

ORIGINAL CONTRIBUTION

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Green synthesized hydroxamic acid administered in high dose disrupts the antioxidant balance in the hepatic and splenic tissues of albino rats

Chiagoziem A. Otuechere^{1*} , Adewale Adewuyi² and Oluwabukola Bankole¹

Abstract

Background: Hydroxamic acids are currently being used in diverse biological activities. We investigated the effect of hydroxamic acid, synthesized from *Cyperus esculentus* seeds, on the antioxidant status of the liver, spleen, and kidney of Wistar rats.

Methods: Twenty male rats were randomly divided into three treatment groups using hydroxamic acid at doses of 5, 15, and 50 mg/kg and a control group using distilled water. Rats were sacrificed 24 h after a seven-day repeated oral dosing. After that, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and the levels of glutathione (GSH) and malondialdehyde (MDA) were investigated in the organs.

Results: Our data showed that MDA levels remained unaltered in the liver, spleen, and kidney. However, it was found that hydroxamic acid at the dose of 50 mg/kg significantly increased SOD activity but depleted CAT, GPx activities and GSH levels in the liver when compared to the control groups. In splenic tissue, SOD activity and GSH levels were significantly diminished. Contrarily, in the kidney, treatment of rats with 50 mg/kg hydroxamic acid did not affect SOD activity, but GPx activity was increased while GST activity was decreased when compared to the controls.

Conclusion: Overall, hydroxamic acid may enhance antioxidant enzyme activities in the liver and kidney. However, caution is required at higher doses to forestall oxidative stress in the hepatic and splenic tissues.

Keywords: Antioxidant status, *Cyperus esculentus*, Hydroxamic acid, Liver, Spleen

Introduction

The biosynthesis of hydroxamic acid compounds presents high potentials due to their versatile applications and biological activities. Hydroxamic acids are groups of naturally occurring and synthetic weak organic acids with general formula R-CO-N-OH. They are common in the tissues of plants and metabolites of bacteria and fungi. Hydroxamic acids are generally synthesized by the chemical reaction of an alkyl or aryl ester with hydroxylamine in high alkaline medium. Fatty hydroxamic acids

have been synthesized via refluxing of triacylglycerides, palm olein, palm stearin, or corn oil with hydroxylamine hydrochloride [1]. Fatty hydroxamic acid derivatives have also been synthesized from palm kernel oil in a biphasic hexane/aqueous medium using Lipozyme TL IM as a catalyst [2]. Metalloenzymes such as carbonic anhydrase, leukotriene A4 hydrolase, matrix metalloprotease, urease, and histone deacetylase have been implicated in the etiology of cancer due to their roles in stimulating cell division. Consequently, the metal-scavenging effect of hydroxamic acids, which could block the activities of these enzymes, has been exploited as a pharmacotherapeutic strategy against cancer and other allied ailments [3]. Specifically, hydroxamic acid compounds have found

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applications as histone deacetylase inhibitors [4], inhibitors of metallo- β -lactamase [5], antitumor [6], anti-trypanocidal [7] and antibacterial agents [8]. Their antiradical, chelating, and antioxidant properties have also been reported [9]. Suberoyl bis-hydroxamic acid has been reported to exert anti-growth effects in several malignancies, including breast cancer [10]. Furthermore, the hydroxamic acids could be incorporated into polymers for use as ion-exchange resins or for water treatment [11].

There are several ways of synthesizing hydroxamic acid compounds. The most important route is via the N-alkylation of O-substituted hydroxylamine using various alkylating agents. However, this synthetic strategy is expensive and potentially toxic as by-products are released in the process [3]. As an alternative, research on the possible bio-applications of green synthesized compounds is gaining currency. Silver nanoparticles prepared from *Salvia officinalis* extract have been reported to possess antioxidant and anti-inflammatory activities [12]. Similarly, azomethine synthesized from cinnamaldehyde, obtained from the bark of a cinnamon tree, has been shown to have antidepressant activity [13]. In this study, we have synthesized hydroxamic acid from the seeds of *Cyperus esculentus* (common name Tiger nut). Mineral and vitamin analyses of the seeds of *Cyperus esculentus* revealed the presence of essential elements (phosphorus, magnesium, potassium, calcium, etc) and water-soluble vitamin C [14]. Volatile constituents of *Cyperus esculentus* such as p-Vinylguaicol and cyprotundone, with antioxidant activities have also been identified [15]. Recently, Nwozo and Nwawuba [16] reported the antioxidant enzymes enhancement potential of *C. esculentus* oil in a rat model of experimental Type 2 diabetes. The objective of this study is the green synthesis of hydroxamic acid and the evaluation of its effects on antioxidant homeostasis in key organs (liver, kidney, and spleen) of rats prior to its utilization as a useful pharmaceutical compound.

Methods

Green synthesis of hydroxamic acid

The seeds of *Cyperus esculentus* were purchased from Festac Town market in Lagos State, Nigeria and identified at the Herbarium Unit, Botany Department University of Ibadan. Hydroxamic acid was synthesized from *C. esculentus* seeds, as previously described [17]. Briefly, oil extracted from the seeds of *C. esculentus* was reacted with 2.75 g of hydroxylamine hydrochloride and 16.5 mL of water, followed by addition of potassium hydroxide, and ethanol. The mixture was gently warmed for 10 min. After cooling, the resulting solid was filtered and dried to give hydroxamic acid. The infrared spectra of hydroxamic acid have been previously reported [17].

Animals

Experiments were performed on healthy, male Wistar rats weighing approximately 270–320 g. Animals were obtained from the primate colony of the Department of Veterinary Pathology, University of Ibadan, Nigeria. All animals received humane care by guidelines governing the handling of laboratory animals as outlined by the Redeemer's University Committee on Ethics for Scientific Research. Before dosing, they were acclimatized for 14 days and subjected to natural photoperiod of 12 h light/12 h dark cycle. The animals were housed in stainless cages with the temperature maintained at $25 \pm 2^\circ\text{C}$. Rats were fed on a commercial pelleted diet (Ladokun Feeds Ibadan, Nigeria) and drinking water ad libitum.

Experimental design

Twenty rats were randomized into four groups of five animals each and were treated as follows for seven days:

- Control: administered 1 ml distilled water, orally, once, daily
- Treatment groups 1–3: administered 5, 15, and 50 mg/kg hydroxamic acid, respectively, orally, once, daily. These doses represented low, medium, and high doses of exposure, as reported previously [17].

Tissue processing

At the termination of studies, rats were sacrificed by cervical dislocation. Liver, kidney, and spleen tissues were harvested, rinsed in ice-cold potassium chloride solution and homogenized in phosphate buffer (0.1 M, pH 7.4). Homogenates were centrifuged at 12000 g, and the supernatants were used for biochemical assays.

Estimation of superoxide dismutase (SOD) activity

The SOD activity was determined using the method of Misra and Fridovich [18]. Briefly, 3 mL of freshly prepared epinephrine was added to a reaction mixture containing 2.5 mL of sodium carbonate buffer and 0.02 mL of homogenate (Liver, spleen, and kidney). Change in absorbance was monitored at 480 nm at intervals of 60 s for 180 s.

Estimation of catalase (CAT) activity

CAT activity in liver, kidney, and spleen homogenates was determined by the procedure of Sinha [19]. Briefly, homogenates (0.1 mL) were incubated with 0.5 ml of H_2O_2 in the presence of 0.01 M phosphate buffer (pH 7.4). The reaction was terminated by adding 5% dichromate solution. The reaction mixture was incubated at 100°C for 15 min. The CAT activity was measured in terms of the amount of H_2O_2 consumed.

Estimation of glutathione peroxidase (GPx) activity

The activity of GPx was evaluated by the method of Rotruck et al. [20]. Briefly, an assay mixture containing 0.5 mL sodium phosphate buffer, 0.1 mL sodium azide, 0.2 mL glutathione, 0.1 mL H₂O₂ and 0.2 mL of homogenates (liver, kidney, and spleen) was incubated for 3 min at 37 °C, before termination of reaction with 0.5 mL of trichloroacetic acid (TCA). After centrifugation, 4 mL disodium hydrogen phosphate and 1 mL of 5, 5'-Dithio-bis (2-nitrobenzoic) acid (DTNB) were added to the supernatant and absorbance was measured at 412 nm.

Estimation of glutathione S-transferase (GST) activity

GST activity was determined, according to Habig et al. [21]. In this procedure, assay mixture containing 0.03 mL of homogenates (liver, kidney, and spleen), 2.79 mL phosphate buffer, 0.15 mL of 1-chloro-2, 4,-dinitrobenzene and 0.03 mL of GSH were mixed by inversion and immediately read at 340 nm against sample blank 60-s interval for 180 s.

Estimation of glutathione (GSH) level

For the quantification of GSH, absorbance recorded at 412 nm, as previously described by Beutler et al. [22]. Briefly, a reaction mixture containing 0.2 mL of homogenates (liver, kidney, and spleen), 3.3 mL of sulfosalicylic acid and 1.5 mL distilled water allowed to stand for 5 min and filtered to obtain a supernatant. Subsequently, 0.5 mL DTNB in phosphate buffer, was added to the reaction mixture, and measured spectrophotometrically after 15 min.

Estimation of malondialdehyde (MDA) level

The MDA level was estimated according to a previous protocol [23]. 0.4 mL of homogenates (liver, kidney, and spleen) was mixed with 1.6 mL of Tris-KCl buffer to which 0.5 mL of TCA and TBA were added. The reaction mixture was incubated in a water bath for 45 min at 80 °C, then cooled and subjected to centrifugation at 3000 g. Absorbance was measured at 532 nm. A molar extinction coefficient of $1.56 \times 10^{-5} \text{ M}^{-1} \text{ cm}^{-1}$ was utilized in computing the MDA levels.

Estimation of Total protein

The protein concentrations in homogenates (liver, kidney, and spleen) were assayed according to the method of Gornall et al. [24].

Statistical analysis

Data are expressed as the mean \pm standard error of the mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) and complemented with Duncan's multiple comparisons between control and treated animals in all groups using Graph Pad

Prism statistical software. *P-values* less than 0.05 were considered to indicate statistical significance.

Results

Effect of hydroxamic acid on defensive antioxidant enzymes

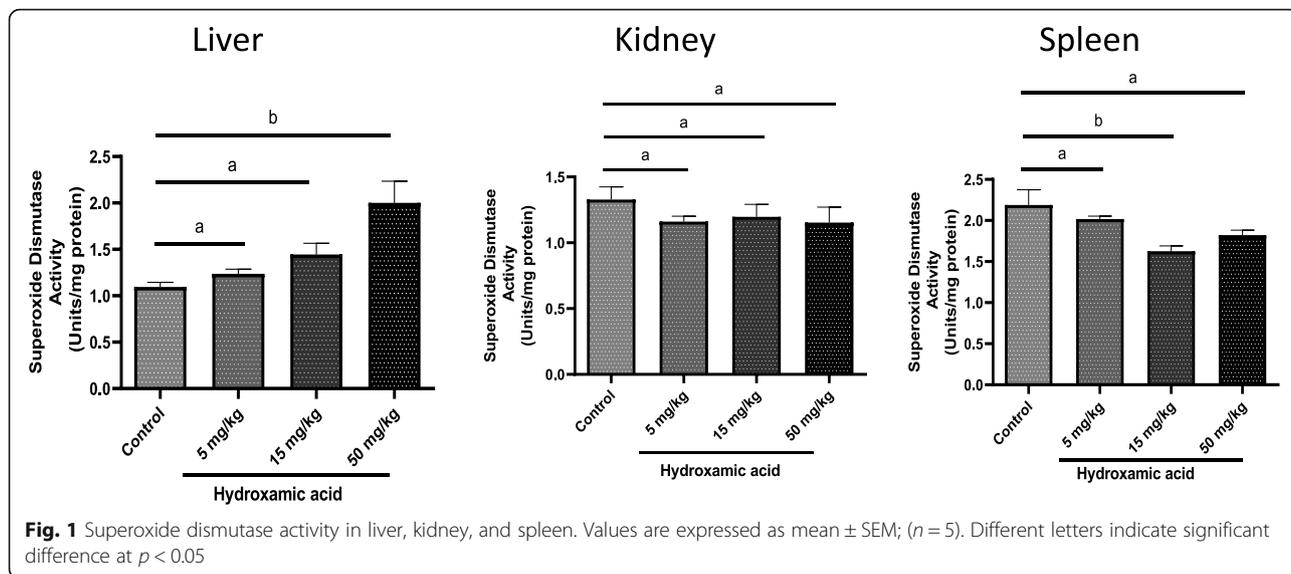
The activity of SOD in liver, kidney, and spleen of rats treated with 5, 15, and 50 mg/kg hydroxamic acid, respectively, is shown in Fig. 1. The low and medium doses (5 and 15 mg/kg) of hydroxamic acid did not affect SOD activity in the liver and kidney. However, the 50 mg/kg hydroxamic acid significantly ($p < 0.01$) elevated the SOD activity in the liver by 83.5% compared to the control group. Contrariwise, there was a significant decrease in SOD activity in the spleen of rats. Figure 2 showed that the treatment of rats at all doses of hydroxamic acid did not elicit any significant change in the kidney and spleen CAT activities, whereas treatment with 15 and 50 mg/kg hydroxamic acid caused significant ($p < 0.05$) and dose-dependent decrease in CAT activity in the liver compared to their respective controls.

Effect of hydroxamic acid on glutathione antioxidant defenses

Figure 3 indicates the changes in GPx activities in liver, kidney, and spleen of rats exposed to hydroxamic acid at the doses of 5, 15, and 50 mg/kg. At the 50 mg/kg treatment dose, there was a significant ($p < 0.01$) increase in GPx activity in the kidney. Furthermore, hydroxamic acid, at all doses, significantly ($p < 0.01$) inhibited GPx activity in the liver. However, administration of hydroxamic acid at all treatment doses did not significantly alter the GPx activities in the spleen. Figures 4 and 5 summarize the GST activities and GSH levels in rats exposed to hydroxamic acid. The changes in GST and GSH revealed different effects of hydroxamic acid in the specific organs. While the GST activities remained unchanged in the spleen, intoxication of rats with hydroxamic acid at the 50 mg/kg dose elicited a significant ($p < 0.05$) depletion of GST activity in the kidney compared with the control group. Contrariwise, administration of hydroxamic acid at the 5 and 15 mg/kg doses elicited a significant ($p < 0.01$) induction in GST activity in the liver. At the 5 mg/kg hydroxamic acid dose, there was a non-significant increase in GSH levels in the liver and spleen. However, the splenic GSH levels were significantly diminished ($p < 0.05$) following treatment of rats with 50 mg/kg hydroxamic acid (Fig. 5).

Effect of hydroxamic acid on malondialdehyde levels

There was no treatment-related changes in the liver, kidney, and spleen MDA levels following the administration of hydroxamic acid at the 5, 15, and 50 mg/kg doses (Fig. 6).



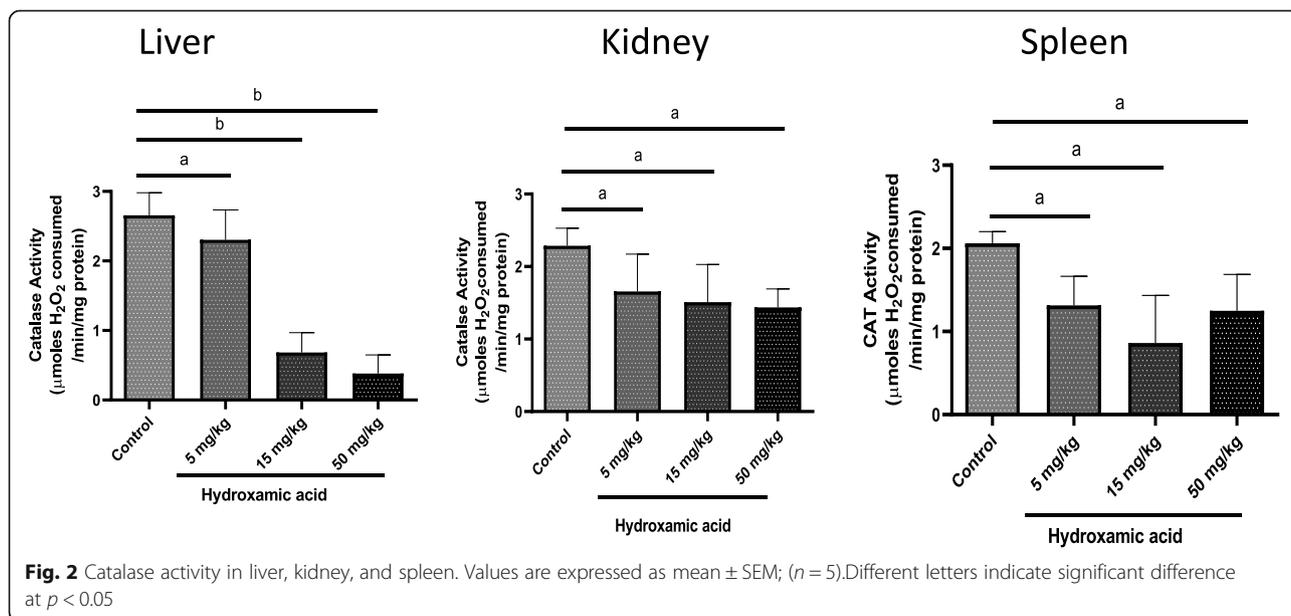
Discussion

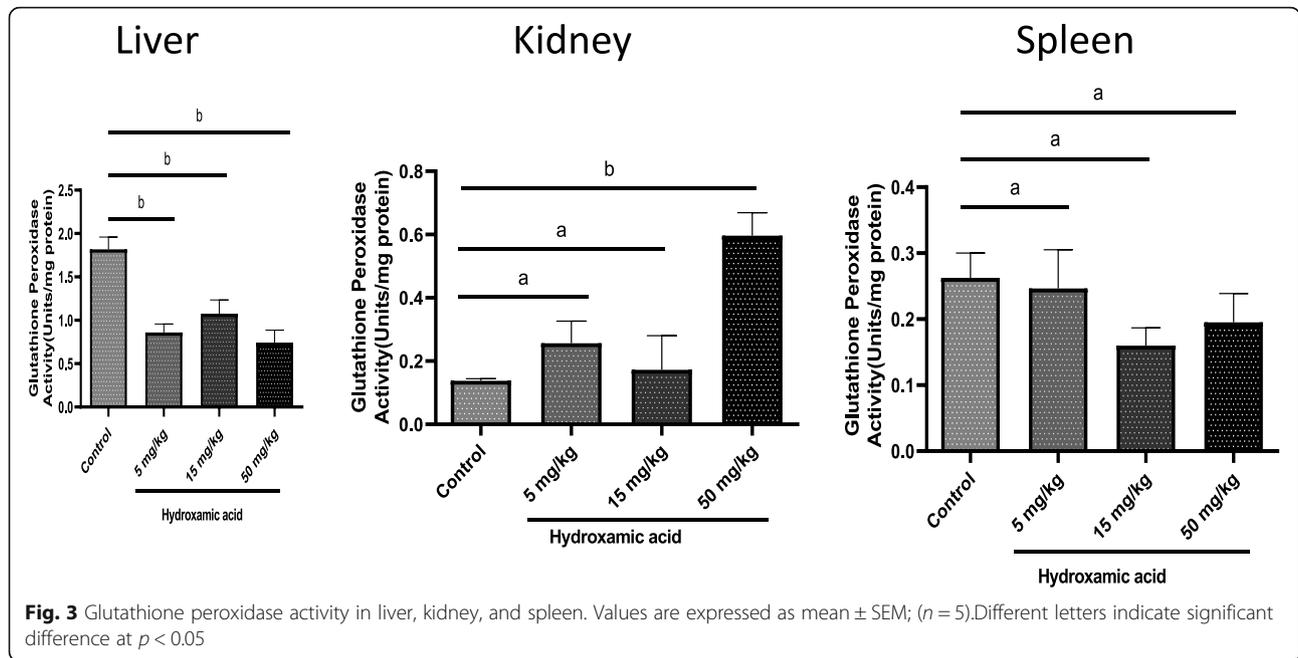
The present study evaluated the effect of hydroxamic acid on antioxidant homeostasis in the liver, kidney, and spleen of rats after short-term treatment. The results revealed that hydroxamic acid caused an imbalance in hepatic and splenic antioxidant markers, especially at the high dose treatment of 50 mg/kg.

Antioxidant defenses, ubiquitous in aerobic organisms, comprise of antioxidant molecules that serve an innate protective role by scavenging free radicals [25]. These antioxidant defense molecules, including GSH, GST, CAT, SOD, GPx, glutathione reductase (GR) and glucose-6-phosphate

dehydrogenase, can serve as indicators of oxidative stress in an organism [26, 27].

In the primary antioxidant defense mechanism, SOD catalyzes the dismutation of superoxide ion to hydrogen peroxide, which is then eliminated by CAT [28]. The present investigation showed that short-term administration of hydroxamic acid elicited alterations in the hepatic and splenic SOD activities as well as hepatic CAT activity. The increase in the hepatic activity of SOD indicates its induction by hydroxamic acid. An increase in SOD activity has been reported to be beneficial in the event of increased free radical generation [28]. On the other

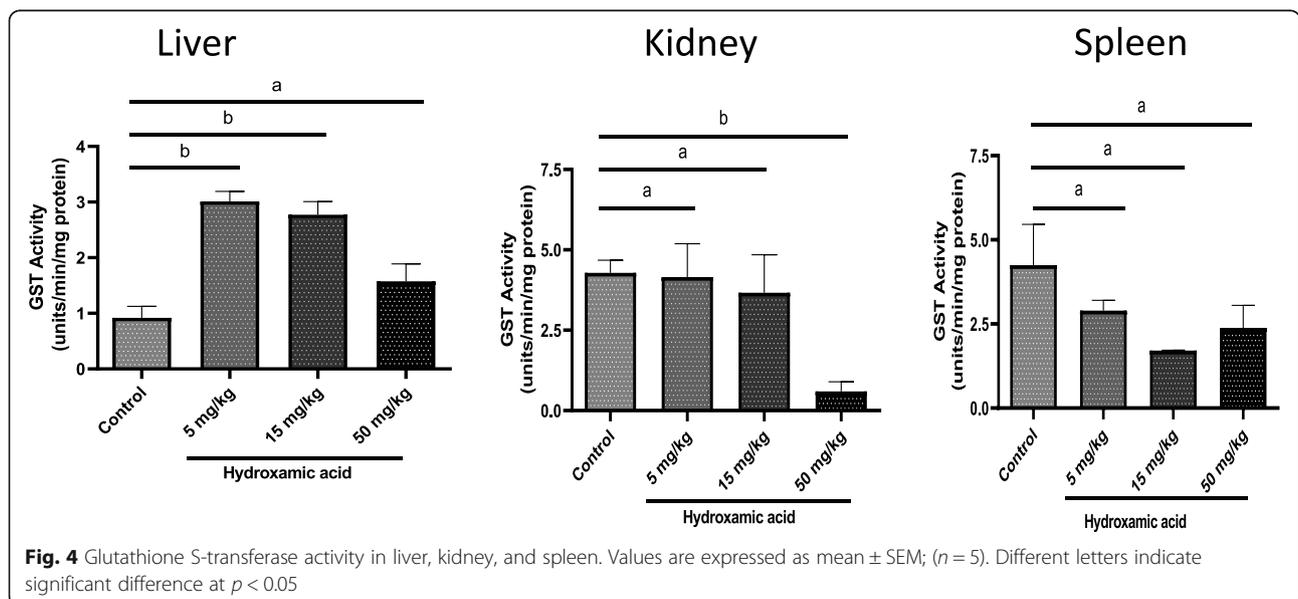


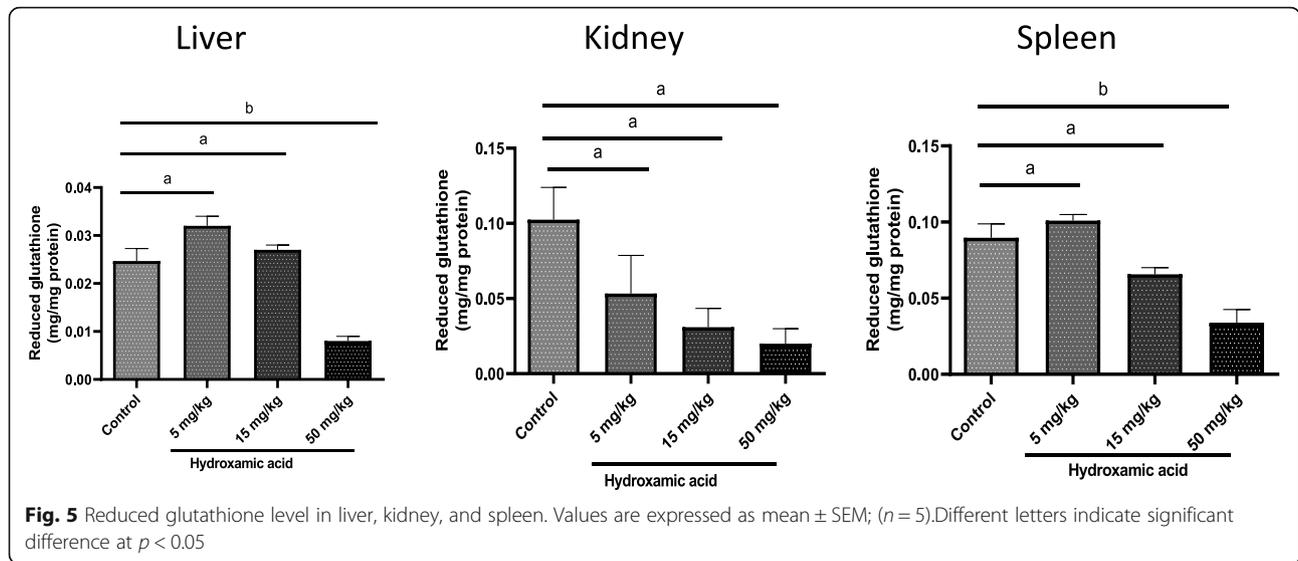


hand, the decrease of CAT activity in response to higher doses of hydroxamic acid can reduce the protection against free radicals. A similar trend of a concomitant increase in SOD and decrease in CAT activities has also been observed in the testes of rats treated with cyclophosphamide [29]. It is also plausible that the depletion of splenic SOD activity following exposure to hydroxamic acid could affect its immune function, as suggested by Estruel-Amades and co-workers [30].

The glutathione redox cycle, which includes GSH, GPx, and GR, plays a vital role in the detoxification of ROS, to protect cells from the potential toxicity and carcinogenesis

[31]. On the other hand, GST is a family of isoenzymes that catalyze the conjugation of GSH with a wide variety of organic peroxides (including lipid peroxides) to form more water-soluble products that are readily excreted from the system [32]. The highest dose of a hydroxamic acid (50 mg/kg) caused a significant decrease in GST activities in the kidney. The reduced activity of GST in this organ may partly be due to the lack of its substrate, GSH, and also because of oxidative modification of its protein structure. However, liver GST activity increased significantly at the doses of 5 and 15 mg/kg. The organs' differences in enzyme activities might result from different

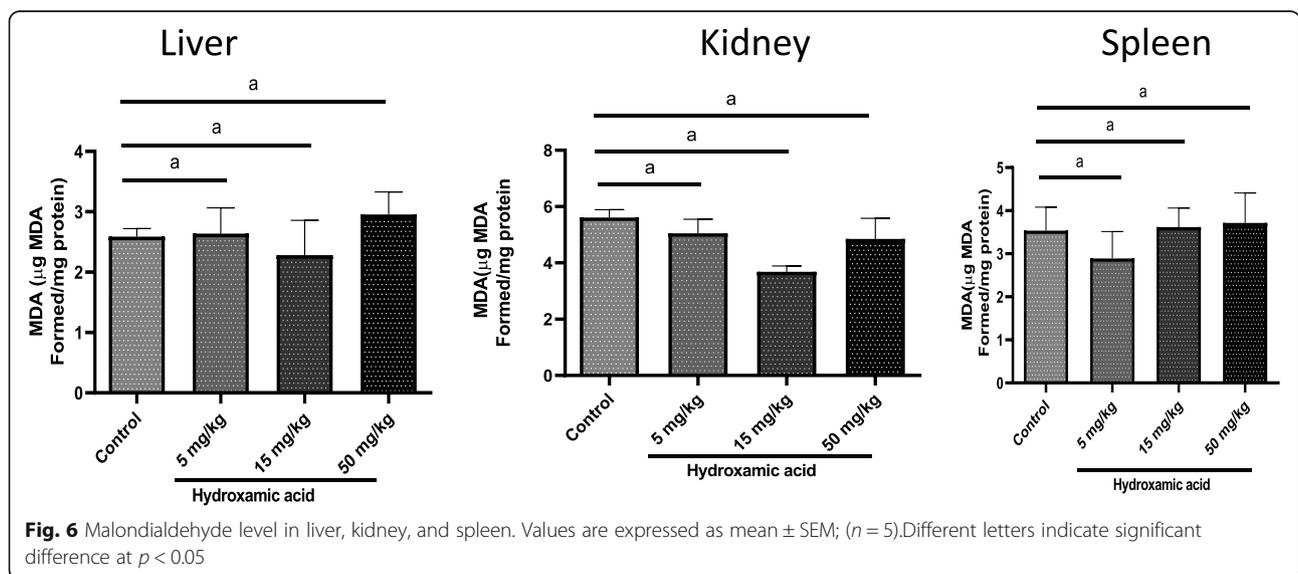




metabolic rates and protein turnover. Another study [33] has reported a significant increase in GST activity in the liver, testes, and spermatozoa of rats treated with *Garcinia kola*. The inter-organ differences in response to antioxidant principles were further observed with a significant decrease in liver GPx across all treatment doses as against the significant increase observed in renal GPx activity in rats exposed to 50 mg/kg hydroxamic acid. This observed increase in renal GPx activity agreed with a previous study reporting an elevated GPx activity in the kidney of rats treated with nanocellulose synthesized from *Khaya senegalensis* seeds [34].

Furthermore, GSH depletion was prominent in the liver and spleen. In a similar study, Celik and co-workers

[35] reported that two plant growth regulators, abscisic and gibberellic acids, elicited alterations on the antioxidant defense systems in various tissues of the rats. By their studies, they reported that abscisic acid significantly depleted GSH levels in the spleen after a 50-day continuous oral dosing in rats. It has been suggested that the depletion of GSH in tissues may be as a result of its increased utilization by antioxidant enzymes such as GST [35]. In this study, administration of hydroxamic acid did not significantly alter the MDA levels in the organs. Our results were in disagreement with another study which reported that linoleyl hydroxamic acid decreased lipid peroxidation levels in blood, heart, liver, and lungs in rats under acute hypoxic hypoxia [36].



Conclusion

Hydroxamic acid synthesized from *Cyperus esculentus* seeds did not perturb the antioxidant balance at the lower doses of treatment; however, at the highest dose, the hepatic and splenic tissues may be vulnerable to oxidative stress. Overall, our study provided useful toxicological data to support the safe bio-applications of hydroxamic acid. Subsequent research emphasizing molecular mechanisms and morphological studies will further enhance understanding of the effects of hydroxamic acid exposure on vital organs.

Abbreviations

ANOVA: One-Way Analysis of Variance; CAT: Catalase; GPx: Glutathione Peroxidase; GR: Glutathione Reductase; GSH: Reduced Glutathione; GST: Glutathione S-Transferase; SOD: Superoxide Dismutase; TCA: Trichloroacetic acid; TP: Total proteins

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CAO and AA designed the study; Author OB carried out the laboratory work. Authors CAO and AA supervised the work and participated in manuscript preparation. Authors approved the manuscript submission.

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Availability of data and materials

The dataset supporting the conclusions of this article is included as Figures uploaded as additional files.

Ethics approval and consent to participate

The principles governing the use of laboratory animals as laid out by the Redeemer's University, Ede Committee on Ethics for Scientific Research. The approved study code was RUN09–10/2435.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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