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Ethyl acetate fraction of *Diaphananthe bidens* leaf extract ameliorates chronic immobilization stress-induced oxidative damage in the rat brain

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ABSTRACT
Chronic immobilization stress causes oxidative damage in the brain by increasing reactive oxygen species, leading to lipid peroxidation and a decrease in antioxidant enzyme activities. These changes can result in depression, anxiety, and neurodegenerative disorders. This study evaluated the prophylactic and curative effects of the ethyl acetate fraction of D. bidens leaf extract against stress-induced damage in rat brains. The leaves of D. bidens were collected, shade-dried, and extracted with 70 % ethanol, then sub-fractionated using vacuum-liquid chromatography. Subsequently, the phytochemical and total phenolic contents were assessed. Rats were divided into prophylactic and curative groups (n = 36 each), subjected to 6-hourly daily restraint for 60 days without food or water in individual wire mesh cages suitable for rats. Graded doses of 93, 186, and 372 mg/kg, representing half, the same, and double the effective dose of the extract, were administered orally alongside 15 mg/kg quercetin as a reference standard. After phenobarbital administration, rat brain tissues were harvested, and tissue homogenates were used to measure lipid peroxidation biomarkers and antioxidant enzyme activities. Chronic stress induction significantly ($p<0.05$) increased malondialdehyde (MDA) levels and decreased superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx-1) activities in the vehicle control groups. The administration of the ethyl acetate fraction of D. bidens reduced MDA levels and restored enzyme activities dose-dependently, with 372 mg/kg showing superior effects compared to quercetin, especially in prophylactic treatment. The ethyl acetate fraction of D. bidens alleviates immobilization-induced oxidative stress through antioxidant mechanisms, providing better prophylactic benefits with potential neuroprotective effects.
Keywords: *Diaphananthe bidens, neuroprotection, antioxidant enzymes, chronic immobilization stress, oxidative damage, Reactive oxygen species.*

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INTRODUCTION

The World Health Organization defined stress as a state of mental tension arising from a different situation[1]. Annually, about 12 billion workers are lost globally due to stress-related conditions, such as depression and anxiety [2]. Immobilization stress is defined as the physiological and psychological response that occurs when animals are restricted or unable to move freely, resulting from various factors such as injury, physical confinement, or restrictive conditions[3,4]. Known also as restraint stress, immobilization stress can significantly affect physical health, leading to issues like cardiovascular diseases, obesity, a weakened immune system, and generally impacting overall well-being [3,5].

Immobilization stress (Restraint stress) could be acute or chronic restraints and induces rapid activation of the hypothalamic-pituitary-adrenal (HPA) axis [6], and in turn elevates glucocorticoids and heightened reactive oxygen species (ROS) production in the brain [7]. When this occurs, oxidative stress develops, a key pathological feature of neurological diseases [8].The elevation of ROS increases lipid peroxidation production activity in the brain's hippocampus and prefrontal cortex, which reduces the endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [9]. Superoxide dismutase (SOD) acts to protect the cell from oxidative damage by scavenging

peroxide radicals, converting O_2^- into H_2O_2 and O_2 [10]. Catalase (CAT), on the other hand, acts to convert peroxide radicals to water and molecular oxygen in two distinct steps (11), and GPx facilitate the reduction of peroxide radicals or organic peroxide (ROOH) into water or alcohol, whereby the glutathione (GSH) is converted into oxidized form (GSSG) in the presence of GSH, protecting the polyunsaturated fatty acids within the cell membrane[12,13]. In the brain, the cellular antioxidants work synergistically with non-enzymatic antioxidants such as vitamins C and E to scavenge both the reactive oxygen species (ROS) and reactive nitrogen species (RNS) [14].

Various forms of stress, including oxidative stress generated from excessive release of reactive oxygen species, physiological stress from hormonal imbalances, and psychological stress from mental health conditions like anxiety and depression, can lead to or exacerbate conditions like neurodegenerative diseases, cardiovascular disease, and metabolic disorders [15,16].

Recently, global attention has been directed towards finding natural antioxidant therapeutics from plants and herbs that could be effective, non-toxic and less costly than synthetic antioxidants like butylated hydroxytoluene, which offers limited efficacy due to toxicity and poor bioavailability [17].

Phytochemicals are a strong group of compounds that are secondary metabolites of plants, which include a wide range of chemical compounds, such as polyphenols, flavonoids, steroidal saponins, organosulphur compounds, and vitamins[18]. These plant secondary metabolites have been reported to possess varying ranges of pharmacological and biological activities, including antioxidants, neuroprotective, cardioprotective, neuro-anti-inflammatory, and anti-ageing, among others[19–21].

Diaphananthe bidens (Afzel. Ex Sw) Schltr, commonly known as the wax orchid and belonging to the family *Orchidaceae*, is an epiphyte with a tough, wire-like stem. It has been reported to contain various secondary metabolites such as polyphenols, flavonoids, alkaloids, saponins, steroids, tannins, and polysaccharides [22,23]. Plant decoctions are reported to possess anti-inflammatory [24], anti-hyperglycaemic [25], hepatoprotective [26], and Immunomodulatory [27] activities, with no toxic effects observed in the liver, kidney, blood, or gonadal hormones[28]. The impact of the plant's ethyl acetate fraction on immobilization stress-induced changes in the rat brain remains uninvestigated.

MATERIAL AND METHODS

Plant material

In June 2023, *Diaphanathe bidens* leaves were collected from Nsukka, Enugu State, Nigeria, and authenticated by Taxonomist Mr. Alfred Ozioko of the Bioresources Development and Conservation Project (BDCP), Nsukka. Voucher specimen BDCP/H8911 was deposited in the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu Campus. The 12 kg of fresh leaves were cleaned, shade-dried for 28 days, and the resulting 6.7 kg dry sample was pulverized using a Gx160 Delmar 5.5HP mechanical grinder (Honda Motor Co., Ltd., Japan) for solvent extraction.

Animals

Adult Swiss albino rats, aged three months (190–200 g) of both sexes from the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Science, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The animals ate pelletized feed (Vital Feeds, Nigeria) and had free water. The animals were housed in standard cages in Nnamdi Azikiwe University's Pharmacology & Toxicology Animal House. The animals acclimatized for 14 days before the study. The animal experiment followed the care and use of laboratory animals by the Nnamdi Azikiwe University-Animal Research and Ethical Committee with an approval number **NAUA/AREC/2024/0088**.

Reagents and apparatus

Folin-Ciocalteu's reagent (LobaChemie, India), Hydrogen peroxide (Avondale Laboratories, England), thiobarbituric acid (TBA) (Guangdong Guanghua Chemical Factory Co., Ltd, China). HCl, Potassium dichromate and potassium ferricyanide were products of Hopkin and Williams Ltd, England. Catalase kits (Mybiosource, USA), Glutathione peroxidase kit and superoxide dismutase (Bioassay Technology Laboratory, China). Ethyl acetate, ethanol, butanol, hexane, dichloromethane and freshly prepared distilled water. Visible Spectrophotometer (721G, Zhejiang Top Cloud-Agri Technology Co., Ltd., China), refrigerator (Haier Thermocool), electronic weighing balance (WANT Balance Instrument Co. Ltd, China), centrifuge (Filtertech, Inc. Manilius, NY), water bath (Bio Technics, Mumbai, India) and separating funnel, syringes, micropipette tips, test tubes, Eppendorf tubes, evaporating dish, sieve mesh, beakers, measuring cylinder, stirring rods, filter paper, hand gloves,Agilent gas chromatography-mass spectrometry, GC-MS (Agilent Technologies, USA) and ELISA (Dynex Technologies, USA).

Extraction and phytochemical analysis

The 6kg of pulverized *D. bidens* leaf was macerated in 30L of 70% ethanol with intermittent shaking for 72 hours, filtered and concentrated using rotary evaporator at 40^0C. Thereafter, 100 g of the extract was

dissolved in distilled water and put through Liquid-Liquid chromatography and fractionated using ethyl acetate to separate soluble fractions using separating funnel. The remaining fraction is called water (aqueous) fraction [29,30]. The total phenolic content (TPC) and qualitative phytochemical analysis were evaluated using the standard methods [31–33]

Vacuum-Liquid (VL) Chromatography of the Ethyl acetate Fraction

The ethyl acetate fraction of D. bidens (1.5 g) was subjected to VLC separation using a 5 L sintered column packed with Silica gel (200 – 400 mesh size) to a 10 cm bed size. The column was eluted with 500 mL each of hexane: ethyl acetate (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10) and dichloromethane: Methanol (10:0, 8:2, 6:4, 4:6, 2:8, 0:10). The resulting VLC fractions were screened for total phenolic contents [34,35].

Experimental design

Dosage Selection

The median effective dose (ED_{50}) of the ethyl acetate fraction of the extract was selected and used for the study because it showed the highest therapeutic activity at 186 mg/kg compared to other fractions and contained the highest total phenolic content, which was determined from the immunosuppressive activity of the extract. Three doses, 186mg/kg (ED_{50} as determined from the immunosuppressive study), 93 mg/kg (half the ED_{50}), and 372 mg/kg (double the ED_{50}) were used in the immobilization stress-induced study [27].

Induction of chronic immobilization stress

Rats were exposed to stress from 9 am -3 pm in the animal house, and restraint stress was accompanied by placing the individual animals in wire mesh cages of their sizes attached to a wooden board for 60 days and deprived of food and water during the stress exposure period[36].

Grouping and Treatment

Seventy–two (72) rats were randomly selected and grouped into

i. Prophylactic study, and
ii. Curative study of 36 rats in each study group.

The thirty-six (36) Rats of each study group were divided into six (6) groups of six (6) rats.

In each study group (prophylactic and curative), group 1 served as the naïve uninduced group and received 10mg/kg 5% tween 20, and group 2 served as the negative induced control and received 10 mg/kg 5% tween 20. Group 3 received 15mg/kg quercetin and served as the reference standard. Groups 4, 5 and 6 received the ED_{50}, 186mg/kg, half ED_{50}, 93mg/kg, and double ED_{50}, 372mg/kg, and served as treatment groups. Treatment was done orally for 60 days, 1 hour before (pre-induction of stress) for the prophylactic study groups and immediately after (post-induction of stress) 6 hours for the curative study. On the 61st day, rats were sacrificed by intraperitoneal injection of 50mg/kg/w phenobarbital.

Preparation of brain homogenate

The brain tissues from each animal were quickly removed and washed with 0.9% cold-ice sterile physiological saline. A 10% homogenate was prepared in 0.1M sodium phosphate buffer, pH 7.4 and centrifuged using a refrigerated centrifuge at 3000 Xg for 15 minutes at 40 °C to remove cellular debris. The supernatant was used to determine the immobilized stress markers.

Determination of brain tissue antioxidant biomarkers

Determination of superoxide dismutase (SOD)

The brain SOD was evaluated using competitive ELISA kit micro-plated with rat SOD1[37]. Briefly, 50µL of standard solution and brain tissue homogenate samples were measured into a right wells, and 50µL of biotinylated detection Ab added. The mixture was incubated at 37^{0}C for 45 minutes, then washed three times with 350µL of wash buffer to remove excess conjugate and unbound samples or standards. Thereafter, 100µL of Avidin-HRP conjugate was added to each well and incubated at 37^{0}C for another 30 minutes followed by washing the mixture more than five times, after which , 90µL of substrate reagents was added and put on the dark at 37^{0}C for 15 minutes for enzyme-substrate reaction. A 50µL of stop reagent was added to each well after the reaction and the colour change was measured at 450nm using a spectrophotometer, and the SOD1 of the samples was determined by comparing the optical density (OD) with that of the standard curve.

Determination of brain tissue Catalase (CAT) **enzyme**

The CAT enzyme brain tissue homogenate was evaluated using a competitive ELISA kit microplated with polyclonal anti-CAT and CAT-HRP conjugate[38]. Briefly, the supernatant of a 100 µL sample and buffer were incubated together with the CAT-HRP conjugate in a pre-coated plate for one hour. Serial fold dilutions of the reference standard (0 – 50 ng/mL) were also prepared and added to their respective wells. After a 1h incubation period at 37°C, the well was decanted and washed five times using the wash buffer. The wells were then incubated with a substrate for HRP enzyme (50 µl) for 20 minutes at 37°C. The product of the enzyme-substrate reaction formed a blue-coloured complex. Finally, a stop solution (50 µl) was added to stop the reaction, which then turned the solution yellow. The intensity of colour was measured

spectrophotometrically at 450nm in a microplate reader. The intensity of the colour was inversely proportional to the CAT concentration. A standard curve was plotted relating the intensity of the colour (O.D.) to the concentration of standards. The CAT concentration in each sample was interpolated from this standard curve.

Determination of brain tissue glutathione peroxidase (GPx) enzyme.

The brain tissue GPx was evaluated using a Sandwich ELISA kit pre-coated with rat GPx antibody [38]. Briefly, a 40 µL of samples and the standards were added in their respective wells followed by addition of 10 µL of biotinylated rat GPX1 antibody to all the wells, then 50 µL of streptavidin-HRP added, and were incubated for 1h at 37°C followed by 5 times washing with washing buffer. Then 50 µL of substrates A and B were added and incubated for 10 minutes at 37°C in the dark. The optical density (OD) of each well was determined immediately using a microplate reader at 450 nm within 10 minutes after adding the acidic stop solution. The standard calibration curve was plotted using average OD for duplicate standard concentrations prepared by serially diluting the stock solution to get concentrations ranging from 120 ng/mL – to 7.5 ng/mL). OD was plotted on the Y-axis while the corresponding concentration was plotted on the X-axis. The best-fit curve was drawn through the points on the graph.

Determination of brain tissue lipid peroxidation biomarker (Malondialdehyde, MDA)

Malondialdehyde (MDA) in the brain tissue homogenate was estimated by the modified thiobarbituric acid method of Draper and Hadley (1990) as described by [39] using an MDA assay kit. Briefly, 5mL test tubes were labelled appropriately for the samples, standard and blank, all in duplicates. 50 µL of the sample, standard and absolute ethanol were placed in their appropriate labelled sample, standard and blank test tubes, respectively. Then 50 µL of Clariant was added to all the test tubes, followed by the addition of 1.5 ml of acid reagent and then 500 µL of the dilute chromogenic agent. The contents of the test tubes were mixed well, covered with a plastic cover and incubated in the water bath at 95 ^{0}C for 40 minutes. After incubation, the test tubes were cooled with running water and centrifuged at 3000 Xg revolutions per minute for 10 minutes. The supernatants were collected from the centrifuged test tubes. The absorbance of the supernatants was read at 532 nm against a distilled water blank. The MDA level of each sample was calculated using the formula below;

$$MDA\ (nmol/mg\ protein) = \frac{Absorbance\ of\ Sample - Absorbance\ of\ Blank}{Absorbance\ of\ Standard - Absorbance\ of\ Blank} \times 10$$

Statistical analysis

The results were presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) using SPSS 20.0 was used to analyze differences between means. P values less than 0.05 were taken to be statistically significant. Further, multiple post-hoc comparisons were done using Turkey's test. Graphical plots were done using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Phytochemical analysis and total phenolic content of *D. bidens* leaf extract

The phytochemical analysis and total phenolic content of the *D. bidens* extract revealed the presence of bioactive compounds such as flavonoids, polyphenols, alkaloids, tannins, polysaccharides, among others (Table 1), with ethyl acetate fractions having the highest total phenolic content of 328.2mg/GAE/g with 40 % yield compared to other fractions. Further fractionation of the ethyl acetate sub-fractions of *D. bidens* with vacuum-liquid chromatography using different solvent ratios showed that the hexane and ethyl acetate ratio (H: E, 3:7) with code S8 have the highest total phenolic content of 528.4 mg/GAE/g (Table 2). The findings that *D. bidens* leaf extract contains these bioactive compounds with a high total phenolic content are in agreement with the works of [26, 25, 24]. Phytochemicals, including polyphenols, also present in *D. bidens* have been reported to act as antioxidants and anti-inflammatories in the brain during restraint stress, and modulate stress-related pathways, such as the HPA-axis, neurotransmitter balance, and scavenge free radicals and suppress pro-inflammatory cytokines, leading to the decrease oxidative stress, neuro-inflammation and improve brain functions [40-42].

Table 1: Phytochemical analysis and total phenolic content of the extract and fractions

Phytocompounds	Extract		Ethyl acetate Fraction		Water Fraction
Flavonoids	**+**		**+**		**+**
Saponins	**+**		**+**		**+**
Tannins	**+**		**+**		**+**
Reducing sugar	**+**		**+**		**+**
Steroids	**+**		**-**		**-**
Terpenoids	**+**		**+**		**-**
Alkaloids	**+**		**+**		**+**
Glycosides	**+**		**+**		**+**
TPC (mgGAE/g)	175.3		328.2		94.1
Yield (%)	2.56[a]		40.32[b]		18.29[b]

+ Present, - absent. a - calculated from 6 kg pulverized leaves, b – calculated from 100 g extract. TPC = Total phenolic content, mg/GAE/g = milligram/Gallic equivalent/gram.

Table 2: Vacuum-liquid chromatographic sub-fractions of the ethyl acetate fraction

Sample code	Solvent	Weight (mg)	TPC (mgGAE/g)
S1	H: E 10:0	13	21.5
S2	H: E 9:1	17	56.3
S3	H: E 8:2	55	151.8
S4	H: E 7:3	93	218.5
S5	H: E 6:4	106	237.4
S6	H: E 5:5	124	266.9
S7	H: E 4:6	189	358.2
S8	H: E 3:7	215	523.4
S9	H: E 2:8	192	395.1
S10	H: E 1:9	133	269.7
S11	H: E 0:10	124	242.5
S12	D: M 10:0	89	198.6
S13	D:M 8:2	52	133.3
S14	D:M 6:4	38	107.9
S15	D:M 4:6	22	72.7
S16	D:M 2:8	18	50.4
S17	D:M 0:10	15	29.3

H: E = n-Hexane: Ethyl acetate and D:M = Dichloromethane: Methanol

Prophylactic and curative effects of ethyl acetate fraction of *D. bidens* leaf extract on immobilization induced chronic oxidative stress in rats' brain

The induction of the immobilization induced chronic oxidative stress significantly ($p<0.05$) elevated the brain lipid peroxidation biomarker -malondialdehyde (MDA), coupled with the significant reduction in antioxidant enzymes in negative control groups compared to naïve uninduced groups in both prophylactic and curative studies, showing effective evidence of immobilization induction (Fig. 1a &2e).

Lipid peroxidation is a complex reaction initiated when free radicals seize an electron from a lipid molecule due to excessive production of ROS during oxidative stress [36,43]. This process generates fatty acid radicals, which subsequently interact with additional fatty acids, resulting in the formation of lipid peroxides and an increase in the concentration of fatty acid radicals [8,44]. Malondialdehyde, MDA, is a by-product and an index of lipid peroxidation during oxidative stress, representing a terminal product of the peroxidation of polyunsaturated fatty acids within brain cells [3]. The brain tissue requires plenty of oxygen, and a significant amount of it is converted to ROS. The overproduction of ROS in the brain due to chronic restraint stress decreases antioxidant enzyme activities, causes mitochondrial dysfunction, and induces neuroinflammation [17].

In the prophylactic study, treatment with ethyl acetate fraction at all tested doses produced a significant ($p<0.05$) reduction in brain tissue MDA level compared to the negative control group (Figure 1a). However, compared to the reference drug – quercetin, lower doses (93 and 186 mg/kg) of the ethyl acetate fraction showed significantly lower activity for lipid peroxidation, while 372 mg/kg showed better activity that was significant ($P<0.05$) compared to the reference drug. Unlike other treatment groups and the reference drug control group, only 372 mg/kg dose of the ethyl acetate fraction was able to resist immobilization-induced

lipid peroxidation as indicated by the non-significant (P>0.05) difference between the MDA concentration of this group of animals and naïve uninduced control. Graded doses of the ethyl acetate fraction showed significant (p<0.05) graded effects compared with each other.

Reactive oxygen species significantly contribute to the loss of brain cells by modulating brain lipids, RNA, DNA, and proteins, as well as processing nucleic acid oxidation and lipid peroxidation within the cell [8,15]. Induction of chronic immobilization stress significantly increased reactive oxygen species (ROS) in the brain, as evidenced by an increase in brain malondialdehyde, a biomarker of lipid peroxidation, and a decrease in antioxidant enzymes (GPx, CAT, and SOD). However, prophylactic treatment with the ethyl acetate fraction of *D. bidens* leaf significantly reversed the trend in a dose-dependent manner, noting that the extract could offer protective abilities in the brain through the mechanism of free radical scavenging properties of the plants. The free radical scavenging property of the plant has been reported by [23,26].

Glutathione peroxidase (GPx-1) in the brain acts primarily as an antioxidant defense system, protecting brain cells from oxidative stress and neuronal damage by neutralizing harmful peroxides, maintaining redox homeostasis, and preventing damage from excessive production of reactive oxygen species that are important for brain functions and plasticity [12,45,46]. Treatment with the ethyl acetate fraction showed a significant increase in brain tissue of GPx-1 enzyme activity compared to negative control (Fig. 1b). Similar activity/effect was recorded by 372 mg/kg dose of the ethyl acetate fraction compared to the reference drug control (quercetin 15 mg/kg) group with no significant (P>0.05) difference between both groups. Catalase (CAT) enzymes also act primarily in the brain to protect the neuronal cells from oxidative stress caused by ROS by hydrolyzing toxic hydrogen peroxide into harmless products, water, and oxygen, which is important in maintaining neuronal homeostasis that is linked to reducing neurodegenerative diseases [47]. Treatment with the ethyl acetate fraction also showed increased brain tissue CAT enzyme activity compared to the negative control; however, significant effects were only recorded byin the 186 and 372 mg/kg doses treatment groups (Fig.1c). The reference drug showed a similar effect as a 372 mg/kg dose of ethyl acetate fraction with no significant (p>0.05) difference between the two groups. However, 93 and 186 mg/kg doses of ethyl acetate fraction recorded significantly lower concentrations than the reference drug. Superoxide dismutase (SOD) in the brain acts as a potent antioxidant by neutralizing superoxide radicals released from metabolic processes due to oxidative stress, and regulates cell signaling pathways by converting superoxide radicals to hydrogen peroxides that act as a second messenger, thus protecting neurons from oxidative damage and apoptosis. The same trend of result was also replicated for the SOD enzyme activity, except for the fact that a 372 mg/kg dose of the ethyl acetate fraction recorded a non-significant (p>0.05) difference in SOD enzyme activity compared to the naïve control group (Fig.1D).

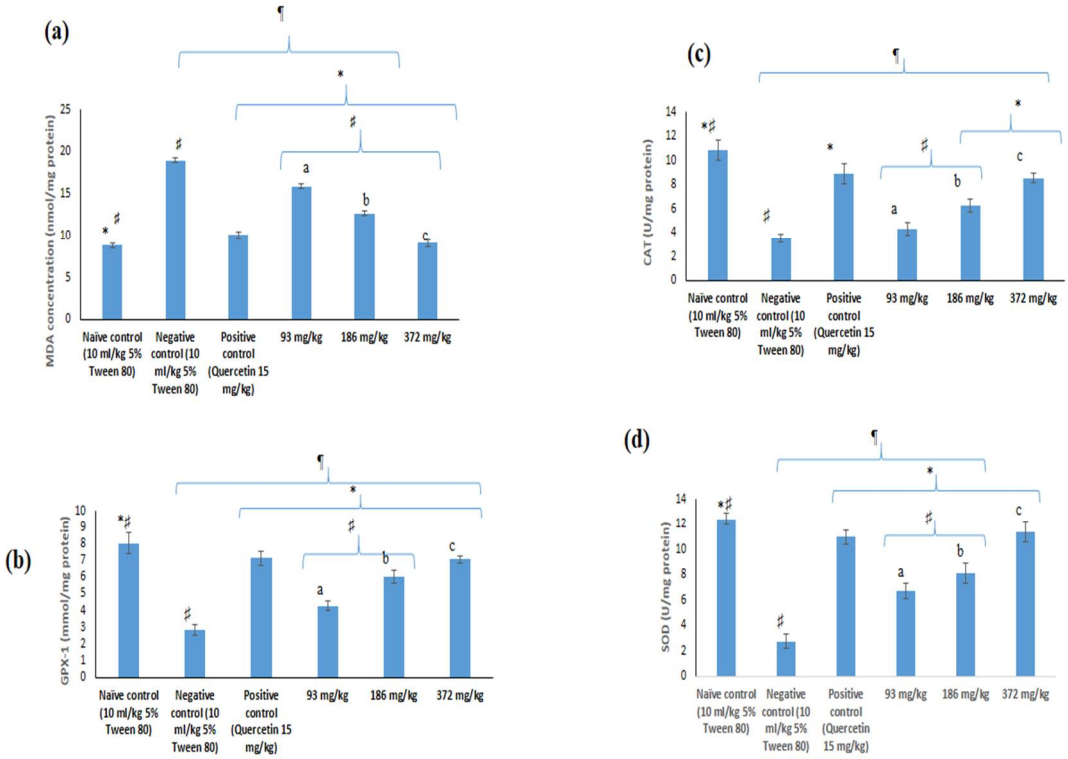

Figure 1: Prophylactic effect of ethyl acetate fraction of *D. bidens* on Immobilization induced chronic oxidative stress

Where: ¶ P<0.05 compared to naïve control; * P<0.05 compared to vehicle control group; # P<0.05 compared to Reference drug – Quercetin 15 mg/kg; different letter alphabet (a,b, c) P<0.05 compared to the doses of the ethyl acetate.

The curative effect of the ethyl acetate fraction following immobilization-induced chronic oxidative stress was evident from the dose-related reduction in brain tissue MDA level (Fig. 2e). Compared to the vehicle (negative) control group, 186 and 372 mg/kg doses of the ethyl acetate fraction showed a significant ($p<0.05$) reduction effect. The effect produced by 186 mg/kg was similar to that produced by the reference drug, while the effect produced by the 372 mg/kg dose was significantly ($p<0.05$) better than that produced by the reference treatment. Analysis of the effect of treatment on antioxidant enzymes revealed that the extract at 186 mg/kg and 372 mg/kg showed a significant increase in glutathione peroxidase (GPx-1) enzyme activity (Fig. 2f) in brain tissue and a non-significant ($p>0.05$) increase at all tested doses on superoxide dismutase (SOD) and catalase (CAT) enzyme activities compared to the vehicle control group (Fig. 2 g & h).

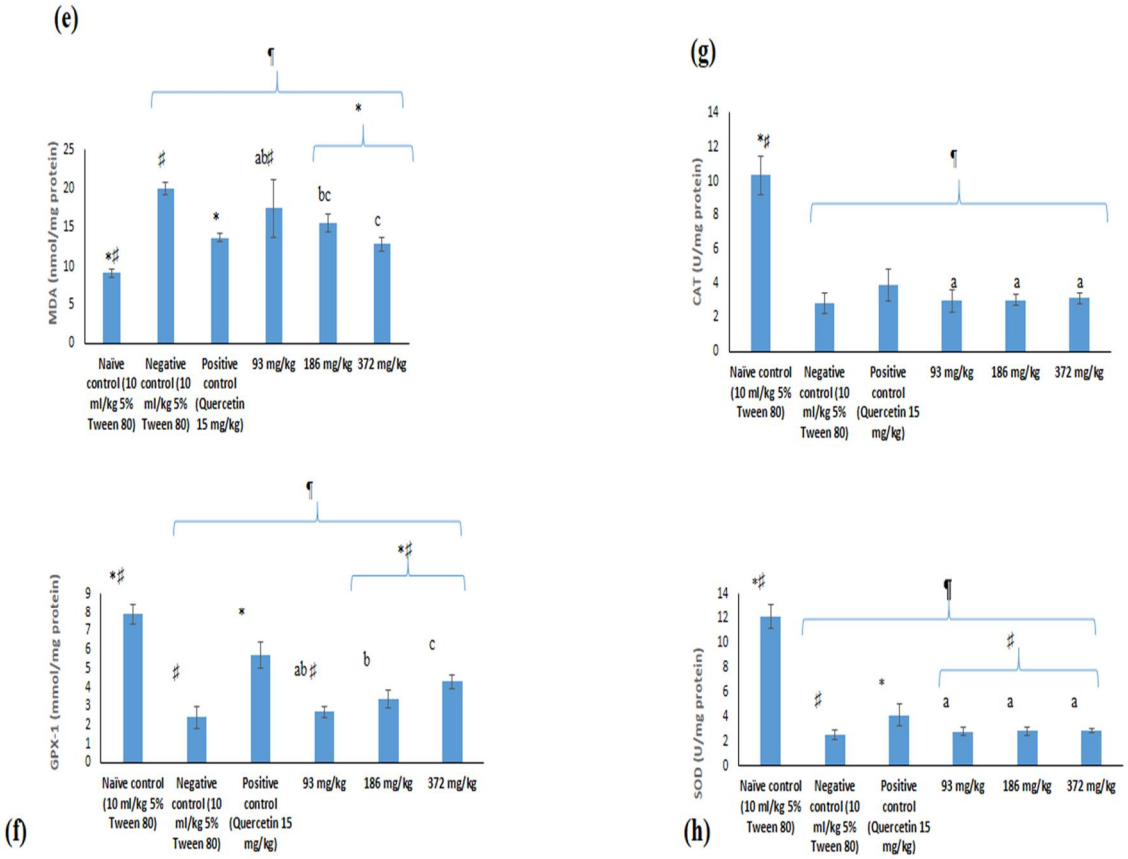

Figure 2: **Curative effect of ethyl acetate fraction of *D. bidens* on immobilization-induced chronic oxidative stress**
Where: ¶ P<0.05 compared to naïve control; * P<0.05 compared to vehicle control group; # P<0.05 compared to Reference drug – Quercetin 15 mg/kg; different letter alphabet (a,b, c) P<0.05 compared to the doses of the ethyl acetate.

However, the prophylactic treatment of the ethyl acetate fraction of *D. bidens* leaf extract offered better ameliorative neuroprotection compared to curative (thereapeutic) treatment groups. This improvement could be a result of the plant extract triggering pro-oxidant signalling activities, which in turn activate antioxidant mechanisms of the plant. This study is consistent with [15, 48,49] which reported significant increase in lipid peroxidation biomarkers of rodents brain tissues on exposure to chronic immobilized induced stress with a reduced antioxidant enzymes activities, but on treatment with different plants extracts, the trend reversed.
Antioxidant enzymes have played an important role in the body's defense against oxidative damage caused by excessive release of reactive oxygen species. These antioxidant enzymes, glutathione peroxidase, catalase, and superoxide dismutase, work synergistically to protect against brain injuries as a result of the accumulation of ROS from oxidative stress, contributing to a reduction in neurodegenerative disorders and enhanced brain functions [11]. Therefore, phytochemicals, especially polyphenols and phenols extracted from various plants have been reported to possess antioxidant activities [19,50,51]. Flavonoids also found in *D. bidens* have been reported to neutralize reactive oxygen species via scavenging ability by donating an electron to ROS such as superoxide and hydroxy radicals, which in turn prevent lipid peroxidation and damage to neuronal proteins and DNA [52]. It reaches the blood-brain barrier, suppresses cyclooxygenase enzymes, inhibits pro-inflammatory cytokine release, reduces ROS production through the NADPH oxidase pathway, and offers neuroprotection and anti-apoptotic protection through stress-activated pathways [53,54]. Other naturally occurring phytochemicals, including tannins, saponins, alkaloids, and terpenoids, are present in the ethyl acetate fraction of *D. bidens*. These compounds have been reported to cross the blood-brain barrier and exert antioxidant activities by suppressing oxidative stress, which inhibits

neuroinflammatory activities and offers protection by modulating redox homeostasis in the hippocampus and substantia nigra of the midbrain [55–58].

CONCLUSION:
This study has shown that the ethyl acetate fraction of *Diaphananthe bidens* leaf extract contains novel phytocompounds, such as flavonoids, tannins, saponins, alkaloids, and other polyphenols, with potential neuroprotective properties against chronic immobilization stress-induced oxidative damage in rat brains. The ethyl acetate fraction of *D. bidens* has a better ameliorative effect on chronic immobilization stress-induced oxidative damage in prophylactic treatment compared to curative treatment by inhibiting lipid peroxidation biomarker, malondialdehyde, and enhancing antioxidant enzyme activities through different mechanisms in a dose-related manner.

ETHICAL APPROVAL
Ethyl acetate fraction of *Diaphananthe bidens* leaf extract ameliorates chronic immobilization stress-induced oxidative damage in the rat brain was approved by members of Nnamdi Azikiwe University Animal Research Ethics Committee (NAU-AREC) with approval number, (**NAU/AREC/2024/0088**).

Conflict of Interest: There are no conflicts of interest, the research was done with utmost integrity and transparency.

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AUTHORS' CONTRIBUTION:
Mba Ogbonnaya Conceptualized and carried out the research
Ikechukwu S. Mbagwu proofread and made useful inputs throughout the work
Emeka E. Ilodigwe and Roseline N. Asomugha supervised the research work
Lotanna D. Ajaghaku and Uchechukwu H. Orji provided laboratory assistance throughout the experiment

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