Neuroprotective effect of *Costus afer* on low dose heavy metal mixture (lead, cadmium and mercury) induced neurotoxicity via antioxidant, anti-inflammatory activities

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**ABSTRACT**

Humans are constantly exposed to heavy metals due to their ubiquity in the environment. Hence, this study investigated the possible protective effect of *Costus afer* aqueous leaf extract (CALE) against low dose heavy metal mixture (LDHMM)-induced neurotoxicity. Male albino rats were divided into 6 equal groups. Group 1 served as the normal control receiving only deionized water. Group 2 served as the toxic control receiving on metal mixture (20 mg/kg PbCl$_2$, 1.61 mg/kg CdCl$_2$ and 0.40 mg/kg HgCl$_2$), groups 3, 4 and 5 were co-treated with metal mixture and CALE (750, 1500 and 2250 mg/kg body weight, respectively) and group 6 was treated with metal mixture and ZnCl$_2$. All treatments were administered through oral gavage for 90 days. Oxidative stress biomarkers [malondialdehyde (MDA), superoxide dismutase (SOD), glutathione content (GSH) and catalase (CAT)], inflammatory cytokines [interlukin-6 (IL-6) and interlukin-10 (IL-10)], histopathological changes and heavy metal concentration were determined in brain of rats. Results indicated that LDHMM significantly increased (p < 0.05) the lipid peroxidation marker (MDA) and the pro-inflammatory cytokine (IL-6), while lowered levels of the oxidative biomarkers (SOD, CAT and GSH) and anti-inflammatory cytokine (IL-10). Also, LDHMM caused some histopathological changes such as reactive gliosis and glia cell proliferation. LDHMM elevated the lead, cadmium and mercury concentrations in the brain. Severity of the distorted cortical parameters were ameliorated by CALE administration. The CALE induced significant protective effect on LDHMM-mediated neurotoxicity in a dose-dependent manner which may be a result of its antioxidant anti-inflammatory and metal chelation mechanisms.

1. Introduction

Neurotoxicity refers to any adverse effect to the nervous system, caused by either a physical, chemical or biological agent thereby inhibiting the ability of an organism to live or adapt to its surrounding [1]. The deleterious effect arising from short-term exposure to neurotoxicants may be compensated by the brain, but a chronic exposure even at low concentration may cause delayed brain damage [2]. Pb, Hg, Cd and As are reported to show their neurotoxic effects [3,4] through common mechanisms, such as the production of reactive oxygen species (ROS) and interaction with micronutrients [3,5,6].

As a result of its capacity to scavenge ROS, an antioxidant- rich plant might be used to protect against its toxicity. Due to the polyphenolic content of many plants across the globe, herbal medicine has been proved to prevent degenerative diseases [7]. *Costus afer* (ker gawl) is an herbaceous plant belonging to the family Zingiberaceae. It has been revealed by many authors to have a wide range of therapeutic effects. The pharmacological activities associated with *Costus afer* include antioxidant property, hepatoprotectivity, nephroprotectivity, antidiabetic role [8,9] and antinociceptive role [10].

However, investigation into the possible protective effect of *Costus afer* aqueous leaf extract (CALE) on low dose heavy metal mixture (LDHMM) - mediated neurotoxicity has not been reported. Thus, the study evaluated the possible neuroprotective role of CALE in LDHMM.
pulverized leaves were pressed and the extract was separated. The 
Ezejiofor and Orisakwe [9]. After vigorous shaking of the mixture, the 
constant agitations at intervals following the previous work done by 
water in a stoppered container and allowed to stand for 24 h with 
2.5. Sample collection 
any form of contamination and were pulverized. Then, 250 g of the 
and authentication of the plant material was done by Mr. A.O. Ozioko 
Ikwerre Local Government Area of Rivers State, Nigeria. Identification 
and given the voucher number [INTERCEDDO/033].

2.2. Preparation of the plant extract

Fresh leaves of Costus afer were collected, washed clean to eliminate 
any form of contamination and were pulverized. Then, 250 g of the 
pulverized leaves was weighed and macerated in 500 ml deionized 
water in a stoppered container and allowed to stand for 24 h with 
constant agitation at intervals following the previous work done by 
Ezejiofor and Orisakwe [9]. After vigorous shaking of the mixture, the 
pulverized leaves were pressed and the extract was separated. The 
filtered liquid was then stored in a refrigerator at 4 °C. The extract was 
redundant after the fourth day of treatment and fresh preparation was 
made. This process was continuous over the 90 days of treatment.

2.3. Animal husbandry

Forty-two young male Wistar rats, approximately 8 weeks old and 
weighing 100–200 g bought from the animal house of the Department of 
Experimental Pharmacology and Toxicology, University of Port 
Harcourt, Choba, Rivers State, Nigeria were used for the study. The test 
animals were kept for fourteen days to adapt in polypropylene cages 
under 25 ± 2 °C, with relative humidity of 55–64 % and light and dark 
conditions (12/12 h) following the previous work of Ezejiofor and Ori 
sakwe [9]. The protocol for the experiment was approved by the Uni 
versity of Port Harcourt Research Ethics Committee and the reference 
number UPH/CEREMAD/REC/04 was assigned. The animals were given 
standard feed and deionized water ad libitum.

2.4. Experimental design

Weight matched rats were divided into six groups of seven rats each. 
Group 1 served as control and received only deionized water, while 
group 2 was treated with heavy metal mixture (PbCl₂, 20 mg/kg; CdCl₂, 
1.61 mg/kg; HgCl₂, 0.40 mg/kg only) (Sigma Aldrich WGK Germany) 
according to the study by Institórás et al. [11]. Rats belonging to groups 
3, 4 and 5 received the metal mixture and Costus afer extract at 
750 mg/kg, 1500 mg/kg and 2250 mg/kg respectively according to 
Ezejiofor and Orisakwe [9]. Group 6 received the metal mixture and 
0.80 mg/kg of an antioxidant metal ZnCl₂

2.5. Sample collection

After the 90 days of treatment period, animals were sacrificed under 
ether anesthesia. The brain was weighed after excision. The relative 
brain weight was obtained by;

Relative weight = Absolute weight × 100

Final body weight

The frontal cortex of the brain was processed for the assay of heavy 
metals and other biomarkers.

2.6. Metal analysis

Acid digestion of the brain was done by using 6 ml of Nitric acid and 
2 ml of perchloric acid after isolating and weighing the organ. After 
aicification, the samples are placed for 30 min before heating at 105 °C 
until digestion is completed. The solution was then filtered with What 
mann filter paper to get a clear solution. The solution was later made up 
to 15 ml (final volume) with distilled water. Solar thermo elemental 
flame Atomic Absorption Spectrometer (Model SG 71906) was used to 
determine the levels of lead, cadmium and mercury in the frontal cortex 
of the brain.

2.7. Antioxidant analysis

2.7.1. Catalase (CAT) activity

Catalase activity was estimated by slightly modifying the method by 
Clairborne [12]. This method is supported by the principle that catalase 
in the sample preparation split hydrogen peroxide which can be 
measured spectrophotometrically at 240 nm [12].

2.7.2. Estimation of testicular reduced glutathione (GSH) level

The reduced glutathione level was estimated using the procedure 
explained by Sedlak and Lindsay [13].

2.7.3. Estimation of Superoxide dismutase (SOD) activity

Superoxide dismutase activity was evaluated by using the method 
described by Misra and Fridovich [14]. This is principally based on 
the ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2.

2.7.4. Lipid peroxidation marker (MDA) activity

The MDA level was assayed by using the procedure of Ohkawa and 
Ohishi [15]. Under acidic medium, MDA reacts with the chromogenic 
reagent, 2-thiobarbituric acid (TBA), to form a pink coloured complex at 
532 nm absorbance.

2.9. Estimation of Inflammatory cytokines [Interlukin-6 (IL-6) and 
Interlukin-10 (IL-10)]

The activities of IL-6 and IL-10 were assayed using 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 0.052 ng/l and 1.51 pg/ml respectively. 
This process is principally based on immobilization.

2.10. Determination of hormone profile levels

The enzyme linked immunosorbent assay kit (IEMA/ELISA; EIA) - 
Accubind Elisa Microwells, Monobind Inc Lake Forest; CA 92630.USA 
was used in carrying out the activities of follicle stimulating hormone 
(FSH), luteinizing hormone (LH), testosterone (TET) and prolactin 
(PRL).

2.11. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using 
SPSS package (23.0) version (SPSS Inc, USA); while multiple compari 
sions were done with Duncan’s multiple comparison method at 5 % 
significant level. All the results were expressed as Mean ± Standard 
deviation (S.D). A statistical tool, XLSTAT 2016 (Version 6 statistical 
package) was used to develop the regression models [16]. Pearson’s 
rank correlation of the inter-elemental relationship between toxic metals 
within brain of rats was also done.
3. Results

After 12 weeks of treatment, animals treated with only the metal mixture showed a marked elevation in brain weight (p < 0.05) in contrast to the control rats (treated with only deionized water). Rats that received a combination of heavy metal mixture and *Costus afer* at (750, 1500 and 2250 mg/kg respectively) and rats co-administered with zinc had significantly reduced brain weight compared to the heavy metal-mixture-exposed rats (Table 1).

Measuring the pro- and anti-inflammatory cytokine levels in the brain is essential in assessing the inflammatory status after metal mixture exposure. Co-administration of *Costus afer* and zinc significantly reduced and increased (p < 0.05) the levels of pro- and anti-inflammatory cytokines (IL-6 and IL-10) respectively when compared to those in heavy metal-mixture-treated group (Fig. 1).

The MDA level was assessed in order to evaluate the oxidative status of the brain. Heavy metal mixture treatment at a dose of 20 mg/kg PbCl2, 1.61 mg/kg CdCl2 and 0.40 mg/kg HgCl2 body weight for 90 consecutive days induced changes in the oxidative state of the brain tissue. There was a marked increase in MDA level (p < 0.05) in the brain tissue of heavy metal mixture-intoxicated rats compared to those of rats in the control group (Fig. 2). Rats co-treated with *Costus afer* and zinc however, showed a reduction in MDA level compared to metal-mixture-treated rats.

Concerning the effect on (GSH) and enzymatic (SOD and CAT) antioxidant system activities in brain, result in Fig. 2 shows that the exposure to low dose heavy metal mixture induced a marked decline (p < 0.05) in GSH content, SOD and CAT levels when compared with control rats. Rats co-treated with *Costus afer* and zinc showed elevation in GSH, SOD and CAT levels when compared with the heavy metal mixture-treated animals, representing the potent antioxidant capacities of *Costus afer* and zinc in the brain following heavy metal mixture-induced oxidative damage.

The concentrations of Pb, Cd and Hg in the frontal cortex of the brain were significantly increased (p < 0.05) in metal mixture-treated rats compared to the control rats (Table 2). However, the co-administration with *Costus afer* and zinc significantly reduced the levels of these heavy metals. Also, the result shows that the group treated with only heavy metal mixture showed the highest concentration of heavy metals (Pb = 78.906 ± 10.389, Cd = 1.543 ± 0.049 and Hg = 5.191 ± 0.287) compared to the control group (treated with deionized water only) having (Pb = 0.122 ± 0.012, Cd = <0.001 ± 0.000 and Hg = <0.001 ± 0.000).

Pearson’s rank correlation showed the inter-elemental relationship between toxic metals within brain of rats with strong positive correlation (r > 0.80) between (a) Cd and Pb (b) Hg and Pb (c) Hg and Cd. All correlations were significant at p < 0.01 (Fig. 3).

The four independent parameters (Pb, Cd, Hg and *Costus afer*) formed the input data and were allowed to pass through multiple linear regressions for purpose of model calibration. The output indicates zero coefficients for β2 and β3. Thus, subsequent model calibration excluded the three constant parameters which formed the input data. In effect, y becomes a function of *Costus afer* variable for a given set of Pb, Cd, and Hg variables. Subsequently, concentration of catalase (y) against *Costus afer* (x) was subjected into trial models of linear, quadratic and exponential options (Table 3). The process is repeated for other parameters and the best with respect to goodness of fit (R²), mean square error (MSE) and root mean square error (RMSE) values were selected and summarized as shown in Table 4 for all experiments carried out on the brain.

To verify the calibrated models, it was imperative to compare the observed against predicted testicular catalase level (Fig. 4) with corresponding goodness of fit, R² = 0.974. Similarly, the verification was repeated for other test parameters in the brain. Hence, each model is essential to forecast the applicable dependent variable for a given independent variable (*Costus afer*) at constant Pb, Cd and Hg concentrations as used in this study.

The histologic observations in the frontal cortex of the brain showed no obvious change in rats treated with only deionized water and reactive gliosis was observed in rats treated with only the heavy metal mixture. Mild signs of reactive gliosis was observed in rats co-treated with 750 mg/kg of *Costus afer* while mild gial cell proliferation was obvious in rats co-treated with 1500 mg/kg of *Costus afer*. Rats co-treated with 2250 mg/kg of *Costus afer* and 0.80 mg/kg of zinc showed no obvious histologic change (Table 5).

4. Discussion

Lead (Pb), cadmium (Cd), and mercury (Hg) are some of the most toxic metals humans are exposed to which target essential organs including brain [17] are of significant public health concerns [18]. In this study rats exposed to low dose heavy metal mixture of Pb, Cd and Hg showed an increase in the absolute weight of the brain compared to the control group. Since a significant increase or decrease in either absolute or relative weight of an organ after administering a chemical signifies the noxious effect of that chemical [19,20], this study confirms that the brain could be a target organ over a long-term exposure to low dose mixture of Pb, Cd and Hg. Treatment with *Costus afer* reversed the effect of the low dose heavy metal mixture on the absolute and relative weights of the brain in a dose dependent manner. The concomitant treatment with zinc also caused a significant decrease (p < 0.05) in the absolute brain weight compared to the toxic control group which is akin to the effect of *Costus afer* extract. *Costus afer* like zinc may be protective against low dose heavy metal mixture induced neurotoxicity in rat.

Similarly, long-term exposure to low dose heavy metal mixture of Pb, Cd and Hg caused an imbalance in immune regulation leading to a marked increase (p < 0.05) in the pro-inflammatory cytokine [interlukin-6 (IL-6)] and a significant reduction (p < 0.05) in anti-inflammatory cytokine [interlukin-10 (IL-10)] compared to the control group. On the other hand, these observations were reversed in a dose dependent manner by concomitant treatment with *Costus afer*. Interestingly both *Costus afer* and zinc an essential trace element produced the same anti-inflammatory effects on the brain. The up-regulation of anti-inflammatory cytokine [interlukin-10 (IL-10)] and down regulation of pro-inflammatory cytokine [interlukin-6 (IL-6)] by *Costus afer* suggests beneficial role of this plant extract. Cytokines attract immune cells to the site of injury or infection. Due to the ability of oxidative stress to induce several health problems, it activates persistent neuroinflammation, distinguished by the development of pro-inflammatory mediators locally produced by host cells, thus engaging the intrinsic immune system [21]. When the generation of free radicals surpasses the counteracting effects of endogenous antioxidants, ROS becomes extremely noxious to the central nervous system. Neuroinflammation supports oxidative stress and add to loss of cells and permanent neuronal dysfunction [22], even though neuroinflammation can add to the outcome of unremitting oxidative stress. The pro-inflammatory cytokines including IL-6 are potentially essential or deleterious, depending on their concentration, site and duration of action [23] and they draw...
out immune responses in the central nervous system (CNS) during inflammation. Probably, elevated pro-inflammatory cytokine signaling may encourage ROS production resulting to oxidative damage, and this may be one mechanism that relates inflammation to neuropsychiatric diseases [21].

In this study, there were severe biochemical and neurochemical changes in the brain resulting from exposure to low dose heavy metal mixture of Pb, Cd, and Hg. Rats treated with only low dose heavy metal mixture (20 mg/kg PbCl\(_2\), 1.61 mg/kg CdCl\(_2\) and 0.40 mg/kg HgCl\(_2\) mg/kg body weight) for 90 days showed oxidative stress marked by increase in MDA content and decrease in the activities of CAT, SOD and GSH. These observations are in line with the works of Radwan et al. [24] who reported that exposure to a heavy metal caused a marked elevation in MDA level and decrease in SOD, CAT and GSH contents in the brain. Our findings are also consistent with the previous works of Karaca and Eraslan [25] and Abdel Moneim [26] which reported that exposure to heavy metals could lead to reactive oxygen species (ROS) generation, causing an elevation in MDA level, sulphydryls depletion, changes of antioxidant cellular defenses and DNA damage. The elevation in the lipid peroxidation marker could be from overproduction of the superoxide anions which stifle the antioxidant enzymatic system [27]. The decreased activities of SOD and CAT levels may be because of binding of...
the heavy metals with the sulphhydryl group of these enzymes and the substitution of endogenous redox metals which changes these enzyme configurations leading to their inhibition [28]. The depletion in the GSH content may be attributed to the use of GSH in scavenging the generated free radicals. The combined effect of increased MDA and decreased SOD, CAT and GSH in our present study could lead to neurodegeneration as a result of heavy metal mixture exposure. Nevertheless, the results

Table 2
Concentration of heavy metals (mg/kg) in frontal cortex of the brain of male albino rats after exposure to heavy metal mixture with or without Costus afer and zinc.

| Treatment                | Cadmium (Cd) | Mercury (Hg) | Lead (Pb) |
|--------------------------|--------------|--------------|-----------|
| Deionized H₂O (only)     | <0.001 ± 0.000a | <0.001 ± 0.000a | 0.122 ± 0.012a |
| Metal mixture (only)     | 1.543 ± 0.049b | 5.191 ± 0.287c | 78.906 ± 10.389c |
| +750 mg/kg CA            | 0.274 ± 0.200b | 3.873 ± 0.389b | 39.278 ± 5.966b |
| Metal mixture            | 0.055 ± 0.033a | 1.534 ± 0.385a | 12.063 ± 1.308a |
| +1500 mg/kg CA           | 0.002 ± 0.001a | 0.007 ± 0.001a | 3.507 ± 1.560a |
| Metal mixture +2250 mg/kg CA | 0.002 ± 0.001a | 0.018 ± 0.021a | 2.538 ± 1.289a |
| Metal mixture +Zinc      | 0.002 ± 0.001a | 0.018 ± 0.021a | 2.538 ± 1.289a |

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; where CA = Costus afer.

Table 3
Summary of regression models for catalase (CAT) in brain.

| Model Type | Equation | R² | MSE   | RMSE |
|------------|----------|----|-------|------|
| Exponential | y = 10.324e0.000x | 0.887 | 0.012 | 0.110 |
| Linear     | y = 0.000x + 0.267 | 0.801 | 0.029 | 0.171 |
| Quadratic  | y = 2E-07x² -0.000x + 0.379 | 0.974 | 0.008 | 0.087 |

*The best model with respect to R², MSE and RMSE values. A repetition of regression models was carried out on other parameters and the best with respect to R², MSE and RMSE values was selected and summarized as shown in Table 4.

Fig. 3. Inter-elemental correlation among toxic metals within brain of rats showed strong positive correlation (r > 0.80) 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.
Toxicology Reports 7 (2020) 1032–1038

...noteworthy. Although the exact mechanism is obscure but chelation of (Cd) and mercury (Hg) following the administration of these metals by an active ingredient of this natural antidote is clear.

...and 2250 mg/kg body weight) significantly alleviated the brain oxidative status induced by heavy metal mixture exposure. This could be suggested that treatment with varying doses of *Costus afer* (750, 1500 and 2250 mg/kg body weight) significantly alleviated the brain oxidative status induced by heavy metal mixture exposure. This could be ascribed to its antioxidant properties.

...and zinc. Costus afer contains 4.09–4.75 mg/100 g zinc [35] which may suggested that treatment with varying doses of *Costus afer* (750, 1500 and 2250 mg/kg body weight) significantly alleviated the brain oxidative status induced by heavy metal mixture exposure. This could be ascribed to its antioxidant properties.

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Table 4

| Parameters                  | Model Type | Model Equations                  | R²   | MSE  | RMSE  |
|-----------------------------|------------|----------------------------------|------|------|-------|
| SOD                         | Quadratic  | \( y = 4.0 \times 10^{-6} - 9E^{-6} \times x \) | 0.996| 0.000| 0.009 |
| GSH                         | Quadratic  | \( y = 9E^{-6} + 0.000x + 1.56 \times 10^{-6} \) | 0.989| 0.007| 0.083 |
| MDA                         | Quadratic  | \( y = -6E^{-6} + 2E^{-6} \times x + 0.883 \) | 0.987| 0.001| 0.031 |
| Inflammatory cytokines      |            |                                  |      |      |       |
| IL-6                        | Quadratic  | \( y = 1E^{-6} \times x - 0.013x^2 + 32.29 \) | 0.995| 1.352| 1.163 |
| IL-10                       | Linear     | \( y = 0.013x + 2.25 \)           | 0.997| 0.729| 0.854 |

Where \( y \) = concentration of the parameter analyzed, \( x \) = *Costus afer* dose, MSE = Mean Squared Error, RMSE = Root Mean Squared Error.

Table 5

| Treatment groups            | Histopathologic Observations                        |
|-----------------------------|-----------------------------------------------------|
| Deionized water (only)      | No obvious histologic change                        |
| Heavy metal mixture (750 mg/kg CA) | Reactive gliosis observed                           |
| Heavy metal mixture + 1500 mg/kg CA | Mild signs of reactive gliosis observed              |
| Heavy metal mixture + 2250 mg/kg CA | Mild glial cell proliferation observed               |
| Heavy metal mixture + 0.80 mg/kg ZnCl₂ | No obvious histologic change                        |

*CA = *Costus afer* and ZnCl₂ = Zinc chloride.

Fig. 4. Predicted brain catalase level (nMole/mg tissue) against observed brain catalase level; where \( y = \) Catalase levels; \( x = \) *Costus afer* concentrations served as input values.

Verifying the calibrated models, it was necessary to compare observed against predicted brain catalase level with corresponding goodness of fit, \( R^2 = 0.974 \). The modeled brain catalase values for *Costus afer* at varying doses of *Costus afer* in exposed groups of male albino rats at 0 mg/kg, 750 mg/kg, 1500 mg/kg and 2250 mg/kg were 0.380, 0.372, 0.589 and 1.031, while the observed values were 0.360, 0.430, 0.530 and 1.050 respectively. Similarly, the verification was done for other parameters in the brain. Hence, each model can be used to predict the applicable dependent variable for a given independent variable (Costus afer) at constant Pb, Cd and Hg concentrations as used in this study.
5. Conclusion

Low dose heavy metal mixture caused oxidative damage in the brain which is evidenced by a change in antioxidant markers (SOD, CAT and GSH) and an increase in the lipid peroxidation marker (MDA). There was up regulation of pro-inflammatory and down regulation of anti-inflammatory cytokines, elevated levels of lead, cadmium and mercury in the brain and histopathological changes including reactive gliosis following exposure to low dose heavy metal mixture. The Costus afer aqueous leaves extract (CALE) significantly reversed all these effects in dose dependent fashion. Costus afer and zinc may be beneficial in low dose heavy metal mixture-induced neurodegeneration in rats by its antioxidant and anti-inflammatory mechanisms.

CRediT authorship contribution statement

Brilliance O. Anyanwu: Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Writing - original draft, Writing - review & editing. Chinna N. Orish: Methodology, Project administration, Validation, Visualization. Anthonet N. Ezejiofor: Methodology, Project administration, Supervision, Validation, Visualization. Ify L. Nwaagazie: Data curation, Formal analysis, Software. Orish E. Orisakwe: Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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