Flavonoid-rich fractions from *Clerodendrum volubile* and *Vernonia amygdalina* extenuates arsenic-invoked hepato-renal toxicity via augmentation of the antioxidant system in rats

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**S U M M A R Y**

Arsenicosis remains a global health concern due to devastating health effects. *Clerodendrum volubile* and *vernonia amygdalina* have tremendous bioactivities against oxidative stress-related diseases. The study, therefore, appraised the effects of flavonoids fractions from C. volubile and V. amygdalina (FCV and FVA respectively) against arsenic-induced oxidative stress in rats. Thirty male Wistar rats (120 ± 10 g) were divided into six groups of five each; Control (distilled water), arsenic alone (40 ppm sodium arsenite), arsenic + FCV (100 mg/kg), arsenic + FVA (100 mg/kg), arsenic + FCV and FVA (50 mg/kg each), and arsenic + vitamin C (100 mg/kg). The treatment commenced after four-week long arsenic exposure and lasted another four weeks. Blood, liver and kidneys of the rats were collected after sacrifice following an overnight fast. Arsenic caused significant (p<0.05) reductions in the total thiols levels in the plasma, liver, and kidneys, as well as the lowering of catalase and glutathione peroxidase activities.
Contrariwise, malondialdehyde and nitric oxide levels, as well as superoxide dismutase activities increased in the non-treated arsenic exposed group. FCV and FVA, both singly or in combination, abrogated the oxidative stress indices and enhanced the antioxidant species in the treated groups. Groups treated with vitamin C also showed improved antioxidant status with concomitant reductions in oxidative stress markers. This study concludes that flavonoids fractions from C. volubile and V. amygdalina could be a viable weapon against arsenic-induced hepatorenal oxidative stress in rats.

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1.0. Introduction

Due to its well-established toxicity and known threats, management of arsenicosis is of utmost importance to the public and also in the research field. Arsenic is a toxic metalloid that can exist in both the organic and inorganic form, with the inorganic form mainly trivalent meta-arsenite (As$^{3+}$) and pentavalent meta-arsenite (As$^{5+}$) [1,2]. Historically, arsenic served as a homicidal agent, and in the past years has been used as pesticide and herbicides [3]. Human exposure to the inorganic and organic forms of arsenic might be through contaminated water, air, or soil or consumption of exposed marine animals and plants [4]. The trivalent form of the inorganic arsenic appeared to be the most toxic due to its tendency to deplete the glutathione level by forming bonds with its thiol group [1,6,11], thereby inducing oxidative stress and causing tissue damage [5]. Human exposure to arsenic from groundwater is currently the one of the biggest environmental health problem worldwide, putting an estimated hundred million people at risk of arsenic-induced ailments [6,7]. More alarmingly, epidemiological studies of human exposure to high level of arsenic continue to soar, and correlates positively with high prevalence of different cancers, kidney, liver, and skin diseases [6,8,9]. Further, recent epidemiological evidences suggests a strong correlation between arsenic exposure and the ever-increasing cases of obesity, implying a broader spectrum of possible debilitating effects of arsenic exposure [10]. Oxidative stress is an occurrence caused by a disproportion between production of reactive species such as reactive oxygen species (ROS) or reactive nitrogen species (RNS), and the detoxifying proficiency of a biological system [12,13]. Besides, in vivo and in vitro studies have demonstrated the induction of hepatocellular and renal damage due to arsenic exposure in experimental animals and humans [14,16]. Arsenic detoxification occurs in the liver by oxidative methylation, and glutathione conjugation followed by excretion by the kidneys [15]. Inorganic arsenite (iAsIII) is converted to monomethylarsonic acid (MMAv) and dimethylarsonic acid (DMAv), and methylated in the presence of glutathione (GSH) or other thiol compounds [15,14]. Arsenic is quickly cleared from the blood and sequestered in many tissues [17]. The stored arsenic metabolites are subsequently reabsorbed through endocytosis in the proximal tubule, failure to eliminate the metabolites can lead to renal failure [18]. Arsenic exposure collapses the liver defence system following the depletion of GSH to pave the way for MMAv and DMAv accumulation, from which oxidative stress may ensue [19]. Thus, the liver and kidney represents two vital organs which are susceptible to arsenic accumulation and toxicity. The increased risks of heavy-metal exposure and oxidative stress-mediated organ failures, around the world have facilitated the research of medicinal plants with antioxidative properties such as the Clerodendrum volubile and Vernonia amygdalina.

Clerodendrum volubile is one of the species of the Clerodendrum genus [20]. The plant is popularly known in the South-west region of Nigeria as “Eweta”, “Marugbo”, or “Obnette” [21]. Due to its medicinal properties, it is locally used in the treatment of oedema, dropsy and arthritis [22,23]. Vernonia amygdalina is another a popular plant grown throughout tropical Africa. It is locally known as “bitter-
leaf” in Nigeria, while it is called “Ewuro” by the Yoruba speaking people of South Western Nigeria [24,25]. It is traditionally used due to its medicinal properties for the treatment of diabetes, nausea, dysentery, among others [24]. It has been shown that these two plant extracts contain flavonoids from which their acclaimed antioxidative properties originated from [24,27,26,28]. However, there has been a dearth of information on the prophylactic effects of the flavonoids extracts of both plants extracts on arsenic-induced oxidative stress in male rats. This study thus, investigated the effects of flavonoid fractions of C. volubile (FCV) and or V. amygdalina (FVA) on arsenic-intoxicated rats.

2.0. Materials and methods

2.1. Chemicals and reagents

Sodium arsenate (NaAsO₂), Chloroform, methanol, n-hexane, dichloromethane, ethylacetate, hydrochoric acid, sodium hydroxide, silica gel, sucrose, Tris Base, ethanol, EDTA, 5,5-dithiobis (nitrobenzoic acid), reduced glutathione, trichloroacetic acid, Folin-Ciocauteu’s reagent, sodium carbonate, and copper sulfate pentahydrate used are products of Sigma Chemical Co (St. Louis, MO, USA). Methylated spirit and Vitamin C were obtained from Pharmaceuticals Nigeria Limited, (Lagos, Nigeria); all other chemicals were of the pure grade available.

2.2. Plant collection and authentication

Clerodendrum Volubile was obtained from a personal farm in Ijoko, Sango Ota, Ogun State, Nigeria, while Vernonia Amgydalina was obtained from a domesticated farm in Odo-Eran, Abeokuta, Ogun State, Nigeria. Plants were collected at dawn, rinsed slightly with clean water and air-dried under a shade. Dried samples were pulverized using a blender and kept in an airtight container for further use. Fresh samples were taken to the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria for authentication.

2.2.1. Isolation of flavonoids from plant materials

Extraction of the plants was done according to the method of Anila and Vijayalakshmi [28]. Briefly, pulverized plants were extracted with absolute methanol in the water bath at 30°C for 60 minutes. It was then evaporated to dryness using a rotary evaporator after which the extract was then reconstituted in distilled water. Thereafter, liquid–liquid extraction was carried out in a separating funnel using n-hexane, n-hexane–dichloromethane (70:30), and ethyl acetate. To 100 mL extract reconstituted extract, 100 mL of n-hexane was added and allowed to separate, the n-hexane layer was then decanted. The procedure was repeated using n-hexane: dichloromethane. The ethyl acetate fraction was then evaporated to dryness. Ethyl acetate fraction was weighed and adsorbed on silica gel and transferred to a column of silica gel equilibrated with n-hexane. Elution was carried out using chloroform: ethyl acetate (50:50), ethyl acetate (100%), ethyl acetate: methanol (75:25), ethyl acetate: methanol (50:50). In each case, 20 mL of fractions was collected in 100 tubes. Isolate was then concentrated using a rotary evaporator at 60°C.

2.2.2. Confirmatory test for flavonoid

Preliminary test for flavonoid was carried on the ethyl acetate fraction. Briefly 1 mL of ethyl acetate fraction was put in a test tube and few drops of dilute sodium hydroxide solution was added. An intense yellow colour which became colourless on the addition of a few drops of dilute hydrochloric acid appeared; indicating the presence of flavonoids [28].

2.3. Experimental animals and treatment protocol

Thirty male Wistar rats (120 ± 10 g) were purchased from a reliable animal farm, housed in separate cages under ambient conditions. They were acclimatized for two weeks, with all conditions of animal
experimentation conforming to the guidelines outlined by the National Research Council [29], and approved (FUNBCH180641) by the Ethical Committee of the Department of Biochemistry, College of Biosciences, Federal University of Agriculture, Abeokuta.

The animals were divided into six groups of five each. Group 1 (control) received distilled water for 8 weeks, while Group 2 (Arsenic) received arsenic only (as 40 ppm NaAsO₂) via drinking water for 8 weeks. The other groups (3–4) also received arsenic. However, Group 3 was treated with FCV (100 mg/kg), Group 4 with FVA (100 mg/kg), Group 5 with combined FCV and FCA (50 mg/kg each), while Group 6 animals were treated with vitamin C at 100 mg/kg dose. The different treatment regimens commenced after four weeks of arsenic intoxication. The doses of FCV and FCA was based on the results obtained from the acute oral lethal toxicity test (LD₅₀) (Results not presented). There was no mortality at 5000 mg/kg body weight for both the FCV and FCA. Therefore, 1/50th of 5000 mg, which equates 100 mg/kg was used in the in vivo study.

2.4. Sacrifice and preparation of samples

The animals were euthanized after an overnight fasting. The blood was collected via the abdominal artery into heparinized tubes and centrifuged at 3000 rpm for 10 minutes to obtain the plasma. The liver and kidney tissues were excised and frozen. One part of each tissue was homogenized in 9 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4), containing 0.25 M sucrose and 0.1 mM EDTA, with a motor-driven Teflon homogenizer. The resulting post-nuclear factions were used for biochemical assays.

2.5. Biochemical assays

2.5.1. Total thiols (TSHs) levels

TSHs levels in the plasma, liver, and kidneys were determined using the spectrophotometric method of Ellman [30], in which DTNB complexes with the thiol groups to form a yellow complex measured at 412 nm.

2.5.2. Catalase (CAT) assay

This was done as described by Shangari and O’Brien [31] and involves reacting the excess hydrogen peroxide (H₂O₂) with ammonium molybdate to form a yellow complex that can be measured at 405 nm. One unit of CAT is equal to the amount of enzyme that decomposes one micromole of hydrogen peroxide per minute under specified conditions at 25 °C.

2.5.3. Superoxide dismutase (SOD) assay

The activity of SOD was measured using Marklund and Marklund [32] which is based on the inhibition of pyrogallol auto-oxidation by SOD, which can be monitored at 420 nm. One unit (U) of SOD activity is defined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%, and expressed as U/mg protein.

2.6.4. Glutathione peroxidase (GPx) assay

The GPx activity was measured according to the method of Rotruck et al. [33], based on the rate of oxidation of glutathione by peroxidase, at 412 nm. The enzyme activity is expressed as U/mg protein, with one unit defined as the amount of enzyme catalyzing the oxidation of one micromole of hydrogen peroxide per minute under specified conditions.

2.5.5. Nitric oxide (NO) assay

The levels of NO was determined according to the method of Rao [34], based on the formation of reddish-pink colour following the reaction nitrites and nitrate compounds with the Griess reagent (containing equal volumes of 1% sulphanilic acid, in 5% phosphoric acid, and 0.1% N-(1-naphthylethylenediamine), measurable at 520 nm.
2.6.6. Malondialdehyde (MDA) assay

The level of MDA, a marker of lipid peroxidation, was determined in the serum and homogenates of tissues, as thiobarbituric acid reactive substances (TBARS), according to the method of Beuge and Aust [35].

2.6.7. Total protein determination

Total protein was determined using commercial kit purchased from Randox Laboratories Limited (Crumlin, United Kingdom) [36].

2.7. Statistical analysis

Results were analysed with one-way ANOVA, followed by Duncan’s multiple range tests ($p<0.05$), and expressed as mean ± standard error mean (S.E.M), using Statistical Package for Social Sciences (SPSS) version 20.0. Graphs were plotted with using Graph Pad Prism (version 6.0).

3.0. Result

3.1. Effects of FCA, FCV and vitamin C on antioxidant system of arsenic-exposed rats

Presented in Figs. 1–4 are the effects of FCV, FVA, and Vitamin C on the plasma, the liver and kidney antioxidants (TSHs, CAT, GPx and SOD) in rats exposed to arsenic.

In comparison with the control group, the levels of TSHs in the plasma, liver and kidney of arsenic groups were significantly depleted ($p < 0.05$) by 33.9%, 43.2%, and 30.4% respectively. Fig. 1a shows that

![Graphs showing effects of flavonoid-rich extracts from C. volubile (FCV), V. amygdalina (FVA), and Vitamin C (Vit C) on plasma, liver and kidney total thiols (TSHs) concentrations in rat exposed to arsenic. Values represents mean ± standard error of mean ($n = 5$). Bars with different letters differ significantly ($p<0.05$).]
treatments of arsenic-intoxicated rats with FCV, FCV + FVA, and Vitamin C significantly (p < 0.05) increased the depleted TSHs levels in the plasma, by the percentage increments of 22.6, 83.4, and 85.1 respectively. Fig. 1b likewise depicts the treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C significantly (p < 0.05) increased the depleted TSHs levels in the liver by the 34.1%, 134.2%, 104.8% and 47.4% when compared to the arsenic group respectively. A significant (p < 0.05) increase of the depleted levels of TSHs in the kidney was also depicted in Fig. 1c which showed percentage increase of 73.4, 85.2, 43.7 and 28.6 on treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C respectively.

Presented in Fig. 2 is the specific activity of catalase in the plasma, liver and kidney of arsenic-intoxicated animals in comparison with the control group which were significantly (p < 0.05) decreased by 17.9%, 29%, and 30.6% respectively in the untreated arsenic group. Fig. 2a shows that treatments of arsenic-intoxicated rats with FCV, FVA, and Vit C significantly (p < 0.05) increased decreased catalase activities in the plasma, by 61.8%, 25.3%, and 15.8% when compared to arsenic group respectively. Also, there was no significant difference (p > 0.05) when (Arsenic + FCA + FVA) group was compared to arsenic group. Fig. 2b likewise depicts the treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C significantly (p < 0.05) increased the depleted catalase specific activities in the liver by the 80.4%, 74.2%, 40.5% and 102.1% when compared to the arsenic group respectively. A significant (p < 0.05) increase of the depleted activities of catalase in the kidney was also depicted in Fig. 2c which showed percentage increment of 43.9, 42.5, 61.7 and 65 on treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C respectively.

Presented in Fig. 3 is the specific activity of GPx in the plasma, liver and kidney of arsenic-intoxicated animals. When comparison with the control group. There were significantly (p<0.05) decreased activities of 32.5%, 27.7%, and 23.6% respectively. Fig. 3a shows that treatments of arsenic-
intoxicated rats with FCV, FCV + FVA, and Vit C restored the activity back to normal. Fig. 3b likewise depicts the treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C significantly \((p < 0.05)\) increased the decreased GPx activity in the liver by the 25.6%, 18.1%, 21.9% and 43.1% when compared to the arsenic group respectively. Fig. 3c also shows a significant \((p < 0.05)\) increase in the depleted GPx activity of the kidney by the percentage increase of 8.8, 9, 2.8 and 7.8 on treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C respectively.

Fig. 4 shows the specific activity of SOD in the plasma, liver and kidney of arsenic positive groups when compared with the control group. A significantly increased \((p < 0.05)\) activities of 33.3%, 35.9%, and 60.8% respectively were observed. Fig. 4a shows that treatments of arsenic-intoxicated rats with FCV, FVA, and FCA + FVA showed no significant difference \((p < 0.05)\) when compared to arsenic group while treatment of arsenic-intoxicated rats with Vit. C showed a significant decrease \((p < 0.05)\) by 20.3% when compared to arsenic group. Fig. 4b shows that treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C restored the activity back to normal. Fig. 4c shows that treatments of arsenic-intoxicated rats with FCV, FVA, and Vit C showed no significant difference \((p < 0.05)\) when compared to arsenic group while treatment of arsenic-intoxicated rats with FCV + FVA showed a significant decrease \((p < 0.05)\) by 28.6% when compared to arsenic group.

3.2. Effects of flavonoid-rich extracts from *C. volubile* (FCV), *V. amygdalina* (FVA), and Vitamin C (Vit C) on oxidative damage biomarkers

In Figs. 5 and 6, the effects of FCV, FVA, and Vitamin C on the plasma, the liver and kidney oxidative damage biomarkers (NO and MDA) in rats exposed to arsenic are shown.

The plasma, liver and kidney NO concentrations of arsenic untreated in comparison with the control group which were significantly \((p < 0.05)\) increased by 13.8%, 36.6%, and 28.3% respectively. Nevertheless, the treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C significantly
(p < 0.05) decreased the plasma, liver, and kidney NO concentration by the 15.4%, 8.3%, and 5.3%, and 6.1% when compared to the arsenic group respectively (Fig. 5).

Presented in Fig. 6 is the plasma, liver and kidney MDA concentrations of arsenic—exposed rats in comparison with the control group, significantly (p < 0.05) increased level of 30.9%, 39.9%, and 59.9% respectively was recorded. However, treatments of arsenic-intoxicated rats with FCV, FCV + FVA, and Vit C restored the MDA concentration back to normal. Indeed, FCV, FVA, FCV + FVA, and Vit C significantly (p < 0.05) decreased the MDA concentration in the liver, by the percentage decrements of 33.3, 63.9, 71.6 and 32.1 when compared to arsenic group respectively. Fig. 6c likewise depicts the treatments of arsenic-intoxicated rats with FCV, FVA, FCV + FVA, and Vit C significantly (p < 0.05) decreased the MDA concentration in the kidney by the 31.3%, 52.2%, 51.3% and 82.1% when compared to the arsenic group respectively.

4.0. Discussion

Chronic arsenicosis arising from contaminated drinking water and food products preludes the onset of myriads of multi-systemic diseases [37]. Chronic exposure to arsenic correlates positively with the incidence of cancers of the organs such as the liver, kidney, lungs, and the skin. Undeniably, arsenic-contaminated groundwater represents a worldwide public health concern, as there are no currently approved regimens for the management of arsenic toxicity [37]. Some of the strategies to manage arsenic include metals chelation therapy with the use of synthetic chemicals such as dimercaptosuccinic acid (DMSA), and \( \alpha \)-penicillamine [1]. Other methods include the use of vitamins A, C, and E, while the use of medicinal plants has also been reported [38,37]. Considering the relative safety, low cost and availability of medicinal plants, they have been employed in the management of different
types of pathologies from time immemorial [39]. Although studies have revealed the beneficial effects of plants extracts in the reduction of arsenic burdens in the body, most studies failed to isolate the active phytochemical(s) that elicited the purported activities [37]. This study therefore elucidated the effects of flavonoid-rich fractions of C. volubile and V. amygdalina on the hepatic and renal antioxidant system of male rats exposed to sub-chronic level of arsenic.

In the present study, arsenic caused a significant (p<0.05) decrease in the total thiol levels in the plasma, liver, and kidneys of rats when compared with the Normal control group. Thiol (SH-group), usually present in glutathione, metallothionein, and other thiol-containing proteins, is critical for elimination of endogenously-derived free radicals. Thus, it constitutes an integral hub of cellular antioxidant system that is responsible for maintenance of redox stability of the cell [40]. There might be an increase in the total thiol content at the onset of oxidative stress due to an adaptive homeostatic response of the cell. However, when the oxidative stress attains an overwhelming degree, thiol content decreases significantly due to inability to replenish its pool [41]. Considering the affinity of heavy metals such as arsenic for thiol-containing compounds, we unsurprisingly observe a decrease in its levels in the groups exposed to arsenic. This lowered level of thiols content following arsenic exposure is consistent with other studies [42,40]. Biotransformation of xenobiotics occurs exclusively in the liver. The kidney is responsible for elimination of waste products of metabolism, acid-base balancing, osmotic, and redox status maintenance [40,5]. These metabolic roles of the kidney and liver predispose them to heavy metal toxicity due to accumulation. Arsenic-induced inhibition of antioxidant and xenobiotic-detoxifying enzymes that have thiol groups at their active sites, by trivalent methylated arsenicals is a well-known mechanism of arsenic toxicity [43]. Therefore, reductions in liver, kidney, and plasma thiol levels, as observed in this study, might be due to the irreversible binding of the metabolized arsenicals to the SH-group of the liver and kidney proteins [43]. Because these xenobiotic-detoxifying enzymes depend on and requires the electron-donating potential of reduced glutathione (a

Fig. 5. Effects of flavonoid-rich extracts from C. volubile (FCV), V. amygdalina (FVA) and Vitamin C (Vit C) on plasma, liver and kidney nitric oxide (NO) concentration in rat exposed to arsenic. Values represents mean ± standard error of mean (n = 5). Bars with different letters differ significantly (p<0.05).
thiol-containing tripeptide), and other thiol-rich compounds for their effects, their activity is impaired by lack of substrate [5]. Regardless, flavonoid fractions of C. volubile and V. amygdalina (FCV and FVA) either singly or, in combination, reversed the decrements of total thiol levels in the plasma, liver and kidneys. Notably, the combined effects of the two fractions produced effect that is more beneficial rather than the individual administration in the plasma, suggesting a synergistic effect. However, in the liver and kidney, FVA treated group showed increased total thiol levels more considerably than every other group, including the vitamin C treated group. Adetutu et al. [44] alluded the ability of V. amygdalina to ameliorate arsenic-induced toxicity to the presence of flavonoids and polyphenols. Similar mechanism might be playing out here. To the best of our knowledge, this study is the first to investigate the prophylactic effects of Clerodendrum volubile on oxidative stress induced by arsenic in rats. We showed here that flavonoid fraction of Clerodendrum volubile (FCV) also possess the ability to increase total thiol levels, especially in the kidney. The antioxidants effects of flavonoids relate directly to their structure, and depends on the number of hydroxyl groups they possess [45]. The hydroxyl groups are excellent scavengers of free radicals, can limit the oxidation process in the body [46]. Although, in this study, we did not isolate one particular flavonoid, the combined effects of the different flavonoids from the isolates might be responsible for the observed effect, suggesting a better plasma bioavailability and synergistic beneficial effects (Fig. 1). Similarly, flavonoids from Moringa leaf extract increased the total thiol levels in the kidneys of rats following ischemic reperfusion [47].

Nitric oxide (NO) performs many physiological functions; including vasodilation of the endothelial system, anti-inflammatory, and immunomodulatory effects, when produced at normal concentration [48]. Conversely, under oxidative stress condition, superoxide radical reacts with NO to form peroxynitrite radical (ONOO−) – a devastating and highly reactive radicals that can react with, and destroy cell membrane, as well as inactivate the endothelial nitric oxide synthase (eNOS) - the isoform which is
critical for endothelial functions [49]. NO levels increased in the plasma, liver and kidneys of the arsenic untreated groups in comparison with the normal controls (Fig. 2). This observation is consistent with another study [50] where arsenic increased brain NO levels following arsenic exposure. Besides, Zhou et al. [51] observed that, exposure of human keratinocytes to arsenic caused a significant increase in peroxynitrite radicals. The observation might be due to the combination of superoxide radical with NO, which consequently leads to the inhibition of the DNA repair system. Consistently, administration of FCV and FVA abolished these increments in the NO levels in the treated group. Indeed, there was no significant difference between the group treated with the combination of the fractions and vitamin C. Noteworthy; FCV appeared to be more therapeutic in the plasma, while there was no significant difference between all the treated groups in the liver and kidneys. NO is produced in mammalian cells by different enzyme isoforms: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS). The latter is responsible for the pro-inflammatory effect of NO and oxidative stress [52]. Increased oxidative stress tends to reduce the bioavailability of NO necessary for its regulatory activities, thereby shifting the equilibrium toward the iNOS isoform and consequently causing tissue damage. This phenomenon is otherwise known as “NO mopping” [13]. The modulation of NO levels by flavonoids appears to be dependent on the prevailing oxidative status of the stress. Benito et al. [53] observed that, flavonoid–rich diets feeding increased NO level in the rat aorta, thereby, authenticating the vasodilatory effect of flavonoids. However, Chaturvedi et al. [54] observed a significant increase of NO in the brain of rats exposed to metronidazole. This observed increment, in this instance, was due to over-expression of inducible nitric oxide synthase (iNOS) protein level in the brain, but was abolished by quercetin (a flavonoid). Flavonoids are excellent donor of electrons capable of protecting the cells from oxidative stress-imposed electron abstraction from biomolecules. Therefore, reductions in NO level as observed in our results suggest that flavonoids fractions nullified the production of NO by inhibiting the expression of iNOS [54]. Fan et al. [55] showed that flavonoids derived from *Phyllolobium chinense* produced similar NO-lowering effect by specific inhibition of iNOS in Zebrafish model.

Malondialdehyde (MDA), an index of oxidative damage to lipids [13], increased dramatically in the plasma, liver and the kidneys of the arsenic exposed group (Fig. 3). The increment is consistent in studies involving heavy metals intoxication [56,57,5], and might be due to arsenic–induced increased free radicals production [5]. Indeed, lipid peroxidation due to electron abstraction by free radicals is common during heavy metals toxicity [5,58,59]. Furthermore, excessive production of ROS due to impaired mitochondrial function has been reported during exposure to arsenic in drinking water [58]. The ROS so generated thus, caused elevation of MDA in the rat brain due to peroxidation of the mitochondrial membrane lipids. However, the groups treated with FCV and FVA significantly showed decreased MDA levels. There was no significant difference between the group treated with FVA and the combination of FCV and FVA. However, vitamin C treated group showed the lowest level of MDA in the kidney. Mulberry leaf-derived flavonoids also deplete MDA level in a dose-dependent manner following heat shock in water Buffalos [60]. These data suggest that flavonoids from C. volubile and V. amygdalina possess protective effects against oxidative stress-induced biomolecule damage due to arsenic exposure.

The cellular antioxidant systems operate in tandem; in that, it encompasses enzymatic machineries such as SOD, CAT, and GPx that synergistically rid the cells of oxidative species that are capable of disrupting cellular homeostasis [13,59]. Superoxide radicals generated due to oxidative stress are taken up by SOD and converted to hydrogen peroxide and water. The former is in turn taken up by CAT or GPx and converted to oxygen and water (CAT) or the respective alcohol and water (GPx) depending on which enzyme catalyzed the reaction, though, GPx is more suited to hydroperoxides [11,61]. Our data showed that SOD increased copiously in the arsenic exposed group when compared with the Normal control (Fig. 6). Although, the flavonoid fraction did not reverse the increased SOD activity in the plasma, the group treated with vitamin C had a normalized SOD activity. However, FCV and vitamin C-treated groups normalized the activities in the liver while the combination of the two flavonoids produced the ameliorative effect in the kidney. Intriguingly, the CAT and GPx activities significantly decreased following arsenic exposure (Figs. 4 and 5). This reduction is consistent with other studies [62,40]. Indeed, the two enzymes are heme-containing proteins which are susceptible to arsenic binding. Heme-containing enzymes are particularly susceptible to heavy metals-induced deactivation.
Probable mechanism includes oxidation of the central iron atom from Fe2+ to Fe3+ via the Fenton reaction [63]. Although the mechanism through which arsenic generates free radicals remains yet enigmatic, the radicals so generated, including NO., OONO-, H2O2, dimethylarsinic and dimethyl peroxyl radicals, and superoxide radicals are capable of inhibiting antioxidant enzymes [64,63]. Indeed, superoxide radicals, in the presence of superoxide dismutase produces H2O2 that initiates the Fenton reaction, culminating into the production of the cytotoxic hydroxyl radicals (OH.) that can eventually inhibit the enzymes [65]. Strategies to limit or abrogate heavy metals-induced free radical challenge on the iron could be through inhibition of H2O2 formation, chelation of ferrous ion or trapping of the radicals formed [63]. The most effective compounds that are capable of protecting the cells against heavy metals-induced toxicity are the thiol compound. Unsurprisingly, our data showed that total thiol levels decreased in the arsenic exposed group. This decrease might be responsible for the inhibition of CAT and GPx as observed in this study. Furthermore, reduced glutathione (a co-substrate for GPx) levels decreased in the arsenic exposed group (results not presented). This suggests that arsenic might have made GSH unavailable because of the formation of arsenic-GSH complex [59]. Remarkably, FCV or FVA abolished the arsenic-induced oxidative stress by augmenting the activities of the antioxidant enzymes either singly or in combination (i.e. FCV + FVA). Although FCV-treated group showed better enhancement in plasma CAT activity, there was no significant difference between the activities in the liver and kidney for either FCV or FVA. The GPx activity increased significantly following treatment with both flavonoid fractions, without any significant difference between either plants. This ability to quench superoxide radicals as well as modulation of enzymatic activities by flavonoids is well-documented elsewhere [45]. Probably be due to the presence of multiple hydroxyl groups (electron-donating groups) in their structures [46,45]. Alternatively, another possible mechanism might be through induction of antioxidant response element (ARE/NRF2) systems by flavonoids, which stimulate the transcription and translation of the cellular antioxidant enzymes [66]. Therefore, we posed that the flavonoid fractions from C. volubile and V. amygdalina possesses the ability to abolish oxidative stress invoked by arsenic exposure in rats due to its enormous in vivo anti-ROS, and antioxidant effects. Data from our study suggest that flavonoids derived from C. volubile and V. amygdalina, possess tremendous bioactivities against oxidative stress-related complications. Further studies focusing on the exact flavonoid (s) responsible for these effects are required to elucidate the molecular target of such phytochemical (s).

4.1. Conclusion

Conclusively, this study validates the hepato-reno protective effects of FCV and FVA against oxidative damages imposed by arsenic exposure. This mechanism is via attenuation of oxidative stress indices, and the concomitant enhancement of the antioxidant molecules/enzymes either singly, or in combination. The observed effects are likely due to the presence of electron-donating sites in their flavonoid structures that quenches radicals’ production or due to their ability to modulate the activity of the antioxidant enzymes. Therefore, flavonoids fractions from C. volubile and V. amygdalina could be a viable weapon against arsenic-induced hepato-renal oxidative stress in rats.

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Conflict of Interest

We declare no known/unknown competing interests.

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