

Hepatoprotective Effect of *Costus Afer* On Trace Metal Mixture Treated Rats Mediated By Regulation of Oxidative Stress Markers, Inflammatory Cytokines And Bio-Metal Chelation

Brilliance Onyinyechi Anyanwu

University of Port Harcourt

Anthonet N. Ezejiofor

University of Port Harcourt

Ify L. Nwaogazie

University of Port Harcourt

Orish Ebere Orisakwe (✉ orishebere@gmail.com)

University of Port Harcourt <https://orcid.org/0000-0002-5806-9858>

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Abstract

Technological developments have led to exposure to various substances that are harmful to the environment and public health, including heavy metals. In the environment, these grades of metals are usually diverse mixtures shown to cause physiological, biochemical and neurological dysfunctions in humans and laboratory animals. Cadmium, Lead, and mercury have been envisaged to exhibit their hepatotoxic effects by oxidative induction damage and synthesis of reactive oxygen species (ROS). The current work evaluated the protective activity of aqueous leaf extract of *Costus afer* (ALECA)) on liver damage arising from exposure to trace metal mixture (TMM): cadmium chloride (CdCl_2), lead chloride (PbCl_2), and mercury chloride (HgCl_2). Five groups of weight matched Sprague Dawley rats were treated for 90 days. Metal mixture and deionized water were used to treat the 2 groups of rats whereas the other 3 groups were treated with various doses of the ALECA through oral gavage alongside the metal mixture. Hepatic function parameters, oxidative biomarkers, inflammatory cytokines, Morphological changes and trace metals (concentrations were monitored in the liver). TMM treatment resulted in significant increase in ALT, AST, ALP, bilirubin, IL-6, MDA, but decreased albumin, total protein, IL-10, SOD, CAT and GSH levels. TMM also caused some morphological changes and increased the heavy metal (Pb, Cd and Hg) concentrations in the liver. The leaf extract gave a reasonable protective effect on the hepatotoxicity caused by trace metal mixture - through the mechanisms of metal chelation, anti-inflammatory, and antioxidant although this depends on the dosage to the rats. ALECA may be beneficial in the management of liver toxicity.

Introduction

Trace metals occur from both natural and human activities. The continued use of trace metals in the industry, agriculture, medicine and technology has posed both public and eco-health concern (Tchounwou et al. 2012). Metals are systemic toxic agents known to cause several organs damage even at low exposure levels. Lead, Mercury, and Cadmium can be hepatotoxic (Rana et al. 2018). These elements are ranked among the precedence metals that are of enormous concern to the public health (WHO, 2010).

Uptake and elimination of extraneous compounds are the vital functions of the liver (Anyanwu et al. 2018). Hepatotoxicity refers to impairment to the hepatocyte ensuing from overload of chemicals and exogenous compounds which include heavy metals (Singh et al. 2011). Owing to the dominance of these metals in our ecosystem, the long-term effect of a combination of lead, cadmium and arsenic have been studied and liver toxicity recorded by Bhattacharjee et al (2016). Treatment of rats with lead and cadmium mixture have also resulted in liver damage (Yuan et al. 2014).

Hitherto, focus on the unfavorable health effects of metals is largely based on human case studies of chronic high exposure seen in metal industry or in densely polluted environments. Occupational and environmental exposures to metals is a common. There is a need to carry out more studies on the consequences of chronic environmental trace metal and their mixtures in animal models that can be

extrapolated to humans (Kentson et al. 2018). Due to the necessity of mimicking real-life situation, it becomes important to evaluate the toxic consequences of various trace metal exposures.

The chelators from modern medicine have been effective in treating the acute toxicity of metal poisoning, elimination of metal from soft tissues, and formation of non-toxic complexes. Despite their contribution in the treatment of metal poisoning, they also have some drawbacks which include toxicity issues, availability and affordability concerns. These disadvantages constitute the need to provide effective and safe pre-treatment therapy. The knowledge of the relevance of natural antidotes as chemo-preventive agents in metal poisoning being deemed as 'generally regarded as safe' GRAS, affordability and availability has gained traction recently.

Different pharmacological properties of *Costus afer* has given rise to the study of its safe bioactive compounds that may show promise as drug. Protection of the liver from noxious trace metal mixtures is considered worthwhile given the importance. The anatomical proximity of the liver to the intestines predisposes the liver to toxic assault. Hepatotoxicity is usually characterized by increased membrane permeability and changes in enzyme levels. In a hydrocarbon-mediated liver damage, there was a substantial decrease in serum ALP and AST within normal at 400 mg/kg of aqueous leaf extract of *Costus afer* (ALECA) compared to rat treated with 200 mg/kg of the extract through oral gavage (Ukpabi et al. 2012; Boison et al. 2019). The stem extract of *Costus afer* was protective in alcohol-mediated liver damage in rats, suggesting a possible benefit in alcohol liver cirrhosis (Tonkiri et al. 2014; Boison et al. 2019).

Previous studies have also shown that aqueous leaf extract of *Costus afer* (ALECA) may be organo-protective (kidney and testis) in lead-mediated kidney and testis damages via antioxidant mechanisms (Tchamgoue et al. 2018, Ezejiofor and Orisakwe, 2019a; Boison et al. 2019). As much as various studies have reported on exposure of single heavy metal studies, there remain insufficient information on the heavy metal mixtures which represent the real situation of these toxicants in various environmental matrices. Classical and synthetic metal chelators which have become the mainstay of the antidotal management of metal intoxication together with the numerous side effects are non-existent and expensive especially in developing countries (Amadi et al 2019). This study focuses on investigating the hepatoprotective action of aqueous leaf extract of *Costus afer* (ALECA) on male albino rats exposed to trace metal mixture (TMM) of lead, cadmium and mercury.

Materials And Methods

Harvesting of *Costus afer*

Samples of *Costus afer* were collected from a farmland in the University of Port Harcourt, Rivers State, Nigeria, in an area free from air pollution due to vehicular traffic. Mr. A. O. Ozioko, who is a staff of Botany Department, University of Nigeria, Nsukka helped to verify the plant for its authenticity prior to its usage.

Preparation of the *Costus afer* leaf extract

Leaves of *Costus afer* were washed to remove sand particles, pulverized and stored. Two hundred and fifty grams 250g of the pulverized leaf samples were macerated in 500ml deionized water for 24 hours amidst continuous agitations after the method of Ezejiofor and Orisakwe (2017). The mixture was shaken while the pulverized leaves pressed to obtain the extract which was stored in a refrigerator at 4⁰C (Anyanwu et al. 2020a). The process was repeated after every four days of treatment to obtain fresh extract to replace the redundant extract all through the 90 days treatment period (Anyanwu et al. 2020a).

Determination of the aqueous leaf extract of *Costus afer* (ALECA) dosage

A total of 12 rats separated into four equivalent groups that received *Costus afer* 1000 , 2000 , 4000 5000 mg/kg respectively and were observed for 24 hours for any change in physical features or death. At the end of the treatment, no death or change in physical features was recorded. This implied that *Costus afer* has no toxic effect.

To determine the dose used for this study, an assumed dose was used since the extract was confirmed to be relatively safe. Thus, we assumed the dose of 3000 mg/kg and then used 25% of the assumed dose as the low dose, 50% of the assumed dose as the medium dose and 75% of the assumed dose as the high dose.

Phytochemical screening of the plant material

The *Costus afer* leaves were assayed to examine the inherent phytochemical constituents using the method of Harbone, (1998); Sofowora, (2006); Trease and Evans, (2009).

Animal care handling

The study used thirty-five male albino rats that were about 8 weeks old with 100-200g weights procured from the Animal House of the Faculty of Pharmacy, University of Port Harcourt, Nigeria. The study utilized the animal husbandry procedure established in previous studies by Ezejiofor and Orisakwe (2017) and Anyanwu et al. (2020b).

Design of the experiment

Five groups of seven rats each based on their weight were used for this study. The first group was as the standard control on which only deionized water was administered whereas the second group received only trace metal mixture (PbCl₂, 20 mg/kg; CdCl₂, 1.61 mg/kg; HgCl₂, 0.40 mg/kg) (Sigma Aldrich WGK Germany) (Institoris et al. 2006 and Anyanwu et al. 2020a). Groups 3, 4 and 5 received trace metal mixture TMM and aqueous leaf extract of *Costus afer* (ALECA) at 750, 1500 and 2250 mg/kg respectively (Anyanwu et al. 2020a). These treatments were done four times in a week for 90 consecutive days through oral gavage.

Necropsy

After 90 days, the rats were anaesthetized with and sacrificed. The samples were collected according to the procedures recorded by Anyanwu et al. (2020a; 2020b).

Preparation of liver homogenate

Liver tissue was homogenized in buffer (pH 7.4). The supernatant was collected after centrifugation (3000 rpm for 15mins at 4⁰C) for inflammatory and antioxidant analysis.

Metal analysis

About 6ml and 2ml of nitric acid and perchloric acid respectively were used for the acid digestion of the liver after isolating the weighed organ. The samples were left for 30 mins after acidification before being heated at 105⁰C until digestion was completed. Whatmann filter paper Number (1) was used for filtration so as to obtain clearer samples. The solution was later made up to 15 ml (final volume) with deionised water. All the glassware were thoroughly washed and rinsed before use. Calibration curves of Pb, Cd and Hg as previously described by Anyanwu et al. (2020a; 2020b). Solar thermo elemental flame Atomic Absorption Spectrometer (Model SG 71906) was used to determine the levels of Pb, Cd and Hg at a detection limit of <0.001 mg/kg. Standard operating parameters were set and the hollow cathode lamps for Pb, Cd and Hg (Model SG 71906) were employed as radiation source and fuel was air acetylene. All the samples and standard were run in duplicate.

Hepatic biomarkers

Estimation of alanine aminotransferase (ALT) and Aspartate transaminase AST

The alanine aminotransferase (ALT) and Aspartate transaminase AST activities of the liver samples were assayed employing a Randox kit (Reitman and Frankel 1975).

Estimation of ALP

The ALP function was determined with the aid of standard diagnostic kits (Randox Laboratories Ltd, UK) using the calorimetric endpoint (Klein et al., 1960).

Estimation of total and direct bilirubin

This was done using standard diagnostic kits in a calorimetric process (Randox Laboratories Ltd, UK; Jendrassic and Groff, 1938)

Estimation of total protein and albumin

This was done using a standard diagnostic kit

Antioxidant analysis

CAT activity

CAT activity was assayed by adapting the method of Clairborne (1995).

Estimation of hepatic glutathione (GSH) level

Glutathione (GSH) level was estimated after the method of Sedlak and Lindsay (1968) .

Estimation of SOD activity

Following the method of Misra and Fridovich (1972), superoxide dismutase was determined.

Lipid peroxidation marker (MDA) activity

Following the method of Ohkawa and Ohishi (1979), the MDA was evaluated.

Evaluation of Inflammatory cytokines [Interlukin-6 (IL-6) and Interlukin-10 (IL-10)]

Enzyme-linked immunosorbent assay (ELISA) was employed. The enzyme linked immunosorbent assay kit (ELISA -Bioassay Technology Laboratory, 1713 Junjiang Inter. Bldg. 218 Ningguo Rd. Yangpu Dist. Shanghai, China) with the sensitivity of 0.052ng/L and 1.51pg/ml respectively as outlined in Anyanwu et al. (2020a) were used.

Histopathological examination

Tissues were soaked in 10% formaldehyde, sectioned and treated with haematoxylin and eosin H&E. The H&E treated tissues were finally examined with a microscope at 200x magnification following the procedures outlined in Anyanwu et al. (2020a).

Statistical analysis

The analysis of variance (ANOVA) was applied to the sequence of observations for the purpose of comparative analysis at 5% significance. Multiple comparisons were carried out with Duncan's multiple comparison method. The principal component analysis (PCA) was employed to select the principal factors (or independent variables) for developing the multiple regression equations. In this study, the use of PCA was accomplished using XLSTAT 2016 (Nwaogazie, 2011; Anyanwu et al. 2020b).

Results

The phytoconstituents of aqueous leaf extract of *Costus afer* (ALECA) is shown on Table 1.

Table 1: Quantitative phytochemical screening (mg/100g) of the aqueous leaf extract of *Costus afer* (ALECA)

Chemical constituents	Content
Alkaloids	4.3±0.10
Phenolic compounds	1.23±0.05
Saponins	2.9±0.08
Tannins	2.72±0.11
Flavonoids	25±0.13

1. Rats treated with with the trace metal mixture TMM only, had a considerable increase in liver weight ($p < 0.05$) compared to the normal control rats (received only deionized water). Rats that received both aqueous leaf extract of *Costus afer* (ALECA) and trace metal mixture (TMM)

had decreased liver weight (Table 2). The absolute and relative weightsof liver of rats that received only trace metal mixture (TMM) was 9.4 ± 1.35 g and 3.62 ± 0.47 % respectively while the rats administered with only deionized water had 5.08 ± 0.95 g and 2.45 ± 0.41 % respectively.

Table2: Effect of aqueous leaf extract of *Costus afer* on the absolute and relative weight of liver treated with trace metal mixture (TMM)

Treatment	*Absolute (g)	Relative (%)
Deionized H ₂ O	5.08 ± 0.95^a	2.45 ± 0.41^a
TMM	9.4 ± 1.35^c	3.62 ± 0.47^c
TMM + 750 mg/kg ALECA	8.48 ± 1.13^{bc}	3.52 ± 0.42^{bc}
TMM + 1500 mg/kg ALECA	8.36 ± 0.52^{bc}	3.15 ± 0.17^{bc}
TMM + 2250 mg/kg ALECA	7.86 ± 0.22^b	3.06 ± 0.08^b

*Values = Mean $\pm$ SD, N=5., data with different superscripts (a, b, c) are significantly different from each other ($p < 0.05$), data with the same superscripts are not significantly different; TMM = Trace metal mixture

The tests for liver function were done to evaluate the likely protective role of *Costus afer* treatment from metal mixture exposure. Treatment with trace metal mixture (TMM) caused significant elevation in aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase levels, bilirubin (total and direct), and a decrease in total protein and albumin, whereas the rats that received both aqueous leaf extract of *Costus afer* (ALECA) and trace metal mixture (TMM) showed reduction in the liver enzyme markers and a rise in total protein and albumin in comparison to metal mixture-treated albino rats (Fig. 1). The aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, total

bilirubin, direct bilirubin, total protein and albumin levels in rats treated with only trace metal mixture (TMM) were significantly different (172 μ /l, 77.8 μ /l, 222.2 μ /l, 32.6 mg/dl, 15.2 mg/dl, 43.8 g/l, 25.4 g/l, $p < 0.05$) respectively, from the groups that received both aqueous leaf extract of *Costus afer* (ALECA) and trace metal mixture (TMM).

Figure 2 is the parallel coordinates plot showing clustering of liver function parameters interaction in different treatment groups. The Fig consists of three classes; class 1 (gp 1 and 5), class 2 (gp 2 and 3), and class 3 (gp 4). The factor loading of liver function variables on significant principal components which was done sequel to the Varimax rotation is presented in Table 3 whereas the correlation plot is shown in Fig. 3. An illustration of the differentiation of parameters and the interactions among liver function parameters in different groups is depicted in Fig. 4. A 3-component system showing 95.35% of total variance was obtained after statistical principal component analysis.

The co-ordinate plot of liver function parameters showing the association between the variables consists of 3 classes. The result showed that class 1 rats had high total protein and albumin levels with low aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, total bilirubin and direct bilirubin, while class 2 rats had high aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, total bilirubin and direct bilirubin levels with low total protein and albumin levels. Class 3 rats were of the intermediate class showing that they had midrange values for all the liver function parameters.

The Principal component analysis (PCA) provided a better interpretation of the data, having grouped the connected variables as one. The male albino rats administered with trace metal mixture (TMM) of Pb, Cd and Hg, seven principal factors (F1-F7) explained 100% cumulative variations in the liver function parameters. The first two components (PC1 and PC2) accounted for more than 92% of total variance, while PC3 gave rise to only 2.80% of the total variance. Factor 1 (F1) had Eigen value > unity explaining 87.34% of total variation of the data set and was regarded as being significant, while factors 2 and 3 (F2 and F3) had Eigen values < unity explaining 7.95% of the total variation. (Anyanwu et al. 2020a).

Strong positive correlations (≥ 0.88) were perceived between these parameters (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, total bilirubin and direct bilirubin) whereas a weak association ($r = 0.6617$) was observed between the Total protein and albumin. Also, both factors had negative correlations with aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, total bilirubin and direct bilirubin which is an indication that a reduction in total protein and albumin would likely lead to an increase in aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, total bilirubin and direct bilirubin and vice versa. All the variables were printed in bold in PC1 (Table 2) whereas aspartate aminotransferase had the highest loading on PC1 after Varimax rotation. The principal components were extracted with the aid of a 3D graphing software to plot the correlated variables.

Table 3

Loadings of liver function variables on significant principal components after Varimax rotation

Liver function parameter	*PC1	PC2	PC3
aspartate aminotransferase (U/L)	0.9105	0.0017	0.0280
alanine aminotransferase (U/L)	0.9443	0.0023	0.0129
alanine phosphatase (U/L)	0.9071	0.0020	0.0036
total bilirubin	0.9192	0.0075	0.0004
direct bilirubin (mg/dl)	0.9258	0.0008	0.0093
total protein (g/l)	0.8008	0.0787	0.1166
ALBUMIN(g/l)	0.7059	0.2677	0.0250
Eigen value	6.1136	0.3607	0.1958
Variability (%)	87.3368	5.1532	2.7973
Cummulative (%)	87.3368	92.4900	95.2873
*Bold figures are highly correlated coefficients; and thus, the principal factors. AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alanine phosphatase, T.BIL = total bilirubin, D.BIL = direct bilirubin, T.Protein = total protein			

An assessment of the inflammatory status after treatment with trace metal mixture TMM was done by evaluating the pro- and anti-inflammatory cytokines level in the liver. The co-treatment using *Costus afer* significantly decreased the levels of pro- and also increased ($p < 0.05$) the anti-inflammatory cytokines (IL-6 and IL-10) in the liver tissue in comparison to in the trace metal mixture TMM-treated group (Fig. 5), suggestive of anti-inflammatory activity of aqueous leaf extract of *Costus afer* (ALECA). The pro- and anti-inflammatory cytokine levels (IL-6 and IL-10 respectively) of rats administered with the trace metal mixture TMM only were significantly different (61.8 Pg/g tissue and 14.7 Pg/g tissue, $p < 0.05$) from the inflammatory cytokines seen in rats co-treated with *Costus afer*.

The oxidative status in the hepatocyte subsequent to the metal mixture treatment using the lipid peroxidation marker, MDA level was evaluated. The 90-day treatment which was done with the following metal mixture in the following dosage PbCl_2 - 20mg/kg, CdCl_2 - 1.61mg/kg, HgCl_2 – 0.40mg/kg body weight induced oxidative reaction in the organ (liver tissue). The MDA level increased significantly ($p < 0.05$) in the hepatocyte of trace metal mixture TMM -treated rats in comparison to those of the normal control group (Fig. 6). A significant decrease in oxidant level was observed in the rats treated with *Costus afer* and metal mixture when compared to those treated with only the trace metal mixture TMM.

With respect to how the treatment affects the non-enzymatic glutathione (GSH) and enzymatic superoxide dismutase and catalase (SOD and CAT) activities in the liver tissue. Treatment with trace metal mixture TMM resulted in a significant decrease ($p < 0.05$) in glutathione and superoxide dismutase and catalase levels in comparison with control. Rats that received aqueous leaf extract of *Costus afer*

(ALECA) plus trace metal mixture TMM had elevated levels of glutathione and superoxide dismutase and catalase in comparison to rats that received only trace metal mixture TMM.

Heavy metal concentration on the liver of the rat samples

The concentration of trace metals (lead, cadmium and mercury) in the liver were notably elevated ($p < 0.05$) in the liver of the rats treated with the trace metal mixture TMM- in comparison to the control (Table 4). Treatment of rats with aqueous leaf extract of *Costus afer* (ALECA) plus trace metal mixture TMM resulted in significant reduction in trace metals (lead, cadmium and mercury) levels in comparison to rats that received only trace metal mixture TMM. In addition, the group exposed to the trace metal mixture TMM only, had the highest level of trace metals (lead = 90.992 ± 13.284 , cadmium = 0.78 ± 0.133 and mercury = 0.305 ± 0.0439) in comparison to the control group. Pearson's rank correlation analyses indicate the inter-trace metal relationship among trace metals in liver of rats showed strong positive correlation ($r > 0.90$) between metals such as: Pb and Cd, Pb and Hg, as well as Cd and Hg. All correlations were significant at $p < 0.01$ (Fig. 7).

Table 4
Levels of trace metals (mg/kg) in the liver

Treatment	Cadmium (Cd)	Mercury (Hg)	Lead (Pb)
Deionized H ₂ O (only)	$< 0.001 \pm 0.000^a$	$< 0.001 \pm 0.000^a$	0.392 ± 0.552^a
TMM (only)	0.782 ± 0.133^c	0.305 ± 0.039^c	90.992 ± 13.284^c
TMM + 750 mg/kg ALECA	0.143 ± 0.046^b	0.051 ± 0.020^b	31.007 ± 6.017^b
TMM + 1500 mg/kg ALECA	0.033 ± 0.024^a	0.009 ± 0.001^a	8.857 ± 4.849^a
TMM + 2250 mg/kg ALECA	0.003 ± 0.002^a	0.001 ± 0.001^a	3.500 ± 1.141^a
*Values are expressed as (Mean $\pm$ SD). Values in the same column with different superscripts are significantly different from each other ($p < 0.05$) and those with the same superscripts in the same column are not significantly different; TMM = Trace metal mixture			

Histopathology of the liver

The liver sections of six different groups were sectioned and presented in (Fig. 8 (G1), (G2), (G3), (G4), and (G5) representing groups treated with deionized H₂O, metal mixture (MM), (MM + 750 mg/kg ALECA, MM + 1500 mg/kg ALECA, and MM + 2250 mg/kg ALECA.

Regression Model Development

XLSTAT 2016 software was employed for the model development in this study. Lead, Cadmium, Mercury, and ALECA were considered as the input data and were used for the calibration of the model using multiple linear regressions. Zero coefficients for x_2 and x_3 were observed as outputs and thus subsequent calibrations were done without the 3 constant parameters (input data).. In other words, y is made a

function of ALECA variable for a given set of the heavy metal variables namely: Lead, Cadmium, and Mercury. A plot of catalase concentration vs ALECA concentration was modelled using linear, quadratic and exponential options (Table 5). The R^2 , MSE and RMSE values were determined and shown in Table 6 for the process.

For verification of the calibrated models, the observed model was compared with predicted model on the liver catalase level (Fig. 9) with corresponding $R^2 = 0.935$.

The quadratic and exponential models for the liver catalase level was R^2 , MSE and RMSE of (0.935, 1.513 and 1.230) and (0.880, 1.022 and 1.011) respectively. The linear model best predicted the process with R^2 , MSE and RMSE values of. 0.935, 0.760 and 0.872 respectively. A repetition of the regression models was done for other liver parameters and based on R^2 , MSE and RMSE, the best models were selected. To verify the calibrated models, it was important to compare observed against predicted liver catalase level with corresponding $R^2 = 0.999$. The modeled liver catalase values for ALECA plus TMM treated groups at 0 mg/kg, 750 mg/kg, 1500 mg/kg and 2250 mg/kg were 2.680, 4.770, 6.860 and 8.950, while the observed values were 2.360, 5.640, 6.080 and 9.180 respectively. Similarly, the verification was equally done on other parameters in the liver. These models could help predict the residual values of liver function parameters, antioxidant profile and inflammatory cytokines in the liver of male albino rats at any treatment dose using ALECA to a high precision provided the levels of lead, cadmium, and mercury remains constant as considered in this work.

Table 5
Regression models of catalase

Type of Model	Equation	R^2	MSE	RMSE
Exponential	$y = 2.801e^{0.000x}$	0.880	1.022	1.011
*Linear	$y = 0.002x + 2.68$	0.935	0.760	0.872
Quadratic	$y = -8E-08x^2 + 0.003x + 2.635$	0.935	1.513	1.230
NB: * The model with the best fit.				

A replication of regression models was done on other biomarkers and the best with respect to R^2 , MSE and RMSE values was selected and summarized as shown in the Table 6 below;

Table 6

Model equations for antioxidant biomarkers, inflammatory cytokines and other test parameters in the liver

Biomarkers	Type of Model	Model Equations	R ²	MSE	RMSE
SOD	Quadratic	$y = 8\text{E-}08x^2 + 7\text{E-}05x + 0.237$	0.999	0.000	0.011
GSH	Quadratic	$y = 1\text{E-}07x^2 + 0.000x + 0.814$	0.999	0.000	0.018
MDA	Quadratic	$y = 3\text{E-}08x^2 - 0.000x + 0.645$	0.996	0.001	0.025
IL-6	Quadratic	$y = 4\text{E-}06x^2 - 0.032x + 62.33$	0.996	5.618	2.370
IL-10	Exponential	$y = 13.98e^{0.000x}$	0.991	5.387	2.321
AST	Quadratic	$y = -9\text{E-}06x^2 - 0.013x + 171.7$	0.999	1.152	1.073
ALT	Linear	$y = -0.021x + 80.32$	0.978	14.904	3.861
ALP	Exponential	$y = 222.6e^{-2\text{E-}0x}$	0.996	7.730	2.780
T. Bilirubin	Quadratic	$y = -8\text{E-}07x^2 - 0.007x + 32.75$	0.998	0.450	0.671
D. Bilirubin	Exponential	$y = 15.53e^{-5\text{E-}0x}$	0.995	0.174	0.417
T. Protein	Quadratic	$y = 2\text{E-}06x^2 + 0.006x + 43.72$	0.999	0.128	0.358
Albumin	Quadratic	$y = -7\text{E-}07x^2 + 0.007x + 25.56$	0.995	0.512	0.716
Where y = concentration of the parameter analyzed, x = <i>Costus afer</i> dose, MSE = Mean Squared Error, RMSE = Root Mean Squared Error, AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alanine phosphatase, T.BIL = bilirubin(total), D.BIL = bilirubin (direct), T.Protein = total protein(total), SOD = superoxide dismutase, GSH = reduced glutathione, MDA = malondialdehyde, IL-6 = interleukin-6, and IL-10 = interleukin-10					

Discussion

According to Jarup (2003), most health challenges can be attributed to trace metals such as lead, cadmium, and mercury. These metals have also been associated with infertility, neurotoxicity, osteoporosis, diverse organ failures in humans (Yuan et al. 2014; Youness et al. 2012; Jang and Hoffman, 2011). The liver helps in the metabolism and excretion and hence their susceptibility to the adverse effects of foreign compounds (Anyanwu et al. 2018). Given the ubiquity of metals, some researchers have investigated the effects of chronic exposure of low doses of a mixture of cadmium and mercury (Hamdy et al 2018, Mumtaz et al. 2019). They posited that long term exposure to trace metal mixture even at low affected the liver of the rats adversely (Hamdy et al 2018, Mumtaz et al. 2019).

Majority of the actions of ALECA can be attributed to the inherent antioxidant properties of *Costus afer*. The alkaloids, saponins, flavonoids, tannins, and phenols, are the phytoconstituents on the leaf extracts

of *Costus afer* and may be responsible for its biological activity. Flavonoids are water soluble polyphenolic compounds with fifteen atoms that are antioxidant in nature (Kurhekar, 2018) (Ezejiofor and Orisakwe, 2017). Natural phenolic compounds have antioxidant and anti-inflammatory effects. Furthermore, tannins are water-soluble polyphenolic compounds within plants of secondary metabolism and are usually of two classes: the hydrolysable and condensed tannins (Haslam, 1998; Ezejiofor and Orisakwe, 2017). Their solutions are acidic with a characteristic astringent taste; they are also known to have strong antimicrobial activities in addition to other physiological effects which help to enhance blood clotting, as well as anti-hypertensive, anti-hyperlipidemic and immunomodulatory (Wang and Xue, 2010). Saponins are glycosides that are usually foamy in nature and have good medicinal value owing to their therapeutic action. Desai et al. 2009 reported that they help to protect plants from pathogenic attacks giving rise to their antimicrobial activity. Alkaloids are nitrogenous compounds which are used as supplements, ingredients, supplements and for medical and pharmaceutical purposes (Ziegler and Facchini, 2008; Kurek, 2019).

A significant increase ($p < 0.05$) was observed in weights of liver treated with the trace metal mixture TMM (only) in comparison to the control rats. However, a significant decrease weight (absolute and relative) was observed in the samples administered with aqueous leaf extract of *Costus afer* (ALECA) (750, 1500 and 2250 mg/kg) respectively in a dose-dependent manner in corroboration with the study of Bhattacharjee et al. (2016) who reported a marked elevation in the weight of the liver in rats treated with a trace metal mixture of Pb, Cd and As. Kluwe (1981), Simmoni et al. (1995) and Orisakwe et al. (2004) further highlighted that increase or decrease in weights of organs after treatment with a chemical substance suggest toxicity. Hence, this indicates that the liver could be a target organ for trace metal mixture toxicity. The increased weight of liver observed in the present work corroborates the work of Ahmad et al. (2007). This current study indicates that the aqueous leaf extract of *Costus afer* (ALECA) may be hepatoprotective.

The significant increment ($p < 0.05$) seen in the rats that received trace metal mixture TMM with respect to ALT, AST, ALP, total and direct bilirubin levels which formed the markers of injury to the hepatocyte could arise from increased membrane permeability and cell loss due to membrane oxidation (Iniaghe et al. 2008; Ramachandran and Jaeschke 2018). Liver cell death (hepatocellular necrosis) or the membrane lesions increase the AST and ALT serum levels; flow into the bloodstream from the liver, in line with an increase in enzyme levels of metal mixture intoxicated rats. These observations indicate cell loss and integrity loss of functional cellular membranes in the liver in accordance with the studies of Zhang et al. 2018 and Ramachandran and Jaeschke 2018. increased level of bilirubin is another marker of abnormal liver function (Kang 2013). The liver is a chemical laboratory of the body involved in oxidation and metabolic conversion of fatty acid, production of cholesterol and phospholipids and the elimination of specific classes of serum lipoprotein (Kio et al. 2018). Decreased total protein and albumin levels evident in rats treated with trace metal mixture TMM only could be as a result of inhibition of protein synthesis and metabolism (Yousuf 2002) which gave rise to hepatotoxicity (Nagao and Sata 2010).

Many previous studies described trace metals as immune-suppressors that result in elevated levels of TNF- α , IL-1 β and IL-6(pro-inflammatory cytokines). The elevated level of pro-inflammatory cytokine (IL-6) in this study may be due to heightened production of reactive oxygen species (Gao 2012; Guyot et al. 2015). Oxidative stress could be associated with an excessive production of the pro-inflammatory cytokines (Gao 2012). The concomitant administration of aqueous leaf extract of *Costus afer* (ALECA) and trace metal mixture TMM resulted in dose dependent reduction in levels of pro-inflammatory cytokine (IL-6) and increase in anti-inflammatory cytokine IL-10. Inflammation is caused by several factors, such as a group of secreted polypeptides called cytokine which control host responses to lesions, some are anti-inflammatory cytokines, while others function as pro-inflammatory cytokines (Dinarello 2010; Niederreiter and Tilg 2018). An elevation in the pro-inflammatory cytokine (IL-6) as well as a decrease in the anti-inflammatory cytokine (IL-10) were observed in the rats exposed to trace metal mixture TMM only. These effects were alleviated upon administration of aqueous leaf extract of *Costus afer* (ALECA) in a dose-dependent manner suggestive of anti-inflammatory potency of aqueous leaf extract of *Costus afer* (ALECA).

Elevated levels of antioxidant enzymes were seen in rats that received aqueous leaf extract of *Costus afer* (ALECA) plus trace metal mixture TMM in comparison to those that received only trace metal mixture TMM which may suggest that aqueous leaf extract of *Costus afer* (ALECA) has antioxidant and hepatoprotective effects.

This observation is in corroboration with work of Ezejiofor and Orisakwe (2017). Superoxide dismutase is the first defense interval for the conversion of superoxide radical anion to the production of free radicals involved in hydrogen peroxide, whereas catalase serve as the second antioxidant protection mechanism by reducing hydrogen peroxide to oxygen and water (Younus, 2018). Thus, aqueous leaf extract of *Costus afer* (ALECA) increased antioxidant enzymes levels which is in agreement with the previous study protective effects of *Costus afer* on the liver by Ezejiofor and Orisakwe (2017).

Despite the fact that the exact mechanism of the metal mixture toxicity is unclear, the observations in this study indicates that treatment with trace metal mixture TMM elicits reactive oxygen species (ROS) generation and impairment cellular antioxidant capacity. This could result in an imbalance between free radical species and the body's resistance against cellular damage (Koivula and Eeva 2010). Therefore, supplementation of antioxidant molecules would be exogenously beneficial in cell antioxidant protection to neutralize heavy metal poisoning. Aqueous leaf extract of *Costus afer* (ALECA) contains antioxidant phytochemicals such as phenolics and flavanoids (Ezejiofor et al.2017). This study has shown the protective effect of aqueous leaf extract of *Costus afer* (ALECA) over trace metal mixture TMM -induced toxicity in rats.

Malondialdehyde, a marker of lipid peroxidation, showed a substantial increase ($p < 0.05$) in the liver of rats that received trace metal mixture TMM in comparison with groups that received aqueous leaf extract of *Costus afer* (ALECA) plus trace metal mixture TMM. Malondialdehyde is a marker used to measure the level of oxidative stress in an organism (Singh et al. 2014). A similar finding revealed an elevation in lipid

peroxidation in the liver after metal poisoning (Ezejiofor and Orisakwe 2017). This suggests that peroxidative injury may be involved in the development of complications of severe heavy metal toxicity.

The significant decrease in trace metals namely lead, cadmium and mercury following administration of aqueous leaf extract of *Costus afer* (ALECA) is prominent. Chelation of these trace metals by some phytoconstituents of ALECA may be a plausible mode of action notwithstanding that actual mechanism remain largely unknown. Only recently some researchers have opined that mopping of free radicals and chelation of metal chelation are essential features in management of oxidative stress (Policegoudra et al. 2010, Jomova and Valko 2011; Sarpong-Kumankomah et al. 2018). There is a need for further study in this regard to ascertain the actual route of metal elimination.

Pearson's rank correlations of trace metals lead, cadmium, and Mercury in the liver show strong significant relationships ($r > 0.90$) between cadmium and lead ($r = 0.940$, $p < 0.05$, $n = 18$), Mercury and lead ($r = 0.982$, $p < 0.05$, $n = 18$), Mercury and Cadmium ($r = 0.960$, $p < 0.05$, $n = 18$) in the liver. This strong association signifies a close physiological connection (Krebs et al., 2014). The strongest association was observed between liver Mercury and liver Lead. This relationship between these trace metals could as a result of the similarity of their oxidative states which makes them exhibit similar chemical properties.

Certain histological changes, such as hepatocyte necrosis and inflammation and severe dysplasia of hepatocytes, were observed in the rats that received trace metal mixture in comparison to the rats that received only deionized water. Hepatic necrosis, inflammation and cytoplasmic swelling are common symptoms of liver damage (Butler et al 2018). The protective effect of the *Costus afer* was observed in the rats co-treated with ALECA at doses of 750, 1500, and 2250 mg/kg. Whereas the rats which received low dose aqueous leaf extract of *Costus afer* (ALECA) plus trace metal mixture showed hepatocyte necrosis and cytoplasmic swelling, higher doses of aqueous leaf extract of *Costus afer* (ALECA) plus trace metal mixture showed mild cytoplasmic swelling and mild hepatocyte inflammation. The dose dependent histomorphological protection of aqueous leaf extract of *Costus afer* (ALECA) against trace metal mixture mediated damage may confer on ALECA a beneficial role

Conclusion

Trace metal mixture exposure may be of significant health effect arising from oxidative damage, inflammation and distortion of histoarchitecture of the liver in rat model. The observed attenuation of destructive effects of TMM by effects tended to be attenuated by the aqueous leaf extract of *Costus afer* (ALECA) which could proffer some hope as an alternative remedy and circumvent the major drawbacks of notable chelators: increase in toxicity, inaccessibility and high-priced associated with modern medicine. Therefore, *Costus afer* could be a potential hepatoprotective agent.

Declarations

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Ethical Approval: The protocol for the experiment was approved by the University of Port Harcourt Research Ethics Committee and the reference number UPH/CEREMAD/REC/04 was assigned.

-Consent to Participate: Not applicable

-Consent to Publish: All authors have given their consent for publication

-Authors Contributions BOA: Data curation, Bench work and draft manuscript preparation; ANE: supervision and bench work; ILN: data analysis and modeling and OEO: conceptualization, supervision and manuscript preparation

-Funding: BOA received tuition waiver from World Bank in the course of PhD her training

-Competing Interests: Authors confirm that there was no conflict of interest

-Availability of data and materials: All data have been provided

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Figures

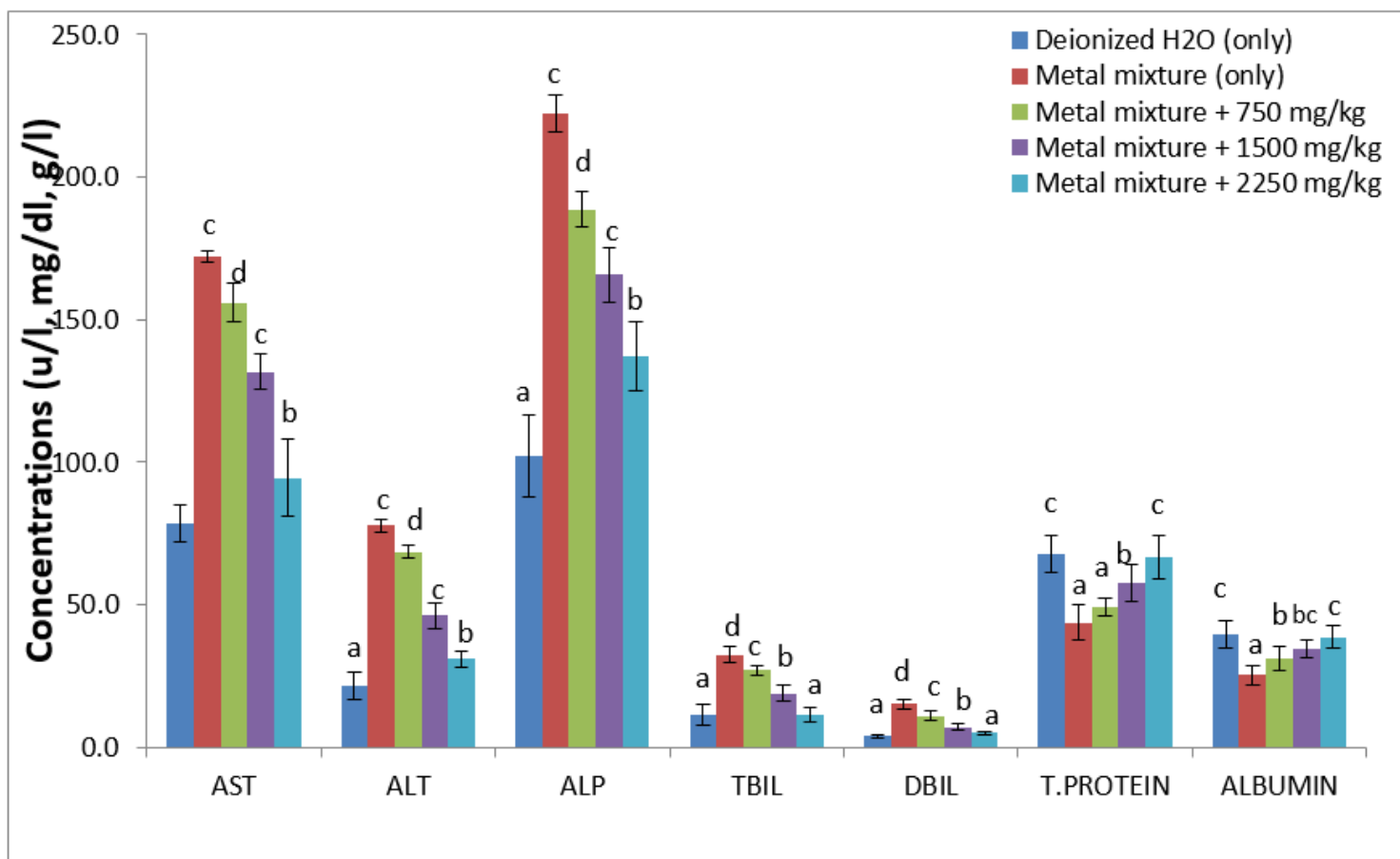


Figure 1

Effect of *Costus afer* extract on serum hepatic enzymes of male albino rats treated with metal mixture. Values with different superscripts (a, b, c, d) are significantly different from each other ($p < 0.05$), while those with the same superscripts are not significantly different. T.Protein = total protein, TBIL = total bilirubin, AST = aspartate aminotransferase, ALP = alanine phosphatase, ALT = alanine aminotransferase, DBIL = direct bilirubin,

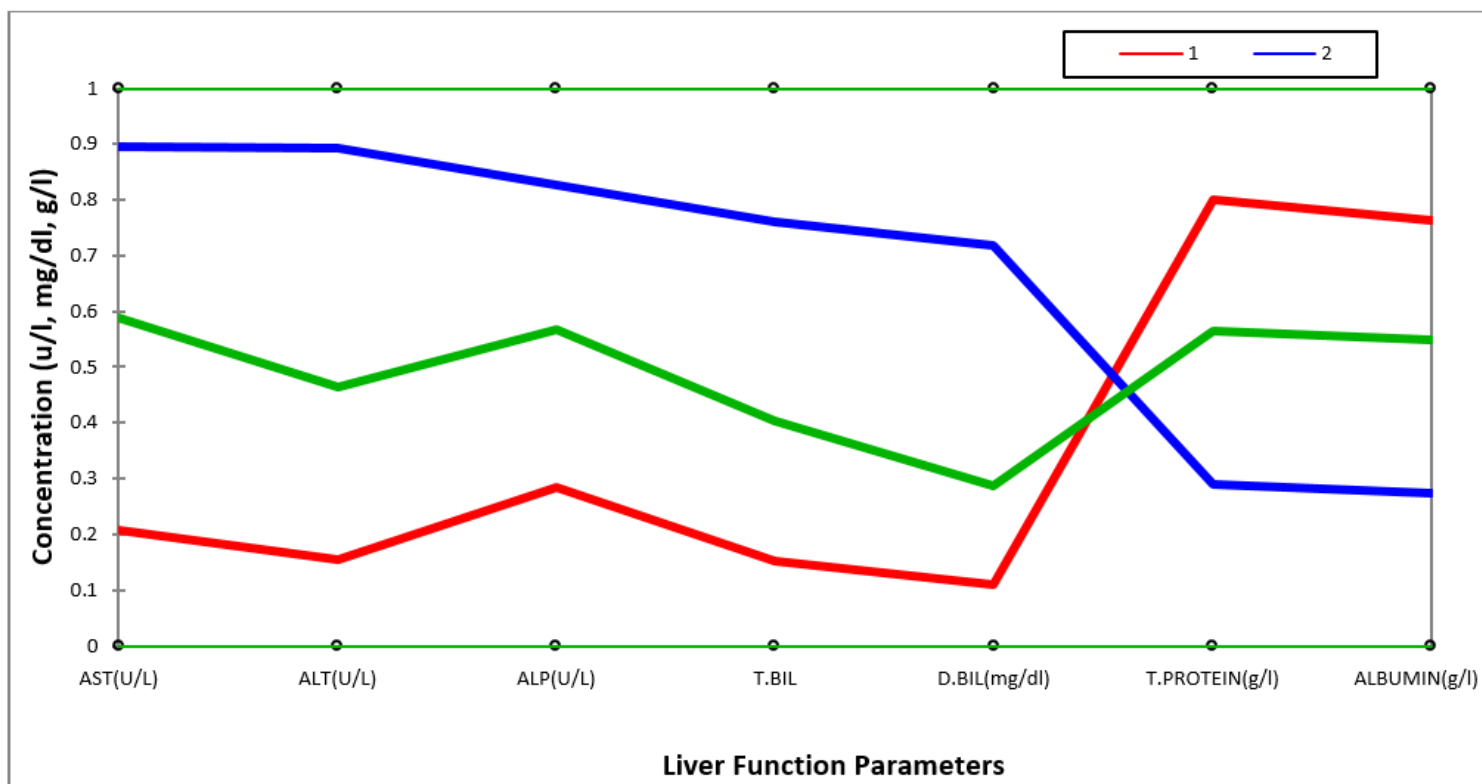


Figure 2

Plot of clusters of liver function parameters interaction in groups; T.Protein = total protein , TBIL = total bilirubin, AST = aspartate aminotransferase, ALP = alanine phosphatase, ALT = alanine aminotransferase, DBIL = direct bilirubin,

Variables (axes F1 and F2: 92.49%)

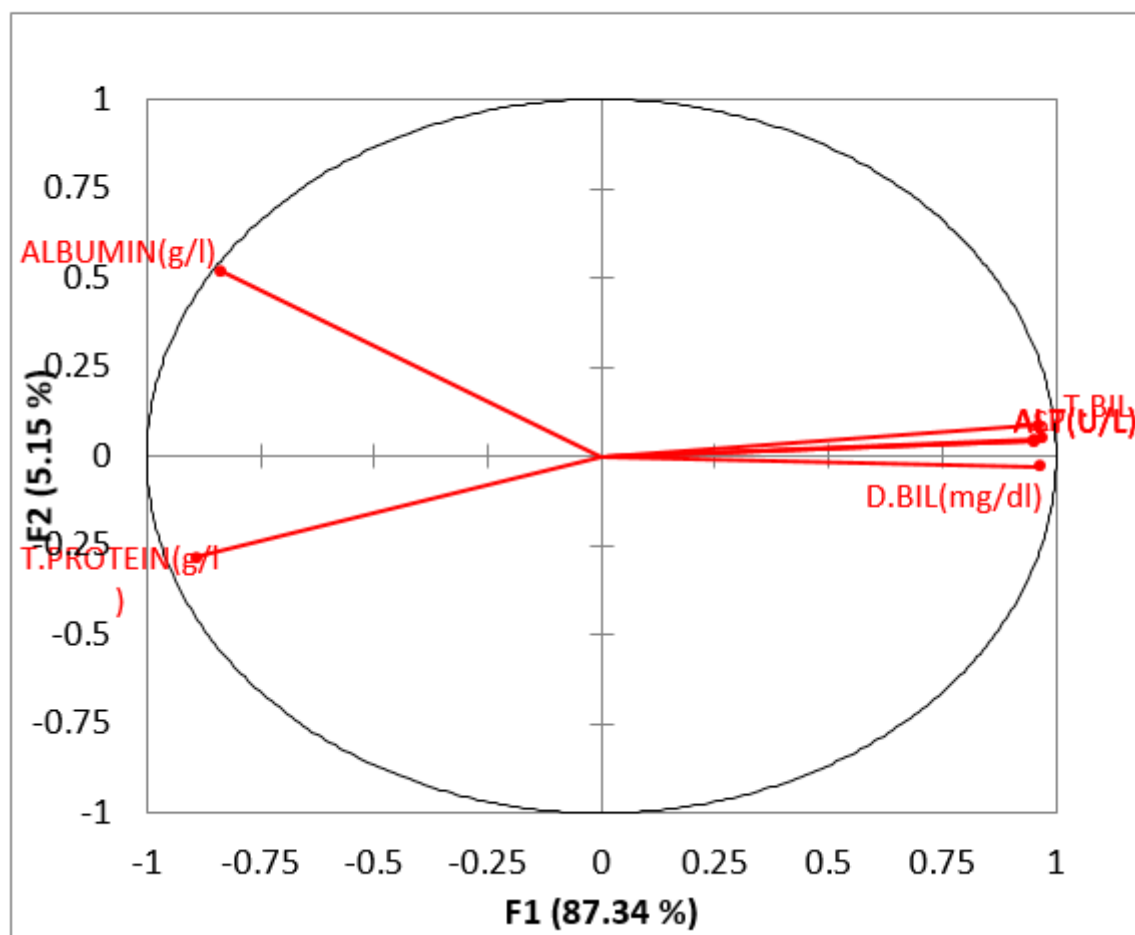


Figure 3

Correlation plot of liver function parameters on male albino rats against the generated factors (F1 and F2). T.Protein = total protein , TBIL = total bilirubin, AST = aspartate aminotransferase, ALP = alanine phosphatase, ALT = alanine aminotransferase, DBIL = direct bilirubin,

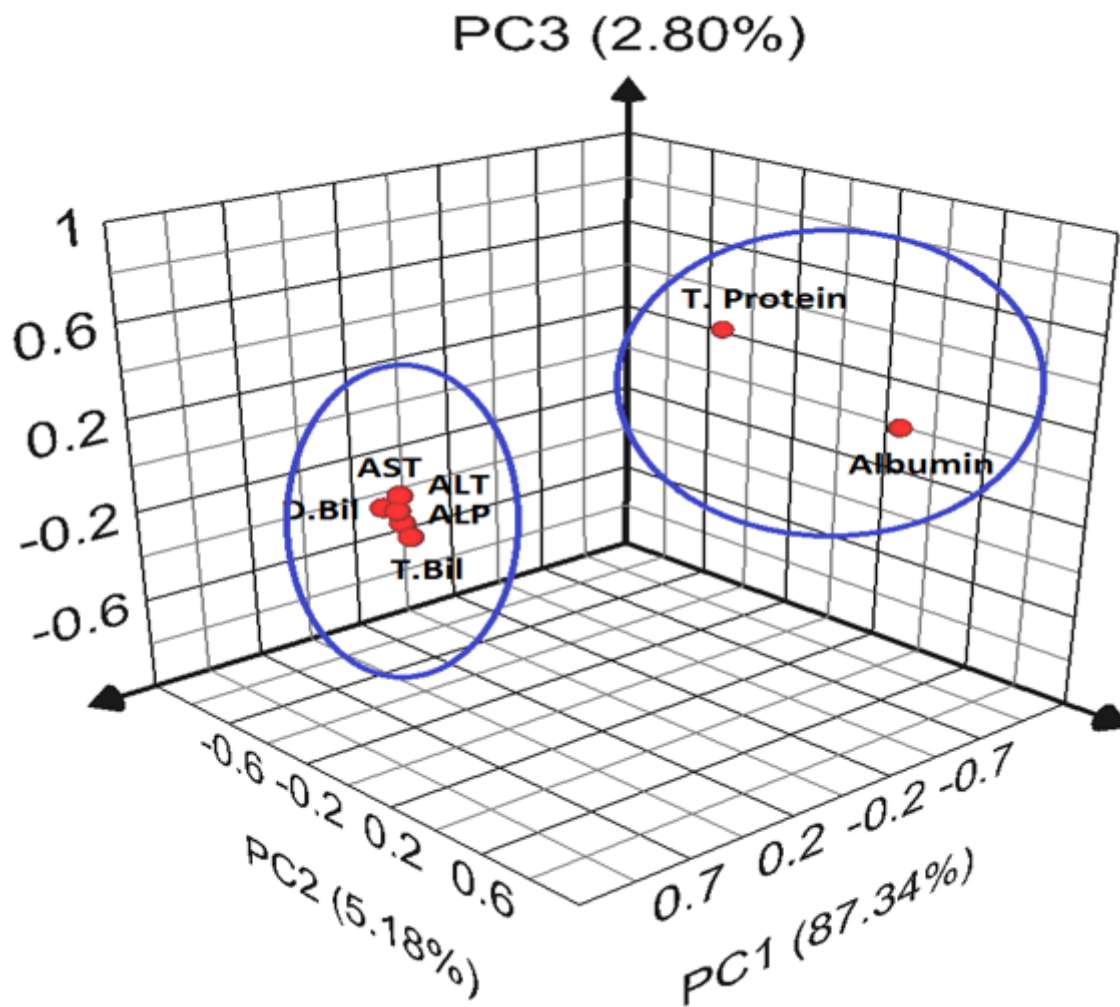


Figure 4

Score plot showing the differentiation of parameters associated with interactions among liver function parameters in different groups. A three-component system explaining 95.35% of total variance was observed after PCA. AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alanine phosphatase, T.BIL= total bilirubin, D.BIL = direct bilirubin, T.Protein = total protein

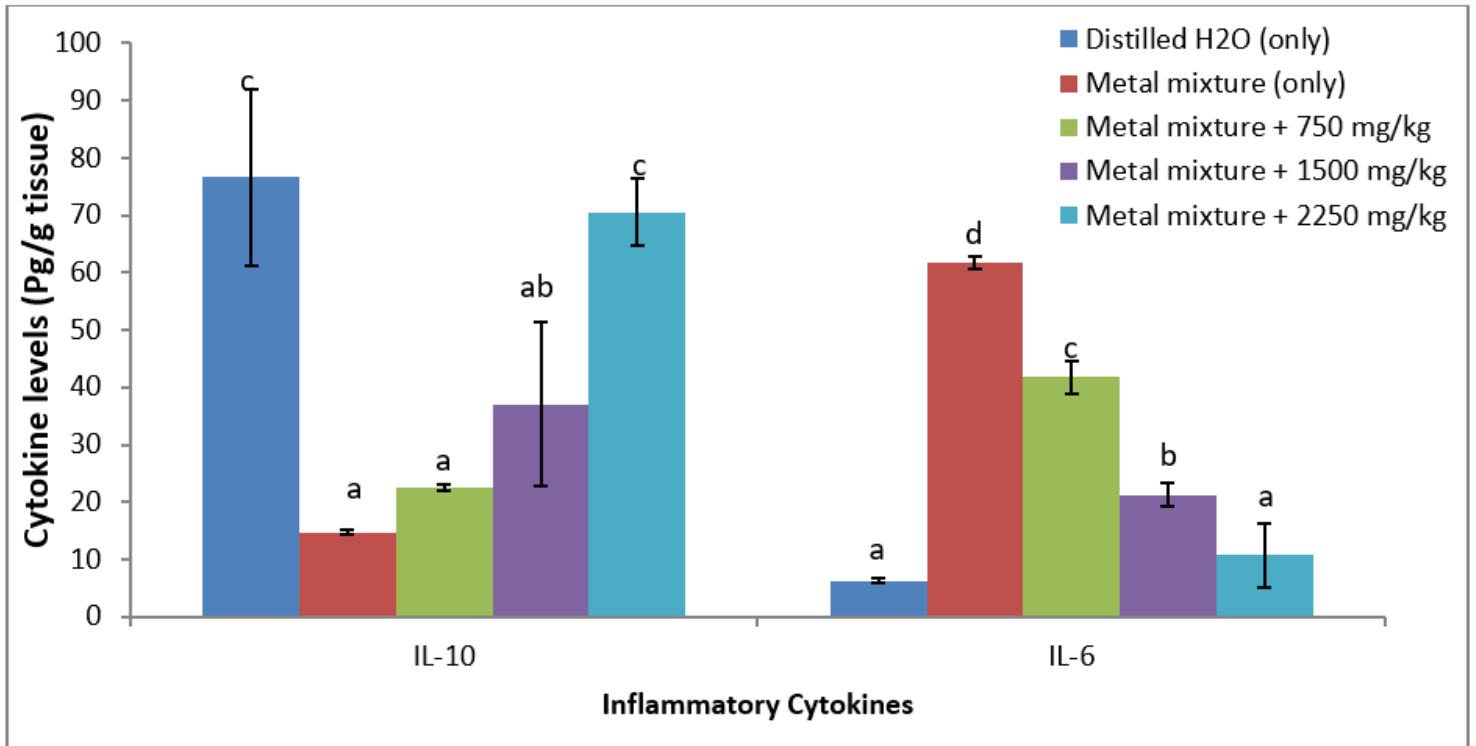


Figure 5

Effect of *Costus afer* on anti-inflammatory cytokines (interlukin-10 (IL-10) and proinflammatory cytokines interlukin-6 (IL-6) on liver of male albino rats treated with trace metal mixture TMM. Values with different superscripts (a, b, c) are significantly different from each other ($p < 0.05$), while those with the same superscripts are not significantly different.

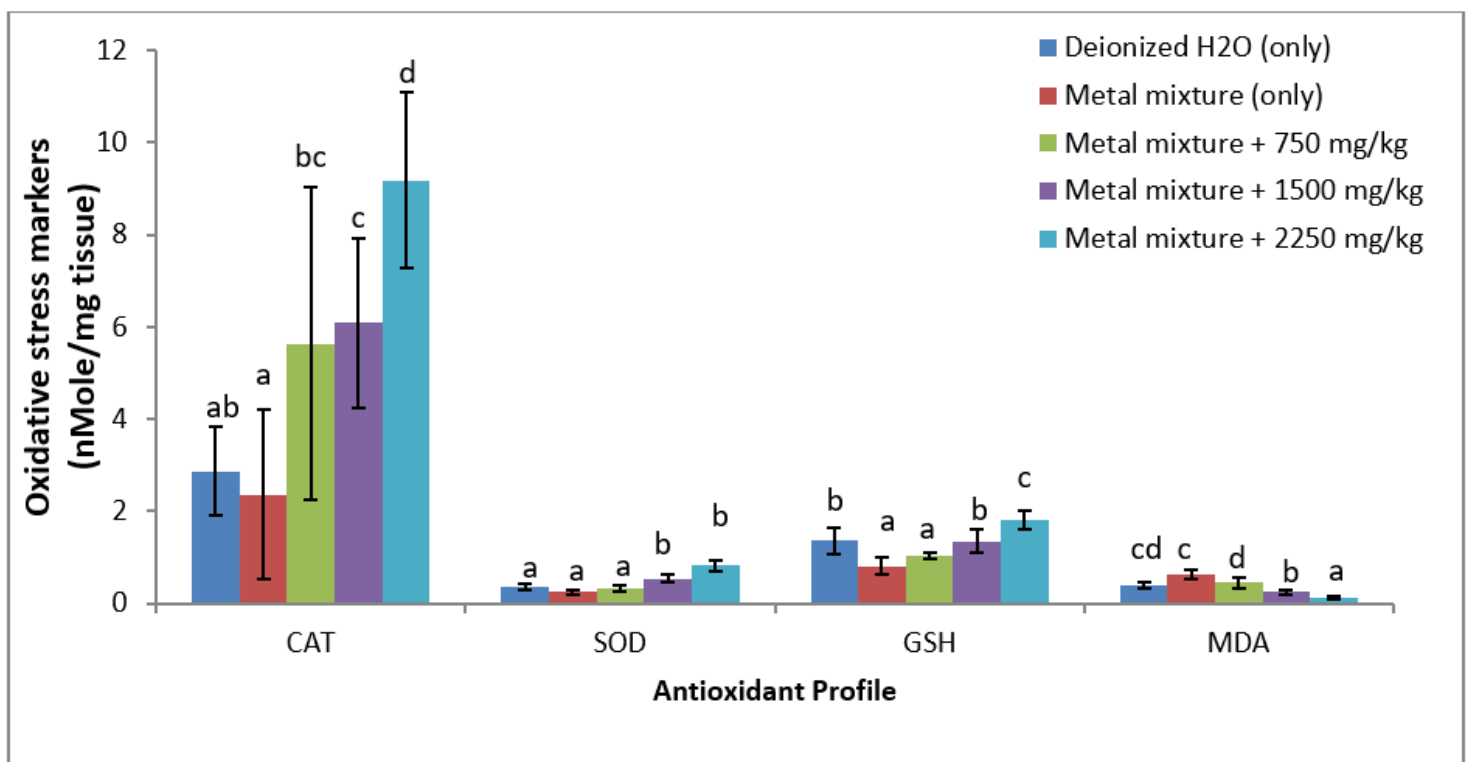


Figure 6

Effects of Costus afer on oxidative stress markers of the liver of male albino rats treated with metal mixture. Values with different superscripts (a, b, c, d) are significantly different from each other ($p < 0.05$), while those with the same superscripts are not significantly different. CAT = catalase, SOD = superoxide dismutase, GSH = reduced glutathione, and MDA = malondialdehyde

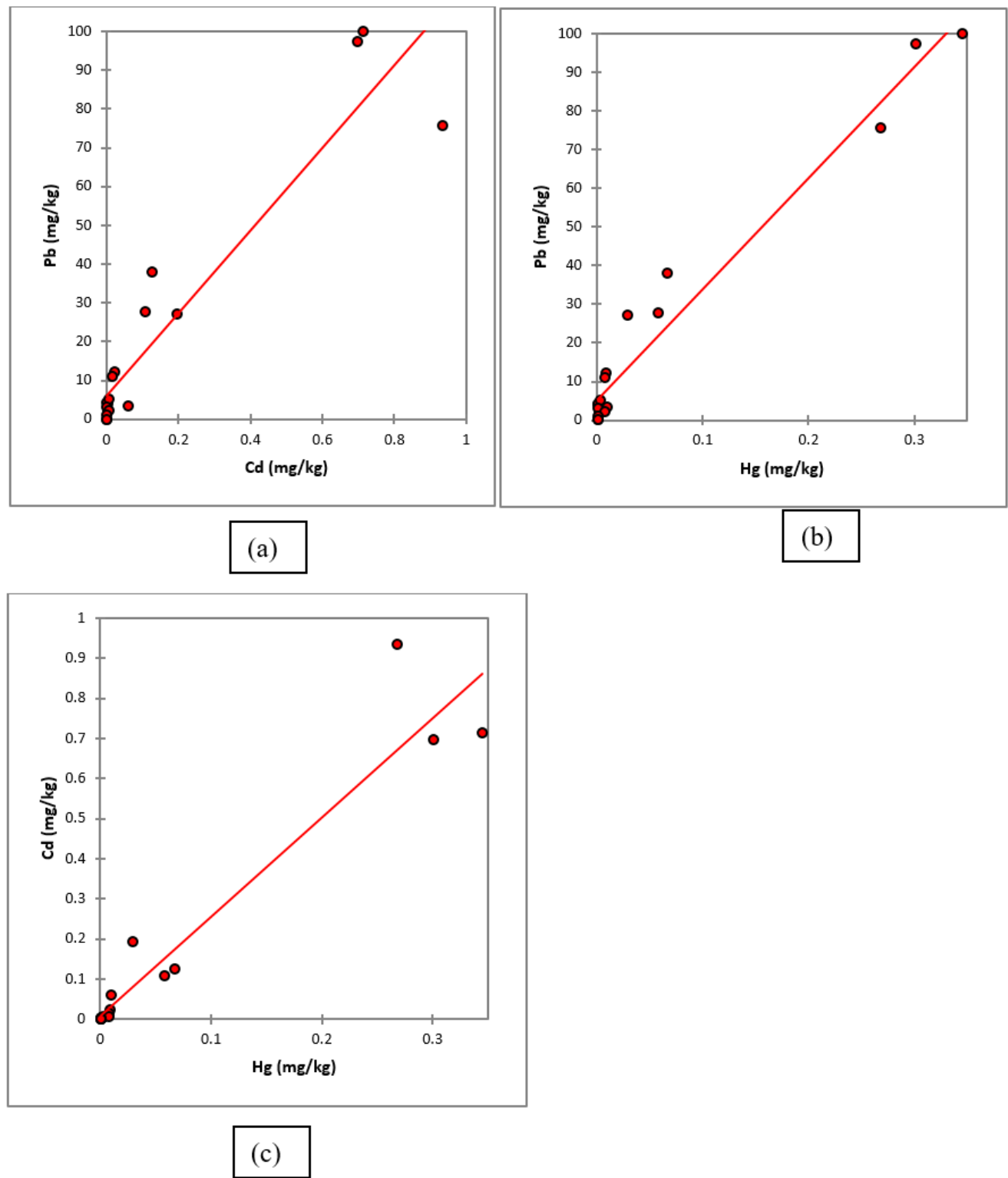


Figure 7

Inter-trace metal correlation among trace metals in the liver of rats showed strong positive correlation ($r > 0.90$) between metals such as (a) Cd and Pb (b) Hg and Pb (c) Hg and Cd during the study. All correlations were significant at $p < 0.01$.

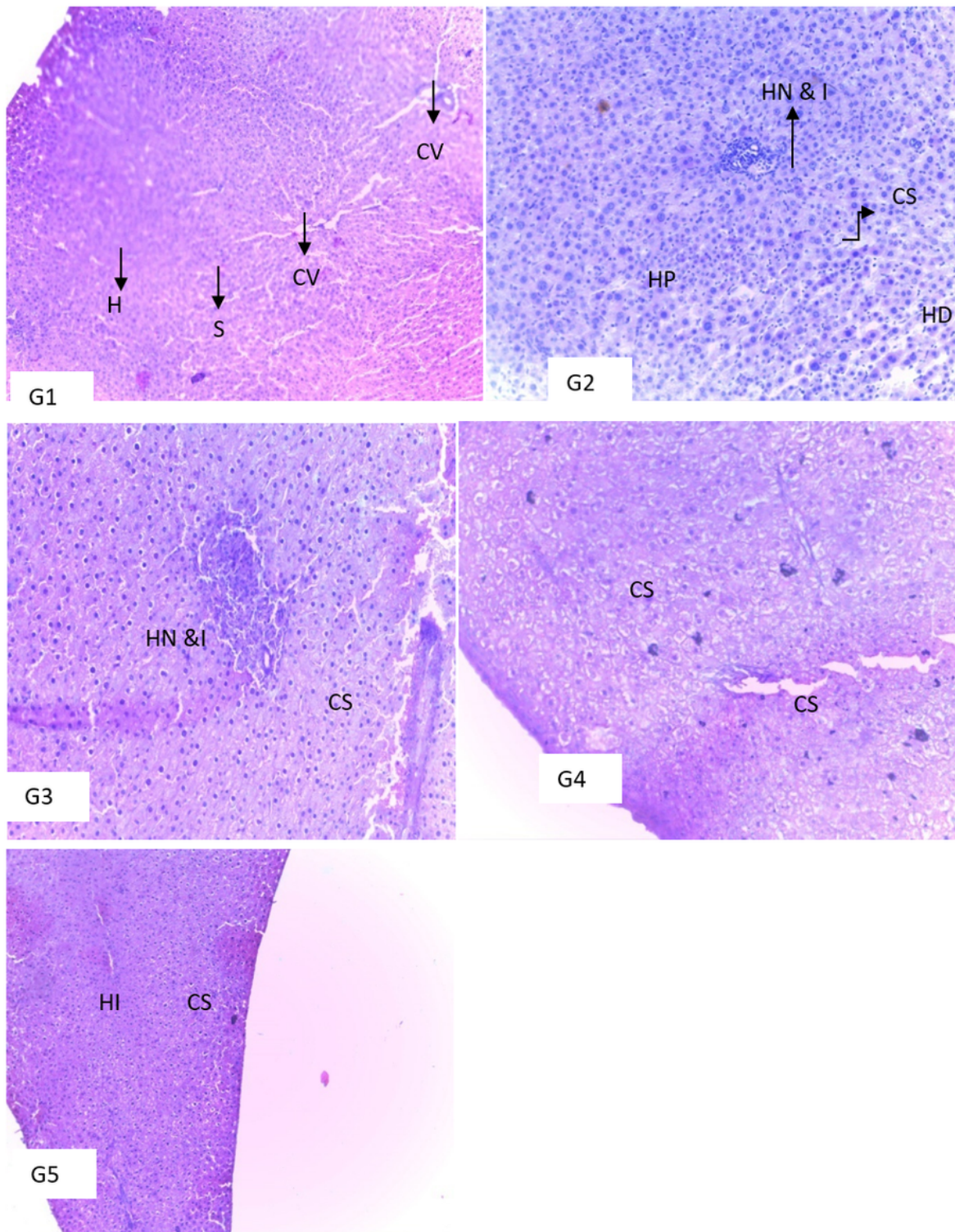


Figure 8

Histopathology of the liver: G1 (H20), G2 (TMM), G3 (TMM + 750 mg/kg ALECA), G4 (MM + 1500 mg/kg ALECA), G5 (TMM + 2250 mg/kg ALECA). Staining was done with haematoxylin and eosin magnification

X 200 in all panels. CS (Cytoplasmic Swelling); HN & I (Hepatocyte Necrosis and Inflammation); CV (Cytoplasmic Vacuolation); FC (Fatty change); HP (Hepatocyte Pleomorphism); HD (Hepatocyte Dysplasia); S (Sinusoids); H (Hepatocytes).

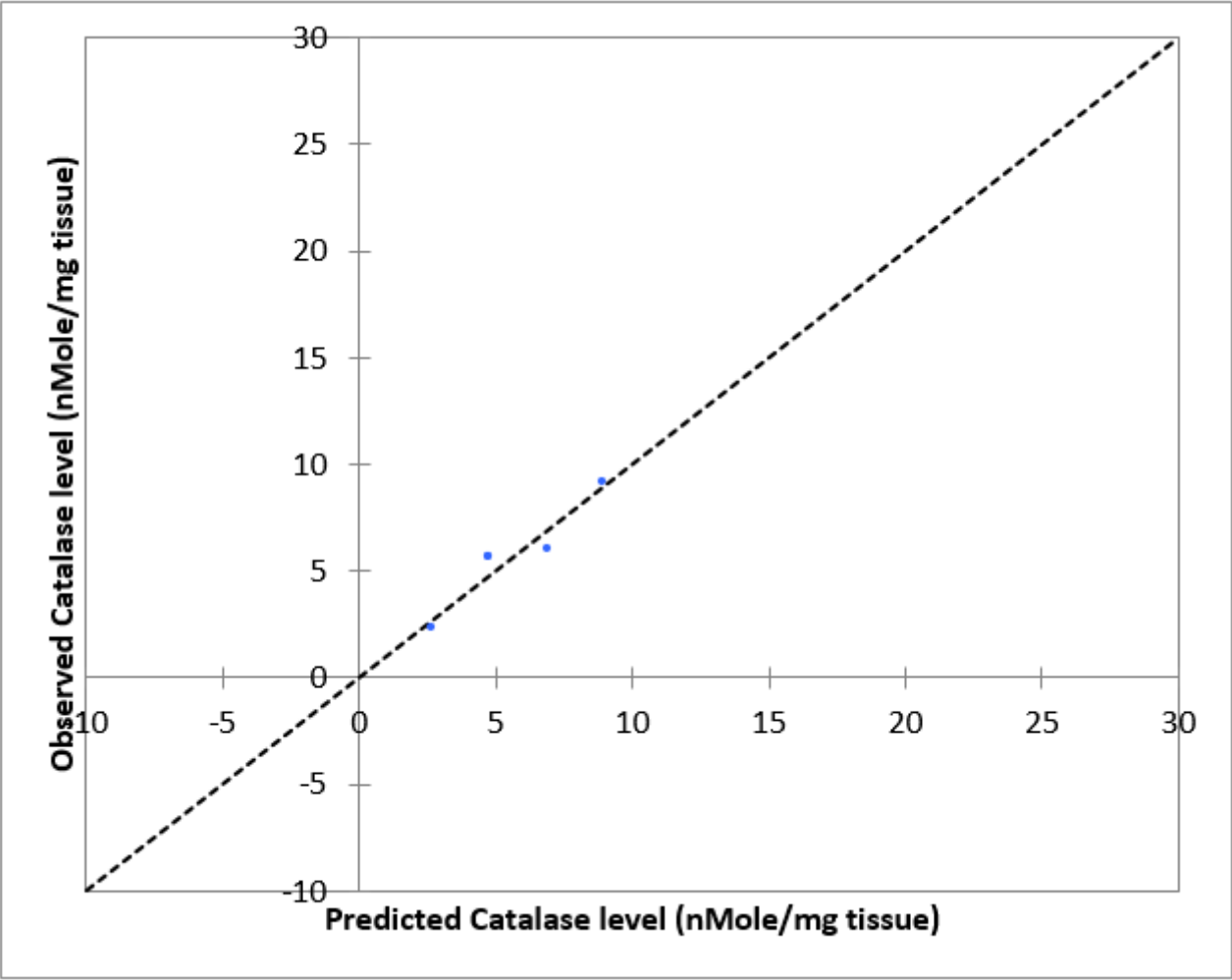


Figure 9

Predicted liver catalase level against observed liver catalase level (nMole/mg tissue); where y = Catalase levels; x = ALECA concentrations served as input values.

Supplementary Files

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