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ORIGINAL PAPER

Hepatoprotective effect of *Costus afer* (Lin) on toxic metal mixture treated rats mediated by regulation of oxidative stress markers, inflammatory cytokines and bio-metal chelation

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ABSTRACT

Introduction and aim. Cadmium, lead, and mercury have been considered to exhibit their hepatotoxic effects by oxidative induction damage and the generation of reactive oxygen species (ROS). The current work evaluated the protective activity of aqueous leaf extracts of *Costus afer* (ALECA) on liver damage arising from exposure to toxic metal mixture (TMM): 1.61 mg/kg cadmium chloride ($CdCl_2$), 20 mg/kg lead chloride ($PbCl_2$), and 0.40 mg/kg mercury chloride ($HgCl_2$).

Material and methods. Five groups of weight-matched Sprague Dawley rats were treated for 90 days. Metal mixtures 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 with TMM. Hepatic function parameters, oxidative biomarkers, inflammatory cytokines, morphological changes, and metal levels in the liver were monitored.

Results. Treatment with TMM resulted in significant increases in alanine transaminase, aspartate transaminase, alkaline phosphatase, bilirubin, interleukin 6, malondialdehyde, but decreased albumin, total protein, interleukin 10, superoxide dismutase, catalase, and glutathione levels. TMM also caused some morphological changes and increased the concentrations of heavy metals (Pb, Cd, and Hg) in the liver.

Conclusion. ALECA showed beneficial effects against TMM-induced hepatotoxicity via metal chelation, anti-inflammatory, and antioxidant mechanism. ALECA may be beneficial in the management of liver toxicity.

Keyword. *C. afer,* environment, exposure, heavy metal mixture, hepatotoxicity

Introduction

Trace metals occur from both natural and human activities. The continued use of metals in various industries like agriculture, medicine, technology, etc. has posed both public and eco-health concerns.[1] Metals are systemic toxic agents known to cause organ damage even at low exposure levels. Lead (Pb), mercury (Hg) and cadmium (Cd) can be hepatotoxic.[2] These elements are

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ranked among metals of enormous concern to the public health (WHO).[3] Pb is a toxic metal found naturally in the Earth's crust.[4] The negative consequences of exposure to Pb exposure have been shown in some organs such as the brain, liver, kidney, heart, and systems, for example, reproductive, musculoskeletal, developmental and immunological systems.[5] Inorganic Hg induces neurotoxic, hematotoxic, cardiovascular, hepatotoxic, genotoxic and nephrotoxic effects.[6-12] Acute exposure to Cd affects the lungs by causing irritation, while chronic exposure leads to accumulation in the kidneys, liver and other organs resulting in dysfunction of the organs.[13-14]

Uptake and elimination of extraneous compounds are vital functions of the liver.[15] Hepatotoxicity implies hepatocyte impairment arising from the overload of chemicals and exogenous compounds that include heavy metals.[16] Owing to the dominance of these metals in the ecosystem, the long-term effect of a combination of lead, cadmium, and arsenic have been studied and liver toxicity reported by Bhattacharjee et al.[17] Treatment of rats with lead and cadmium have also resulted in liver damage.[18] Until now, the focus on the unfavorable health effects of metals is largely based on human case studies of chronic high exposure seen in the metal industry or in densely polluted environments. Occupational and environmental exposure to metals is common. Therefore, more studies on the consequences of chronic environmental trace metals and their mixtures in animal models that can be extrapolated to humans.[19] To mimic real-life situations, it is imperative to evaluate the toxicities of metal mixtures.

Chelation with meso-2,3-dimercaptosuccinic acid (Succimer or DMSA) and D, L-2,3-dimercapto-1-propanesulfonic acid (Dimaval or DMPS); the 2,3-dimercaptopropanol (British Anti Lewisite, BAL or Dimercaprol), Ethylenediamine-tetracetic acid (EDTA) and D-penicillamine remain the mainstay of the treatment of metal poisoning.[20,21] Despite their contribution in the treatment of metal poisoning, they also have some drawbacks, including toxicity issues, availability, and affordability concerns. These disadvantages constitute the need to provide effective and safe pretreatment therapy. Understanding the relevance of natural antidotes, considered 'generally considered safe' GRAS and given their affordability and availability, as chemopreventive agents in metal poisoning has gained traction recently.

The different pharmacological properties of *C. afer* have led to the study of its bioactive compounds that may show promise as drugs. Protection of the liver from noxious trace metal mixtures is considered worthwhile given its importance. The anatomical proximity of the liver to the intestines predisposes the liver to toxic attack. Hepatotoxicity refers to liver damage resulting from various chemicals and xenobiotics, including heavy metals and their metabolites.[22] Due to the ubiquity of metals in the environment, Bhattacharjee et al. evaluated the effects of long-term exposure at a low dose to a mixture of Cd, As, and Pb and concluded that chronic exposure to of heavy metal mixture at a very low environmentally relevant dose produced hepatotoxic effects in albino rats.[17] Hepatoxicity has also been reported by Yuan et al. to be among the toxicities resulting from the mixture of Pb and Cd on Sprague Dawley rats.[18] Hepatotoxicity is usually characterized by increased membrane permeability and changes in enzyme levels. Other liver damage, reported substantial decrease in serum ALP and AST at 400 mg/kg of *C. afer.*[23] The stem extract of *C. afer* was protective in alcohol-mediated liver damage in rats, suggesting a possible benefit in alcohol-mediated liver cirrhosis.[23-24] *C. afer* is a herbaceous, perennial and unbranched rhizomatous herb that belongs to the Zingiberaceae family. It grows up to 4 m high and has been proven in many studies to have strong therapeutic effects.[25] It is commonly found in shady or moist forests or riverbanks of tropical West Africa, including Cameroun, Ghana, and Nigeria.[26] It is commonly known as bush cane or ginger lily. Among Igbos in Nigeria, it is commonly called 'Okpoto', 'Okpete Ohia' or "Okpete".[27] The Hausas call it 'Kakizuwa', Yoruba call it "Tete-egun" and the Efik calls it 'Mbritem' 27 *C. afer* has been evidenced to have many therapeutic effects in humans and animals. Reports on the phytochemical analysis of *C. afer* revealed that the plant is rich in steroidal saponins, flavonoids, alkaloids, tannins, terpenoids, sapogenins, oxalates, furans, furan derivatives, and starches without any form of toxicity.[27] These phytochemicals are rich in antioxidants. Pharmacological activities associated with *C. afer* include antioxidant property, hepatoprotective, nephroprotective, antidiabetic, and antinociceptive role.[28-30]

Previous studies have shown that ALECA may be organoprotective (kidney and testis) in lead-mediated kidney and testes damage through antioxidant mechanisms.[23,31,32] As much as various studies have reported exposure of single heavy metal studies, there is insufficient information on the heavy metal mixtures that represent the real situation of these toxicants in various environmental matrices. Classical and synthetic metal chelators, which have become the mainstay of antidotal management of metal intoxication together with the numerous side effects, are scarce and expensive especially in developing countries.[33]

Aim

This study focuses on investigating the hepatoprotective action of ALECA in male albino rats exposed to lead, cadmium, and mercury.

Material and methods

Harvesting of C. afer

Samples of *C. afer* leaves were collected, in the month of July 2021, from a farmland in the University of Port Harcourt, Rives state, Nigeria, in an area free of air pollution

due to vehicular traffic. Mr. A. O. Ozioko, of the Botany Department, University of Nigeria, Nsukka, helped verify the plant for its authenticity prior to its use.

Preparation of ALECA

The leaves of *C. afer* were washed to remove sand particles, pulverized, and stored. Two hundred and fifty grams of the pulverized leaf samples were macerated in 500 ml of deionized water for 24 hours amidst continuous agitations after the method of Ezejiofor and Orisakwe.[29] The mixture was shaken and filtered using Whatman No 1 filter paper to obtain the extract with a yield of 0.11g/ml which was stored in a refrigerator at 4°C.[34] The process was repeated after every four days of treatment to obtain fresh extract throughout the 90 days treatment period.[34]

Determination of the ALECA dosage

A total of 12 male albino rats of approximately 8 weeks old with 100-200 g weights separated into four equivalent groups that received *C. afer* 1000, 2000, 4000 and 5000 mg/kg bw respectively were observed for 24 hours for any change in physical characteristics or death. This administration was carried out by oral gavage and at the end of the treatment no death or change in physical features was recorded. A previous study from our lab showed that subchronic administration of ALECA was not toxic.[29]

To determine the dose used for this study, given the safety of ALECA, 3000 mg/kg bw ALECA was chosen and then 25% of 3000 mg/kg bw as the low dose, 50% of 3000 mg/kg bw as the medium dose and 75% of the 3000 mg/kg bw as high dose.

Phytochemical screening of the plant material

The phytochemical constituents of *C. afer* were tested to confirm the existence of tannins, alkaloids, saponins, flavonoids, and phenolic compounds using the standard procedures of Trease and Evans.[35]

Animal care handling

The study used 25 male albino rats that were about 8 weeks old with 100-200 g weights procured from the Animal House of the Faculty of Pharmacy, University of Port Harcourt Rivers state, Nigeria. The study used the animal husbandry procedure established in previous studies by Ezejiofor and Orisakwe and Anyanwu et al.[29,36] Rats were kept under standard laboratory conditions with ambient temperature (25±2°C), relative humidity (55-64%) and 12-hour light-dark condition cycles. The rats were acclimatized for 2 weeks and fed standard rat chow (Sander Nigeria Ltd) with water *ad libitum*. The protocol for the study was allowed by the University of Port Harcourt and was assigned the reference number UPH/CEREMAD/REC/04.

Design of the experiment

Twenty-five rats consisting of five weight matched male albino rats per group of each were used for this study. The first group was as control which received only deionized water whereas the second group received only TMM ($PbCl_2$, 20 mg/kg; $CdCl_2$, 1.61 mg/kg; $HgCl_2$, 0.40 mg/kg) (Sigma Aldrich WGK Germany).[34,37] Groups 3, 4 and 5 received a toxic metal mixture TMM and ALECA at 750, 1500 and 2250 mg/kg respectively, according to a previous study from our laboratory.[34] These treatments were done five times in a week to mimic occupational exposure for 90 consecutive days by oral gavage.

Necropsy

On the 91st day, the animals were sacrificed under ether anesthesia. The liver was harvested, washed in ice-cold saline, blotted with Whatman No.1 filter papers, and weighed afterward to get the absolute weight. Normal saline and formalin were used for storing the samples for biochemical, histopathological, oxidative markers, and inflammatory analysis. These samples were collected according to the procedures recorded by Anyanwu et al.[34,36]

Preparation of liver homogenate

Two grams of liver sample was homogenized in cold phosphate buffer (5 mM, pH 7.4). The supernatant was collected after centrifugation for inflammatory and antioxidant analysis.

Metal digestion (acid digestion method) and analysis

About 6 ml and 2 mL 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 min after acidification before being heated at 105°C until digestion was complete. Whatman filter paper Number (1) (pore size 11 μm) of was used for filtration to obtain clearer samples. The solution was later made up to 15 ml (final volume) with deionized water. All glassware was thoroughly washed and rinsed before use. Calibration curves for Pb, Cd, and Hg as previously described by Anyanwu et al.[34,36] The 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 samples and standards were run in duplicate.

Hepatic biomarkers

Estimation of alanine aminotransferase (ALT) and aspartate transaminase (AST)

The ALT and AST activities of the liver samples were tested using a Randox kit.[38]

Estimation of alkaline phosphatase (ALP)
The ALP function was determined with the aid of standard diagnostic kits (Randox Laboratories Ltd, UK) using the colorimetric endpoint.[39]

Estimation of total and direct bilirubin
This was done using standard diagnostic kits in a colorimetric process (Randox Laboratories Ltd, UK).[40]

Estimation of total protein and albumin
A hepatocellular injury is indicated by a decrease in total protein.[41] The level of total protein in serum was estimated using standard diagnostic kits (Randox Laboratories Ltd, UK). Similar to total protein, a reduction in albumin level also signifies liver injury.[42] With the standard diagnostic kits (Randox Laboratories Ltd, UK), the albumin level in the serum was determined.

Antioxidant analysis
Estimation of catalase (CAT) activity
CAT activity was assayed by adapting the method of Clairborne.[43]

Estimation of liver glutathione (GSH) level
The GSH level was estimated after the method of Sedlak and Lindsay.[44]

Estimation of superoxide dismutase (SOD) activity
Following the method of Misra and Fridovich, SOD was determined.[45]

Lipid peroxidation marker (MDA) activity
Following the method of Ohkawa and Ohishi, the MDA was evaluated.[46]

Evaluation of Inflammatory cytokines [interlukin-6 (IL-6) and interlukin-10 (IL-10)]
Enzyme-linked immunosorbent assay (ELISA) was employed. The ELISA kit (Bioassay Technology Laboratory, Shanghai, China) with a sensitivity of 0.052 ng/L and 1.51 pg/mL, respectively, as described in Anyanwu et al.[34]

Histopathological examination
The tissues were soaked in 10% formaldehyde, sectioned, and treated with hematoxylin 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.[34]

Statistical analysis
Analysis of variance (ANOVA) was applied to the sequence of observations for the purpose of comparative analysis at 5% significance. Multiple comparisons were performed with Duncan's multiple comparison method. Principal component analysis (PCA) was employed to select the principal factors (or independent variables) for the development of the multiple regression equations. In this study, the use of PCA was carried out using XLSTAT (Microsoft, Redmond, Washington, USA).[36,47] The annotation on the bar graphs indicates whether there is a significant difference between two groups. Groups that have different alphabets signify that there is a significant difference in the mean concentration, while groups with the same alphabet signify that there is no significant difference in the mean concentration.

Results

Phytoconstituents of ALECA
The phytoconstituents of ALECA are shown in Table 1. Flavonoids were the main phytoconstituents in ALECA (25±0.13 mg/100 g) which accounted for approximately 70% of the total chemical constituents. Alkaloids, tannins, and saponins were also found in ALECA.

Table 1. Quantitative phytochemical screening (mg/100 g) of ALECA*

Chemical constituents	Content
Alkaloids	4.3±0.1
Saponins	2.9±0.08
Tannins	2.72±0.11
Flavonoids	25±0.13

* values are expressed as mean±SD, n=5

Effect of ALECA on body weight, absolute and relative weight of liver
Treatment with TMM did not decrease the body weight of the rats. There was an increase in body weight in all the groups. Rats treated with TMM only had a considerable increase in liver weight ($p<0.05$) compared to normal control rats (received only deionized water). Rats that received both ALECA and TMM had decreased liver weight compared to the TMM only treated rats (Table 2).

Table 2. Effect of *C. afer* on body weight, absolute and relative weight of liver of male albino rats treated with TMM*

Treatment	Absolute (g)	Relative (%)	Body weight (g)
Deionized H_2O (only)	5.08 ± 0.95^{a}	2.45 ± 0.41^{a}	I=118.2±7.46; F=207.1±42.61 % diff.=139.1
Metal mixture (only)	9.4 ± 1.35^{c}	3.62 ± 0.47^{c}	I=194.2±10.74; F=260±10 % diff.=62.7
Metal mixture + 750 mg/kg	$8.48\pm1.13^{b,c}$	$3.52\pm0.42^{b,c}$	I=174.2±8.19; F=248.3±25.63 % diff.=78.7
Metal mixture + 1500 mg/kg	$8.36\pm0.52^{b,c}$	3.15 ± 0.17^{bc}	I=158.1±4.77; F=247.1±33.02 % diff.=104.1
Metal mixture + 2250 mg/kg	7.86 ± 0.22^{b}	3.06 ± 0.08^{b}	I=150.6±3.03; F=228.6±30.37 % diff.=44.1

* values are expressed as mean±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, initial weight, F – final weight, % diff. – % difference

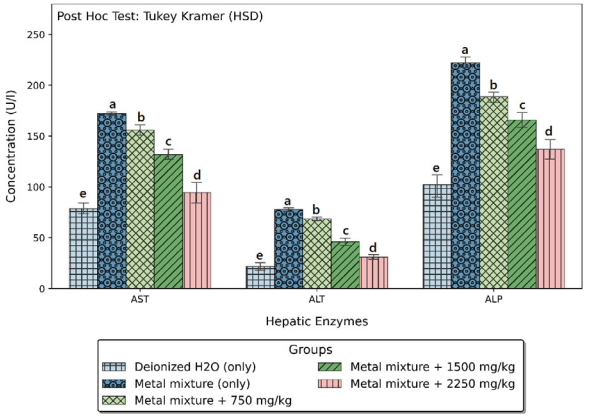

Fig. 1. Effect of ALECA on serum liver enzymes of male albino rats treated with TMM, values with different superscripts (a, b, c, d) are significantly different from each other (p<0.05), while those with the same superscripts are not significantly different

Effect of C. afer extract on serum liver enzymes, bilirubin and proteins

Liver function were done to evaluate the likely protective role of *C. afer* treatment from exposure to the metal mixture. Treatment with TMM caused a significant elevation in AST, ALT, and ALP levels relative to the control Figure 1. There was a significant increase in bilirubin (total and direct) in the TMM treated only compared with control Figure 3. In Figures 1 and 3, there were significant reductions in liver enzyme concentrations (ALT, AST and ALP) and bilirubin (total and direct) in rats that received both ALECA and TMM. Figure 2 showed a significant decrease in total protein and albumin when rats were treated with TMM only compared to the control group. Treatment with ALECA caused a significant increase in total protein and albumin when the ALECA concentration exceeded 750 mg/kg. AST, ALT and ALP, total bilirubin, direct bilirubin, total protein and albumin levels in rats treated with only TMM were significantly different (172 µg/L, 77.8 µg/L, 222.2 µg/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 ALECA and TMM.

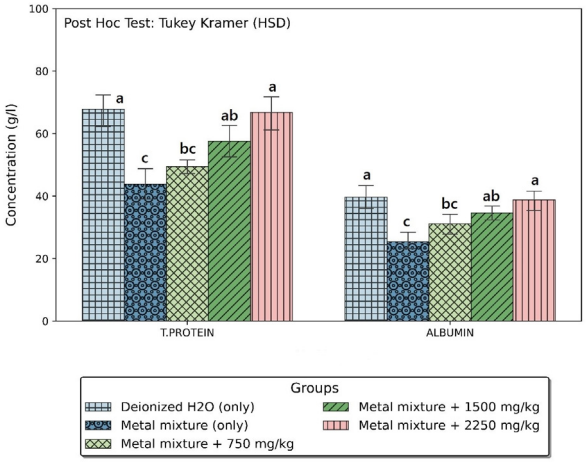

Fig. 2. Effect of ALECA on serum liver enzymes of male albino rats treated with TMM, values with different superscripts (a, b, c, d) are significantly different from each other (p<0.05), while those with the same superscripts are not significantly different, total protein

Effect of C. afer on anti-inflammatory cytokines and pro-inflammatory cytokines on the liver

An evaluation of the inflammatory status after TMM treatment by evaluating the pro- and anti-inflammatory cytokines in the liver. Cotreatment with *C. afer* significantly decreased the levels of pro- and increased (p<0.05) the anti-inflammatory cytokines (IL-6 and IL-10) in liver tissue respectively in comparison to the TMM-only treated group (Fig. 4), suggesting anti-inflammatory activity of ALECA. Pro and anti-inflammatory cytokine levels (IL-6 and IL-10, respectively) of rats administered with TMM only were significantly different (61.8 pg/mg of tissue and 14.7 pg/mg tissue, p<0.05) from the inflammatory cytokines seen in rats cotreated with *C. afer*.

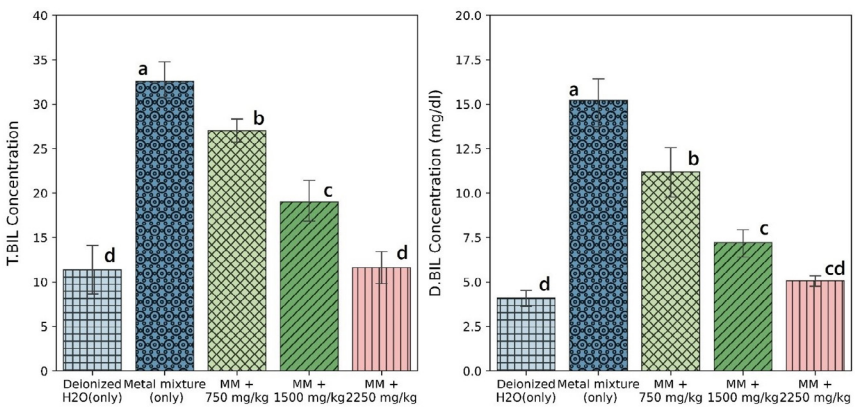

Fig. 3. Effect of ALECA on serum liver enzymes of male albino rats treated with TMM, values with different superscripts (a, b, c, d) are significantly different from each other (p<0.05), while those with the same superscripts are not significantly different, TBIL total bilirubin, DBIL – direct bilirubin

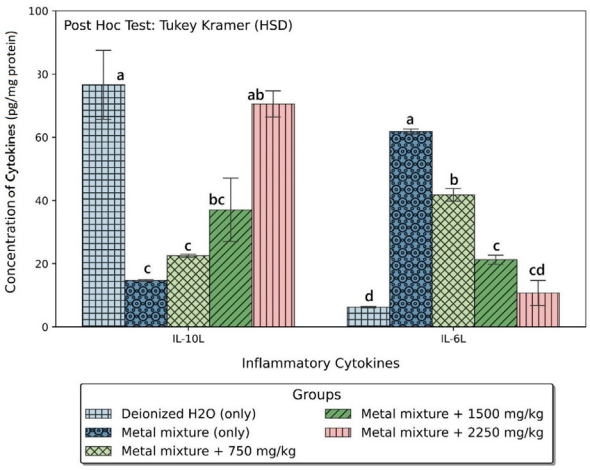

Fig. 4. Effect of *C. afer* on anti-inflammatory cytokines (interlukin-10 (IL-10) and pro-inflammatory cytokines interlukin-6 (IL-6) on the liver of male albino rats treated with TMM, values with different superscripts (a, b, c) are significantly different from each other ($p<0.05$), while those with the same superscripts are not significantly different.

Effects of C. afer on markers of liver oxidative stress

The oxidative status in the hepatocyte was evaluated after treatment with the metal mixture using the lipid peroxidation marker, MDA level. The 90-day treatment that was done with the following metal mixture at the following dosage $PbCl_2$ – 20 mg/kg, CdCl2 1.61 mg/kg, $HgCl_2$ – 0.40 mg/kg body weight induced oxidative reaction in the liver. The liver MDA level increased significantly ($p<0.05$) in rats treated with TMM only compared to those of the control group (Table 4). A significant decrease in MDA level was observed in the rats treated with *C. afer* and the metal mixture when compared to those treated with only TMM. Treatment with TMM resulted in a significant decrease ($p<0.05$) in GSH, SOD, and CAT levels compared to the control. Rats that received ALECA plus TMM had higher levels of GSH, SOD, and CAT compared to rats that received only TMM.

Table 3. Effects of *C. afer* on liver oxidative stress markers of male albino rats treated with a metal mixture

Treatment	CAT (U/mg)	SOD (U/mg)	GSH (µmol/g)	MDA (nmol/g)
Distilled H_2O (only)	2.87±0.95^{b}	0.35±0.06^{c}	1.36±0.28^{b}	0.39±0.08^{b}
Metal mixture (only)	2.36±1.85^{b}	0.24±0.06^{c}	1.82±0.2^{c}	0.64±0.1^{a}
Metal mixture + 750 mg/kg ALECA	5.64±3.39^{a,b}	0.33±0.06^{c}	1.35±0.24^{b,c}	0.45±0.110^{b}
Metal mixture + 1500 mg/kg ALECA	6.08±1.84^{a,b}	0.54±0.08^{b}	1.04±0.07^{b}	0.24±0.04^{c}
Metal mixture + 2250 mg/kg ALECA	9.18±1.9^{a}	0.82±0.12^{a}	0.81±0.18^{a}	0.12±0.03^{c}

* 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.

Heavy metal concentration on the liver of the rat samples

The concentration of trace metals (Pb, Cd, and Hg) in the liver was markedly elevated ($p<0.05$) in the liver of rats treated with the TMM compared to the control (Table 5). Treatment of rats with ALECA plus TMM resulted in a significant reduction in trace metal levels (Pb, Cd, and Hg) compared to rats that received only TMM. Furthermore, the TMM group only had the highest level of metals (Pb=90.992±13.284, Cd=0.78±0.133 and Hg=0.305±0.0439) in comparison to the control group. Pearson's rank correlation analyzes indicate the inter-trace metal relationship between metals in liver of rats showed strong positive correlation ($r>0.90$) between metals such as: Pb and Cd, Pb and Hg, and Cd and Hg. All correlations were significant at $p<0.01$ (Fig. 5).

Table 4. Metal levels (mg/kg) in the liver

Treatment	Cadmium (Cd)	Mercury (Hg)	Lead (Pb)
Deionized H_2O (only)	<0.001^{a}	<0.001^{a}	0.392±0.552^{a}
TMM (only)	0.782±0.133^{b}	0.305±0.039^{b}	90.992±13.284^{c}
TMM + 750 mg/kg ALECA	0.143±0.046^{a}	0.051±0.02^{a}	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±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.

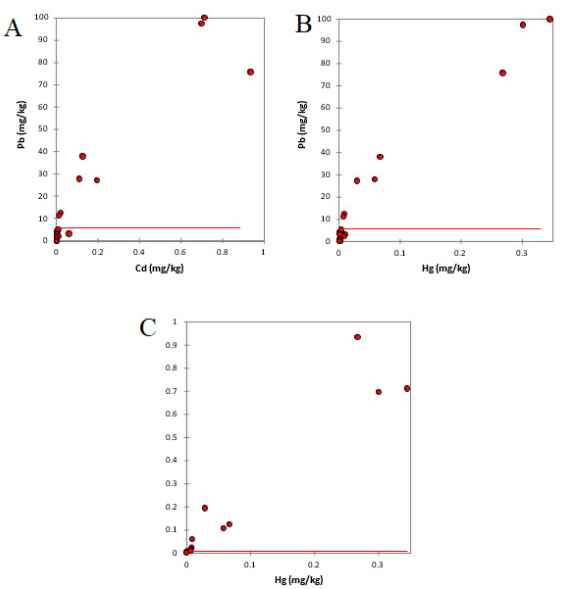

Fig. 5. The correlation among metals in the liver of rats showed a strong positive correlation ($r>0.90$) between metals such as (a) Cd and Pb (b) Hg and Pb (c) Hg and Cd during the study. All correlations were significant at $p<0.01$

Histopathology of the liver

The liver sections of six different groups were sectioned and presented in (Fig. 6 (G1), (G2), (G3), (G4) and (G5)

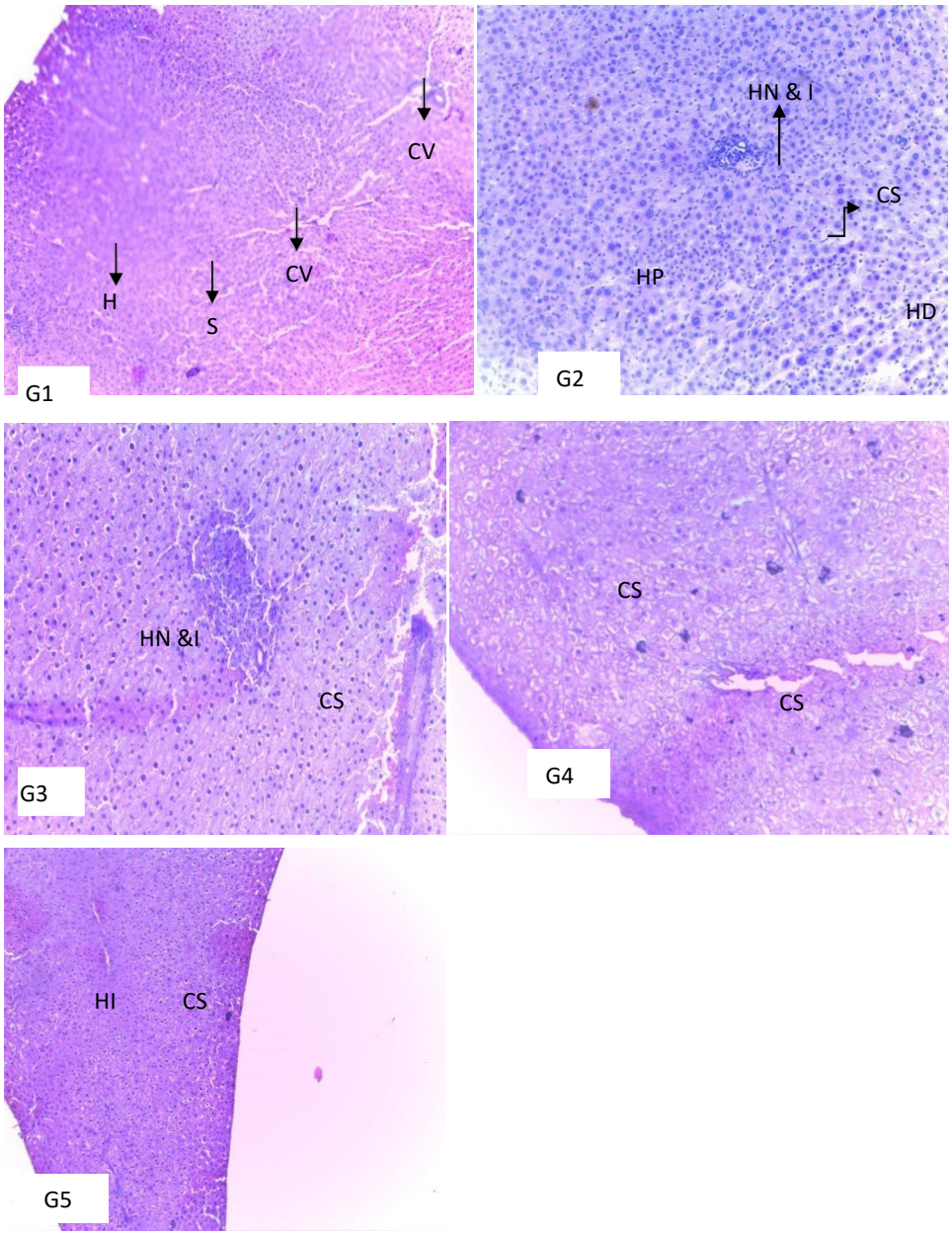

Fig. 6. Histopathology of the liver: G1 (H_2O), G2 (TMM), G3 (TMM + 750 mg/kg of ALECA), G4 (MM + 1500 mg/kg of ALECA), G5 (TMM + 2250 mg/kg ALECA). Staining was performed with H&E magnification X 200 in all panels, cytoplasmic swelling, HN & I – hepatocyte necrosis and inflammation of HN and I hepatocytes, cytoplasmic vacuolation CV, fatty change, HP – hepatocyte pleomorphism, HD hepatocyte dysplasia, S – sinusoids, H – hepatocytes

representing groups treated with deionized H20, metal mixture (MM), (MM 362 + 750 mg/kg ALECA MM + 1500 mg/kg ALECA, and MM + 2250 mg/kg ALECA. The rats treated with TMM only showed cytoplasmic swelling; hepatocyte necrosis and inflammation); cytoplasmic vacuolation; fatty change; hepatocyte pleomorphism and dysplasia of hepatocytes. These histological changes were reduced by ALECA. Fig. 7 is a synoptic capture or graphic illustration of the antioxidant and anti-inflammatory mechanism suggested of ALECA in metal mixture induced hepatotoxicity in rats.

Discussion

According to Jarup, most health challenges can be attributed to Pb, Cd, and Hg.[48] These metals have also been associated with infertility, neurotoxicity, osteoporosis, and various organ failures in humans.[18,49-50] The liver helps in metabolism and excretion and therefore its susceptibility to the adverse effects of foreign compounds.[15] Given the ubiquity of metals, some researchers have investigated the effects of chronic exposure of low doses of a mixture of cadmium and mercury.[51,52] They posited that long-term exposure to trace metal mixture even at low affected the liver of rats adversely.[51,52]

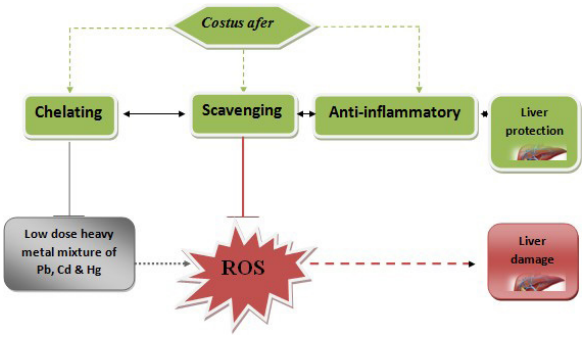

Fig. 7. Summary of the suggested mechanism

Majority of the actions of ALECA can be attributed to the inherent antioxidant properties of *C. afer*. Alkaloids, saponins, flavonoids, tannins, and phenols are the phytoconstituents of ALECA and may be responsible for its biological activity. Flavonoids are water-soluble polyphenolic compounds with 15 atoms that are antioxidant in nature.[29,53] 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: hydrolyzable and condensed tannins.[29,54] Their solutions are acidic with a characteristic astringent taste; they are also known to have strong antimicrobial activities in addition to other physiological effects that help to enhance blood clotting, as well as antihypertensive, antihyperlipidemic, and immunomodulatory.[55] Saponins are glycosides that are usually foamy in nature and have good medicinal value due to their therapeutic action. Desai et al. reported that they help protect plants from pathogenic attacks, giving rise to their antimicrobial activity[56] Alkaloids are nitrogenous compounds that are used as supplements, ingredients, supplements and for medical and pharmaceutical purposes.[57,58]

A significant increase ($p<0.05$) was observed in weights of liver treated with the TMM (only) in comparison to the control rats. However, a significant reduction in weight (absolute and relative) was observed in the samples administered with ALECA (750, 1500 and 2250 mg/kg) respectively in a dose-dependent manner in corroboration with the study by Bhattacharjee et al. that reported a marked elevation in the weight of the liver in rats treated with a trace metal mixture of Pb, Cd and As. Kluwe, Simmoni et al., and Orisakwe et al. further highlighted that an increase or decrease in organ weight after treatment with a chemical substance suggests toxicity.[59-61] Hence, this indicates that the liver could be a target organ for TMM toxicity. The increased liver weight observed in the present work confirms the work of Ahmad et al.[62] This current study indicates that ALECA may be hepatoprotective.

The significant rise ($p<0.05$) seen in rats that received TMM with respect to ALT, AST, ALP, total and direct bilirubin levels that formed the markers of injury to the hepatocyte could have arisen from increased membrane permeability and cell loss due to membrane oxidation[63-64] Liver cell death (hepatocellular necrosis) or membrane lesions increase serum levels of AST and ALT serum levels, flow into the bloodstream from the liver, in line with increased enzyme levels of metal mixture intoxicated rats. These observations indicate cell loss and loss of functional cellular membrane integrity in the liver in accordance with the studies by Zhang et al. and Ramachandran and Jaeschke.[64-65]

The increased level of bilirubin is another marker of abnormal liver function66 The liver is a chemical laboratory of the body involved in oxidation and metabolic conversion of fatty acids, the production of cholesterol and phospholipids and the elimination of specific classes of serum lipoprotein.[67] The apparent decrease in total protein and albumin levels in rats treated with TMM only could be due to inhibition of protein synthesis and metabolism that gave rise to hepatotoxicity.[68,69]

Many previous studies described trace metals as immune suppressors that provoke elevated levels of TNF-α, Il-1β and IL-6 (pro-inflammatory cytokines). The elevated level of IL-6 in this study may be due to increased production of reactive oxygen species.[70,71] Oxidative stress could be associated with an excessive production of pro-inflammatory cytokines.[70] The concomitant administration of ALECA and TMM resulted in dose-dependent reduction in levels of IL-6 and an increase in IL-10. Inflammation is caused by several factors, such as a group of secreted polypeptides called cytokines that control host responses to lesions; some are anti-inflammatory cytokines, while others function as pro-inflammatory cytokines72-73 An elevation in the pro-inflammatory cytokine (IL-6) and a decrease in the anti-inflammatory cytokine (IL-10) were observed in rats exposed to TMM only. These effects were alleviated upon administration of ALECA in a dose-dependent manner suggestive of anti-inflammatory potency of ALECA.

Elevated levels of antioxidant enzymes were observed in rats that received ALECA plus TMM compared to those that received only TMM, which may suggest that ALECA has antioxidant and hepatoprotective effects. This observation is in corroboration with the work of Ezejiofor and Orisakwe.[29] SOD is the first defense against the conversion of superoxide radical anion to the production of free radicals involved in hydrogen peroxide, while CAT serves as the second antioxidant protection mechanism by reducing hydrogen peroxide to oxygen and water.[74] Thus, ALECA increased antioxidant enzyme levels, which agrees with the previous study protective effects of *C. afer* in the liver by Ezejiofor and Orisakwe.[29]

Although the toxicity of the exact mechanism of the metal mixture is unclear, the observations in this study indicate that treatment with TMM elicits ROS generation and alters cellular antioxidant capacity. This

could result in an imbalance between free radical species and the resistance against cellular damage.[75] Therefore, supplementation of antioxidant molecules would be exogenously beneficial in the protection of cell antioxidants to neutralize heavy metal poisoning. ALECA contains antioxidant phytochemicals such as phenolics and flavonoids.[29] This study has shown the protective effect of ALECA on TMM-induced toxicity in rats. MDA, a marker of lipid peroxidation, showed a substantial increase ($p<0.05$) in the liver of rats that received TMM compared to the groups that received ALECA plus TMM. MDA is a marker used to measure the level of oxidative stress in an organism.[76] A similar finding revealed an elevation in lipid peroxidation in the liver after metal poisoning.[29] This suggests that peroxidative injury may be involved in the development of complications of severe heavy metal toxicity.

The significant decrease in Pb, Cd, and Hg following administration of ALECA is prominent. The chelation of these trace metals by some phytoconstituents of ALECA may be a plausible mode of action notwithstanding the fact that the actual mechanism remains largely unknown. Only recently have some researchers opined that mopping of free radicals and metal chelation are essential characteristics in management of oxidative stress.[77-79] There is a need for further studies in this regard to ascertain the actual route of metal elimination. Pearson's rank correlations of Pb, Cd, and Hg in the liver show strong significant relationships ($r>0.90$) between Cd and Pb ($r=0.940$, $p<0.05$, $n=18$), Hg and Pb ($r=0.982$, $p<0.05$, $n=18$), Hg and Cd ($r=0.960$, $p<0.05$, $n=18$) in the liver. This strong association indicates a close physiological connection.[80] The strongest association was observed between liver Hg and liver Pb. This relationship between these trace metals could be due to the similarity of their oxidative states, which makes them exhibit similar chemical properties.

Certain histological changes, such as necrosis and inflammation and severe dysplasia of the hepatocytes, were observed in rats that received trace metal mixture compared to the rats that received only deionized water. Hepatic necrosis, inflammation, and cytoplasmic swelling are common symptoms of liver damage.[81] The deleterious histomorphological features in the TMM treated rats are consistent with the oxido-inflammatory effects of metals in the liver. The protective effect of *C. afer* was observed in the rats co-treated with ALECA at doses of 750, 1500, and 2250 mg/kg. Whereas rats which received low dose ALECA plus trace metal mixture showed hepatocyte necrosis and cytoplasmic swelling, higher doses of ALECA plus trace metal mixture showed mild cytoplasmic swelling and mild hepatocyte inflammation. The dose-dependent histomorphological protection of ALECA against trace metal-mixture-mediated damage may confer on ALECA a beneficial role. Perhaps some of the limitations of the present study which will be addressed in follow-up studies are the use of crude extract of *C. afer* and an in-depth mechanistic consideration of the beneficial effect of *C. afer* in heavy metal-mediated hepatotoxicity.

Conclusion

The suggested mechanism of action is shown in Fig. 10. Exposure to trace metal mixtures may be of significant health effect arising from oxidative damage, inflammation, and distortion of the liver in rat model. The observed attenuation of the destructive effects of TMM by the effects tended to be attenuated by ALECA, which could provide some hope as an alternative remedy and circumvent the major drawbacks of notable chelators: increase in toxicity, unavailability, and prohibitive cost. *C. afer* may have promise in the management of chronic hepatotoxicity arising from metal exposure.

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Declarations

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Author contributions

Conceptualization, O.E.O.; Methodology, B.O.A. and A.N.E.; Validation, B.O.A., A.N.E. and O.E.O.; Formal Analysis, B.O.A., A.N.E. and O.E.O.; Investigation, B.O.A., A.N.E. and O.E.O.; Data Curation, B.O.A., A.N.E. and O.E.O.; Writing – Original Draft Preparation, B.O.A. and O.E.O.; Writing – Review & Editing, D.N.A. and O.E.O.; Supervision, A.N.E. and O.E.O.

Conflicts of interest

Authors declare no conflict of interest.

Data availability

All data have been provided.

Ethics approval

The protocol for the study was permitted by the University of Port Harcourt Research Ethics Committee and was assigned the reference number UPH/CEREMAD/REC/04.

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