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Cobalt Chloride-induced Hepatic and Intestinal damage in rats: Protection by ethyl acetate and chloroform fractions of *Ocimum gratissimum*

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Abstract

Cobalt chloride is known to produce symptoms of diarrhea, vomiting and other gastrointestinal disturbances. We investigated the potential roles of the ethyl acetate and chloroform fractions of *Ocimum gratissimum* (OG), traditionally used to treat diarrhea and other gastrointestinal disorders in protection against cobalt chloride ($CoCl_2$)-induced liver and intestinal damage. Wistar albino rats were given $CoCl_2$ (350 ppm) in drinking water for 7 days, alone or concurrently with either fractions of OG at 100 and 200mg/kg each. Gallic acid (120 mg/kg) was administered to a group of rats as a standard flavonoid. Biochemical indices of oxidative stress, antioxidant enzyme activities, the levels of pro-inflammatory cytokines (Interleukin 1β; IL-1β and Tumor necrosis factor, TNF-α) were evaluated and the histological appearance of the liver and intestinal mucosa was investigated. $CoCl_2$ produced significant elevations ($p<0.05$) in hydrogen peroxide (H_2O_2), malondialdehyde (MDA), IL-1β, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP). This was accompanied with significant reductions ($p<0.05$) in reduced glutathione (GSH), glutathione peroxidase (GPX) and glutathione S-transferase (GST) activities. Liver sections of rats exposed to $CoCl_2$ had poor architecture and areas of necrosis with several dead hepatocytes, while some appeared with hyperchromic nuclei. Intestinal mucosa showed significant loss of absorptive epithelial cells with $CoCl_2$ exposure. Treatment with the fractions from OG produced reduction in H_2O_2, MDA and IL-1β levels; reduced serum activities of ALT, AST and ALP; restoration of GSH levels and improved activities of GPX and GST. The fractions significantly preserved the hepatic and intestinal architecture.Our results indicate that the fractions of OG exhibited considerable hepatic and intestinal protection by reduction in levels of oxidants and pro-inflammatory cytokines, enhancement of antioxidant enzyme activities and preservation of tissue integrity and might thus be very useful agents in protecting the liver and intestines during concurrent exposure to Cobalt chloride.

Keywords: Cobalt, hepatotoxicity, intestines, *Ocimum gratissimum*, Gallic acid, antioxidant.

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1. Introduction

Cobalt is an element present in the earth's crust and is a component of animal and human tissues. Cobalt ions are beneficial in trace amounts in vivo being a component of the corrin nucleus of Vitamin B12 [1]. Cobalt and its salts have found wide applications in industries in the manufacture of paints, varnishes pottery, ceramics and glass; as a foam stabilizer in beer; and as a catalyst in the petrochemical industry. Occupational exposures to cobalt have been reported to have mutagenic and carcinogenic effects [2, 3, 4, 5]. Internal exposures to toxic levels of Co released from metallic implant devices are part of recent health concerns in orthopaedic surgery [6, 7].

Cobalt chloride has been reported to induce hepatotoxicity [8, 9]; nephrotoxicity [10]; cardiotoxicity [11]; reproductive toxicity [12]. Different cobalt compounds have been reported to produce symptoms of diarrhea, vomiting and other gastrointestinal disturbances [13]. A growing body of evidence has attributed the underlying mechanism of these injuries to be due to the excessive production of reactive oxygen species (ROS).

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When ROS are present in concentrations greater than that which can be decomposed by antioxidant defense systems such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX) or reduced glutathione (GSH), they tend to produce cellular damage [8, 14, 15]. Cobalt, like some other metal ions can catalyze the conversion of hydrogen peroxide into hydroxyl radical ($HO^{\cdot}$)by the Fenton reaction [16]. Therefore, when high amounts of cobalt ions are present, the $HO^{\cdot}$formed by the metal-catalyzed Fenton reactions are greatly increased, with excessive amounts causing deleterious oxidative injury to lipids, proteins and DNA [17].

Several research efforts have recently focused on dietary strategies to mitigate the toxic effects of individuals at high risk of heavy metal exposure [18]. Heavy metal toxicity is unique in that metals are usually non-biodegradable and therefore persist in the environment. Dietary modifications which include essential phytochemicals may be helpful in preventing the absorption of these metals into the body or limiting the extent of damage done to body tissues.

Ocimum gratissimum, of the family Labiatae is a small erect plant found throughout the tropics and subtropics [19]. It is a widely used culinary in salads, soups, vinegars, etc. [20]. It has been used in traditional medicine as a tonic and anti-diarrhoeal agent. It is also used in the treatment of conjunctivitis by direct instillation into the eye; the oil from the leaves is applied in combination with alcohol as a lotion for skin infections and is also taken internally for the treatment of bronchitis [21]. It is also used for the management of upper respiratory tract infections, cough, pneumonia and fever [22, 23]. Phytochemicals e.g. flavonoids, tannins, alkaloids, saponins, terpenes from *Ocimum gratissimum* have been reported to possess a variety of beneficial activities.

Flavonoids are particularly beneficial naturally-occuring compounds widely distributed in many plants, including *Ocimum gratissimum*. High concentrations of flavonoid contents in leaves of *Ocimum gratissimum* has been reported [20]. Flavonoids are known to prevent oxidant injury by scavenging oxygen radicals, protecting against lipid peroxidation and chelation of metal ions [24, 25, 26]. The flavonoid-rich fractions of different plants have been studied against hepatotoxicity in rats [28, 29, 30] and against metal-induced damage in various tissues [28, 30]. The protective effects of certain natural agents against cobalt-chloride induced toxicity have also been reported [8, 10]. The present study was designed to evaluate the protective effects of ethyl acetate and chloroform fractions from the leaves of *Ocimum gratissimum* on cobalt-chloride-induced injury to liver and small intestinal tissues in Wistar albino rats.

2. Materials and methods

2.1 Chemicals

Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$) was obtained from Tianjin Kermel® Chemical Reagent Co, China. Gallic acid, Epinephrine, glutathione, 5, 5'-dithiobis-2-nitrobenzoic acid, hydrogen peroxide, thiobarbituric acid, and trichloroacetic acid, were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and were obtained from British drug houses (UK).

2.2 Extraction of *Ocimum gratissimum* fractions

Leaves of *Ocimum gratissimum* were obtained from local markets within Ibadan, Nigeria. The plant was identified and authenticated at the Department of Botany, University of Ibadan, Nigeria. The voucher specimen (UIH-22398) was deposited at the herbarium. The leaves were air-dried and grounded with an electric blender. The dried and powdered leaves (700g) were extracted successively with n-hexane and methanol using a Soxhlet apparatus. The solvents were then removed under reduced pressure and sticky residues were obtained. The crude methanol extract after removal of the solvent was dissolved in 10% sulfuric acid solution and subsequently partitioned with chloroform and ethyl acetate to give chloroform-, and ethyl acetate-soluble fractions respectively. The chloroform and ethyl acetate fractions were evaporated under reduced pressure and concentrated in vacuo to obtain flavonoid rich fractions 9.122g and 1.608g, respectively. The appropriate amounts of the chloroform and ethyl acetate fractions were weighed and suspended in 10% Dimethyl sulphoxide (DMSO) prior to administration to rats.

2.3 Animal Experimental protocol

Forty nine male Wistar albino rats (weighing 150-200g), about 8-10 weeks of age, were obtained from the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan, Nigeria. The animals were kept in plastic cages in a well-ventilated animal house under controlled light cycle (12 h light/12 h dark)

and fed with commercial rat chow and water *ad libitum*. The standard diet were purchased from Ladokun feeds, Ibadan, Nigeria and contains carbohydrates (68%), protein (21%), fat (3.5%), vitamins (2%), minerals (2%) and fibre (6%). All the animals were handled humanely under guidelines criteria outlined in principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985). The animals were divided into seven groups each containing seven rats as shown in Table 1.

2.4 Animal necropsy

Under light diethyl ether anesthesia, blood was obtained from the retro-orbital plexus into small plain sample bottles. The rats were thereafter sacrificed on the 8th day by cervical dislocation. The liver and small intestines were harvested and separated from attachment of mesentery and other tissues. The small intestine was opened along the entire length and was cleaned of debris and digesta. The liver and intestines were all rinsed in normal saline, blotted dry and weighed. Small portions of the tissues were cut and put in 10% formalin for histopathological examination. Serum samples were obtained from the blood by centrifugation at 3000 rpm for 10 minutes. The serum samples were stored at -20^{O}C prior to analysis for enzymes (Alanine transaminase, ALT; aspartate transaminase, AST and alkaline phosphatase, ALP) and pro-inflammatory cytokines (IL-1β and TNF-α).

2.5 Biochemical assays

The liver and intestines were homogenised in 50 mMTris–HCl buffer (pH 7.4) containing 1.15% potassium chloride, and the homogenate was centrifuged at 12 000 g for 15 min at 4°C. The supernatant was collected for the estimation of different biochemical parameters. Protein concentration was determined by the method of Lowry et al. [31]. Hydrogen peroxide generation was assessed by the method of Wolff [32]. Lipid peroxidation was quantified as malondialdehyde according to the method described by Farombi*et al* [33] and expressed as micromoles of MDA per gram tissue. Catalase activity using hydrogen peroxide as substrate was assayed according to the method of Clairborne [34]. Superoxide dismutase was assayed by the method described by Misra&Fridovich [35]. Glutathione S-transferase was assayed by the method of Habig*et al* [36]. Glutathione peroxidase (GPX) activity was measured by the method of Rotruck et al., [37], in which hydrogen peroxide was used as substrate. Reduced glutathione was determined at 412 nm using the method described by Jollow et al. [38]. Serum activities of aspartate aminotransferase and alanine aminotransferase according to Reitmann& Frankel [39] and alkaline phosphatase were all determined using kits from Randox Laboratories Limited, Crumlin, UK).

Table 1. Experimental design

Group	No of Rats	Treatment	Route of administration	Dosage	Days							
					1	2	3	4	5	6	7	8
A (control)	7	10% DMSO	Oral	Average volume of other treatments	√	√	√	√	√	√	√	S
B	7	Cobalt chloride	Drinking water	350ppm	√	√	√	√	√	√	√	S
C	7	Cobalt chloride +	Drinking water	350ppm	√	√	√	√	√	√	√	S
		EAOG	Oral gavage	100mg/kg	√	√	√	√	√	√	√	S
D	7	Cobalt chloride +	Drinking water	350ppm	√	√	√	√	√	√	√	S
		EAOG	Oral gavage	200mg/kg	√	√	√	√	√	√	√	S
E	7	Cobalt chloride +	Drinking water	350ppm	√	√	√	√	√	√	√	S
		COG	Oral gavage	100mg/kg	√	√	√	√	√	√	√	S
F	7	Cobalt chloride +	Drinking water	350ppm	√	√	√	√	√	√	√	S
		COG	Oral gavage	200mg/kg	√	√	√	√	√	√	√	S
G	7	Cobalt chloride +	Drinking water	350ppm	√	√	√	√	√	√	√	S
		Gallic acid	Oral gavage	120mg/kg	√	√	√	√	√	√	√	S

EAOG = Ethyl acetate fraction of *Ocimum gratisimum*; COG = Chloroform fraction of *Ocimum gratissimum*; √ = administered; S = sacrificed.

2.6 Determination of serum Interleukin 1 beta (IL-1β) and Tumor necrosis factor (TNF-α)

Serum levels of IL-1β and TNF-α were measured using commercially purchased Enzyme-linked immunosorbent (ELISA) assay kits (Abcam® Biotech, UK), according to the manufacturer's instructions. The concentrations of the cytokines were determined from standard curves and expressed as picogram per ml.

2.7 Histopathology

Small portions of liver, duodenum and ileum were collected and immediately put in 10% formal saline buffer for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5–6μm in thickness were made and stained with haematoxylin and eosin for histopathological examination [40].

3. Statistical analysis

Data obtained were expressed as mean ± standard deviation. Means across the different groups were compared using one-way ANOVA. Test of significance between two groups was done with Student's t- test. All data were analyzed with SPSS (student version 7.5; SPSS Inc., Surrey, UK). Values less than 0.05 were considered statistically significant.

4. Results

Following the administration of cobalt chloride to the rats, we observed a reduction in appetite in the groups given cobalt chloride, irrespective of the treatments administered. However, no animals died through the duration of the experiment.

4.1 Effects of fractions of *Ocimum gratissimum* and Gallic acid on relative weights of liver and intestines in $CoCl_2$-treated rats.

The relative weights of the liver and intestines in each group of rats are presented in Table 2. The data shows significant reduction ($p<0.05$) in the weights of the intestines and liver in the group exposed to cobalt chloride when compared with the control. There were no significant differences in the relative liver and intestinal weights in the groups treated with either the fractions of OG or Gallic acid, when compared to rats given cobalt chloride alone. However, there were significant reductions in the relative weights of the intestines in almost all the different groups given cobalt chloride when compared to the control group.

4.2 Effects of fractions of *Ocimum gratissimum* and Gallic acid on Serum activities of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) in $CoCl_2$-treated rats

As presented in Table 3, cobalt chloride caused significant increases ($p<0.05$) in the serum activities of ALT, AST and ALP, when compared with the control. Gallic acid showed considerable protection against cobalt chloride hepatotoxicity causing significant reduction ($p<0.05$) in AST and ALP activities when compared with rats exposed to cobalt chloride only. The higher dosage (200mg/kg) of the chloroform fraction of *Ocimum gratissimum* produced the best protection among the different dosages of the fractions producing significant reductions ($p<0.05$) in ALT and AST activities.

Table 2. Effect of the ethyl acetate and chloroform fractions of *Ocimum gratissimum* and Gallic acid on relative weights of liver and intestine in $CoCl_2$ intoxicated rats

Treatment groups	Relative liver weight (g/100g)	Relative intestinal weight (g/100g)
Control	3.55±0.72	3.27±0.79
$CoCl_2$ only (350ppm)	3.05±0.30[a]	2.75±0.48[a]
$CoCl_2$ + EAOG (100mg/kg)	3.37±0.15	2.34±0.31[a]
$CoCl_2$ + EAOG (200mg/kg)	3.09±0.34	2.68±0.51[a]
$CoCl_2$ + COG (100mg/kg)	2.99±0.25[a]	2.09±0.24[a]
$CoCl_2$ + COG (200mg/kg)	2.94±0.19[a]	2.14±0.30[a]
$CoCl_2$ + gallic acid (120mg/kg)	3.24±0.28	2.45±0.55[a]

$CoCl_2$ = Cobalt chloride; EAOG = ethyl acetate fraction of *Ocimum gratissimum*; COG = Chloroform fraction of *Ocimum gratissimum*;
[a] Values differ significantly from control ($p<0.05$)

Table 3. Effect of the ethyl acetate and chloroform fractions of *Ocimum gratissimum* and Gallic acid on serum ALT, AST, ALP and hepatic and intestinal antioxidant enzymes in $CoCl_2$-intoxicated rats

Parameters	Tissues	Treatment groups						
		Control	$CoCl_2$ only (350ppm)	$CoCl_2$ + EAOG (100mg/kg)	$CoCl_2$ + EAOG (200mg/kg)	$CoCl_2$ + COG (100mg/kg)	$CoCl_2$ + COG (200mg/kg)	$CoCl_2$ + Gallic acid (120mg/kg)
ALT (U/L)	Serum	4.82 ± 1.12	7.64 ± 1.60[a]	9.58 ± 0.85	7.32 ± 1.65	6.63 ± 0.38	5.12 ± 0.6[b]	6.36 ± 1.83
AST (U/L)	Serum	51.67 ± 7.21	59.41 ± 4.65[a]	65.60 ± 6.40	60.54 ± 3.17	56.34 ± 1.66	45.24 ± 8.26[b]	28.66 ± 6.37[b*]
ALP (U/L)	Serum	166.52 ± 21.19	309.73 ± 65.87[a]	249.32 ± 51.95	243.19 ± 30.66	274.47 ± 20.50	272.01 ± 46.52	135.85 ± 22.73[b]
SOD	Liver	0.16±0.05	0.15±0.03	0.19±0.02	0.19±0.03	0.15±0.03	0.16±0.02	0.15±0.02
	Intestine	0.30±0.04	0.29±0.04	0.24±0.03[b]	0.28±0.03	0.23±0.02[b]	0.27±0.02	0.26±0.02
CAT	Liver	52.52±2.73	48.37±5.54	53.99±4.55	52.91±6.87	45.70±5.54	49.51±4.06	51.66±2.76
	Intestine	81.34±5.52	69.53±8.98[a]	61.18±6.31	75.18±6.62	64.47±3.60	69.12±4.99	71.49±4.68

$CoCl_2$ = Cobalt chloride; EAOG = ethyl acetate fraction of *Ocimum gratissimum*; COG = Chloroform fraction of *Ocimum gratissimum*; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; SOD, superoxide dismutase (units/mg protein); CAT, catalase (micromole H_2O_2 consumed per minute per mg protein).

[a] Values differ significantly from control ($p<0.05$)

[b] Values differ significantly from Cobalt chloride alone ($p<0.05$)

* Values also differ significantly at $p<0.01$

4.3 Effects of fractions of *Ocimum gratissimum* and Gallic acid on serum levels of Interleukin 1 beta (IL-1β) and Tumor necrosis factor (TNF-α) in $CoCl_2$-treated rats

Serum Interleukin levels (IL-1β and TNFα) in the rats exposed to cobalt chloride and treated with either Gallic acid or fractions of *Ocimum gratissimum* are presented in Fig. 1 (I & II). There was significant increase ($p<0.05$) in levels of IL-1β following exposure to cobalt chloride compared to control. Both fractions of *Ocimum gratissimum* at the different dosages, as well as Gallic acid, caused significant reductions in IL-1β up to levels of normal control rats. TNFα was significantly reduced ($p<0.05$) in rats exposed to cobalt chloride alone or in combination with the ethyl acetate fraction of OG, compared to the control group. However, levels of TNF-α obtained for the groups treated with cobalt chloride and either the chloroform fractions of OG or Gallic acid were similar to that of control rats.

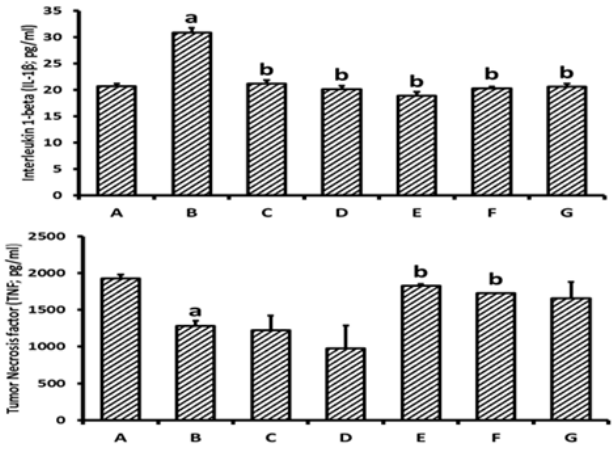


Figure 1. Effect of the ethyl acetate and chloroform fractions of *Ocimum gratissimum* and Gallic acid on serum Interleukin 1β (IL-1β) and Tumor necrosis factor (TNF-α) in $CoCl_2$ intoxicated rats.

A = Control; B = $CoCl_2$ only; C = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (100mg/kg)*; D = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (200mg/kg)*; E = $CoCl_2$ + chloroform fraction of *Ocimum gratissimum (100mg/kg)*; *F* = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (200mg/kg)*; *G* = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (120mg/kg)*.

[a] Values differ significantly from control ($p<0.05$)

[b] Values differ significantly from Cobalt chloride alone ($p<0.05$)

4.4 Effects of fractions of *Ocimum gratissimum* and Gallic acid on oxidant levels in liver and intestines of $CoCl_2$-treated rats

As shown in Fig. 2a, cobalt chloride produced significant increase ($p<0.05$) in hydrogen peroxide concentration in both liver and intestines, when compared with the control. This effect was significantly ameliorated ($p<0.0$) by both ethyl acetate and chloroform fractions of *Ocimum gratissimum*. Gallic acid also produced a reduction in H_2O_2 generation, although the effect was not statistically significant.

The MDA concentration in liver and intestines are presented in Fig. 2b. MDA level was not significantly altered in the liver of both exposure and treatment groups. However, cobalt chloride exposure caused significant increase in MDA level in the intestines, compared to the control. Treatment with the chloroform fraction of OG at 200mg/kg and Gallic acid (120mg/kg) ameliorated the increased MDA levels.

4.5 Effects of fractions of *Ocimum gratissimum* and Gallic acid on antioxidant systems in liver and intestines of $CoCl_2$-treated rats

Results presented in Figs. 2c, d and e show the concentrations of GSH, activities of GPX and GST, respectively in the liver and intestines. There were significant reductions ($p<0.05$) in GSH concentration and GPX activity in both liver and intestines of cobalt chloride-treated rats compared to control. These effects were significantly reversed ($P<0.05$) in rats treated with fractions of OG and Gallic acid. There was also reduction in the activity of GST in the liver and intestines, which was significantly reversed ($p <0.05$) especially by the higher dosage of the chloroform extract of OG (200mg/kg) and Gallic acid.

The effects of cobalt chloride and fractions of *Ocimum gratissimum* and Gallic acid on liver SOD and catalase are presented in Table 2. Reductions in CAT activity was obtained in both liver and intestine, although this was

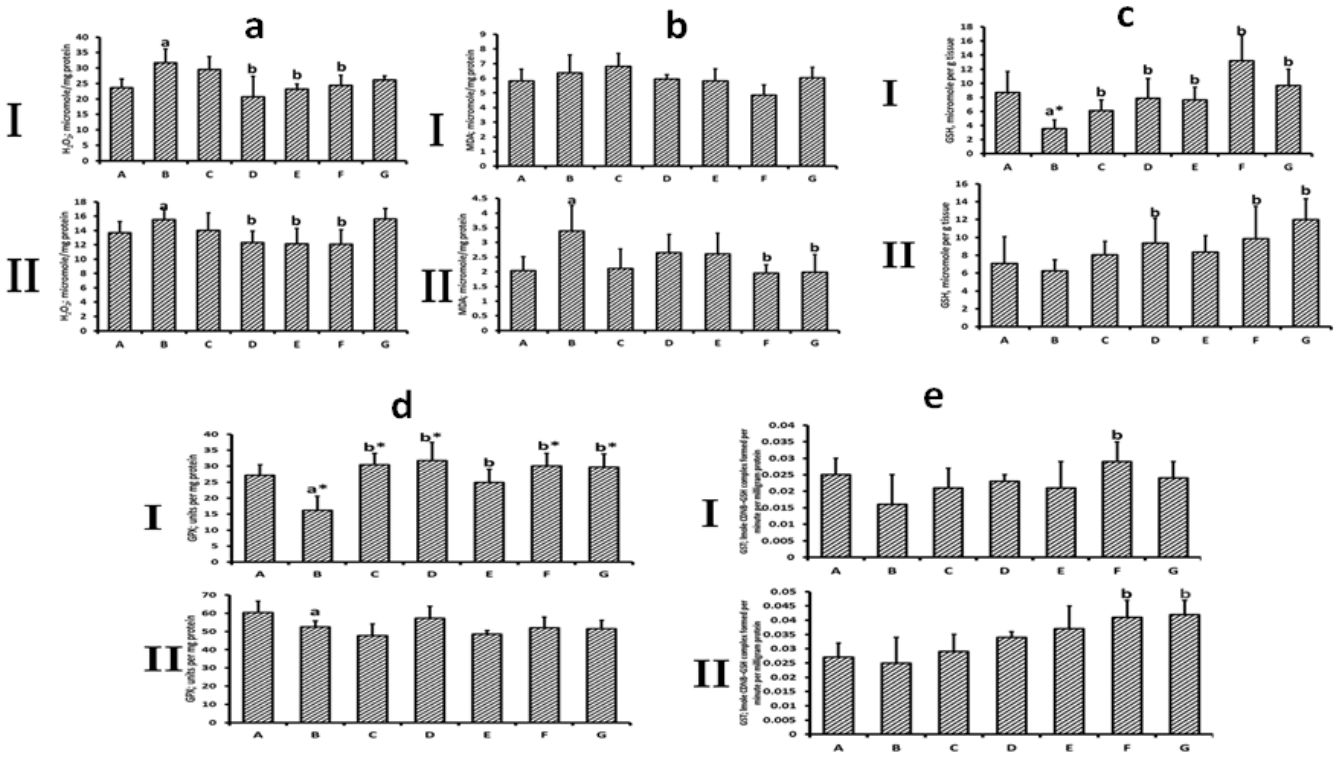


Figure 2. Effect of the ethyl acetate and chloroform fractions of *Ocimum gratissimum* and Gallic acid on some oxidant concentrations and glutathione-related enzymes in (I) liver and (II) small intestines in $CoCl_2$ intoxicated rats.

I = Liver; II = Small intestine; A = Control; B = $CoCl_2$ only; C = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (100mg/kg)*; D = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (200mg/kg)*; E = $CoCl_2$ + chloroform fraction of *Ocimum gratissimum (100mg/kg)*; *F* = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (200mg/kg)*; *G* = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (120mg/kg)*.

[a] Values differ significantly from control ($p<0.05$)

[b] Values differ significantly from Cobalt chloride alone ($p<0.05$)

* Values also differ significantly at $p<0.01$

only statistically significant ($p < 0.05$) in the intestines. Treatment with fractions of OG or Gallic acid, however, showed CAT activities not significantly different from those of the cobalt chloride group. SOD activity was not significantly altered in the liver of rats from all the different groups, while fractions of OG were also not able to significantly improve SOD activity.

5. Microscopy

Histological changes in the liver in all the groups are presented in Fig. 3a. The liver sections in control rats showed normal architecture with no congestions in the central venules and sinusoids and the hepatocytes showed normal morphology. In contrast, liver from rats treated with $CoCl_2$ alone had considerably poor architecture showing areas of necrosis with several dead hepatocytes and others showing bizarre morphology, while some had hyperchromic nuclei. Treatment with fractions of OG (especially at the higher doses) or Gallic acid produced very well preserved liver morphology resembling that of control.

Sections from the duodenum and ileum in all the groups are summarized in Figs. 3b and 3c. The tissues from control rats showed normal mucosa with villi which appear normal and the intestinal glands and muscularis layers were also normal. In rats given $CoCl_2$ alone, intestinal injury was marked with only few intact villi and majority showing depletion of absorptive epithelial cells. The intestinal glands and muscularis externa were, however, still normal. Considerable preservation of the intestinal mucosa and epithelium was obtained with fractions of OG, in both duodenum and Ileum.

6. Discussion

Exposure to cobalt occurs through four media: water, soil, food and air [41]. Oral administration of cobalt chloride in drinking water in this study was done to simulate natural exposures to toxic concentrations of $CoCl_2$ in drinking water or food. In such cases of oral exposure, the gastrointestinal tract and liver represent the first sites of substantial exposure to the chemical or its metabolites. We therefore assessed the nature of toxicity exerted on

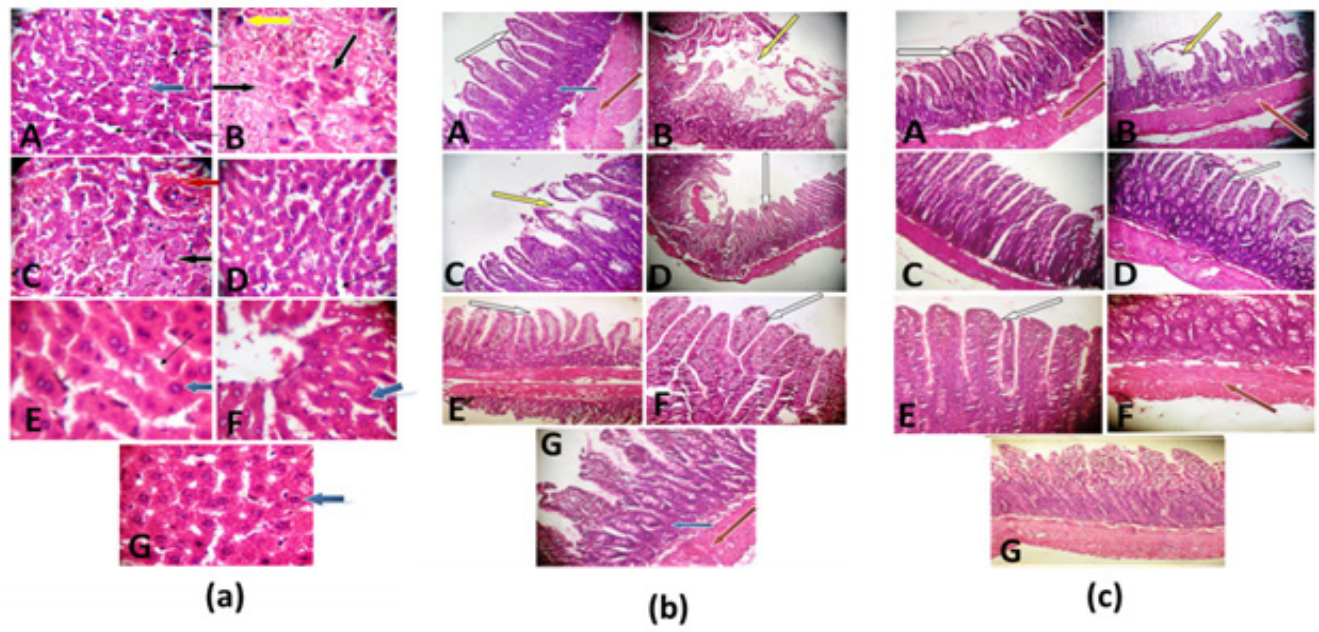


Figure 3. Effects of the ethyl acetate and chloroform fractions of *Ocimum gratissimum* on liver, duodenum and ileum histopathology (H&E; x400) in $CoCl_2$-intoxicated rats.

A = Control; B = $CoCl_2$ only; C = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (100mg/kg)*; D = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (200mg/kg)*; E = $CoCl_2$ + chloroform fraction of *Ocimum gratissimum (100mg/kg)*; *F* = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (200mg/kg)*; *G* = $CoCl_2$ + ethyl acetate fraction of *Ocimum gratissimum (120mg/kg)*.

In the liver, dead hepatocytes and areas of necrosis are indicated by black arrows, hyperchromic nuclei by yellow arrows, and hemorrhage by red arrows. Normal hepatocytes are indicated by blue arrows, while normal, non-congested sinusoids are indicated by the slender arrow. In the intestines, loss of epithelial cells are shown by the yellow arrow, normal intestinal glands by the blue arrow, normal villi by white arrow, and normal muscularis layer are indicated by the red arrow.

the integrity of the intestinal tissues as well as the liver, a major organ responsible for the metabolism of most exogenous compounds. Since metals are persistent in the environment of man and animals, we sought to investigate the probable protective ability of fractions from *Ocimum gratissimum* in mitigating the toxic effects of $CoCl_2$.

A notable finding from our study was the reduction in the relative weights of the intestines and liver in all the rats exposed to $CoCl_2$, compared to control rats. Reduction in organ weights may indicate an effect related to a reduction in appetite observed in rats exposed to $CoCl_2$ during the period of our study. The reduction in weights of the organs could also be related to injuries to these tissues. Histopathology sections of liver tissues exposed to only $CoCl_2$ revealed a generally poor architecture with areas of necrosis and several dead hepatocytes. Intestinal mucosal injury was also revealed in this group of rats by a depletion of absorptive epithelial cells. Previous studies have also observed a lower water and food intake in cobalt-treated rats with corresponding reduction in mean body weights, absolute and relative liver and kidney weights, compared to control rats [9, 10]. Reductions in body weights and liver weights were also reported with cobalt-treated rats. However, in our study, treatment of rats with both fractions of OG produced considerable preservation of the integrity of the hepatocytes and the intestinal mucosa, although the fractions could not significantly improve the weights of the organs.

Further evidence of $CoCl_2$-induced injury to the liver and probably intestines in this study was indicated by significantly elevated serum activities of ALT, AST and ALP in $CoCl_2$-treated rats. Increases in serum activities of these enzymes have been attributed to a compromise or damage to the structural integrity of the liver and other tissues where these enzymes are normally located. Elevations in ALT and AST activities are more specific indicators of liver damage [9]. ALP is an important component of the microvillus membrane in the intestines. It functions in synthesis of cellular protein, cell differentiation, dephosphorylation and glycosylation of membranes [42]. The enzyme is found in high concentrations attached to the brush border membranes of the small intestine. The impairment of the integrity of the intestinal mucosa as well as damage to other tissues, including the liver may have resulted in increased activity in the serum as a result of leakage from normal sites of localization into the blood stream.

Several reports have identified oxidative stress mechanisms as major factors involved in the toxicity of cobalt. Cobalt ions (Co^{2+}) react with hydrogen peroxide to produce hydroxyl radicals by the Fenton reaction [43]. In addition, cobalt ions are known to bind sulfhydryl groups of proteins and many enzymes interfering with their functions. As a result, the antioxidant defense provided by proteins such as GSH and other antioxidant enzymes against toxicant exposure becomes compromised.

In this study, increased intracellular production of ROS with $CoCl_2$ administration was indicated by a significant elevation in hydrogen peroxide in both liver and intestines. Furthermore, significant increases in MDA in the intestines confirmed increased lipid peroxidation, all of which are indications of oxidative stress. Increased lipid peroxidation could also be responsible for disrupted membrane integrity with resultant increases in the extracellular activities of ALT, AST and ALP [44]. There were also concomitant reductions in GSH concentrations, as well as the activities of GPx and GST in rats treated with $CoCl_2$. Depletion in the activities of these antioxidant enzymes and GSH may be due to decreased synthesis or oxidative inactivation of the enzyme protein [10].

Our results showed that both fractions of OG and Gallic acid reverted the decreases in GSH, GPx and GST, while reducing the concentrations of Hydrogen peroxide and MDA produced. The protection offered by fractions of OG especially at the higher dosages compared favorably with that of Gallic acid. Previous phytochemical screening has revealed that the leaves of OG represent a rich source of flavonoids and other phytochemicals such as tannins, alkaloids and saponins, all of which possess considerable antioxidant properties [45]. Previous studies in our laboratory have demonstrated the protective ability of the methanol extracts of OG leaves against intestinal ischemia-reperfusion injury in rats [46].

Quite remarkably, the results of our biochemical assays correlated in many ways with the histological findings in the liver and intestines. As noted earlier, elevations in serum activities of ALT, AST and ALP were indicative of hepatocellular necrosis, as well as depletion of intestinal epithelia observed at histology. In the same vein, elevated levels of H_2O_2 and MDA in rats exposed to cobalt chloride alone were consistent with the generally poor hepatic and intestinal tissue architecture in this group of rats. A largely dose-dependent restoration of oxidant and/or antioxidant balance was obtained with the fractions of *Ocimum gratisimum* and these were found to compare very well with Gallic acid employed as a

standard flavonoid in this study. This latter observation suggests that the favorable effects of these compounds on the antioxidant defense systems may be one of the major mechanisms involved in their preservation of tissue integrity observed at microscopy.

We found significant increases in serum levels of Inteleukin 1 beta (IL-1β) and reduction in Tumor necrosis factor (TNF-α) with $CoCl_2$ administration, compared with control rats. Cytokines play a central role in regulation of the immune system. The balance between the productions of pro- and anti-inflammatory cytokines represents one of the key factors regulating the extent of injury in toxicant exposures. Both IL-1β and TNF-α have been regarded as pro-inflammatory cytokines released early in an inflammatory reaction [47]. $CoCl_2$ has been reported to show mixed effects with both pro- and anti-inflammatory effects [48]. Treatment with fractions of OG and Gallic acid caused significant reductions in IL-1β to levels in normal control rats. In our study, while rats treated with $CoCl_2$ alone had reductions in TNF-α, the chloroform and ethyl acetate fractions had varied effects on serum levels of TNF-α.

The reason for the paradoxical actions of $CoCl_2$ on levels of the cytokines studied in our work is not fully understood. Previous findings by Catelas et al [49] would seem to corroborate our findings. The study indicated that low concentrations of either Co^{2+} (0-10ppm) and Cr^{3+} , in vitro, caused concentration and time dependent increases in TNF-α secretion in macrophages, while decreases in TNF-α secretion were obtained at the highest concentrations tested [49]. Decrease in TNF-α level was thought to be due to the high toxicity of these metals at the high concentrations. Time-dependent considerations, relating to the serum half-life of this cytokine may also be responsible for the reduction in TNF-α, observed after necropsy, as there is a possibility that initial elevations in the earlier stages of the study, could have been followed by a reduction in its levels. Robust discussions relating to time-dependent perturbations in cytokine levels have been discussed elsewhere [50].

In conclusion, the present study demonstrates for the first time, evidence for the effectiveness of the flavonoid-rich fractions of OG in preventing $CoCl_2$-induced hepatic and intestinal damage. This protection involved enhancement of antioxidant systems, reduction in levels of oxidants and an attenuation of the activities of pro-inflammatory cytokines, all of which resulted in a generally well preserved architecture of both the liver and intestines.

7. Conflict of interest

The authors declare that they have no conflict of interest

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed in the conduct of the animal experiments in this study.

8. References

1. Scharf B, Clement CC, Zolla V, Perino G, Yan B, Elci SG, Purdue E, Goldring S, Macaluso F, Cobelli N, Vachet RW, Santambrogio L (2014) Molecular analysis of chromium and cobalt-related toxicity. Scientific reports doi: 10.1038/srep05729.
2. Jensen AA, Tüchsen F (1990) Cobalt exposure and cancer risk. Critical Reviews in Toxicology. 20: 427-437.
3. Beyersmann D, Hartvig A (1992) The genetic toxicology of cobalt. Toxicology and Applied Pharmacology. 115: 137-45.
4. Bucher JR, Hailey JR, Roycroft JR, Haseman JK, Sills RC, Grumbein SL, Mellick PW, Chou BJ (1999) Inhalation toxicity and carcinogenicoty studies of cobalt sulfate. Toxicological Sciences. 49: 56-67.
5. Lison D, DeBoek M, Verougstraete V, Kirsch-Volders M (2001) Update on the genotoxicity and carcinogenicity of cobalt compounds. Occup Environ Med. 58 (10): 619- 625.
6. Bozic KJ *et al (2009)*The epidemiology of bearing surface usage in total hip arthroplasty in the United States. *J Bone Joint Surg Am* **91**: 1614–1620, doi:10.2106/JBJS.H.01220.
7. Huber M, Reinisch G, Trettenhahn G, Zweymuller K, Lintner F (2009) Presence of corrosion products and hypersensitivity-associated reactions in periprosthetic tissue after aseptic loosening of total hip replacements with metal bearing surfaces. *ActaBiomater* **5**: 172–180, doi:10.1016/j.actbio.2008.07.032.
8. Gonzales S, Polizio AH, Erario MA, Tomaro ML (2005) Glutamine is highly effective in preventing in vivo cobalt-induced oxidative stress in rat liver. World Journal of Gastroenterology 11(23): 3533–8.
9. Garoui EM, Fetoui H, Makni FA, Boudawara T, Zeghal N (2011) Cobalt chloride induces hepatotoxicity in adult rats and their suckling pups. Experimental and Toxicologic pathology 63: 9-15.
10. Garoui EM, Troudi A, Fetoui H, Soudani N, Boudawara T, Zeghal N (2012) Propolis attenuates cobalt-induced nephrotoxicity in adult rats and their progeny. Experimental and Toxicologic pathology 64: 837-846.
11. Clyne N, Hofman-bang C, Haga Y, Hatori N, Marklund SL, Pehrsson SK, Wibom R (2001) Chronic cobalt exposure af-

fects antioxidants and ATP production in rat myocardium. Scand Journal of Clinical and Laboratory Investigation. 61: 609-614
12. Elbetiela A, Al-thani AS, Al-thani RK, Darmani H, Owais W (2008) Effects of chronic exposure to cobalt chloride on the fertility and testes in mice. Journal of Applied Biological Sciences 2(1): 01-06.
13. Speijers GJA, Krajnc EI, Berkvens JM, van Logten MJ (1982) Acute oral toxicity of inorganic cobalt compounds in rats. Food and Chemical Toxicology. 20: 311-314.
14. Datta K, Sinha S, Chattopadhyay P (2000) Reactive oxygen species in health and disease. Natl Med J India. 13: 304–10.
15. Olivieri J, Hess C, Savaskan E, Ly C, Meier F, Baysang G, Muller-Spahn F. et al (2001) Melatonin protects SHSY5Y neuroblastoma cells from cobalt-induced oxidative stress, neurotoxicity and increased beta-amyloid secretion. Journal of Pineal Research 31:320–5.
16. Beyersmann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Archives of Toxicology* **82**: 493–512, doi:10.1007/s00204-008-0313-y.
17. Cannizzo ES, Clement CC, Sahu R, Follo C, Santambrogio L (2011) Oxidative stress, inflamm-aging and immunosenescence. *Journal of Proteomics* **74**: 2313–2323, doi:10.1016/j.jprot.2011.06.005.
18. Zhai Q, Narbad A, Chen W (2015) Dietary strategies for the treatment of cadmium and Lead Toxicity. *Nutrients* 7:552-571
19. Nakamura CV, Ishid K, Faccin LC, Filho BP, Cortez DA, Rozental S, Souza W, Ueda N (2004) In ***vitro*** activities of essential oils from ***Ocimum gratissimum*** against four ***Candida*** species. ***Research in Microbiology*** 155**(7)**: **579-586.**
20. Ijeh II, Njoku OU, Ekenze EC (2004) Medicinal evaluation of extracts of Xylopiaaethipica and Ocimum gratissimum. J Med. Arm. Plant Sci. 26: 41-49.
21. Aruna K, Sivaramakrishina VM (1990) Plants as protective agents against cancer.Indian Journal of Experimental Biology28(11): 108-111.
22. Iwalokun BA, Ghenle GO, Adewole TA, Akinsinde KA (2003) Shigellocidal properties of the Nigerian Medicinal plants: Ocimum gratissimum, Terminalia aicennaoides and Monmercidicabalsamma. J. Health, population and nutrition. 19(4): 331-335.
23. Nangia-Makker P, Tait L, Sheknar MPU, Palmino E, Hogan V, Piechaochi MP, Funasaka T, Raz A (2007) Inhibition of breast tumor growth and angiogenesis by a medicinal herb. Ocimum gratissimum. International Journal of Cancer. 121 (4): 884-894.
24. Inal ME, Akgun A, Kahraman A (2002) Radioprotective effects of exogenous glutathione against whole-body gamma-ray irradiation: age- and gender-related changes in malondialdehyde levels, superoxide dismutase and catalase activities in rat liver. Methods Find ExpClinPharmacol 24:209-12.
25. Laughton MJ, Evans PJ, Moroney MA, Hoult JR, Halliwell B (1991) Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. Biochemical Pharmacology 42:1673-81.
26. Afanas'ev IB, Dorozhko I, Brodskii AV, Korstyuk VA, Potapovitch A (1989) Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochemical Pharmacology 38: 1763-9.
27. Xia D, Fan Y, Zhang P, Fu Y, Ju M, Zhang X (2013) Protective effects of the flavonoid-rich fraction from rhizomes of Smilax glabraRoxb. On Carbon tetrachloride-induced hepatotoxicity in rats. Journal of Membrane Biology. 246: 479-485
28. Nagma NM, Ashraf J, Siddique J, Abdur R (2012) Protective effect of Rutin against Cadmium-induced hepatotoxicity in Swiss albino mice. Journal of Pharmacology and Toxicology 2012; doi: 10.3923/jpt.
29. Jin X, Qian J, Lu Y (2011) The role of hepatoprotective effect of a flavonoid-rich extract of Salvia plebeian R. Br. On carbon tetrachloride-induced acute hepatic injury in mice. Journal of Medicinal Plants research 5(9): 1558-1563.
30. Xia DZ, Yu XF, Liao SP, Shao QJ, Mou HL, Ma W (2010) Protective effect of Smilax glabra extract against lead-induced oxidative stress in rats. Journal of Ethnopharmacol 130: 414–420.
31. Lowry OH, Rosenbrough NJ, Farr AL (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265–75.
32. Wolff SF (1994) Ferrous ion oxidation in the presence of ferric ion indicator xylenol orange for measurement of hydrogen peroxides. Methods in Enzymology. 233: 182–189.
33. Farombi EO, Tahnteng JG, Agboola AO, Nwankwo JO, Emerole GO (2000) Chemoprevention of 2acetylaminofluorene-induced hepatotoxicity and lipidperoxidation in rats by kolaviron – a Garcina kola seed extract. Food and Chemical Toxicology. 38: 353–541.
34. Claiborne A (1995) Catalase activity. In: Greewald AR, editors. Handbook of Methods for Oxygen Radical Research. Boca Raton, FL: CRC Press. 1995; pp 237–242.
35. Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biological Chemistry. 217: 3170–3175.
36. Habig WH, Pabst MJ, Jacoby WB (1974) Glutathione-S-transferase activity: The enzymic step in mercapturic acid formation. Journal of Biological Chemistry 249:130–139.
37. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179:588-90
38. Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR (1974) Bromobenzene-induced liver necrosis: protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology 11: 151–69.
39. Reitmann S, Frankel S (1957) Colorimetric method for the determination of serum transaminase activity. American Journal of Clinical Pathology. 28: 56–68.

40. Drury RA Wallington EA, Cancerson R (1976). Carlton's Histopathological Techniques, fourth ed. Oxford UniversityPress, Oxford/London/New York.
41. Kim JH, Gibb HJ, Howe P (2006) Sheffer M. Cobalt and Inorganic Cobalt Compounds. Geneva: World Health Organization.
42. Jang I, Jung K, Cho J (2000) Influence of Age on Duodenal brush border membrane and specific activities of brush border membrane enzymes in Wistar rats. Exp. Anim. 49(4): 281-287.
43. Wang XY, Yokoi I, Liu JK, Mori A (1993) Cobalt (II) and nickel (II) ions as promoters of free radicals in vivo: detected directly using electron spin resonance spectrometry in circulating blood in rats. Archives of Biochemica et Biophysica 306: 402–6
44. Zou W, Yan M, Xu W, Huo H, Sun L, Zheng Z (2001) Cobalt chloride induces PC12 cells apoptosis through reactive oxygen species and accompanied by AP-1 activation. Journal of Neuroscience Research 64: 646–53.
45. Olayinka OI, Nta HA, Adeagbo G (2011) Effect of natural antioxidant – Ocimum gratissimum in modulating neurodegenerative changes in rats fed with high concentration of lead acetate J. Med. Plants Research. 5(13): 2743-2747
46. Akinrinmade JF, Akinrinde AS (2013) Effect of oral administration of methanolic extracts of Ocimum gratissimum on intestinal ischemia-reperfusion injury in rats. European Journal of Medicinal plants 3(4): 591-602.
47. Dinarello CA (1988) Cytokines: interleukin-1 and tumour necrosis factor (cachectin). In: Gallin JI, Goldstein IM, Snyderman R, Eds. Inflammation: Basic Principles and Clinical Correlates. New York: Raven Press, 1988; pp195–208, 1988.
48. Oh SW, Lee YM, Kim S, Chin HJ, Chae D, Na K (2014) Cobalt chloride attenuates oxidative stress and inflammation through NF-kB inhibition in human renal proximal tubular epithelial cells. Journal of Korean Medicinal Science. 29: S139-145.
49. Catelas I, Petit A, Zukor DJ, Antoniou J, Huk OL (2003) TNF-alpha secretion and macrophage mortality induced by cobalt and chromium ions in vitro-qualitative analysis of apoptosis. Biomaterials. 24 (3): 383-91.
50. Tarrant JM (2010) Blood Cytokines as Biomarkers of In Vivo Toxicity in Preclinical Safety Assessment: Considerations for Their Use. Toxicological sciences 117(1): 4–16