Assessment of Oxidative Stress Indices in Tissues of Cadmium-challenged Female Wistar Rats Treated with Combined Ethanolic Leaf Extract of *Manihot esculenta* and C*itrus sinensis*

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ABSTRACT: This research assessed the oxidative stress indices in tissues of cadmium-challenged female Wistar rats treated with combined ethanolic leaf extract of *Manihot esculenta* and C*itrus sinensis*. Eighteen female rats (150±20g) were divided put into three (3) groups: Group 1 (received feed and water only); Group 2 (received single dose of cadmium, 30mg/kg body weight at the start of the experiment) and Group 3 (received cadmium as in group 2 above and 200mg/kg body weight of combined extract of orange and cassava leaf, daily for two weeks). Cadmium load in the tissues of the rats were analyzed using AAS after acid digestion and oxidative stress indices were analyzed using standard procedures. Data obtained showed that the cadmium tissue load ($\bar{x}$±SD, μg/g tissue) were 0.005±0.0007 (liver), 0.007±0.0004 (kidney); 55.4±5.2 (liver), 28.3±4.6 (kidney); and 13.6±2.3 (liver), 20.7±2.5 (kidney) for Groups 1, 2 and 3 respectively. There was a higher concentration of Cd in the liver and kidney of rats exposed to Cd alone (Group 2) compared to the control (Group 1) and Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis* (Group 3). Exposure to Cd alone caused significant ($p<0.05$) increase in lipid peroxidation and glutathione peroxidase in the liver and kidney compared to the control. Conversely, the administration of Cd alone to rats caused significant ($p<0.05$) reduction in the level of glutathione and the activities of superoxide dismutase and catalase compared to the control in both tissues. Significant amelioration was witnessed in all the parameters in Cd-challenged rats treated with the extract compared to rats challenged with Cd alone. The results show that Cd-induced oxidative stress can be countered effectively by combined leaf extract of *Manihot esculenta* and *Citrus sinensis* which is rich in antioxidant molecules.

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Oxidative stress results when the amount of reactive oxygen species (ROS)/free radicals (superoxide radicals, O_2, hydrogen peroxide, H_2O_2, hydroxyl radicals, OH, and singlet oxygen) in cells exceeds the cell's ability to neutralize them (Sato *et al*., 2013). Free radicals come from both endogenous sources (e.g infection, excessive exercise, aging, inflammation and mental stress) and exogenous sources (e.g heavy metals, drugs, chemical solvents, cigarette smoke, alcohol, environmental pollutants and radiations)

(Valko *et al*., 2007). Over production of ROS activates deleterious consequences on proteins, nucleic acids, lipid and other vital components of the cell and has been implicated in various diseases states such as artherosclerosis, diabetes, cancer, metabolic disorders and cardiovascular system disorders (Al-Gubory *et al*., 2012; Wu *et al*., 2013). Cadmium is a heavy metal that occurs naturally in the environment and has drawn global concerns dues to its huge toxicity (Embugushiki *et al.,* 2013). It is used profusely in manufacturing of

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paints, plastics and batteries and usually gets in contact with humans through vegetables, food, welding activities, cigarette smoke, and beverages (Das *et al*., 2019). Due to the fact that Cd possesses extremely low tolerable exposure limit and a lengthy natural half-life, it bio-accumulates in humans and animal where it brings about severe damage to the liver, kidneys and other organs, tissues and cells mainly through the induction of oxidative stress (Goyer, 1997; Jacopo *et al*., 2020). It has been reported that Cd drains glutathione (an endogenous antioxidant molecule), restrain electron transport process in the mitochondrial, dislodge Zinc and other redox-active metals, and incapacitate antioxidant enzymes which results in enhanced production of reactive oxygen species (ROS) (Mona *et al.,* 2018, Orororo *et al.,* 2018; Geng *et al.,* 2019; Apiamu et al., 2019; Innih *et al*., 2021; Poli *et al*., 2022). Current research in Cd toxicity focuses on finding antioxidants that can treat Cd intoxication in biological systems, with emphasis on the antioxidant potentials of various plant extracts, which contain carotenoids, flavonoids, and other polyphenols that play pivotal roles in antioxidant defense mechanisms against Cd-induced oxidative injury (Orororo *et al*., 2018a; Genchi *et al*., 2022). *Citrus sinensis* (sweet orange) is one of the most produced fruit crops in the world. Its leaves contain important levels of proteins and minerals (Mohamed, 2006; Joshipura *et al.,* 2012). Citrus leaves also contain significant quantities of hesperidin, naringin and other phenolic compounds. *Manihot esculenta* (cassava) is a conventional and staple crop in tropical regions, like Nigeria. Significant quantities of crude protein, vitamins, carotenoids, essential amino acids (such as phenylalanine and methionine), dietary fibres, minerals, B-carotene, phenolics, anthocyanins, and flavonoids abound in cassava leaves (Linn *et al.,* 2018; Agroriches, 2022). According to Hasim *et al*. (2016), these antioxidant molecules can supply electrons to free radicals thereby slowing down their chain reactions and stabilizing free radical components. Multi-plant extracts have been shown to be generally more effective than single plant extracts (Otieno *et al*., 2008; Olajuyigbe and Afolayan, 2013, Efekemo and Orororo, 2022; Orororo *et al*., 2022).Hence, in other to find solution in the amelioration of cadmium toxicity, this study assessed the oxidative stress indices in tissues of cadmium-challenged female Wistar rats treated with combined ethanolic leaf extract of *Manihot esculenta* and C*itrus sinensis*.

MATERIALS AND METHODS

Chemicals: All chemicals and regents used were products of standard suppliers and were of analytical grade.

Plant Materials: The leaves of Orange and cassava were harvested from Kiagbodo, Delta State. The leaves were first separated from the stem, thoroughly washed with distilled water (without squeezing) to remove debris and dust particles, and thereafter dried for a few days at room temperature under shade to avoid inactivation of sensitive chemical components by ultra-violet rays (Clark and Omo-Udoyo,2021). The dried leaves were then evenly pulverized with the aid of a manual blender (Porker Manual Grinder NO. 32) and kept in air-tight containers for further use.

Extraction Method: The extraction was done using a simple maceration method depicted in Figure 1. Extraction was carried out using 200 grams of the powdered leaves immersed with 1 L of ethanol in a stoppered flask at room temperature for 3 days with frequent stirring (Clark and Omo-Udoyo, 2021). After 3 days the mixture wasfiltered using Whatman filter paper No.1 into a conical flask to obtain the ethanolic extract. The remaining residue was re-extracted using the same solvent according to the procedure described above to obtain the second ethanolic extract. This process was repeated five times in other to exhaustively extract the plants components. The extracted contents were concentrated using a rotary evaporator under reduced pressure at 40-degreecelcius to obtain a thick, viscous mass that was then air-dried.
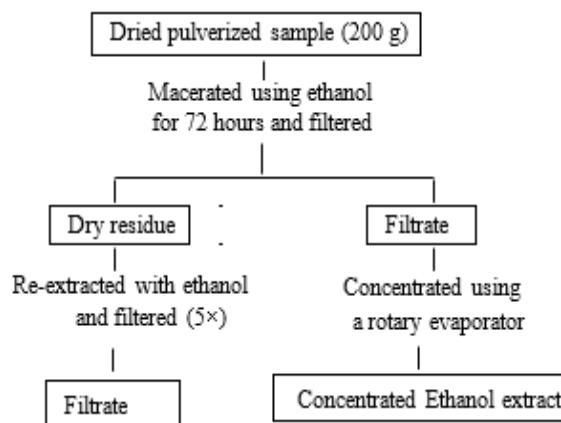

Fig. 1. Flow chart of extraction process

Experimental Animals and Experimental Design: A total of 18 female Wistar rats (150±20g) were used for the experiment. The animals were obtained from the Faculty of Basic Medical Sciences, Delta State University, Abraka and were acclimatized in the animal house of Edwin Clark University, Kiagbodo Delta State for one week before the commencement of the experiment. The animals were handled in accordance with international protocols for handling experimental animals. The animals were divided into three (3) treatment groups:
Group 1 – Control (received feed and water only)

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Group 2 – Cadmium (received single dose of cadmium, 30mg/kg body weight at the start of the experiment)

Group 3 - Cadmium + combined leaf extract (received cadmium as in group 2 above and 200mg/kg body weight of combined extract of orange and cassava leaf, daily for two weeks)

The toxicant (Cd) and the extract were administered to the animals orally by syringe through gastric intubation. At the end of the experimental period, the animals were sacrificed, after a 24 hrs fast via cervical dislocation technique. The liver and kidney were obtained and weighed and 1g portion of each was homogenized in phosphate buffer and centrifuged at 600g for 10mins. Supernatants collected were stored frozen until used for analysis.

Digestion of samples: Exactly 20 ml HNO_3 and $HClO_4$ mixed in a ratio of 4:1 (v/v) was used in digesting weighed portions of the liver and kidney separately into beakers (Asagba, 2010). The samples were heated at 100°C to facilitate the digestion and thereafter, were diluted with distilled water after cooling to reach 100 ml as final volume.

Cadmium analysis: Atomic absorption spectrophotometry was utilized in assessing the Cd load in the digested samples using Cd dissolved in distilled water as standard (Asagba, 2010).

Biochemical assays: Catalase Activity assessment: The activity of catalase in the samples was assessed using the method proposed by Sinha (1972) which rests on the fact that dichromate mixed with acetic acid is converted to chromic acetate upon heating along with hydrogen peroxide. The chromic acetate so formed is thereafter measured spectrophotometically at 570-610nm.

Superoxide Dismutase (SOD) activity assessment: This was done by employing the procedure described by Misra and Fridovich (1972) in which the autoxidation of adrenaline is inhibited by SOD at pH 10.2. One unit of SOD activity is therefore defined as the quantity of the enzyme that can bring about 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

Lipid Peroxidation assessment: Following the method described by Varshney and Kale (1990), lipid peroxidation was determined by measuring the formation of Malonaldehyde (MDA). The assay is based on the understanding that under acidic condition, malondialdehyde (MDA) produced from the peroxidation of membrane fatty acid and food product react with a chromogenic reagent to yield a pink coloured complex which have maximum absorbance at 532nm and fluorescence at 553nm.

Reduced Glutathione (GSH) Assessment: The method of Beutler *et al.* (1963) was followed in estimating the level of reduced glutathione (GSH) based on the fact that the bulk of cellular non-protein sulfhydryl groups is in most instances composed of the reduced form of glutathione. When 5'5'-dithiobis-(2-nutriobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds, a relatively stable (yellow) colour is obtained called 2-nitro-5-thiobenzoic acid, which possesses molar absorption at 412nm.

Glutathione peroxidase (GPx) assessment: This was done according to the method described by Paglia and Valentine (1967). The assay is based on the reaction catalyzed by Glutathione Peroxidase (GPx) in which Glutathione (GSH) is oxidized by Cumene Hydroperoxide. Oxidized Glutathione (GSSG) is converted to the reduced in the presence of Gluthathione Reductase (GR) and NADPH with a concomitant oxidation of NADPH and NADP+. The decrease in absorbance at 340nm is measured.

Statistical Analysis: The results obtained from this study were analysed by one way analysis of variance (ANOVA), followed by Least Significance Difference (LSD) test to ascertain the difference between the mean value of the measured parameters in the respective test and control groups. A significant change was considered acceptable at P<0.05. Results of the biochemical estimations are reported as means ± SD.

RESULTS AND DISCUSSION

Cadmium Load in the tissues of Cd-challenged rats treated with combined leaf extract of Manihot esculenta and Citrus sinensis: In this study an attempt was made to assess the effect of combined leaf extract of *Citrus sinesis* and *Manihot esculenta* on oxidative stress parameters in the tissues of Cd-challenged rats. The study also assessed the possible influence of the extracts on Cd load in the tissues following oral Cd administration. Table 1 shows the Cadmium Load in the tissues of Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis*. There was a higher concentration of Cd in the liver and kidney of rats exposed to Cd alone (Group 2) compared to the control (Group 1) and Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis* (Group 3). However, the liver retained higher concentration of Cd than the kidney.

This observation agrees with earlier reports, which confirmed that the liver and kidney are principle sites of Cd accumulation in the body (Haouem *et al.*, 2007; Koizumi *et al.*, 2008). The fact that the liver retained higher concentration of Cd than the kidney as witnessed in this study also agrees with the reports of Horiguchi *et al.* (1996), Borges *et al.* (2008) and Asagba (2010), which stated that following oral and subcutaneous administration of Cd, there was higher deposition in the liver than in the kidneys. Although, absorbed Cd binds to various macromolecules and proteins such as Metallothionein, there is little or no metabolism of Cd. The liver produces Metallothionein that is sufficient to bind all accumulated Cd. According to Kutlu (2006), the metallothionein-bound Cd is released from the liver into the blood where it is cleared by glomerular filtration in the kidney and taken up by the renal tubules.

There, the metallothionein is cleaved and Cd is released and because, the synthesis of metallothionein in the kidney is lower and insufficient to bind all the free cadmium, tubular damage or cell membrane destruction via activation of oxygen species results (Chang *et al.*, 2009). The result of this study also showed that the administration of the combined leaf extract to Cd-challenged rats significantly reduced Cd load in the liver and kidney compared to the untreated rats (Group 2). This may be due to the effect of the bioactive molecules in the extracts which limited Cd absorption/uptake. It has previously been shown that nutritional deficiencies, such as proteins, trace elements and antioxidants affect Cd absorption (Ferramola *et al.*, 2012).

Table 1: Cadmium Load in tissues of Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis*

Groups	Tissues	
	Liver	Kidney
Group 1 (Control)	0.005 ± 0.0007^a	0.007 ± 0.0004^a
Group 2 (Cadmium only)	55.4 ± 5.2^b	28.3 ± 4.6^b
Group 3 (Cd+ Cassava and Orange Leaf Extract)	13.6 ± 2.3^c	20.7 ± 2.5^b

Values are presented as mean $\pm$ standard deviation (SD). Units are (µg/g tissue) n=3. Values on the same column with different superscripts differ significantly (p<0.05).

Oxidative stress indices in the liver of Cd-challenged rats treated with combined leaf extract of Manihot esculenta and Citrus sinensis: Oxidative stress indices in the liver of Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis* is depicted in Table 2. Exposure to Cd (Group 2) caused profound (p<0.05) elevation in lipid peroxidation (MDA) (141.67±10.4) compared rats not challenged with Cd (65.00±3.60). GPx activity the samples followed a similar trend as that of lipid peroxidation. Conversely, the administration of Cd (Group 2) to rats caused profound (p<0.05) decrease in the level of glutathione (30.33±1.52) and the activities of SOD (32±3.0) and CAT (27.33±3.05) relatively to the control (Group 1). GSH levels (66±3.60) in Cd-exposed rats treated with the extract (Group 3) was appreciably (p<0.05) higher than in rats challenged with Cd alone (Group 2). A similar trend was recorded in the values of SOD (57±3.0) and CAT (48.66±1.52) in Cd-challenged rats treated with the extract compared to those challenged with Cd alone (Group 2). MDA level significantly reduced in Cd-challenged rats treated with the extract compared to Cd alone (Group 2).

Table 2: Oxidative stress indices in the liver of Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis*

GROUPS	Liver				
	GSH	MDA	SOD	CAT	GPx
Group 1 (Control)	61.33 ± 3.21^a	65.00 ± 3.60^a	55.67 ± 2.08^a	45 ± 3.0^a	52.12 ± 2.32^a
Group 2 (Cadmium only)	30.33 ± 1.52^b	141.67 ± 10.4^b	32 ± 3.0^b	27.33 ± 3.05^b	74.34 ± 2.34^b
Group 3 (Cd+ Cassava and Orange Leaf Extract)	66 ± 3.60^a	81.67 ± 3.51^c	57 ± 3.0^a	48.66 ± 1.52^a	50.56 ± 2.15^a

Values are presented as mean $\pm$ standard deviation (SD). Units are µmole/mg protein (MDA, SOD, GSH & GPx) and mmoles H_2O_2 consumed min-1 mg-1 protein (CAT). n=3. Values on the same column with different superscripts differ significantly (p<0.05).

Oxidative stress indices in the kidney of Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis:* Oxidative stress indices in the kidney of Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and C*itrus sinensis* is presented in Table 3. The group exposed to only Cd (Group 2) experienced noteworthy (p<0.05) rise in lipid peroxidation (MDA) (119.66±3.51) relative to the control (47.66±2.08). Whereas, the administration of Cd alone (Group 2) to rats brought about major (p<0.05) reduction in the

level of glutathione (31.33±3.05) and the activities of SOD (18.33±1.52) and CAT (13.60±2.08) compared to the control (Group 1). GSH levels (49.67±2.08) in Cd-challenged rats administered the extract (Group 3) was significantly (p<0.05) higher than in rats challenged with Cd alone (Group 2). Same flow was evidenced in the values of SOD (40.66±2.52) and CAT (18.65±1.53) in Cd-challenged rats given the extract compared to those exposed to Cd alone (Group 2). MDA level was significantly reduced in Cd-challenged rats treated with the extract compared to Cd

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alone (Group 2). A similar result was obtained for the activity of glutathione peroxidase in the samples. GPx activity was significantly increased from 35.74±2.63in the control to 42.34±2.50 in rats challenged in Cd alone (Group 2). Significant amelioration was also witnessed in GPx activity in Cd-challenged rats which received the extract (Group 3) compared to rats challenged with Cd alone (Group 2).

Table 3: Oxidative stress indices in the kidney Cd-challenged rats treated with combined leaf extract of *Manihot esculenta* and *Citrus sinensis*

GROUPS	Kidney				
	GSH	MDA	SOD	CAT	GPx
Group 1 (Control)	64.67±2.51[a]	47.66±2.08[a]	38.66±1.52[a]	26.60±2.52[a]	35.74±2.63[a]
Group 2 (Cadmium only)	31.33±3.05[b]	119.66±3.51[b]	18.33±1.52[b]	13.60±2.08[b]	42.34±2.50[b]
Group 3 (Cd+ Cassava and Orange Leaf Extract)	49.67±2.08[c]	81.33±3.06[c]	40.66±2.52[a]	18.65±1.53[c]	36.78±1.92[a]

Values are presented as mean ± standard deviation (SD). Units are µmole/mg protein (MDA, SOD, GSH & GPx) and mmoles H_2O_2 consumed min-1 mg-1 protein (CAT). n=3. Values on the same column with different superscripts differ significantly (p<0.05).

The significant increase in lipid peroxidation and glutathione peroxidase (GPx) in Cd-challenged rats compared to control and significant decrease in glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in both the liver and kidey, recorded in this study is a clear indication of the Cd-induced oxidative stress. This finding agrees with the confirmed ability of Cd to induce oxidative stress in the rat tissues (Mona *et al.,* 2018, Orororo *et al.,* 2018b; Apiamu *et al*., 2019; Jacopo *et al*., 2020; Innih *et al*., 2021; Renuka *et al*., 2021; Poli *et al*., 2022). Cd, unlike other heavy metals is unable to generate free radicals by itself, however, according to Galan *et al*. (2001), it can generate hydroxyl radical, superoxide radical, and nitric oxide radicals indirectly. Watanabe *et al*. (2004) showed that Cd can generate non-radical hydrogen peroxide which can become a significant source of free radicals. This is because Cd replaces iron and copper from a number of cytoplasmic and membrane proteins like ferritin, which in turn would release and increase the concentration of unbound iron or copper ions. Accoording to Waisberg *et al*. (2003), these free ions participate in causing oxidative stress via the Fenton reactions. Watjen and Beyersmann (2004) showed evidence in support of this proposed mechanism. According to them, copper and iron ions displaced by Cd, reacted with hydrogen peroxide via the Fenton reaction to generate activated oxygen radicals. According to Casalino *et al*, (2002), Cd can bind to the imidazole group of the His-74 in SOD, which is vital for the breakdown of hydrogen peroxide, thus causing its toxic effects. The major cellular enzymatic antioxidants are superoxide dismutase (SOD), which degrades O_2^-, Catalase, and the glutathione (GSH) redox system, which inactivates H_2O_2 and hydroperoxides. GSH forms intermolecular disulphide non-radical end-product called oxidized glutathione (GSSG), which is either exported from the cell or converted to GSH by a reductase reaction that obtains electrons from NADPH (Rania*et al*., 2014). According to Dudley *et al*., (1982), since GSH is abundant in the liver, it is thought to be the first line of defence against Cd hepatotoxicity as Cd binds tightly

to thiol groups. Thus, the depletion of hepatic GSH greatly accelerates Cd-induced hepatotoxicity. This according to Liu *et al*. (2009) suggests that the disruption of the cellular GSH system is a key element for Cd-induced oxidative stress in the liver. Administration of combined leaf extracts of orange (*Citrus sinesis*) and *Manihot esculenta* (cassava) showed a significant reversal effect, in both the liver and kidney, which may be due to the antioxidant properties of the extract which contain significant quantities of flavonoids, Vitamin E and C (Agroriches, 2022). Dai and Mumper (2010) defined antioxidants as biochemical molecules with the ability of delaying, inhibiting or preventing the oxidation of oxidizable materials. This is achieved when they scavenge free radicals and thus reduce oxidative stress. Dai and Mumper (2010) and Miguel (2011) stated the following mechanisms of action of antioxidants in an oxidative sequence: preventing chain initiation by scavenging reactive oxygen and/or nitrogen species (ROS/RNS), e.g., superoxide anion, hydrogen peroxide, hydroxyl radical and peroxynitrite; decreasing localized oxygen concentrations; binding metal ions in such a manner that they will not generate species such as HO*, ferryl or $Fe^{2+}/Fe^{3+}/O^2$, and/or decompose lipid peroxides to peroxyl and alkoxyl radicals; decomposing peroxides by converting them to non-radical products, such as alcohols; and chain-breaking by scavenging intermediate radicals, such as alkoxyl radicals and peroxyl. This prevents continued hydrogen abstraction. Treatment with the combined leaf extracts of *Citrus sinesis* and *Manihot esculenta* may significantly restored oxidative stress indices in both liver and kidney by decreasing or stopping the increased generation of free radicals. This adds to the list of evidences that proofs the ability of natural herbal extracts/formulations to protect cells/tissues against toxicity induced by heavy metals (Aja *et al*., 2020; Etemadi *et al*., 2020; Handan *et al*., 2020).

Conclusion: The ability of Cd to induce oxidative stress was strongly evidenced by the results of this study as the administration of Cd affected the oxidative

stress indices (CAT, MDA, SOD, GSH and GPx) adversely. However, the treatment of Cd-challenged rats with combined extract of cassava and orange leaves extract resulted in positive reduction in the Cd induced changes in the liver and kidney oxidative stress indices, suggesting that it can be used as part of treatment against Cd toxicity which is still a common environmental pollutant.

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