute exposure in *Daphnia magna*¹³⁸.

Plants-based polyphenols (resveratrol, curcumin, and catechin) and α -tocopherol have been reported to possess immunomodulatory^{139,140} and strong antioxidant activities¹⁴¹, promotion, progression and cellular proliferation^{142,143}, and cytotoxicity induced by metals and insecticides^{144,145,118,146}. Concurrent treatment with polyphenols (resveratrol, curcumin, and catechin) or α -tocopherol in present study showed decrease in TUNEL₊ve cells, per cent micronuclei and comet formation in thymocytes evidently suggest the ameliorative potential against flubendiamide and copper-induced toxic effects. Observations are in agreement with our earlier report against splenocytes¹⁸. However, all the tested antioxidants failed except curcumin in preventing thymocytic DNA shearing exposed to xenobiotics.

Cyto- and geno-protective potential of resveratrol and curcumin against flubendiamide and/or copper toxicity in thymocytes reduced the TUNEL₊ve cells, comet formation, micronuclei-induction and reduction in DNA

fragmentation might be due to its unique structure which enables the lipids to conjugate with 3' position of curcumin¹⁴⁷, radical trapping ability as a chain-breaking antioxidant, inhibition of lipid peroxidation¹⁴⁸. Antioxidant action of resveratrol is due to scavenging of hydroxyl, superoxide and other¹⁴⁹⁻¹⁵¹ and prevents lipid peroxidation in cell membranes and DNA lesions and fragmentation¹⁵¹⁻¹⁵³. Thus resveratrol and curcumin can reduce the amount of reactive oxygen species (ROS) in response to oxidative stress¹⁵⁴. Similar, cyto-protective and anti-apoptotic effects of curcumin against other xenobiotics like copper, arsenic, fluoride induced DNA damage have also been reported^{155-158,118}. Although in the present study, we have not researched the precise pathway of cyto- and geno-protective effect of any of the natural phytoconstituents, but possibility of modulation of different above mentioned mechanisms/pathways involved in cyto- and geno-protective effect of resveratrol cannot be ruled out.

Besides these, catechin has ability to chelate copper (II) and decreases expression of proapoptotic genes (Bax, Bad, Mdm2), induces anti-apoptotic genes (Bcl-2, Bcl-w, Bcl-x_L)¹⁵⁹ and free radical scavenging activity by forming stable semiquinone free radicals which prevent the deaminating ability of free radicals. Possible involvement of similar mechanism(s) in protective effect of catechin against flubendiamide and copper-induced cyto-genotoxicity cannot be ruled out as both these xenobiotics are known to cause oxidative stress; therefore, further studies on the mechanistic pathways of geno-protective effect of catechin against these xenobiotics are required¹⁶⁰.

α -tocopherol (α -TOH) is known to protect cellular membranes and lipoproteins from peroxidation and results in formation of an unreactive α -tocopheroxyl radical¹⁶¹ that can be recycled back to active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol¹⁶². Lipophilic nature of α -tocopherol facilitates its entry through membrane and thereby quenches the free radical species, terminates lipid peroxidation chain reaction and interferes with initiation and progression of xenobiotics-induced oxidative damage¹⁶³ and thus prevents oxidative DNA damage¹⁶⁴ and decreases caspase 3 activation¹⁶⁵. Protective effect of α -tocopherol against DNA damage in thymocytes is in the agreement with the findings of Blasiak and Sankowaska¹⁶⁶ and Sharma and Sharma¹⁶⁷ however, they have reported against the toxicity of malaoxon and carbofuran in human lymphocytes, respectively. Protective effect of α -tocopherol against copper-induced cytotoxicity has also been reported by Pourahmad and O'Brien¹⁶⁸ and Mattie and Freedman¹⁶⁹ and antiapoptotic/anti-genotoxic action of α -tocopherol has been reported in rat bone marrow-endothelial progenitor cells¹⁷⁰ and splenocytes¹⁸.

These results along with histological examination and drug metabolizing enzymes activity evidently suggest the ameliorative potential of α -tocopherol against hepato-and nephrotoxicity especially induced by copper, however, it was not found to be so effective against flubendiamide-induced tissue-insults. Our finding on protective and reparative activity of α -tocopherol corroborates well with the observations of several other researchers against certain pesticides^{171,172,16,18} and copper^{21,173,16,18} due to its chain breaking antioxidant activity and free radicals scavenging activity.

Conclusions

Results of the present study concluded that blood biochemical parameters, hepatic and renal oxidative stress biomarkers, drug metabolizing enzyme activities and histological lesions in liver and kidneys of the rats of different intoxicated groups revealed hepato- and nephrotoxic potential of flubendiamide and copper where kidney was found to be more sensitive than liver for toxic insults induced by test xenobiotics, however simultaneous administration of α -tocopherol provided very good ameliorative potential against copper while only marginal protective effect against flubendiamide. Flubendiamide and copper have the potential to induce apoptosis and genotoxicity in thymocytes, also. Polyphenols (resveratrol, curcumin and catechin) and α -tocopherol possess cyto- and geno-protective potential as these significantly decreased the percentage of nonviable cells, TUNEL₊ve cells, and number of comets and micronuclei formed in thymocytes. Overall cyto-geno-protective potential of different natural antioxidants at equimolar concentration (10 μ M) against flubendiamide and copper was found to be curcumin > resveratrol > catechin = α -tocopherol. Based on the present findings, it is evident that natural polyphenols and α -tocopherol hold promising potential in preventing cyto-genotoxic effects of xenobiotics. Therefore, their inclusion in functional foods or their use as nutraceutical seems to hold promising potential in preventing the deleterious effects of environmental pollutants including flubendiamide and copper.

References

1. Central Water Commission, Ministry of Water Resources, River Development and Ganga Rejuvenation, Government of India (CWC, GOI). Status of trace and toxic metals in Indian rivers. River Data Compilation -2 Directorate Planning and Development Organisation New Delhi 110066. (2018).
2. Ali, H., Khan, E. & Ilahi, I. Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. *Journal of Chemistry*. 1-14. <https://doi.org/10.1155/2019/6730305> (2019).
3. Ebbinghaus-Kintscher, U., Lümmlen, P., Lobitz, N., Schulte, T., Funke, C., Fischer, R., Masaki, T., Yasokawa, N. & Tohnishi, M. Phthalic acid diamides activate ryanodine-sensitive Ca^{2+} release channels in insects. *Cell Calcium*. **39**, 21-33 (2006).
4. Das, S.K., Mukherjee, I. & Roy, A. Flubendiamide as new generation insecticide in plant toxicology: A Policy Paper. *Adv Clin Toxicol*. **2**, 000122 (2017).
5. Joint Meeting on Pesticide Residues (JMPR). Report of the joint meeting of the FAO panel of experts on pesticide residues in food and the environment and the WHO core assessment group on pesticide residues at the request of codex committee on pesticide residues Italy.345-382 (2010).
6. Government of India (GOI), Ministry of Agriculture, Department of Agriculture & Cooperation. Directorate of Plant Protection, Quarantine & Storage, Central Insecticide Board & Registration Committee, N.H. IV, Faridabad-121 001, Registered under the Insecticides Act, 1968 (2009). Accessed on January 1 2015. <http://www.cibrc.nic.in/mupi.pdf>

7. Mohapatra, S., Ahuja, A.K., Deepa, M., Jagadish, G.K., Rashmi, N. & Sharma, D. Development of an analytic method for analysis of flubendiamide, des-iodoflubendiamide and study of their residue persistence in tomato and soil. *Journal of Environmental Science and Health, Part B*. Pesticides, Food Contaminants, and Agricultural Wastes. **46**, 246-271(2011).
8. Das, S.K., Mukherjee, I. & Das, S.K. Dissipation of flubendiamide in/on Okra [Abelmoschus esculenta (L.) Moench] Fruits. *Bull Environ Contam Toxicol*. **88**(3), 381-384 (2012). <https://doi.org/10.1007/s00128-011-0491-9>.
9. European Food Safety Authority. Modification of the existing MRL for flubendiamide in strawberries. *EFSA Journal*. **11**(10), 3403, 27 (2013). doi:10.2903/j.efsa.2013.3403.
10. Buddidathi, R., Mohapatra, S., Siddamalliah, L., Manikrao, G. & Hebbar S.S.. Dissipation pattern of flubendiamide residues on capsicum fruit (Capsicum annum L.) under field and controlled environmental conditions. *Journal of Environmental Science and Health, Part B*. Pesticides, Food Contaminants, and Agricultural Wastes. **51**: 44-51 (2016).
11. United State of Environmental Protection Agency (USEPA). Flubendiamide-Notice of Intent to Cancel and other supporting document. (2016). Available online. <https://www.epa.gov/ingredients-used-pesticide-products/flubendiamide-notice-intent-cancel-and-other-supporting>. (Accessed on 09Jan 2020) 11. <https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1132.htm>; <https://www.cropscience.bayer.in/en/Products-H/Brands/Crop-Protection/Insecticide-Fame.aspx>
12. Das, S.K. & Mukherjee, I. Influence of microbial community on degradation of flubendiamide in two Indian soils. *Environ Monit Assess*. **186**, 3213-3219 (2014).
13. Ranjan, A., Dumka, V.K. & Singh, N.D. Effect of flubendiamide, lead and their combined exposure on erythrocytic indices in Water Buffalo calves. *Bull Environ Contam Toxicol*. **92**, 410–414 (2014).
14. Miyahana, K. Micronucleus test of NNI-0001 in mice. Unpublished report No. LSRC-T02-089A from Nihon Nohyaku Co., Ltd, Osaka, Japan. Submitted to WHO by Bayer CropScience AG, Monheim, Germany. (2003).
15. Herbold, B. NNI-0001, micronucleus-test on the male mouse. Unpublished report No. AT01775 from Bayer Health Care, Wuppertal, Germany. Submitted to WHO by Bayer CropScience AG, Monheim, Germany. (2005).
16. Mandil, R., Rahal, A., Prakash, A., Garg, S.K., Gangwar, N.K. & Swain, D.K. Ameliorative potential of α -tocopherol against flubendiamide and copper-induced testicular-insult in Wistar rats. *Chemico-Biological Interactions*. **260**, 91-101(2016).
17. Mandil, R., Rahal, A., Koli, S., Prakash, A., Kumar, R. & Garg, S.K. Protective role of α -tocopherol against flubendiamide and copper-induced chronic toxicity in Wistar rats. *Journal of Veterinary Pharmacology and Toxicology*. **16**(2), 18-27 (2017).

18. Mandil, R., Prakash, A., Rahal, A., Singh, S.P., Sharma, D., Kumar, R. & Garg, S.K. In vitro and in vivo effects of flubendiamide and copper on cytogenotoxicity, oxidative stress and spleen histology of rats and its modulation by resveratrol, catechin, curcumin and α -tocopherol. *BMC Pharmacology and Toxicology*. **21(29)**, 1-17 (2020). <https://doi.org/10.1186/s40360-020-00405-6>
19. El-Demerdash, F.M., Yousef, M.I., Kedwany, F.S. & Baghdadi, H.H. Role of alpha-tocopherol and beta-carotene in ameliorating the fenvalerate induced changes in oxidative stress, hemato-biochemical parameters, and semen quality of male rats. *J Environ Sci Health B*. **39**, 443-459 (2004).
20. Boone, C., Gervais, J.A., Luukinen, B., Buhl, K. & Stone, D. Copper Sulfate Technical Fact Sheet; National Pesticide Information Center, Oregon State University Extension Services. (2012). <http://npic.orst.edu/factsheets/cuso4tech.pdf>.
21. Gaetke, L.M. & Chow, C.K. Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology*. **189**, 147-163 (2003).
22. Central Water Commission, Ministry of Jal Shakti Dept. of Water Resources, River Development and Ganga Rejuvenation. Status of trace and toxic metals in Indian rivers. (2019). <http://cwc.gov.in/sites/default/files/status-trace-toxic-metals-indian-rivers-2019-2.pdf>
23. Tang, H., Xu, M., Luo, J., Zhao, L., Ye, G., Shi, F., Lv, C., Chen, H., Wang, Y. & Li, Y. Liver toxicity assessments in rats following sub-chronic oral exposure to copper nanoparticles. *Environ Sci Eur*. **31**, 30 (2019). <https://doi.org/10.1186/s12302-019-0214-0>
24. Kumar, J., Sathua, K.B. & Flora, S.J.S. Chronic copper exposure elicit neurotoxic responses in rat brain: Assessment of 8-hydroxy-2-deoxyguanosine activity, oxidative stress and neurobehavioral parameters. *Cellular and Molecular Biology* (Noisy-le-Grand, France). **65**, 27-35 (2019).
25. Pal, A. Copper toxicity induced hepatocerebral and neurodegenerative diseases: An urgent need for prognostic biomarkers. *NeuroToxicology*. **40**, 97-101 (2014).
26. Wu, X., Leegwater, P.A.J. & Fieten, H. Canine models for copper homeostasis disorders. *Int J Mol Sci*. **17**, 196 (2016).
27. Hans, Z. & Borchard, S. Mitochondrial copper toxicity with a focus on wilson disease. *Clinical and Translational Perspectives on WILSON DISEASE*. 65-75 (2019).
28. Prá, D., Franke, S.I., Giulian, R., Yoneama, M.L., Dias, J.F., Erdtmann, B. & Henriques, J.A. Genotoxicity and mutagenicity of iron and copper in mice. *Biometals*. **21(3)**, 289-97 (2008) .
29. Claudia, A.G., Miguel, A.R. & Mónica, A.F. LdeM. Does over-exposure to copper ions released from metallic copper induce cytotoxic and genotoxic effects on mammalian cells?. *Contraception*. **81 (4)**, 343-349 (2010).

30. Vernon, E.L. & Jha, A.N. Assessing relative sensitivity of marine and freshwater bivalves following exposure to copper: Application of classical and novel genotoxicological biomarkers. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. **842**, 60-71 (2019).
31. Shen, H.M., Lin, Y., Choksi, S., Tran, J., Chang, L., Jin, T., Karin, M., Zhang, J. & Liu, Z.G. Essential Roles of Receptor-Interacting Protein and TRAF2 in Oxidative Stress-Induced Cell Death. *Mol. Cell. Biol.* **24**, 5914-5922 (2004).
32. Ko, C.H., Shen, S.C., Lee, T.J.F. & Chen, Y.C. Myrecetin inhibit matrix metalloproteinase 2 protein expression and enzyme activity in colorectal carcinoma cells. *Mol. Cancer Ther.* **4**, 281-290 (2005).
33. Lin, H.Y.L., Lansing, F.B., Merilon, J.M., Davis, F.B., Tang, H.Y., Shis, A., Vitrac, X., Krisa, S., Keating, T., Cao, H.J., Bergh, J., Quackenbush, S. & Davis, P.J. Integrin $\alpha\beta 3$ contains a receptor site for resveratrol. *FASEB J.* **20**, 1742-1744 (2006).
34. Tadolini, B., Juliano, C., Piu, L., Franconi, F. & Cabrini, L. Resveratrol inhibition of lipid peroxidation. *Free Radic Res.* **33(1)**, 105-114 (2000).
35. Yavuz, T., Delibas, N., Yildirim, B., Altuntas, I., Candir, O., Cora, A., Karaman, N., Ibrisim, E. & Kutsal A. Vascular wall damage in rats induced by methidathion and ameliorating effect of vitamins E and C. *Arch. Toxicol.* **78**, 655-659 (2004).
36. Ak, T. & Gülçin, I. Antioxidant and radical scavenging properties of curcumin. *Chem Biol. Interact.* **174**, 27-37 (2008).
37. Mukherjee, S., Dudley, J.I., Das, S.K. 2010. Dose-dependency of resveratrol in providing health benefits. *Dose Response*. **8(4)**, 478-500.
38. Fraga, C.G., Galleano, M., Verstraeten, S.V. & Oteiza, P.I. Basic biochemical mechanism behind the health benefits of polyphenols. *Mol Aspects Med.* **31**, 435-445 (2013).
39. Rodriguez-Ramiro, I., Martin, M.A., Ramos, S., Bravo, L. & Goya, L. Comparative effect of dietary flavonols on antioxidant defences and their response to oxidant-induced stress on Caco2 cells. *Eur J Nutr.* **50**, 313-322 (2011).
40. Chen, S.Q., Hu, T., Han, Y., Huang, W., Yuan, H.B., Zhang, Y.T., DU, Y. & Jiang, Y.W. Preventive effects of catechins on cardiovascular disease. *Molecules*. **21**, 1759 (2016).
41. OECD. Considerations for Assessing the Risks of Combined Exposure to Multiple Chemicals, Series on Testing and Assessment No. 296, *Environment, Health and Safety Division, Environment Directorate* (2018)
42. WHO. Assessment of combined exposures to multiple chemicals: report of a WHO/IPCS international workshop on aggregate/cumulative risk assessment. (2009).

43. Tabrizian. Nutritional Medicine: fact & fiction West Aust., HyperhealthCD-Rom. Trace Elements & Other Essential Nutrients Mertz 1986 NY, Websites-Dr P Eck. Dr L Wilson. Interclinical Hair Analysis. (2002).
44. Sorg, D.A. & Buckner, B. A simple method for obtaining venous blood from small laboratory animals. *Proceeding of the society for Experimental Biology and Medicine*. **115**, 1131-1132 (1964).
45. Shafiq-U- Rehman. Lead-induced regional lipid peroxidation in brain. *Toxicol.* (1984).
46. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J. Protein measurement with the Folin phenol reagent. *J Biol Chem*. **193**, 265-275 (1951).
47. Sedlak, J. & Lindsay. R.H. Estimation of total protein bound NPSH groups in tissues with Ellaman's reagent. *Anal Biochem*. **25**, 192-205 (1968).
48. Bergmeyer, H.U. U.V. method of catalase assay, in: Methods of Enzymatic Analysis, third ed vol. III, Dacrfeld Beach, Florida, Basal, Weinheim. 273 (1983).
49. Madesh, M. & Balasubramanian, K.A. Microtiter plate assay for superoxide dismutase using MTT reduction by superoxide. *Indian J biochem Biophys*. **35**, 184-188 (1998).
50. Habig, W.H., Pabst, M.J. & Jacokoby, W.B. Gluuathione-S-transferase: the first enzymatic step in mercapturic acid formation. *J Biol Chem*. **249**, 7130-7139 (1974).
51. Paglia, D.E. & Valantine, W.N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med*. **70**, 158-169 (1967).
52. Omura, T. & Sato, R. The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem*. **239**, 2370-2378 (1964).
53. La Du, B.N., Mandel, H.G. & Way, E.L. The Williams & Wilikins Comparny, Baltimore, Fundamentals of drug metabolism and drug disposition. Eds. 22-43 (1971).
54. Dutton, G.J. & Storey, I.E. Glucuronide test forming enzyme. In: Colowick SP, Kaplan NO (ed.) Methods in enzymology. Academic, New York. **5**, 159-164 (1962).
55. Luna, L.G. Manual of histology staining methods of the Armed Forces Institute of Pathology. 3rd. edn. Mcgraw-Hill Book Co. New York. (1968).
56. Mansour, S.A. & Refaie, A.A. Xenobiotics interaction. 2. An approach to the use of biochemical data measurements interpreting interaction of insecticide the mixtures in rat. *Ad. Pharmacol. Toxicol*. **1 (1)**, 1-20 (2000).
57. Boyse, E.A., Miyazawa, M., Aoki, T. & Old, L.J. Ly-A and Ly-B: two systems of lymphocyte antigens in the mouse. *Proc.roy. Soc. B*. **170**, 175 (1968).

58. Hayashi, M., Sofuni, T. & Ishidata, M.Jr. An application of acridine orange fluorescent staining to the micronucleus test. *Mutat. Res.* **120**, 241-247 (1983).
59. Sambrook, J. & Russel, D.W. Molecular cloning: A laboratory manual. Cold/Spring Harbor Laboratory Press, New York. A1-A12 (2001).
60. Dhawan, A., Bajpayee, M., Pandey, A.K. & Parmar, D. Protocol for the single cell gel electrophoresis/comet assay for rapid genotoxicity assessment, Lucknow, ITRC, 1-10 (2009).
61. Kodama, H. & Fujisawa, C. Copper metabolism and inheritedcopper transport disorders: molecular mechanisms, screeningand treatment. *Metallomics.* **1**, 42-52 (2009).
62. Kabak, Y. B. & Gülbahar, M. Y. Determination of apoptosisin liver and kidney tissues in experimental copper toxicity in rats. *Ankara Universitesi Veteriner Fakultesi Dergisi.* **60(1)**, 39–45 (2012).
63. Mitra, S., Keswani, T., Dey, M. *et al.* Copper-induced immunotoxicity involves cell cycle arrest and cell death in the spleen and thymus. *Toxicology.* **293(1-3)**, 78–88 (2012).
64. Mandil, R., Prakash, A., Rahal, A., Koli, S. & Garg, S.K. Investigation of the influence of alpha-tocopherol on copper, iron, zinc and manganese levels in liver following chronic exposure of flubendiamide and copper to wistar rats. *Journal of Veterinary Pharmacology and Toxicology.* **17 (1)**, 57-62 (2018).
65. Liu, H., Guo, H. Z. J., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X. & Zhao, L. Copper Induces Oxidative Stress and Apoptosis in the Mouse Liver. *Oxidative Medicine and Cellular Longevity* (Hindawi). Article ID 1359164 (2020).
66. Manna, P., Ghosh, M., Ghosh, J., Das, J & Sil, P.C. Contribution of nano-copperparticles to in vivo liver dysfunction and cellular damage: role of IκBα/NF-κB, MAPKs and mitochondrial signal. *Nanotoxicology.* **6 (1)**, 1–21 (2012).
67. Kumar, V., Kalita, J., Misra, U.K. & Bora, H.K. A study of dose response and organ susceptibility of copper toxicity in a rat model. *J. Trace Elements in Med Biol.* **29**, 269-274 (2015).
68. Mohammadyari, A. T. R. S., Maryam, M. & Masoud, N. Exploring vivo toxicity assessment of copper oxide nanoparticle in Wistarrats. *J. Biol. Today's World.* **6**, 124–128 (2014).
69. Arafa, A. F., Ghanem, H. Z., Soliman, M. S. & EL-Meligy, E. Modulation effects of quercetin against copper oxidenanoparticles-induced liver toxicity in rats. *Egypt Pharmaceut J.* **16**:78–86 (2017).
70. El-Magd, M. A. Effect of naringenin and hesperidin in amelioration of copper oxide nanoparticles toxicity in rat liver. *AJMS.* **1(1)**, 14–18 (2018).

71. Abdelazeim, S.A., Shehata, N.I., Aly, H.F. *et al.* Amelioration of oxidative stress-mediated apoptosis in copper oxide nanoparticles-induced liver injury in rats by potent antioxidants. *Sci Rep.* **10**, 10812 (2020).
72. Babaknejad, N., Moshtaghi, A.A. & Shahanipour, K. The toxicity of copper on serum parameters related to renal functions in male Wistar rats. *Zahedan J Res Med Sci.* 29-31 (2015).
73. Ghara, A. R. & Ghadi, F. E. Effect of purslane on kidney failure following copper toxicity in a rat model. *Iranian Journal of Health Sciences.* **6(1)**, 25-32 (2018).
74. Lei, R.H., Wu, C.Q., Yang, B.H., Ma, H.Z., Shi, C., Wang, Q.J., Wang, Q.X., Yuan, Y. & Liao, M.Y. Integrated metabolomic analysis of the nano-sized copper particle-induced hepatotoxicity and nephrotoxicity in rats: A rapid in vivo screening method for nanotoxicity. *Toxicol Appl Pharm.* **232(2)**, 292–301 (2008).
75. Lee, I.C., Ko, J.W., Park, S.H. *et al.* Comparative toxicity and biodistribution assessments in rats following subchronic oral exposure to copper nanoparticles and microparticles. *Part Fibre Toxicol.* **13**, 56 (2016).
76. Galhardi, C.M., Diniz, Y.S., Fiana, L.A., Rodrigues, H.G., Burneiko, R.C.M., Ribas, B.O. & Novelli, E.L.B. Toxicity of copper intake: lipid profile, oxidative stress and susceptibility to renal dysfunction. *Food Chem Toxicol.* **142**, 2053-60 (2004).
77. Seven, P. T., Baykalir, B. G., Seven, I., Tuba, P. A. K, Neşe, B. & Hakan, G. The protective effects of chrysin and flunixin meglumine against excess copper in male rats. *Turk J Vet Anim Sci.* **42**, 376-387 (2018).
78. Hashimyousif, E., Obaid, H. M., Karim, A. .J, Hashim, M. S. & Naimi, R. A. A. Toxicological study of copper sulfate modulated by zinc oxide and coriandrum sativum plant treatment in mice. *Plant Archives.* **19 (1)**, 299-308 (2019).
79. Guo, H., Li, K., Wang, W. *et al.* Effects of Copper on Hemocyte Apoptosis, ROS Production, and Gene Expression in White Shrimp *Litopenaeus vannamei* . *Biol Trace Elem Res.* **179**, 318–326 (2017).
80. Hosseini, M.J., Shaki, F., Ghazi-Khansari, M. *et al.* Toxicity of copper on isolated liver mitochondria: impairment at complexes I, II, and IV leads to increased ROS production. *Cell Biochem Biophys.* **70**, 367–381 (2014).
81. Mladenović, J.M., Paunović, M.G., Matić, M.M., Knežević, V.S., Ognjanović, B.I., Štajn, A.Š. & Saičić, Z.S. Copper-induced changes of lipid peroxidation and hemato-biochemical parameters in rat blood: protective role of flavonoids. *Archives of Biological Science Belgrade.* **66**, 1271-1279 (2014).
82. Halliwell, B. Free radicals and antioxidants: updating a personal view. *Nutr. Rev.* **70**, 257–265 (2012).

83. Møller, P., Jacobsen, N.R., Folkmann, J.K., Danielsen, P.H., Mikkelsen, L., Hemmingsen, J.G. *et al.* Role of oxidative damage in toxicity of particulates. *Free Radic Res.* **44**, 1–46 (2010).
84. Cui, W., Peng, X., Deng, J. L. & Cui, H. M. Effects of dietary high copper on antioxidative function and observation of pathologic lesion in spleen of chick. *Chinese Veterinary Science.* **39**, 338–343 (2009).
85. Li, M., Cui, W., Peng, X., Bai, C. M. & Cui, H. M. Effect of dietary high copper on the antioxidase activities of brain tissue in chickens. *Acta Veterinaria et Zootechnica Sinica.* **41**, 220–223 (2010).
86. Cui, F., Chai, T., Qian, L. & Wang, C. Effects of three diamides (chlorantraniliprole, cyantraniliprole and flubendiamide) on life history, embryonic development and oxidative stress biomarkers of *Daphnia magna*. *Chemosphere.* **169**, 107–116 (2017).
87. Nareshkumar, B., Akbar, S. M., Sharma, H. C., Jayalakshmi, S. K., & Sreeramulu, K. Evaluation of flubendiamide-induced mitochondrial dysfunction and metabolic changes in *Helicoverpa armigera* (Hubner). *Archives of insect biochemistry and physiology*, **96**(1), e21401 (2017).
88. Sun, Q., Lin, J., Peng, Y., Gao, R. & Peng, Y. Flubendiamide Enhances Adipogenesis and Inhibits AMPK α in 3T3-L1 Adipocytes. *Molecules.* **23**(11), 2950 (2018).
89. Hosseini, M.J., Shaki, F., Ghazi-Khansari, M. *et al.* Toxicity of Copper on Isolated Liver Mitochondria: Impairment at Complexes I, II, and IV Leads to Increased ROS Production. *Cell Biochem Biophys.* **70**, 367–381 (2014).
90. Romo, A.I.B., Abreu, D.S., de, F., Paulo, T. *et al.* Hydroxyl radical generation and DNA nuclease activity: a mechanistic study based on a surface-immobilized copper thioether clip-phen derivative. *Chemistry.* **22**, 10081–10089 (2016).
91. Speisky, H., Go´mez, M., Burgos-Bravo, F. *et al.* Generation of superoxide radicals by copper-glutathione complexes: redox consequences associated with their interaction with reduced glutathione. *Bioorg Med Chem.* **17**, 1803–1810 (2009).
92. Quamar, S., Kumar, J., Mishra, A., & Flora, S. J. S. Oxidative stress and neurobehavioural changes in rats following copper exposure and their response to MiADMSA and D-penicillamine. *Toxicology Research and Application*, **3**, 2397847319844782 (2019).
93. Hayes, J.D. & Pulford, D. The glutathione-S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol.* **30**, 445–600 (1995).
94. Josephy, P.D. Genetic variations in human glutathione transferase enzymes: significance for pharmacology and toxicology. *Hum Genomics Proteomics.* 876940–876953 (2010).

95. Banci, L., Bertini, I., Ciofi-Baffoni, S., Kozyreva, T., Zovo, K., Palumaa, P. Affinity gradients drive copper to cellular destinations. *Nature*. **465**, 645–648 (2010).
96. Sharma, Y., Bashir, S., Irshad, M., Nag, T.C. & Dogra, T.D. Dimethoate-induced effects on antioxidant status of liver and brain of rats following subchronic exposure. *Toxicology*. **215**, 173-181 (2005).
97. Rehman, H., Ali, M., Atif, F., Kaur, M., Bhatia, K. & Raisuddin, S. The modulatory effect of deltamethrin on antioxidants in mice. *Clin Chim Acta*. **369**, 61-65 (2006).
98. Sankar, P., Telang, A.G. & Manimaran, A. Protective effect of curcumin on cypermethrin-induced oxidative stress in Wistar rats. *Experimental and Toxicologic Pathology*. **64**, 487-493(2012).
99. Tao, Y., Wang, M.; Chen, E. & Tang, H. Liver Regeneration: Analysis of the main relevant signaling molecules. *Mediators of Inflammation*. 9, 1-9 (2017). pages <https://doi.org/10.1155/2017/4256352>.
100. Frederick, N.B. & Panemangalare, M. Pesticides and Essential Minerals Modify Endogenous Antioxidants and Cytochrome P450 in Tissues of Rats. *J Envi Sci Health, Part B*. **40**, 769-784 (2005).
101. Pourahmad, J. & O'Brien, P.J. A comparison of hepatocytes cytotoxic mechanisms for Cu²⁺ and Cd²⁺. *Toxicology*. **143**, 263-273 (2000).
102. Kim, J.S., Ahn, T., Yim, S.K. & Yun, C.H. Differential effect of copper (II) on the cytochrome P450 enzymes and NADPH-cytochrome P450 reductase: inhibition of cytochrome P450-catalyzed reactions by copper (II) ion. *Biochemistry*. **41(30)**, 9438-9447 (2002). doi:10.1021/bi025908b
103. Parmar, P., Limson, J., Nyokong, T. & Daya, S. Melatonin protects against copper-mediated free radical damage. *J. Pineal Res*. **32**, 237-242 (2002).
104. Tang, H., Xu, M., Shi, F., Ye, G., Lv, C., Luo, J., Zhao, L. & Li, Y. Effects and Mechanism of Nano-Copper Exposure on Hepatic Cytochrome P450 Enzymes in Rats. *International Journal of Molecular Sciences*. **19(7)**, (2018). DOI: 10.3390/ijms19072140.
105. Kim, J.S., Ahn, T., Yim, S.K., Yun, C.H. Differential effect of copper (II) on the cytochrome P450 enzymes and NADPH-cytochrome P450 reductase: inhibition of cytochrome P450-catalyzed reactions by copper (II) ion. *Biochemistry*. **41(30)**, 9438-9447 (2002). doi:10.1021/bi025908b
106. Jorquera, E., Culebras, J.M. & Gonzalez, G. Influence of nutrition on liver oxidative metabolism. *J. Nutr*. **12**, 442-447 (1996).
107. Motoba, K. Absorption, distribution, metabolism and excretion of radiolabeled NNI-0001 following a single oral administration to male and female rats. Unpublished report No. LSRC-M04-115A from Nihon Nohyaku Co., Ltd, Osaka, Japan. Submitted to WHO by Bayer CropScience AG, Monheim, Germany. (2004).
108. King, J.L. & Parke, D.V. Induction, Inhibition and Receptor Interactions. *Methods for Assessing the Effects of Mixtures of Chemicals*. 423-446 (1987).

109. Zhou, S., Yung, C.S., Cher, G.B., Chan, E., Duan, W., Huang, M., & McLeod, H.L. Mechanism-based inhibition of cytochrome P450 3A4 by therapeutic drugs. *Clin. Pharmacokinet.* **44**, 279-304 (2005).
110. Flora, S.J.S., Bhadauria, S., Kannan, G.M. & Singh, N. Arsenic induced oxidative stress and the role of antioxidant supplementation during chelation: A review. *J. Environ.Biol.* **28**, 333-347 (2007).
111. Vakharia, D., Liu, N., Pause, R., Fasco, M., Bessette, E., Zhang, Q. & Kaminsky, L. Effect of metals on polycyclic aromatic hydrocarbon induction of CYP1A1 and CYP1A2 in human hepatocyte cultures. *Toxicol. Appl. Pharmacol.* **170**, 93-103 (2001).
112. Noreault, T.L., Kostrubsky, V.E., Wood, S.G., Nichols, R.C., Strom, S.C., Trask, H.W., Wrighton, S.A., Evans, R.M., Jacobs, J.M., Sinclair, P.R. & Sinclair, J.F. Arsenite decreases CYP3A4 and RXRalpha in primary human hepatocytes. *Drug Metab. Dispos.* **33**, 993-1003 (2005).
113. Kadiiska, M.B., Hanna, P.M., Jordan, S.J. & Mason, R.P. Electron spin resonance for free radical generation in copper-treated vitamin-E and selenium-deficient rats: *in vivo* spin-trapping investigation. *Mol. Pharmacology.* **44**, 222-227 (1993).
114. Moreno, J.J., Castellote, M.C., Queralt, J. 1989. Effects in vitro of copper and zinc on hepatic cytochrome P-450 activities. *Comp. Biochem. Physiol C.* 93, 355-7
115. Nakahama, T., Inouye, Y., Fukuhara, M. 2001. Comparative study on in vitro inhibitory effects of heavy metals on rabbit drug-metabolizing enzymes. *J. Health Sci.* 47, 14-20.
116. Rajpoot, D.S., Prakash, A., Rahal, A., Mandil, R. & Garg, S.K. Differential Modulation of Xenobiotic-Metabolizing Enzymes in Rats Following Single and Concurrent Exposure to Chlorpyrifos, Arsenic, and Ascorbic Acid. *Journal of Toxicology and Environmental Health Part A.* **76(24)**, 1354-1365 (2013). DOI: 10.1080/15287394.2013.853005.
117. Zoheb, S.M., Prakash, A., Rahal, A., Mandil, R., Gangwar, N.K. & Garg, S.K. Curcumin attenuates oxidative stress-induced altered histoarchitecture of testes in experimentally exposed rats to metal mixture (lead, arsenic, cadmium, mercury, iron, and copper) for 28 days. *Toxicological & Environmental Chemistry.* **96(4)**, 660-679 (2014). DOI: 10.1080/02772248.2014.975425.
118. Prakash, A., Khan, S., Kumar, D., Telang, A.G. & Malik, J.K. Concurrent administration of curcumin mitigates arsenic – and chlorpyrifos – induced apoptosis in rat thymocytes. *Adv Anim Vet Sci.* **7**, 407-413 (2014).
119. Siller, F., Quintanilla-Veag, B., Cebrian, M.E. & Albor, A. Effects of arsenite pretreatment on the acute toxicity of parathion. *Toxicology.* **116**, 59-65 (1997).
120. Naraharisetti, S.B., Aggarwal, M., Sarkar, S.N. & Malik, J.K. Concurrent subacute exposure to arsenic through drinking water and malathion via diet 111 male rats: effects on hepatic drug-metabolizing enzymes. *Arch. Toxicol.* **82**, 543-551 (2008).

121. Santos, S., Silva, A.M., Matos, M., Monteiro, S.M. & Álvaro, A.R. Copper induced apoptosis in Caco-2 and Hep-G2 cells: Expression of caspases 3, 8 and 9, AIF and p53. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. **185–186**, 138-146 (2016).
122. Husain, N. & Mahmood, R. Copper(II) generates ROS and RNS, impairs antioxidant system and damages membrane and DNA in human blood cells. *Environmental Science and Pollution Research*. **26 (20)**, 20654-20668 (2019). DOI: 10.1007/s11356-019-05345-1
123. Olgun, S., MGogal,Jr.R.,Adeshina, F., Choudhury, H. & Misraa, H.P. Pesticide mixtures potentiate the toxicity in murine thymocytes. *Toxicology*. **96 (3)**, 181-195 (2004).
124. Smida, A., Ncibi, S., Taleb, J.tr, Saad, A.B., Ncib, S. & Zourgui, L. Immunoprotective activity and antioxidant properties of cactus (*Opuntia ficus indica*) extract against chlorpyrifos toxicity in rats. *Biomedicine & Pharmacotherapy*. **88**, 844-851 (2017). <https://doi.org/10.1016/j.biopha.2017.01.105>Get rights and content
125. Migliore, L. & Coppede, F. Environmental- induced oxidative stress in neurodegenerative disorders and ageing. *Mutat Res Genetic Toxicol Environ Mutagen*. **674**, 73-84 (2009).
126. Lu, J., Zheng,Y.l., Wu, D.M., Sun, D-x., Shan, Q., Fan & S.H.F. Trace amounts of copper induce neurotoxicity in the cholesterol-fed mice through apoptosis. *FEBS Letters*. **580**, 6730-6740 (2006).
127. Tufan, A. C., Akdogan, I., Turgut, G. & Adiguzel, E. Increased Tunel positive cells in CA1, CA2, and CA3 subfields of rat hippocampus due to copper and ethanol co-exposure. *International J. Neuroscience*.**118**, 647-656 (2008).
128. Yi, J., Zhu, R., Wu, J., Wu, J., Xia, W., Zhu, L., Jiang, W., Xiang, S. & Tan, Z. *In vivo* protective effect of betulinic acid on dexamethasone induced thymocyte apoptosis by reducing oxidative stress. *Pharmacological Reports*. **68 (1)**, 95-100 (2016).
129. Vidyashankar, S. & Patki, P.S. Liv.52 attenuate copper induced toxicity by inhibiting glutathione depletion and increased antioxidant enzyme activity in HepG2 cells. *Food Chem Toxicol*. **48**, 1863-1868 (2010).
130. Garriott, M.L., Phelps, J.B. & Hoffman, W.P. A protocol for the *in-vitro* micronucleus test I. Contribution to the development of a protocol suitable for regulatory submissions from an examination of 16 chemicals with different mechanism of action and level of activity. *Mutation Res*. **517**, 123-134 (2002).
131. Corvi, R., Albertini, S., Hartung, T., Hoffmann, S., Maurici, D., Pfuhler, S., Benthem, J. & Vanparys, P. ECVAM retrospective validation of in vitro micronucleus test (MNT). *Mutagenesis*. **7**, 1-13 (2008).
132. Lemos, A.O., Oliviera, N.CD. & Lemos, C.T. In vitro micronuclei test to evaluate the genotoxicity of surface water under the influence of tanneries. *Toxicology in Vitro*. **25**, 761-766 (2011).

133. Prá, D., Franke, S.I., Giulian, R., Yoneama, M.L., Dias, J.F., Erdtmann, B. & Henriques, J.A. Genotoxicity and mutagenicity of iron and copper in mice. *Biometals*. **21(3)**, 289-97 (2008).
134. Cavas, T., Garanko, N.N. & Arkhipchuk, V.V. Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. *Food Chem Toxicol*. **43**, 569-574 (2005).
135. Junn, E. & Mouradian, M.M. Apoptotic signaling in dopamine-induced cell death: the role of oxidative stress, p38 mitogen-activated protein kinase, cytochrome c and caspases. *J. Neurochem*. **78**, 374-383 (2001).
136. Salido, G.M. Oxidative stress, intracellular calcium signals and apoptotic processes. [apoptosis: involvement of oxidative stress and intracellular ca²⁺ homeostasis](#). Springer-Science + Business Media B.V. 1-16 (2009).10.1007/978-1-4020-9873-4
137. Kushawaha, B., Yadav, R.S., Swain, D.K., Rai, P.K. & Garg, S.K. Mercury-induced inhibition of tyrosine phosphorylation of sperm proteins and altered functional dynamics of buck spermatozoa: an *in vitro* study. *Biological Trace Element Research*. **198**, 1–15 (2020).
138. Cui, F., Chai, T., Quian, L., Wang, C. Effect of three diamides (cholrantranilipore, cyantranilipore and flubendiamide) on life history, embryonic development and oxidatuive stress biomarkers of *Daphnia Magna*. *Chemosphere*. **169**, 107-116 (2017).
139. Lee, G.Y. & Han, S.N. The Role of Vitamin E in Immunity. *Nutrients*. **10(11)**, 1614 (2018). doi:10.3390/nu10111614
140. Mileo, A.M., Nisticò, P. & Miccadei, S. Polyphenols: Immunomodulatory and Therapeutic Implication in Colorectal Cancer. *Front Immunol*. **10**, 729 (2019). doi:10.3389/fimmu.2019.00729
141. Pandey, K.B. & Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease . *Oxid Med Cell Longev*. **2(5)**, 270–278 (2009).
142. Gatz, S.A. & Wiesmuller, L. Take a break-resveratrol in action on DNA. *Carcinogenesis*. **29**, 321-332 (2008).
143. Choi, B.W., Lee, H.S., Shin, H.C. & Lee, B.H. Multifunctional activity of polyphenolic compounds associated with a potential for Alzheimer's disease therapy from *Ecklonia cava*. *Phytotherapy Research*. **29(4)**, 549-553 (2015).
144. Weinreb, O., Mandel, S. & Youdim, M.B. Gene and protein expression profiles of anti- and pro-apoptotic actions of dopamine, Rapomorphine, green tea polyphenol (–)-epigallocatechine-3- gallate, and melatonin. *Ann. N. Y. Acad. Sci*. **993**, 351-61 (2003).
145. Fusi, E., Rebucci, R., Pecorini, C., Campagnoli, A., Pinotti, L., Saccone, F., Cheli, F., Purup, S., Sejrsen, K. & Baldi, A. Alpha-tocopherol counteracts the cytotoxicity induced by ochratoxin a in primary porcine

fibroblasts. *Toxins*. **2**(6), 1265–1278 (2010). <https://doi.org/10.3390/toxins2061265>

146. Akter, M., Islam, N.N., Sumit, A.F., Ahsan, N., Hossain, S., Ahmed, M. & Akhand, A.A. Tea extract prevents arsenic-mediated DNA damage and death of murine thymocytes *in vitro*. *Dhaka Univ. J. Pharm. Sci.* **14**, 79-85 (2015).
147. Masuda, T., Maekawa, T., Hidaka, K., Bando, H., Takeda, Y. & Yamaguchi, H. Chemical studies on antioxidant mechanisms of curcumin: analysis of oxidative coupling products from curcumin and linoleate. *J. Agric. Food Chem.* **49**, 2539-2547 (2001).
148. Gutteridge, J.M. & Halliwell, B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann. N.Y. Acad. Sci.* **899**, 136-147 (2000).
149. Martinez, J. & Moreno, J.J. Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. *Biochem Pharmacol.* **59**(7), 865-70 (2000).
150. Losa, G.A. Resveratrol modulates apoptosis and oxidation in human blood mononuclear cells. *Eur J Clin Invest.* **33**(9), 818-23 (2003).
151. Leonard, S.S., Xia, C., Jiang, B.H., Stinefelt, B., Klanforf, H. & Harrish, GK. et al. Resveratrol scavenges reactive oxygen species and effect the radical induce cellular responses. *Bioch Biophys Res Commun.* **309**(4), 1017-1026 (2003).
152. Ahmad, K.A., Clement, M.V., Hanif, I.M. & Pervaiz, S. Resveratrol inhibits drug-induced apoptosis in human leukemia cells by creating an intracellular milieu nonpermissive for death execution. *Cancer Research.* **64**, 1452–1459 (2004).
153. Goswami, S.K. & Das S.K. Resveratrol and chemoprevention. *Cancer Lett.* **284**(1), 1-6 (2009).
154. Chen, J., Tang, X.Q., Zhi, J.L., Cui, Y., Yu, H.M., Tang, E.H., Sun, S.N, Feng, J.Q. & Chen, P.X. Curcumin protect PC12 cells against 1-methyl 4-phenylpyridinium ion–induce apoptosis by bcl-2-mitochondria-ROS-iNOS pathway. *Apoptosis.* **11**, 943-963 (2006).
155. Sriivasan, M., Rajendra, P.N. & Menon, V.P. Protective Effect of Curcumin on g-Radiation- Induced DNA Damage and Lipid Peroxidation in Cultured Human Lymphocytes. *Mutation Res.* **611**, 96-103 (2006).
156. Corona-Rivera, A., Urbina-Cano, P., Bobadilla-Morales, L., Vargas-Lares, J.J., Ramírez-Herrera, M.A., Mendoza-Magaña, M.L., Troyo-Sanromán, R., Díaz-Esquivel, P. & Corona-Rivera, J.R. Protective *in vivo* effect of curcumin on copper genotoxicity evaluated by comet and micronucleus assays. *J. App. Genetics.* **48**, 389-396 (2007).
157. Roy, M., Sinha, D., Mukherjee, S., Paul, S. & Bhattacharya, R.K. Protective effects of dietary phytochemicals against arsenite induced genotoxicity in mammalian V 79 cells. *Indian J. Experi. Biology.* **46**, 690-697 (2008).

158. Khan, N. & Mukhtar, H. Tea and health: studies in humans. *Curr. Pharm. Des.* **19**, 6141-6147 (2013).
159. Levites, Y., Amit, T., Youdim, M.B. & Mandel, S. Involvement of protein kinase C activation and cell survival/cell cycle genes in green tea polyphenol (-) –epigallocatechin 3-gallate neuroprotective action. *J Biol Chem.* **277**, 30574-80 (2002).
160. Ogaly, H.A., Khalaf, A.A., Ibrahim, M.A., Galal, M.K. & Abd-Elsalam, R.M. Influence of green tea extract on oxidative damage and apoptosis induced by deltamethrin in rat brain. *Neurotoxicol. Teratol.* **50**, 23–31 (2015).
161. Sies, H. & Murphy, M.E. Role of tocopherols in the protection of biological systems against oxidative damage. *J Photochem Photobiol. B.* **8**, 211 (1991).
162. Cook, N.R., Albert, C.M., Gaziano, J.M., Zaharris, E., MacFadyen, J., Danielson, E., Buring, J.E. & Manson, J.E. A randomized factorial trial of vitamin C and E and beta carotene in the secondary prevention of cardiovascular events in women: Results from the women antioxidants cardiovascular study. *Arch Intern Med.* **167**, 1610-8 (2007).
163. Kalender, S., Kalender, Y., Ogutcu, A., Uzunhisarcikli, M., Durak, D. & Acikgoz, F. Endosulfan-induced cardiotoxicity and free radical metabolism in rats: the protective effect of vitamin E. *Toxicology.* **202**, 227-235 (2004).
164. Baumeister, P., Korn, G., Berghaus, A., Matthias, C. & Harreus, U. Chemopreventive action of dexamethasone and α -tocopherol in oxidative stress cells. *Cancer Detection and Prevention.* **32(5-6)**, 452-457 (2009).
165. Kim, S.K., IM, G.J., An, Y.S., Lee, S.H., Jung, H.H. & Park, S.Y. The effects of the antioxidant α -tocopherol succinate on cisplatin-induced ototoxicity in HEI-OC1 auditory cells. *International Journal of Pediatric Otorhinolaryngology.* **86**, 9-14 (2016).
166. Blasiak, J. & Sankowaska, D. Genotoxicity of malaoxon: induction of oxidized and methylated bases and protective effect of α -tocopherol. *Pesticide Biochemistry and Physiology.* **71(2)**, 88-96 (2001).
167. Sharma, R.K. & Sharma, B. In-vitro carbofuran induced genotoxicity in human lymphocytes and its mitigation by vitamins C and E. *Dis. Markers.* **32**, 153-63.
168. Pourahmad, J., O'Brien, P.J. 2000. A comparison of hepatocytes cytotoxic mechanisms for Cu⁻² and Cd⁻². *Toxicology.* **143**, 263-273 (2012).
169. Mattie, M.D. & Freedman, J.H. Protective effects of aspirin and vitamin E (alpha-tocopherol) against copper and cadmium-induced toxicity. *Biochem. Biophys. Res. Commun.* **285**, 921-925 (2001).

170. Wu, Z., Zheng, X., Meng, L., Fang, X., He, Y., Li, D., Zheng, C. & Zhang, H. α -tocopherol, especially α -tocopherol phosphate, exerts antiapoptotic and angiogenic effect on rat bone marrow-derived endothelial progenitor cells under high glucose and hypoxic conditions. *Journal of Vascular Surgery*. **67(4)**, 1263-1273 (2018).
171. Durak, D., Uzun, F.G., Kalender, S., Ogutcu, A., Uzunhisarcikli, M. & Kalender, Y. Malathion-induced oxidative stress in human erythrocytes and the protective effect of vitamins C and E *in vitro*. *Environ Toxicol*. **24**, 235-242 (2008).
172. Sodhi, S., Sharma, A., Brar, A.P.S. & Brar, R.S. Effect of α -tocopherol and selenium on antioxidant status, lipid peroxidation and hepatopathy induced by malathion in chicks. *Pestic Biochem Phys*. **90**, 82-86 (2008).
173. Valko, M., Morris, H. & Cronin, M.T.D. Metals, Toxicity and Oxidative stress. *Current Med Chem*. **12**, 1161 (2005).

Declarations

Acknowledgement

Authors are thankful to Dean COVSc and Head Department of Pharmacology and Toxicology for providing the necessary laboratories facilities established in the Department of Pharmacology and Toxicology under Niche Area of Excellence Project funded by ICAR and RKVY project of Govt. of India. The research work was undertaken with routine facilities and funds made available to the Department from ICAR Development Grant. Financial assistance from ICAR is thankfully acknowledged.

Author's contribution

R.M: Executed the research work. A.P: Conceptualization of genotoxicity. A.R: Conceptualization of oxidative stress. S.K: Interactive Index. R. K: Histological study. S. K. G: supervised the study. All the authors have read and approved the final manuscript.

Authors' details

***Rajesh Mandil**, Associate Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut-250110, India

rajesh_mandil@rediffmail.com

Atul Prakash, Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, U.P. Pt. DeenDayalUpadhyay PashuChikitsa Vigyan Vishwavidyalaya Evam Go-AnusandhanSansthan (DUVASU), Mathura-281001, India

dratul2510@rediffmail.com

^b**Anu Rahal**, Principal Scientist, Division of Goat Health, Central Institute for Research on Goat (CIRG), Makhdoom, Farah, Mathura, Uttar Pradesh-281122, India

rahalanu72@gmail.com

Swatikoli, MVScscholar, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, U.P. Pt. DeenDayal Upadhyay PashuChikitsa Vigyan Vishwavidyalaya Evam Go-AnusandhanSansthan (DUVASU), Mathura-281001, India

swati3koli@gmail.com

^a**Rahul Kumar**, Assistant Professor, Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, U.P. Pt. DeenDayal Upadhyay PashuChikitsa Vigyan Vishwavidyalaya Evam Go-AnusandhanSansthan (DUVASU), Mathura-281001, India

rahulpoultrypatho@gmail.com

Satish K. Garg, Ex-Professor and Head, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, U.P. Pt. DeenDayalUpadhyay PashuChikitsa Vigyan Vishwavidyalaya Evam Go-AnusandhanSansthan (DUVASU), Mathura-281001, India

profsatish@gmail.com

Competing interests

The authors declare no competing interests.

Funding

Authors received no financial support for the research, authorship and/or publication of this article. The research work was undertaken with the routine facilities and grants available in the Department and it was a part of PhD thesis

Additional information

Correspondence and requests for materials should be addressed to R.M.

Tables

Tables 1-6 are in the supplementary files section.

Figures

1 (a): Total Protein

1 (b): LPO

1 (c): GSH

1 (d): SOD

1 (e): GST

1 (f): Catalase

1 (g): GPx

Figure 1

(a-g): Effect of α -tocopherol on hepatic and kidneys oxidative stress biomarkers in copper and flubendiamide alone and combined- treated rats.

I: control group, II: vehicle control (corn oil), III: copper-treated with 33 mg/kg, IV: flubendiamide- treated (200 mg/kg), V: group treated with copper (33 mg/kg) + flubendiamide (200 mg/kg), VI: α -tocopherol treated group (100 mg/kg), VII: group treated with copper (33 mg/kg) + α -tocopherol (100 mg/kg), VIII: group treated with flubendiamide (200 mg/kg) + α -tocopherol (100 mg/kg) and IX: group treated with copper (33 mg/kg) + flubendiamide (200 mg/kg) + α -tocopherol (100 mg/kg).

· **Data (Mean $\pm$ SE; N=6) bearing different superscripts in the same column differed significantly ($P<0.05$)**

2 (a) : CYP450 & CYPb5

2 (b): ANDM

2 (c): APH, GST & Microsomal Protein

2(d): UGT

Figure 2

(a-d) Effect of α -tocopherol on the phase-I & II drug metabolizing enzyme levels in copper and flubendiamide-treated rats.

2 (a): CYP 450 (nM /mg microsomal protein); CYPb5 (nM /mg microsomal protein); 2(b): Aminopyrene-N – demethylase (nm formaldehyde formed /min/mg microsomal protein); 2 (c): Aniline-p-hydroxylase (nM p-

aminophenol formed /min/mg microsomal protein); GST (μ M CDNB -GSH conjugate formed /min/mg microsomal protein); Microsomal protein(mg/g tissue) 2(d): **UGT** (nM /min/mg microsomal protein;

I: control group, II: vehicle control (corn oil), III: copper-treated with 33 mg/kg

, IV: flubendiamide- treated (200 mg/kg), V: group treated copper (33 mg/kg) + flubendiamide (200 mg/kg), VI: α -tocopherol treated group (100 mg/kg), VII: group treated with copper (33 mg/kg) + α -tocopherol (100 mg/kg), VIII: group treated with flubendiamide (200 mg/kg) + α -tocopherol (100 mg/kg) and IX: group treated with copper (33 mg/kg) + flubendiamide (200 mg/kg) + α -tocopherol (100 mg/kg).

· **Data (Mean $\pm$ SE; N=6) bearing different superscripts in the same column differed significantly (P<0.05)**

Figure 3

(a-g): Effect of α -tocopherol on the histoarchitectural changes in liver tissue of copper and flubendiamide alone and combined- treated rats

Figure 4

(a-g): Effect of α -tocopherol on the histoarchitectural changes in kidneys tissue of copper and flubendiamide alone and combined- treated rats

Figure 5

TUNEL + Ve cells (40 X) formation in rat thymocytes following *in vitro* exposure to IC₅₀ concentrations of flubendiamide alone (40 μM) and in the presence of resveratrol, catechin, curcumin and α-tocopherol at different concentrations.

Figure 6

TUNEL + Ve cells (40 X) formation in rat thymocytes following *in vitro* exposure to IC₅₀ concentrations of copper alone (40 μ M) and in the presence of resveratrol, catechin, curcumin and α -tocopherol at different concentrations.

Figure 7

Rats thymocytes showing micronuclei formation (100 X) following their *in vitro* exposure to IC₅₀ concentrations of flubendiamide alone (40 μ M) and in the presence of different concentrations of resveratrol, catechin and curcumin and α -tocopherol.

Figure 8

Rats thymocytes showing micronuclei formation (100 X) following their *in vitro* exposure to IC₅₀ concentrations of copper alone (40 μ M) and in the presence of different concentrations of natural antioxidants resveratrol, catechin, curcumin and α -tocopherol.

Figure 9

Rats thymocytes showing DNA fragmentation pattern following their *in vitro* exposure of IC₅₀ concentrations of flubendiamide alone and in resveratrol ((5 and 10μM), catechin (10 and 20μM) curcumin (5 and 10 μM) and α-tocopherol (5, 10 and 20 μM).

RV: Resveratrol

Cath: Catechin

A-T: α-tocopherol

Cur: Curcumin

Flb: Flubendiamide

Dexa: Dexamethasone

Cont: Control

Figure 10

Rats thymocytes showing DNA fragmentation pattern following their *in vitro* effect of IC₅₀ concentrations of copper alone and in presence of resveratrol ((5 and 10μM), catechin (10 and 20μM) curcumin (5 and 10 μM) and α-tocopherol (5, 10 and 20 μM).

RV: Resveratrol Cath: Catechin A-T: α-tocopherol
 Cur: Curcumi) Cu: Copper Dexa: Dexamethasone
 Cont: Control

Thymocytes

Control

DMSO

Dexamethasone (20 μm)

Flubendiamide (40 μM)

Copper (40 μM)

Figure 11

Representative photographs of rats thymocytes showing comet formation following their *in vitro* exposure to median lethal concentrations of flubendiamide and copper.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables.docx](#)