RESEARCH ARTICLE

ISSN: 0331 - 670X

https://doi.org/10.51412/psnnjp.2023.21

The Nigerian Journal of PHARMACY

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THE OFFICIAL ORGAN OF THE PHARMACEUTICAL SOCIETY OF NIGERIA FOUNDED 1927

Vanillic Acid Ameliorates Diethyl Phthalate and Bisphenol S -Induced Cardiotoxicity in rats via abrogating oxidative Stress.

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ARTICLE INFO

Article history:

Received 5 April 2023
Revised 28 April 2023
Accepted 1 June 2023
Online Published 30 September 2023

Keywords:

Bisphenol S,
diethyl phthalate,
vanillic acid,
cardiotoxicity,
oxidative stress.

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ABSTRACT

Background: Neglected home producing toxicants (NHPT) are the endocrine disruptors affecting biological systems. We investigated the effect of vanillic acid on diethyl phthalate (DEP) and bisphenol S (BPS)-induced cardio-toxicity and oxidative stress using rat model. Methods: Rats were exposed to DEP (50 mg/kg) and BPS (50 mg/kg) and treated with vanillic acid (25 and 50 mg/kg) by oral gavage for twenty-one days. Afterwards, they were sacrificed. **Results:** Vanillic acid treatment significantly ($p < 0.05$) protected cardiac tissues architecture by abolishing the DEP+BPS-induced decrease in the activity of all the enzymatic antioxidants. Co-treatment with vanillic acid remarkably ($p < 0.05$) reversed DEP+BPS-induced decreases in glutathione levels, GSH, CAT, GPx, and SOD activities in cardiac tissues, while attenuating DEP+BPS mediated increase in cardio-oxidative damage markers (MDA, AOPP, and NO as well as increase activity of arginase and PDE-5.
Conclusion: Vanillic acid may have exert a cardio-protective effect against DEP+BPS induced cardiotoxicity by decreasing oxidative stress via its antioxidant, free radical scavenging effect.

1. Introduction

Vanillic acid (VA) is a natural compound of phenolic acids family and also a derivative of benzoic acid which is used as a flavoring agent, preservative, and food additive in the food industry. It is a form of vanillin oxide and is produced when vanillin converted to ferulic acid. VA has several pharmacological effects including anti-metastatic [1] anti-melanogenesis [2], antioxidant, anti-angiogenesis [3], and anti-apoptotic effect [4]. Recent study has shown the cardio-protective effect of VA in ischemia-reperfusion through decreasing oxidative stress and improving myocardial dysfunction [5]. Other investigations revealed some phenolic acid such as ferulic acid suppressed TLR4-induced inflammatory responses against acetaminophen-induced liver injury. Diethyl phthalate (DEP) has been found to have diverse acute and chronic toxic effects on several species at different trophic levels and endocrine-disrupting properties [6]. DEP could also be seen as a colorless, odorless, oily

substance used to improve the performance and durability of many products [7]. It is added to plastic polymers as a plasticizer to help maintain flexibility. It has been used in various products, including plastic films, rubber, tape, toothbrushes, automotive components, tool handles, and toys. BPS is a synthetic organic compound widely used as a precursor in polycarbonate plastic products, epoxy resins, and various materials. Bisphenol S (BPS) has been introduced to the industry as a safe alternative to BPA; however, recent studies have shown that different BPS concentrations correlate with oxidative stress [8]. It can be directly released from factories or consumer products and is found in the environment. Consequently, global human exposure mainly results from environmental contamination and dermal contact with bisphenol-containing products or food contamination since bisphenols are primarily used in food and beverage containers [9]. Exposure to BPS for a prolonged period can cause significant alteration in the structure of experimental animals.

Compelling evidence demonstrates that vanillic acid is a bioactive compound, showing a broad range of pharmacological activities and very low cytotoxicity. A previous study showed that vanillic acid protects erythrocytes from peroxyl radicals generated by pro-oxidants [10] and can also form stable complexes with metal ions and reduce the formation of free radicals. Vanillic acid possesses antioxidant [11], anti-inflammatory [12], cardio-protective [13], neuro-protective [14], and anti-diabetic [15] properties. Hence, this study was conducted to 1) elucidate the mechanisms in which DEP and BPS can induce oxidative stress and cardiotoxicity and 2) ascertain if the antioxidant activity of vanillic acid can ameliorate this condition in oxidative stress markers. Therefore, the present attempt has been made to investigate the possible cardio-protective effects of vanillic acid against DEP+BSP-induced cardio-toxicity.

2. Materials and Methods

2.1 Materials

Vanillic acid, diethyl phthalate and bisphenol S, and Dimethyl sulfoxide (DMSO) were purchased from India and Libertas laboratory services limited, Abeokuta. All other chemicals used were of analytical grade.

2.2 Animal model

Experimental animals, female albino Wistar rats weighing 150 g – 200 g, were inbred at the Animal House, Department of Biochemistry, Federal University of Agriculture, Abeokuta, Nigeria. They were housed in a plastic suspended cage placed in a well-ventilated, temperature controlled (25 ◦C) rat house with standard 12-h light/12-h dark cycles. The rats were provided with standard pellet chow and given water *ad libitum*. All animals were acclimatized for one week before the commencement of the experiments.

2.3 Experimental protocol for the study

Twenty-five rats were divided into five groups (n = 5), using a simple randomization method. Five rats per group were used based on 3R (replacement, reduction and refinement) principles (Maestri, 2021), before the experiments, vanillic acid was mixed with the vehicle (sweetened condensed milk diluted in water in a 1:6 ratio). Aliquots of different concentrations (50, 25, and 200 mg/kg bw vanillic acid). However, the doses of BPS, DEP, and vanillic acid were based on previous studies of Administration was for twenty-one (21) days. Dimethyl sulfoxide (DMSO) served as the vehicle for all treatments and administration was by oral gavage. These various vanillic acid dosages have been stated to prevent oxidative stress in rats. Meanwhile, the normal control of diethyl phthalate and bisphenol s alone rats received the vehicle, as detailed in Table 1. Afterwards, a single dose of diethyl phthalate and bisphenol s (50 mg/kg) prepared in sterile injection water was administered intraperitoneally (i.p.) to all the groups (II–V) except normal control group I, which received only sterile injection water i.p.

Table 1 Grouping of experimental animals and their treatments.

GROUPS (n = 5)	TREATMENT
A	DMSO (50 mg/kg b.wt)
B	BPS +DEP (50mg/kg b.wt)
C	BPS + DEP (50mg/kg b.wt) + Vanillic Acid (25mg/kg b.wt)
D	BPS+DEP (50mg/kg b.wt) + Vanillic Acid (50mg/kg b.wt)
E	Vanillic Acid (50mg/kg b.wt)

The rats were sacrificed 24 hours after the last administration. Feed and water were withdrawn from the animal cages 12 hours before sacrifice. The animals were sacrificed by cervical dislocation. The hearts were excised

and rinsed in cold normal saline after which they were blotted dry and weighed.

10% tissue homogenate, was prepared by weighing approximately 0.2g of the excised organ (heart) and homogenizing in 1.8ml of phosphate buffer (pH 7.4) using a Teflon® homogenizer. The resulting homogenate was centrifuged at 4000rpm for ten minutes after which the supernatant was collected into 2ml Eppendorf tube®. Portions of the heart samples were preserved in phosphate buffered formalin for histopathological analysis. The homogenate and the remaining intact organs were stored at low temperature in a chest freezer.

2.4 Principle of the biomarkers

Malondialdehyde (MDA) is formed from the breakdown of polyunsaturated fatty acids and therefore serves as a convenient index to determine the extent of the peroxidation reaction. It has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid on heating in acidic pH to give a pink complex which absorbs maximally at 532nm. The results are expressed as the amount of free MDA produced.

2.5 Analysis of reduced glutathione concentration

Concentration of GSH was determined according to the method described by [17]. The acid soluble sulfhydryl groups of reduced glutathione form a yellow colored complex with dithionitrobenzene (DTNB). The absorbance of the colored complex was measured at 412 nm.

2.6 Determination of glutathione peroxidase activity

The activity of GPx was determined by the method described by [18].

About 0.2 mL of the sample was mixed with 0.2 ml phosphate buffer, 0.1 mL NaN_3, 0.2 mL GSH and 0.1 mL of H_2O_2. The mixture was incubated at 37°C for 10 minutes after which 0.2 mL of TCA was added. The entire solution was centrifuged at 3000rpm for 15 minutes. To 0.4 mL of the resulting supernatant, 0.36 mL of distilled water, 0.8 ml of tris buffer and 20 µL of freshly prepared DTNB were added. The absorbance reading was taken at 412 nm within 5 minutes of adding the DTNB against a blank containing 0.5 ml of water instead of sample.

2.7 Determination of superoxide dismutase activity and catalase activity

The enzyme activity of SOD was determined by the methods described by [19]. This assay was based on the inhibitory effect of SOD on spontaneous autoxidation. One unit of the SOD is the amount which requires 50% inhibition of the rate of autoxidation. Specific activity is defined as the measure of the density of a substance in comparison to the density of water.

The enzyme activity of catalase was estimated according to the method described by [20]. Catalase activity was assessed by incubating the enzyme sample in 1.0 mL substrate (65 mmol/ mL hydrogen peroxide in 60 mmol/L sodium–potassium phosphate buffer, pH 7.4) at 37 °C for three minutes. The reaction was stopped with ammonium molybdate. Absorbance of the yellow complex of molybdate and hydrogen peroxide is measured at 374 nm against the blank.

2.8 Estimation of nitric oxide level and arginase activity

Nitric oxide levels were determined according to the method described by [21]. 120 µL of sulfanilamide solution was added to 120 µL of the sample in a 2.0 mL Eppendorf tube and left to react on ice for 15 minutes. Thereafter, 120 µL of the NED was added to it and the entire solution was incubated for 10 minutes in the dark. The absorbance reading was taken at 520nm against a standard which contained 120 µL of serially diluted 0.1M $NaNO_2$.

The enzyme activity of arginase was determined by measuring the rate of urea production using 9% α-isonitrosopropiophenone in absolute ethanol. 50 µL of the sample was added into 75 µL of tris HCl buffer containing 10 mmol/L $MnCl_2$. The solution was incubated at 37 °C for 10 min to activate the enzyme. Afterwards, the hydrolysis reaction of L-arginine by arginase was performed by incubating the mixture containing the now activated arginase enzyme with 50 mL of L-arginine at 37 °C for 1 hour.

2.9 Determination of type 5 phosphodiesterase activity and advanced oxidation protein products concentration

The enzyme activity of PDE-5 was estimated according to the method described by the method described by [22]. About 800 µl of Tris buffer (pH 7.4) was added to 100 µL of the sample. The solution was incubated at 37°C for 10 minutes. 100 µL of PNPP (Paranitrophenyl phosphate) was added afterwards and the absorbance was read at 400nm at 0-, 1- and 2-minutes' intervals.

The concentration of advanced oxidation protein products was determined according to the method described by [23]. The concentration of AOPPs is estimated by measuring the decrease in concentration of NADH. (It significant plays a crucial role in regulating the intracellular redox state,

especially in the mitochondria and nucleus). About 200 µL of the sample was diluted in 1:5 chloramine - T standard solution and 20 µL of acetic acid. 10 µL of potassium iodide was added and the absorbance reading of the solution was immediately taken at 340 nm against a blank containing 200 µL of phosphate buffered saline, 10 µL of potassium iodide and 20 µL of acetic acid. AOPP concentration was expressed in µmol/L of chloramine - T equivalent. Chloramine - T absorbance at 340 no was linear within the range of 0 to 100 µmol/L.

2.10 Histopathological analysis of the cardiac tissue samples

A portion of the cardiac tissue samples were fixed in 10% phosphate buffered formalin. They were subsequently dehydrated in an increasing concentration of alcohol, cleared twice in xylene with bench top tissue processor and then fixed in paraffin with a tissue embedded. We use 5 µm of the embedded tissues were sectioned and stained with hematoxylin and eosin. The tissues were finally observed at ×100 magnification under a Nikon light microscope and images were captured with a digital camera attached to it.

2.11 Statistical analysis

Data obtained were analyzed by column statistics followed by one-way analysis of variance (ANOVA) using graph pad prism version 6.0. Results were expressed as mean ± Standard Error of Mean (SEM). Statistical significance was assigned as $p < 0.05$.

1. Results

Exposure to DEP and BPS inhibited cardiac GPX activity compared to control. However, following treatment with 25 and 50 mg/kg vanillic acid, there was a significant increase ($p < 0.05$) in cardiac glutathione peroxidase (GPX) activity compared to the DEP+BPS group respectively. In addition, there was also a significant increase ($p < 0.05$) cardiac glutathione peroxidase (GPX) activity in the group treated with vanillic acid only when compared to the control group. **Figure 1** shows the effect of vanillic acid on glutathione (GSH) concentration in cardiac tissues of diethyl phthalate and bisphenol S-exposed rats. There was a significant decrease ($p < 0.05$) cardiac GSH concentration in the DEP+BPS–exposed group compared to the control group. In contrast, exposed groups treated with 25 and 50 mg/kg vanillic acid showed a significant increase ($p < 0.05$) in GSH concentration compared to the DEP + BPS group respectively

Cardiac SOD activity showed a significant decrease ($p < 0.05$) in rats exposed to DEP and BEP compared to the control group. However, there was a significant increase ($p < 0.05$) in cardiac SOD activity in groups treated with 25 and 50 mg/kg vanillic acid, respectively, when compared to the DEP + BPS group.

Similarly, cardiac CAT significantly decreased ($p < 0.05$) in DEP + BPS–exposed group compared to the control group. There were significant increases ($p < 0.05$) in cardiac CAT activity in exposed groups treated with 25 and 50 mg/kg vanillic acid respectively, when compared to the DEP + BPS group.

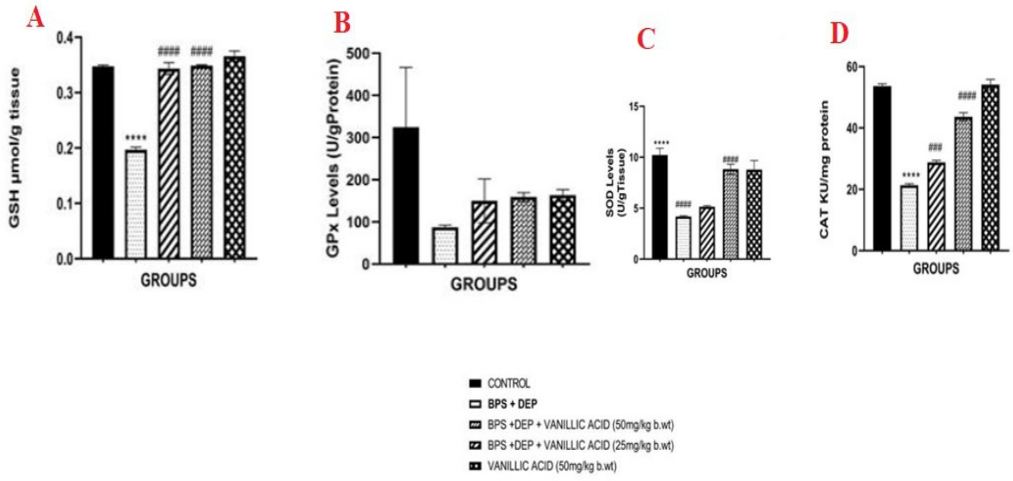

Figure 1 Effect of Vanillic acid pretreatment on diethyl phthalate and bisphenol s mediated decrease in antioxidant in rats in red blood cells concentration. (a) the activities of glutathione s-transferase (b) the activities of glutathione peroxidase (c) the activities of superoxide dismutase, (d) the activities of catalase. Bars represent mean ±SEM (n=5). Bars with different letters are significantly different at $P < 0.05$.

Figure 2 shows the effect of vanillic acid on markers of oxidative damage in the cardiac of DEP + BPS-exposed rats. There was a significant increase ($p < 0.05$) cardiac MDA concentration in the DEP + BPS–exposed group compared to the control group. On the other hand, treatment with vanillic acid (25 and 50 mg/kg) occasioned a marked decrease in cardiac MDA concentration, which followed a dose-dependent trend. Furthermore, there was no significant difference in the group treated with vanillic acid only when compared to the control group.

The effects of exposure to DEP + BPS and treatment with vanillic acid on cardiac NO concentration followed a similar trend. While there was a significant decrease ($p < 0.05$) cardiac NO concentration in the exposed group compared to the control group, treatment with vanillic acid occasioned dose-dependent reduction in reactive nitrogen species (i.e., NO). Meanwhile, no significant difference ($p > 0.05$) was observed in the group treated with vanillic acid only compared to the control group.

The effects of exposure to DEP + BPS and treatment with vanillic acid on arginase activity followed a similar trend. While, there was a significant increase ($p < 0.05$) arginase activity in the exposed group compared to the control group, treatment with vanillic acid occasioned dose-dependent reduction in reactive arginase. Meanwhile, no significant difference ($p > 0.05$) was observed in the group treated with vanillic acid only compared to the control group.

The effects of exposure to DEP + BPS and treatment with vanillic acid on PDE-5 activity followed a similar trend. While there was a significant increase ($p < 0.05$) PDE-5 activity in the exposed group compared to the control group, treatment with vanillic acid occasioned dose-dependent reduction in reactive PDE-5. Meanwhile, no significant difference ($p > 0.05$) was observed in the group treated with vanillic acid only compared to the control group.

The effects of exposure to DEP + BPS and treatment with vanillic acid on AOPP level followed a similar trend. While there was a significant increase ($p < 0.05$) AOPP level in the exposed group compared to the control group, treatment with vanillic acid occasioned dose-dependent reduction in AOPP. Meanwhile, no significant difference ($p > 0.05$) was observed in the group treated with vanillic acid only compared to the control group.

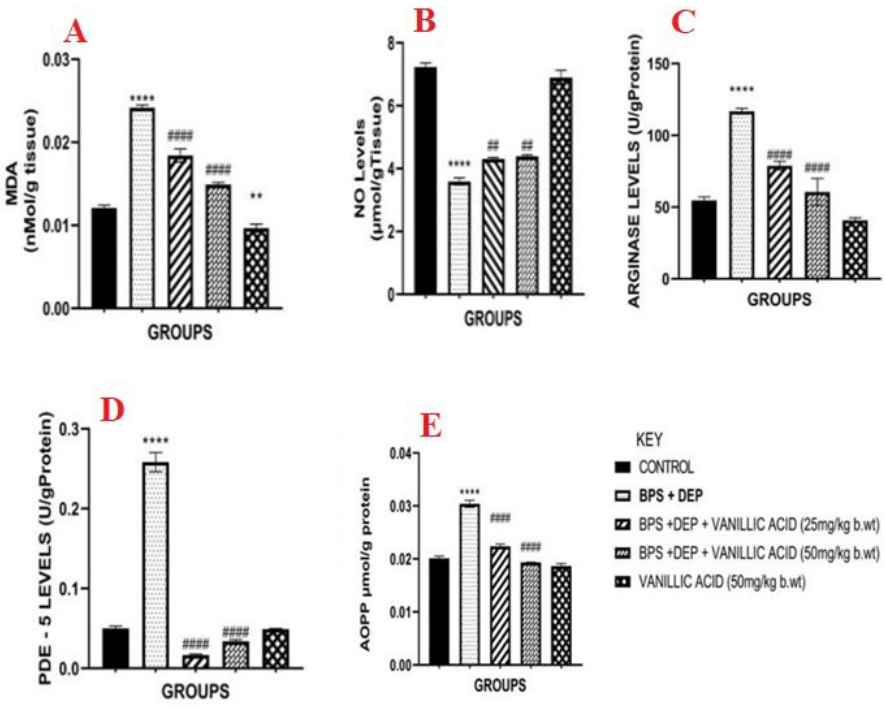

Figure 2 Effects of vanillic acid pretreatment on diethyl phthalate and bisphenol s mediated increase in oxidative stress markers in rats on hematological parameters. (a) MDA (b) Nitric oxide (c) Arginase (d) PDE-5 (e) AOPP. Bars represent mean ±SEM (=5). Bars with different letters are significantly different at $P<0.05$.

While no death was recorded among all groups during this study, section of heart tissues from group A (0.4% DMSO) showed a preserved architecture with a prominent changes were observed in the heart tissues of DEP+BSP exposed groups, showing distorted cardiac muscles fires with deep stained nuclei and nucleated (Figure 3). Upon pre-treatment with vanillic acid there is observable restoration of the heart tissues which implies that the histo-pathological examination shows a slight increase of the nucleated cells.

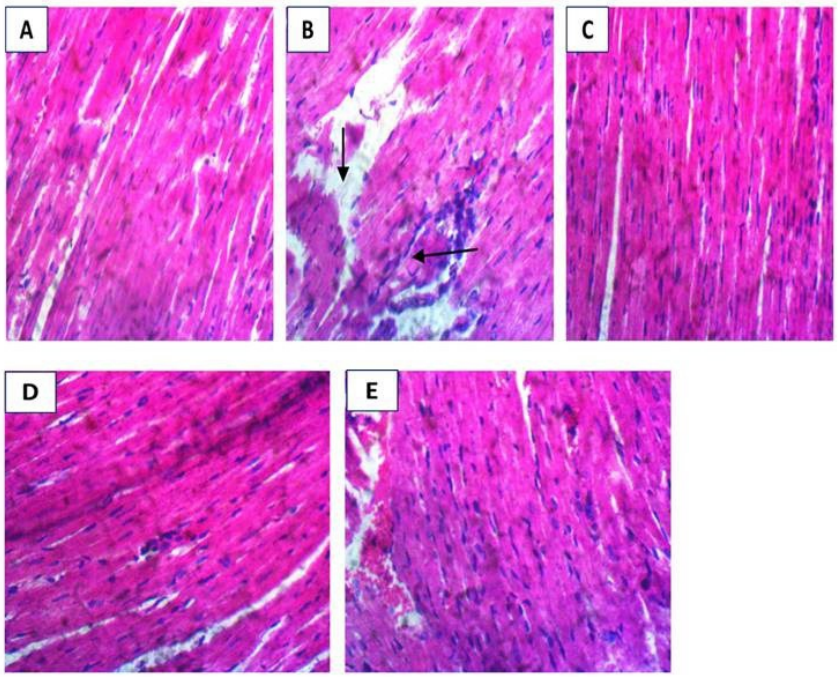

Figure 3: cardiac tissues histology of control and DEP + BPS-exposed rats treated with vanillic acid. Group A: 0.4% DMSO; GROUP B: DEP + BPS; GROUP C: DEP + BPS + vanillic acid (25 mg/kg); GROUP D: DEP + BPS + vanillic acid (50 mg/kg); GROUP E: vanillic acid (50 mg/kg). The black arrow indicates nucleated cells implying that the histoarchitecture of the cardiac tissues is adequately nucleated. Upon treatment vanillic acid with (50 mg/kg) the nucleated cells histopathological examination show slightly increase the nucleated cells.

4. Discussion

Despite the challenges we faced at home with plastics and other endocrine disruptors in our environment. It provokes some biochemical mechanisms proposed for DEP+BSP-induced cardiotoxicity and inflammation including increased levels of free oxygen radicals, alteration of myocardial cell membrane permeability due to lipid peroxidation, mitochondrial oxidative phosphorylation, and changes in electrolyte contents [24]. Different bioactive components contain Phenolic compounds that are commonly used as antioxidant effect; these compounds are present in various plants. Because these compounds have the potential to treat various kinds of human diseases. But the actual mechanism by which they treat the diseases could be as a result of its bioactive components. From a study which was done using murine model of inflammation it was concluded that vanillic acid exerts anti-inflammatory and analgesic action by reducing manufacture of pro-inflammatory cytokines [25].

Glutathione protects cells by reducing ROS such as peroxides and other free radicals while being oxidized to GSSG [26]. This process makes it capable of preventing

cellular damage caused by ROS. Figure 1 shows a decreased concentration of GSH. The activity of GSH implies that lower concentrations may lead to increased level of ROS which may subsequently cause oxidative stress. A significant increase in the concentration of GSH was however, seen after treatment with vanillic acid. This implies that while BPS and DEP increase ROS levels by causing a decrease in the concentration of GSH, vanillic acid may be able to reverse this effect. Glutathione peroxidase is an enzyme that catalyzes the reduction of ROS such as peroxides to their corresponding alcohols and water through the oxidation of GSH [27]. A decrease in the activity of GPx as shown in Figure 1 suggests that the rate at which the ROS are broken down may be greatly decreased causing a possible increase in ROS and consequently, oxidative stress. Figure 1 also shows that while treatment with vanillic acid may not totally reverse the effects of BPS and DEP on GPx activity, it can extensively ameliorate these effects.

Superoxide dismutase is an antioxidant enzyme that catalyzes the breakdown of superoxide radicals (example $HO_2^{\cdot}$) into oxygen and hydrogen peroxide [28]. Reduced activity of SOD would consequently lead to an increase in the level of superoxide radicals and thus, oxidative stress. The result of SOD in Figure.1 also shows that vanillic acid was more effective in restoring the activity of SOD at the higher dose of 50mg/kg body weight compared to the 25mg/kg body weight dose.

Catalase is an enzyme that catalyzes the rapid decomposition of hydrogen peroxide to water and oxygen [29]. (SOD is an antioxidant that protects the heart from ischemia and the lungs from inflammation and fibrosis. Catalase is the key enzyme which uses hydrogen peroxide, a nonradical ROS, as its substrate. This enzyme is responsible for neutralization through decomposition of hydrogen peroxide, thereby maintaining an optimum level of the molecule in the cell which is also essential for cellular signaling processes). It rapidly decomposes the hydrogen peroxide produced by SOD as hydrogen peroxide breaks down to give two hydroxyl radicals ($OH^{\cdot}$) in the absence or at low activity of catalase [27]. From figure. 1 it can be inferred that exposure to BPS and DEP can lead to a possible accumulation of hydrogen peroxide and consequently, an increase in free hydroxyl radicals leading to oxidative stress. The ameliorative effect of treatment with vanillic acid was however found to be significantly more effective at 50mg/kg body weight compared to the 25mg/kg body weight dose. Nitric oxide regulates several vital physiological factors including blood pressure. It controls vascular tone and the contraction of the myocardium [30]. Oxidative stress has been implicated in the damage of various cellular structures and tissues and consequently in the pathology of several diseases including atherosclerosis and other cardiovascular conditions which may lead to or result from it [31]. This study was carried out to investigate the effects of BPS and DEP-induced oxidative stress on cardiac function as well as the ameliorative role of vanillic acid in rat models by examining the resulting changes in the activities and concentrations of known oxidative stress biomarkers. Malondialdehyde is produced as a result of lipid peroxidation of polyunsaturated fatty acids i.e a process through which radical or non-radical oxidants attack polyunsaturated fatty acids [32]. Bio-monitoring of MDA has been used in several studies as a key biomarker for various disease patterns including hypertension and atherosclerosis. Higher levels of MDA have been reported in patients of various categories. The findings suggest the validity of the MDA assay as a reliable tool in finding out the oxidative stress level in the pathology of different diseases [33]. Increase in the concentration of MDA as shown in Figure. would therefore indicate an increased rate of lipid peroxidation. This directly implies an increased activity of the pro-oxidative species and consequently, oxidative stress. Treatment with vanillic acid showed a significant decrease in MDA concentration which was further decreased at a higher dose of 50mg/kg body weight. This result suggests that vanillic acid is able to hinder lipid peroxidation and therefore implies an ability to combat the pro-oxidative species responsible for lipid peroxidation and oxidative stress.

Reduced levels of NO have been linked to several risk factors for cardiovascular disease. According to research by [34], a decrease in NO level as shown in Figure. could lead to hypertension and atherosclerosis which are known risk factors for cardiovascular diseases. Treatment with vanillic acid however did not completely reverse this effect but merely showed a slight ameliorative effect. Arginase is the final enzyme in the urea cycle which catalyzes the conversion of L-arginine to urea and L-ornithine [35]. L-arginine is also required for the synthesis of NO by endothelial NOS. An increased activity of arginase as shown in figure. would consequently lead to a decomposition of the L-arginine required for the synthesis of NO thereby reducing the bioavailability of NO. Several studies have associated increased arginase activity with endothelial dysfunction in vascular vessels [36]. Figure. also shows a significant increase or inhibition of arginase activity after treatment with vanillic acid. This inhibition

was more significant at 50mg/kg body weight. Pernow and Jung [36] showed that inhibition of arginase activity could increase the bioavailability of NO and reduce superoxide levels. It has also been suggested that inhibition of arginase could be an effective treatment strategy in cardiovascular medicine [37].

Type 5 phosphodiesterase hydrolyzes cGMP which is a nitric oxide donor and potent vasodilator [38]. Increased activity of PDE-5 Figure. 2 would imply a speedy hydrolysis of cGMP leading to reduce NO bioavailability and vasoconstriction which could cause myocardial stress due to high blood pressure and hypertension. Treatment with vanillic acid as also shown in the result significantly decreased the activity of PDE-5. Previous research by [38] suggests that the selective inhibition of PDE-5 could protect the heart against high blood pressure induced congestive heart failure. Advanced oxidation protein products are produced as a result of the reaction of proteins with chlorinated oxidants such as hypochlorous acid [27, 38]. Studies also, indicate that higher AOPP levels (Figure 2) have been observed in subjects suffering from coronary artery disease than in healthy subjects. The ability of vanillic acid to decrease AOPP levels as also shown in Figure. implies a relatively lower risk of coronary artery disease as it has been demonstrated that AOPP concentration is directly related to the degree of diseased coronary arteries [38]. The results of histopathological analysis of the cardiac tissues Figure. 3 show lesions, inflammation and multi focal myofiber degeneration of the cardiac tissues of the animals that were exposed to just BPS and DEP indicating severe damage to the cardiac tissues. The control group as well as the treated groups showed no visible signs of tissue damage. The treatment control group (group E) for all parameters shows that vanillic acid has no significant negative effect on the activity or concentration of any of the analyzed parameters.

5. Conclusion

Different doses of vanillic acid pretreatment for 21 days could ameliorate BPS and DEP induced cardio-toxicity by acting as specific ROS and RNS scavenger. This preserve the cytoarchitecture of the heart and oxidative stress in rats via it oxidant free radical scavenging capacity. Vanillic acid demonstrated a significant degree of attenuation which could proffer a promising attribute to its strong antioxidant properties.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors wish to appreciate all the coauthors for their valuable contributions, most especially the technical staff of Department Biochemistry.

Ethical Approval

All the animals received humane care according to the conditions outlined in the 'Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science (NAS) and published by the National Institute of Health. The institution approved an experimental number of the researcher is BCH/20160957

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