African Journal of Pharmaceutical Research & Development

Vol. 13 No.2; pp. 097-108 (2021)

EVALUATION OF THE TOXICITY AND ANTIOXIDANT PROFILES OF METHANOLIC LEAF EXTRACT AND FRACTIONS OF *DIAPHANANTHE BIDENS*

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ABSTRACT

Oxidative stress has been reported to be involved in the development and progression of many diseases. This study was carried out to evaluate the toxicity and antioxidant profiles of *Diaphananthe bidens*. Powdered leaves of *D. bidens* were extracted in methanol by cold maceration and fractionated into n-hexane, ethylacetate and butanol fractions. *In vitro* antioxidant activity was evaluated using 2,2-diphenyl-l-picrylhydrazil (DPPH) while catalase (CAT), superoxide dismutase (SOD) and lipid peroxidation (LPO) assays were used to evaluate the *in vivo* antioxidant activity. The median lethal dose (LD_{50}) of CME and serum liver maker (SLM) enzymes were analyzed following standard protocols. The crude methanol extract (CME) and fractions showed concentration-dependent *in vitro* antioxidant activity with ethylacetate fraction (EAF) showing the highest activity (EC_{50} value of 31.11 µg/ml) which is close to that of the positive control (15.2 µg/ml). The EAF and butanol fraction (BF) at 500 mg/kg showed more than 3-fold increase in CAT and SOD activities compared with the positive control group, ascorbic acid. EAF significantly ($p<0.05$) inhibited LPO in all the fractions. LD_{50} of CME even at a maximum test dose of 5,000 mg/kg, recorded no mortality, and the fractions did not produce significant ($p<0.05$) alteration of SLM enzymes. The study revealed that the extract and fractions *D. bidens* have excellent safety and antioxidant profiles.

KEYWORDS: ***Diaphananthe bidens*; Oxidative stress; Antioxidant; Liver enzymes; Toxicity.**

INTRODUCTION

Many natural products are biologically active and have been used for thousands of years as traditional medicines. Medicinal plants have important chemical constituents called secondary metabolites or phytochemicals that provide protection and defense against predators and pathogens [1]. Free radicals are constantly produced in all living organisms with damaging effects resulting in cell injury and sometimes, death [2]. Oxidative stress is the result of the disparity between pro-oxidants and antioxidants in an organism, which is important in the pathogenesis of several degenerative disorders, such as arthritis, Alzheimer's, cancer, and cardiovascular diseases among other health conditions. Free radicals can damage biomolecules, such as nucleic acids, lipids, proteins, polyunsaturated fatty acids, and carbohydrates, and the DNA leading to mutations. The use of antioxidants is effective in delaying the oxidation of biomolecules [3]. Antioxidants are one of the major complexes found in food that can retard or deter oxidation by preventing the initiation and propagation

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of oxidizing chain reactions [4]. Oxidative stress has been shown in the development and progression of many diseases such as inflammation, coronary heart disease, atherosclerosis [5-12].

Diaphananthe bidens (Afzel. Ex Sw) Schltr. (Orchidaceae) is commonly known as "wax orchid in (English), "Ikori" (Yoruba tribe in Nigeria), and "Bombins" (Sierra Leone). The flowers are salmon-pink, yellowish-pink and white or flesh coloured. The plant belongs to the family Orchidaceae and the order of Asparagales which is characterized by tough wiry stems. It is an epiphyte with an elongate, pendent stem of about 50 cm long and this makes the plant become gracefully pendulous from the branches of the foot hill forest trees. However, locals of South-East Nigeria use decoctions of the plant for the treatment of asthma, diabetes mellitus, and inflammatory diseases. So far, a study has investigated the anti-hyperglycaemic activity of the methanol leaf extract of *D. bidens* in normoglycaemic and streptozotocin-induced hyperglycaemic rats [13], but there is no scientific study carried out to confirm the toxicity and antioxidant profiles of the plant. Therefore, this study was undertaken to evaluate the toxicity and antioxidant profiles of *D. bidens* with the aim of establishing the pharmacological basis of its ethnomedicinal use in the management of oxidative stress.

MATERIALS AND METHODS

Plant material

Fresh, green whole plant (2 kg) of *Diaphananthe bidens* (Afzel. Ex. Sw) Schltr. was collected from the wild in Ede-Oballa in Nsukka Local Government Area, Enugu State, Nigeria in the month of June, 2016. Mr. Felix Nwafor of the Department of Botany, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria authenticated the plant material, and a voucher specimen (PCG/520/A/056) was deposited at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka for reference purposes. The plant name was checked with http://www.theplantlist.org/ on 13 July 2016, and confirmed to be an accepted name. It is called wax orchid by the English, and 'bombins' by Sierra Leoneans. The Yoruba tribe of South-West Nigeria call it 'ikori'.

Extraction and fractionation

Fresh plant materials were washed with clean water, cut into tiny pieces, dried under shade for about four (4) months, and was mechanically pulverized into powders. The powders (1 kg) were cold macerated in methanol (3.5 L) for 48 h with intermittent shaking at 2 h interval, followed by decantation, and use of fresh solvent so as to maintain the extraction gradient. The resulting extract was filtered using Whatman filter paper (No.1), and the filtrate was concentrated to dryness using rotary evaporator at 40 °C under reduced pressure to obtain the crude methanol extract (CME) which was stored in a refrigerator at 10 °C until further use.

Animals care and use protocols

Swiss albino mice (25 – 30 g, BALB/c strain) were employed for this study. The animals were obtained from the Animal House of the Department of Pharmacology and Toxicity, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka. The animals were housed in standard laboratory conditions of 12 h and fed with commercial rodent feed (Guinea feeds Nigeria Ltd) and had free access to food and water *ad libitum*. All animal experiments complied with the ARRIVE guidelines and were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Cage-side clinical observations of the mice were made throughout the study period [14]. The study protocols were approved by the Animal Care and Use Committee of our institution (ethical approval number: FP/PG/019/A/005).

Acute toxicity test

This test was carried out according to guidelines prescribed by Organization of Economic Co-operation and Development (OECD) [15]. Four Swiss albino mice of either sex selected randomly were used in this study. The animals were fasted over-night with free access to water. The crude methanol extract (CME) of *Diaphananthe bidens* at 2000, 3000, 4000 and 5000 mg/kg were administered to the mice by gavage to determine the acute toxic dose. The mice were observed closely for obvious signs of toxicity and mortality for 24 h and for a total of 7 days.

In vitro antioxidant activity screening

Free-radical scavenging activity of the CME and fractions were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) test [16]. An amount (25 µl) of freshly prepared DPPH solution (0.6 mmol) was added to 25 µl of different concentrations of the test solutions with a serial dilution at (15.62, 31.25, 62.5, 125, 250 and 500) µg/ml. The volume of the solution was adjusted with methanol to a final volume of 600

µl. The control tube contains 175 µl methanol and 25 µl of DPPH. Absorbance of the mixtures was measured at 517 nm using UV spectrophotometer (Cary 60 UV-V is spectrophotometer, Agilent, Malaysia). All the tests were performed in triplicates and ascorbic acid was used as the control. The plot of percentage inhibition against concentrations were used to derive the half maximum effective concentration (EC_{50}) of the CME and fractions. All the fractions were also selected for further *in vivo* analysis.

In vivo antioxidant activity screening

Thirty-six Swiss albino mice were divided into six groups of six mice each and treated with the fractions for 14 days [17]. Groups 1 – 4 were treated with 250 and 500 mg/kg of each of ethylacetate, *n*-hexane, butanol, and aqueous fractions; group 5 served as untreated control group, while group 6 received 100 mg/kg of ascorbic acid (standard). Six hours after the last day of the treatment, oxidative stress was induced in all the groups of animals with carbon tetrachloride (CCl_4) (2 ml/kg) by intraperitoneal injection except the untreated control group. Eighteen hours later, blood samples were collected from all the animals through retro-orbital puncture and the liver harvested through surgical dissection. The blood samples collected were centrifuged at 3000 rpm using a centrifuge (Model TGL-20M, China) for 10 min and the supernatant was decanted to get the serum. The harvested liver was rinsed in phosphate buffer saline (pH 7.4), homogenized and centrifuged at 12,000 rpm for 20 min at 25 °C; the supernatant was decanted and stored in a refrigerator. The serum was used for the estimation of serum liver marker enzymes – aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) respectively, while the supernatant of the liver homogenate was used for the determination of liver antioxidant enzymes - [catalase (CAT) and superoxide dismutase (SOD)] and lipid peroxidation.

Evaluation of liver antioxidant enzyme activity and lipid peroxidation

Catalase activity was determined as the ability of the enzyme to degrade hydrogen peroxide [18], and an extinction coefficient for hydrogen peroxide at 240 nm of 40.0 $M^{-1}cm^{-1}$. Superoxide dismutase (SOD) enzyme activity was determined as its ability to inhibit the auto-oxidation of epinephrine and an extinction coefficient for epinephrine at 480 nm of 4020 $M^{-1}cm^{-1}$. Lipid peroxidation was assessed through malondialdehyde (MDA) – an aldehyde product of lipid peroxidation [19]. The supernatant was measured using UV spectrophotometer (Cary 60 UV-Vis spectrophotometer, Agilent, Malaysia) at 532 nm against a blank.

Biochemical assay of serum liver enzymes

The assay to determine the liver enzyme induced by treatment using fractions of *D. bidens* was evaluated using commercial reagent kits (Randox Laboratories limited, United Kingdom). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to the method of Reitman and Frankel, while the alkaline phosphatase (ALP) was determined as already described [20].

Hispathological studies

Histological examination of the liver tissue from an animal from each group was done using haemolysin and eosin (H and E) staining procedure [21, 22].

Statistical analysis

The statistical analysis was assessed using Graphpad Prism version 5.0 for Windows software and presented as mean ± standard deviation (SD). Significance between control, crude and fraction treated groups were determined using student *t*-test and one-way analysis of variance (ANOVA). Differences between means were considered statistically significant at $p<0.05$.

RESULTS

Yield of extraction of the CME and fractions

The percentage yield of the crude methanol extract and fractions of *Diaphananthe bidens* is shown in Table 1. The crude methanol extract of *D. bidens* gave a yield of 37.5 %, the fractions from the extract gave percentage yield ranging from 3 – 10 %. The *n*-hexane fraction gave the least yield of 3.02 %, followed by aqueous and butanol fractions with 8.70 and 9.86 %, ethylacetate fraction gave the highest yield of 10.34 %.

Acute toxicity

The result of the acute toxicity study (LD_{50} determination) of the crude methanol extract of *D. bidens* revealed that the extract is safe. This is because no lethality or toxic reactions were recorded in the study mice after 24 h and after 7 days of observation post-administration of extract and even at a maximum test dose of 5,000 mg/kg. In addition, the animals did not show any significant changes in behaviour, skin defects, breathing, impairment in food intake and water consumption, postural abnormalities or hair loss.

DPPH scavenging activity

The DPPH scavenging activity of CME and the various fractions of *D. bidens* is shown in Fig. 1 using AA (ascorbic acid) as reference. CME, EAF, BF and AQ greatly diminished the deep purple colour of DPPH which is the evidence of their antioxidant activity. Ethylacetate fraction gave the best antioxidant activity with EC_{50} of 31.11 µg/ml which is close to that of the positive control (15.2 µg/ml) by comparison (Table 2). While *n*-hexane produced the least activity with an EC_{50} of 101.07 µg/ml but this is close to 110.25 µg/ml which was produced by the crude extract.

In vivo antioxidant study

Catalase and superoxide dismutase

The result of the *in vivo* antioxidant activity of the fractions of *D. bidens* is shown in Figs. 2 and 3. Ethylacetate fraction produced the highest increase in serum CAT activity at 500 mg/kg followed by butanol and aqueous fractions, while *n*-hexane fraction gave the least serum CAT activity. The serum CAT level of the group treated with ethylacetate fraction at 500 mg/kg was significantly ($p<0.05$) higher than the untreated, *n*-hexane, and aqueous groups but comparable with the group treated with the standard drug (ascorbic acid). Similarly, the fractions also produced dose-dependent increase in the serum level of superoxide dismutase (SOD) activity. The group treated with ethylacetate fraction produced the highest increase in serum SOD activity followed by butanol and aqueous fractions, while *n*-hexane fraction gave the least serum SOD activity. The serum SOD level of the group treated with ethylacetate fraction at 500 mg/kg was significantly ($p<0.05$) higher than the untreated, *n*-hexane, and aqueous groups but comparable with the group treated with the standard drug (ascorbic acid).

Lipid peroxidation

Lipid peroxidation levels indicated by serum malondialdehyde (MDA) level as shown in Fig. 4 was significantly ($p<0.05$) higher in the untreated group compared to other treatment groups and the standard ascorbic acid. However, all the groups treated with the fractions did not produce any significant ($p>0.05$) difference in their serum MDA levels compared to the group treated with the standard drug, ascorbic acid.

Assay of serum liver marker enzymes

Serum liver marker enzymes mobilized by the fractions of *D. bidens* are shown in Table 3. The serum liver enzymes (AST, ALT, and ALP) were significantly ($p<0.05$) elevated in the untreated control group compared to other treated and ascorbic acid (standard) drug. However, rats treated with fractions of *D. bidens* did not provoke enzyme elevation ($p>0.05$) compared to the standard drug and the untreated control group. Liver enzyme levels of the fractions and ascorbic acid groups were comparable.

Hispathological results

The photomicrographs of sections of the liver obtained by histopathological examination are shown in Figures 5 – 10. The figures showed normal liver tissue histology with the hepatic vein (HV), hepatocytes (H), and the hepatic sinusoids (S) showing normal architecture for all the treatment groups administered with ethylacetate, butanol, and aqueous fractions as well as the positive control group which received ascorbic acid. However, the treatment group which received n-hexane fraction showed moderate hypertrophied hepatic vein, while for the untreated group, the hepatocytes were normal but there was a focal area showing cellular degeneration (CD) and remarkable infiltration of inflammatory exudates with lymphocytic involvement.

DISCUSSION

The percentage yield of the crude extract (37.50 %) was appreciable considering that methanol is a polar solvent and will only extract polar constituents of the crushed plant. This is because polar substances would dissolve in polar solvents, while non-polar constituents would dissolve in non-polar solvents [21]. The use of these varying solvents for fractionation is justifiable because the larger the variety of the solvent system, the better the chance of solubilizing a larger amount of biologically active constituents. More phytoconstituents were present in the ethylacetate fraction (10.34 %), followed by butanol (9.86 %) and aqueous (8.70 %) fractions while *n*-hexane gave the least (3.02 %) content of phytocompounds. This suggests that the constituents of the crude methanol extract were better solubilized in ethylacetate compared to the other fractionating solvents.

The acute toxicity study showed that the crude methanol extract of *D. bidens* has a wide safety margin since no death was recorded in the study animals used at a maximum dose of 5,000 mg/kg. Toxicity results from animals are highly essential in judging the safety of crude drugs if they are found to have promising pharmacological activities. From the

Table 1: Extraction yield of the crude methanol extract and Fraction of *D. bidens* leaves

Extract/Fraction	Yield (%)
Crude methanol extract (CME)	37.50
n-hexane fraction (*n*-HF)	3.02
Ethylacetate fraction (EAF)	10.34
Butanol fraction (BF)	9.86
Aqueous fraction (AF)	8.70

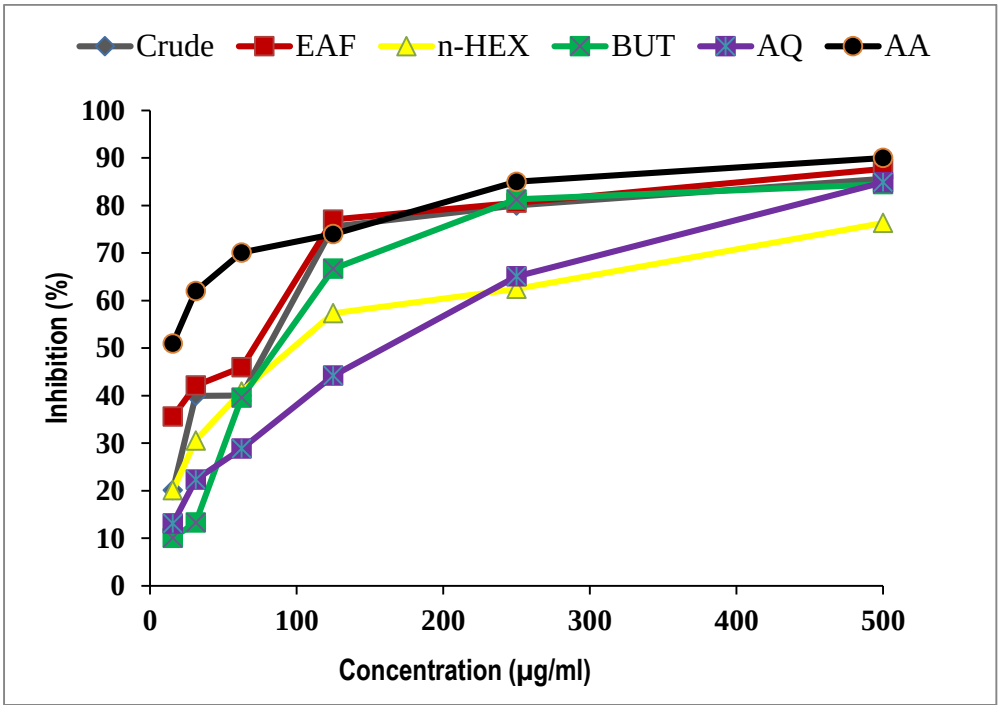

Figure 1: Graph of DPPH scavenging activity of crude extract and fractions of *D. bidens*.
Keys: EAF = ethylacetate fraction, *n*-HEX = *n*-hexane fraction, BUT = butanol fraction, AQ = aqueous fraction, AA = ascorbic acid.

Table 2: Half maximum effective concentration (EC_{50}) of the crude methanol extract (CME) and fractions of *D. bidens*

Test sample	DPPH EC_{50} (µg/ml)
Crude extract	110.25 ± 0.15
n-hexane fraction	101.07 ± 0.02
Ethylacetate fraction	31.11 ± 0.01
Butanol fraction	55.64 ± 0.06
Aqueous fraction	72.15 ± 0.03
Ascorbic acid (positive control)	15.20 ± 0.00

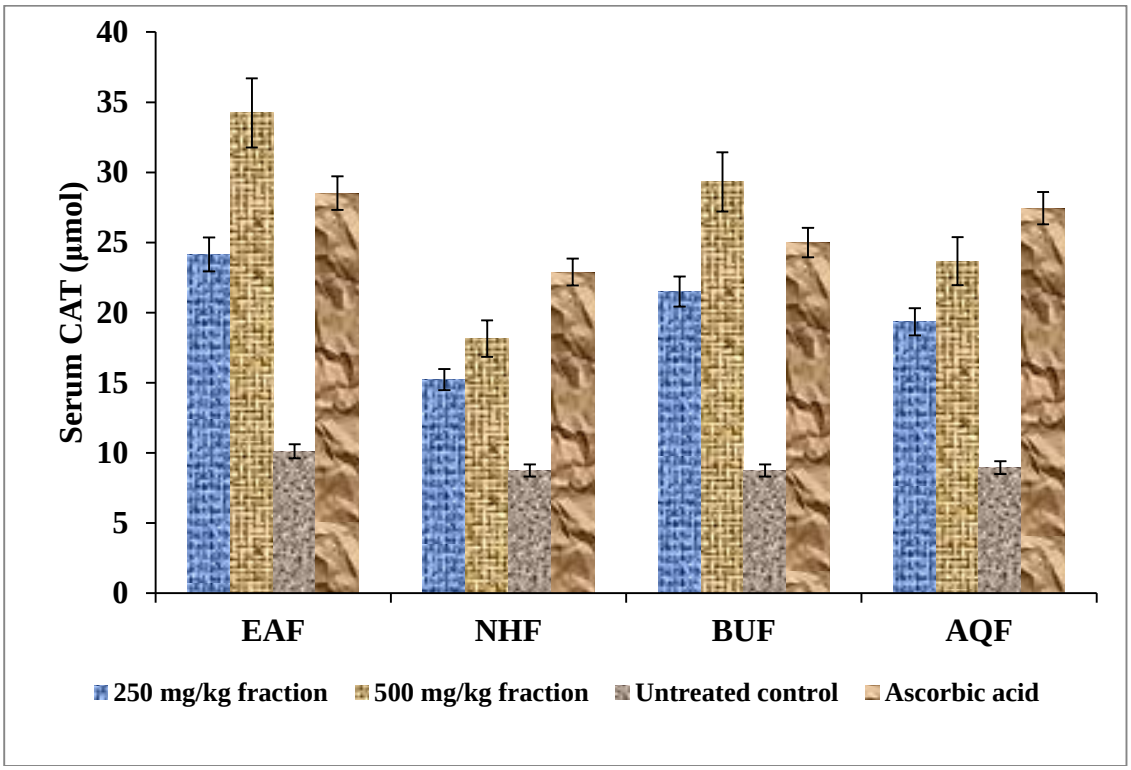

Figure 2: Effect of fractions of *D. bidens* on serum catalase enzyme activity.
Keys: EAF = ethylacetate fraction, NHF = *n*-hexane fraction, BUF = butanol fraction, AQF = aqueous fraction. CAT = Catalase. Each fraction was administered at 250 and 500 mg/kg.

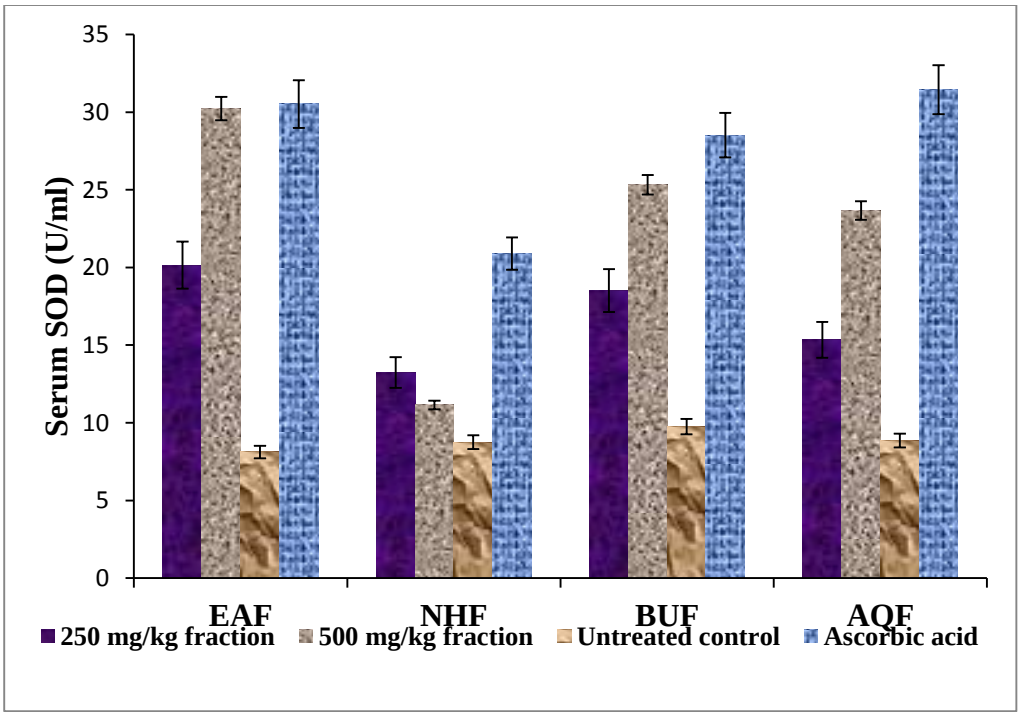

Figure 3: Effect of fractions of *D. bidens* on serum superoxide dismutase enzyme activity.
Keys: EAF = ethylacetate fraction, NHF = *n*-hexane fraction, BUF = butanol fraction, AQF = aqueous fraction. SOD = Superoxide dismutase. Each fraction was administered at 250 and 500 mg/kg.

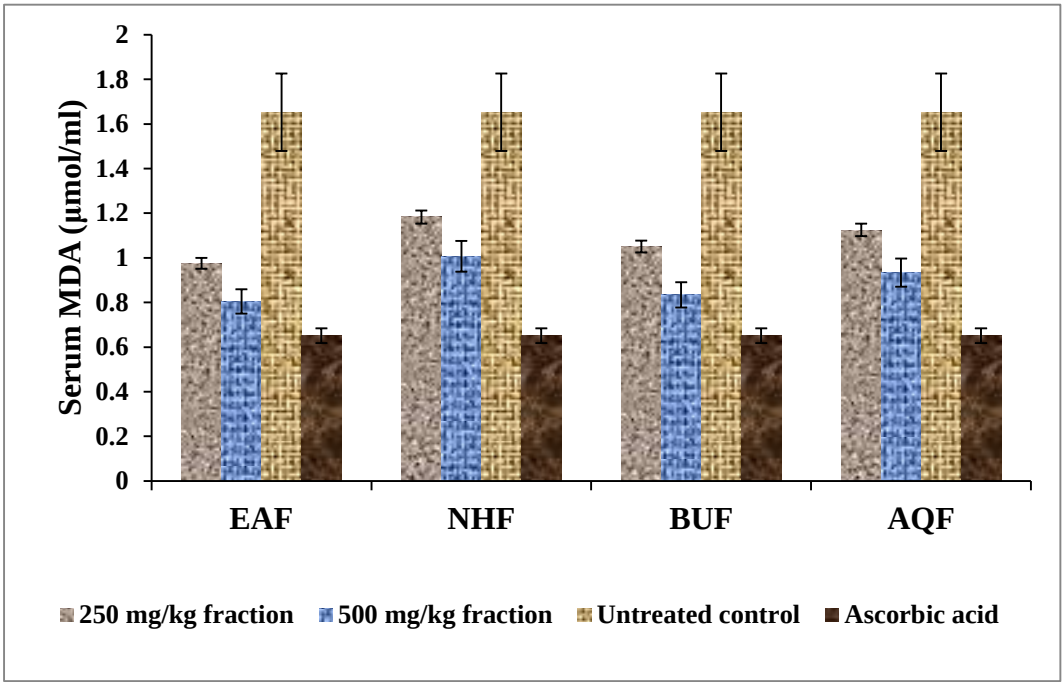

Figure 4: Effect of fractions of *D. bidens* on serum malondialdehyde activity.
Keys: EAF = ethylacetate fraction, NHF = *n*-hexane fraction, BUF = butanol fraction, AQF = aqueous fraction. MDA = Malondialdehyde. Each fraction was administered at 250 and 500 mg/kg.

Table 3: Effect of fractions of *D. bidens* on serum liver marker enzymes

Treatment groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
n-hexane fraction	31.30 ± 2.1	40.70 ± 1.1	80.00 ± 2.5
Ethylacetate fraction	28.33 ± 5.3	38.74 ± 4.1	79.70 ± 1.3
Butanol fraction	32.00 ± 2.2	41.00 ± 1.6	78.30 ± 2.6
Aqueous fraction	31.30 ± 2.2	39.20 ± 1.8	77.30 ± 4.7
Untreated control	38.25 ± 1.5	46.17 ± 2.1	87.12 ± 2.0
Ascorbic acid	33.30 ± 3.7	39.00 ± 2.9	79.33 ± 2.8

Result presented as mean determination ± standard deviation. AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase.

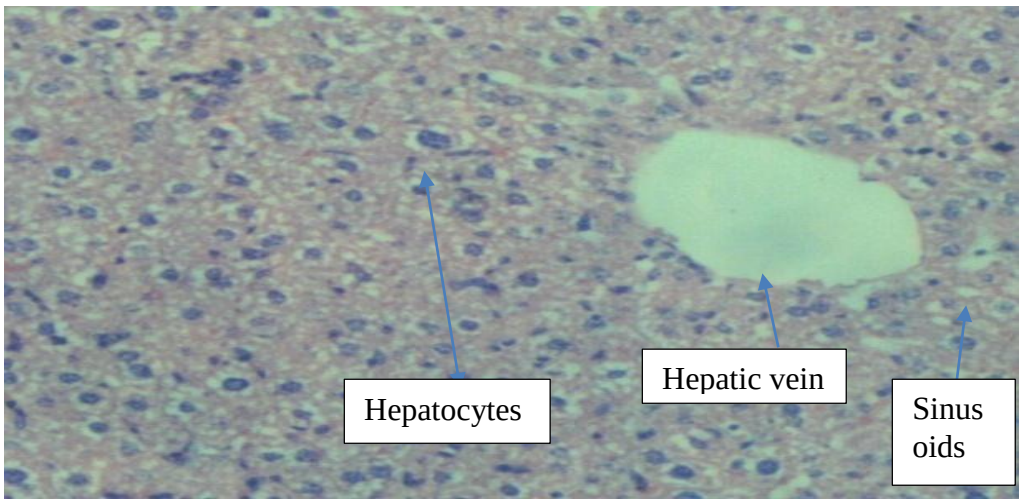

Figure 5: Photomicrograph of liver tissue from rats which received ethylacetate fraction showing normal histological features: hepatic vein (HV), hepatocytes (H) and sinusoids (S) (H&E ×100).

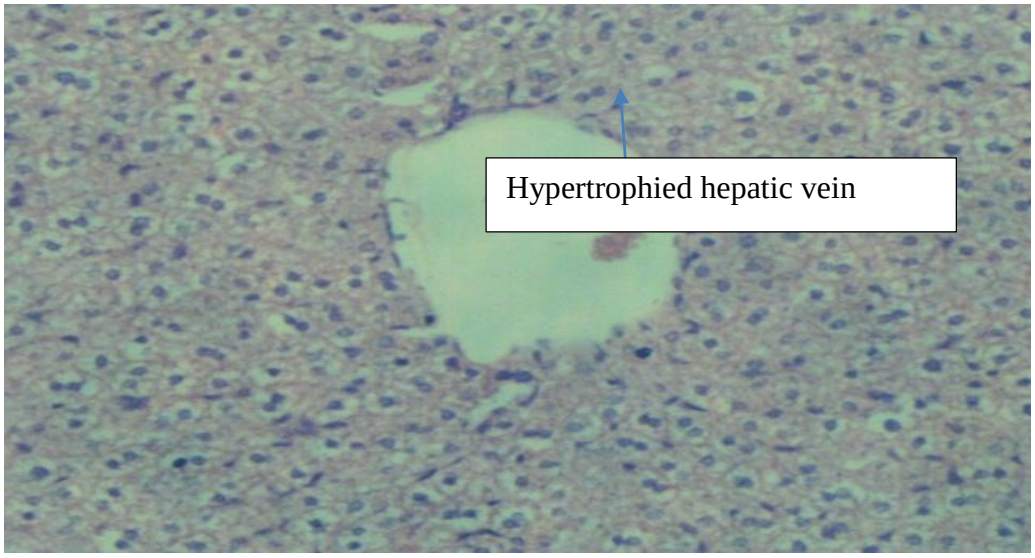

Figure 6: Photomicrograph of liver tissue from rats which received n-hexane fraction showing normal histological features but with moderate hypertrophied hepatic vein (H&E ×100).

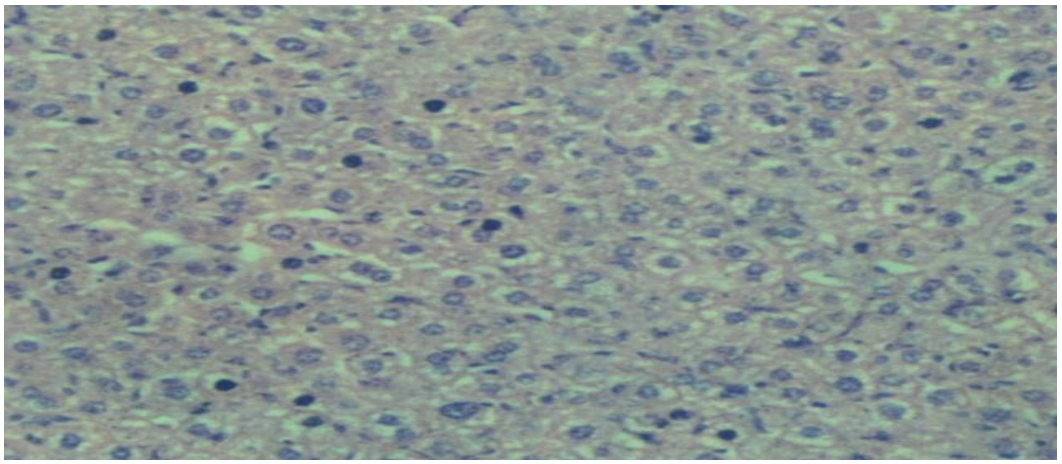

Figure 7: Photomicrograph of liver tissue from rats which received butanol fraction showing normal histological features: hepatic vein (HV), hepatocytes (H) and sinusoids (S) (H&E ×100).

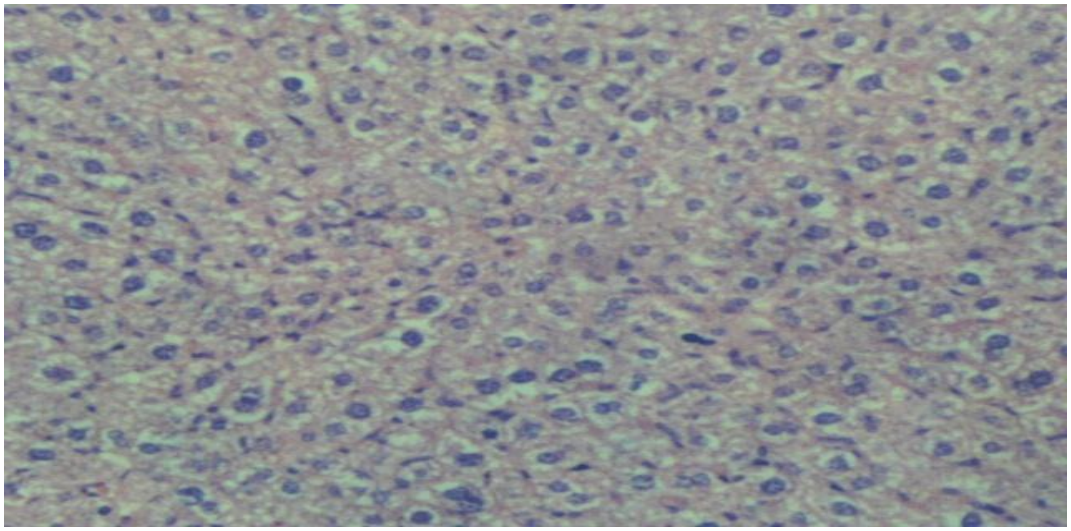

Figure 8: Photomicrograph of liver tissue from rats which received aqueous fraction showing normal histological features: hepatic vein (HV), hepatocytes (H) and sinusoids (S) (H&E ×100).

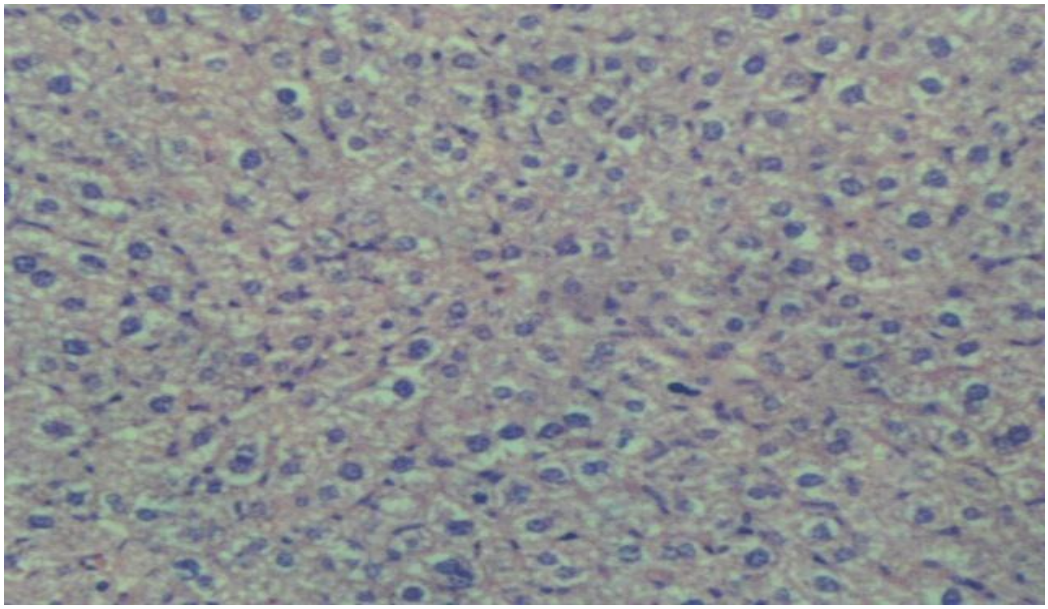

Figure 9: Photomicrograph of liver tissue from rats which received ascorbic acid showing normal histological features: hepatic vein (HV), hepatocytes (H) and sinusoids (S) (H&E ×100).

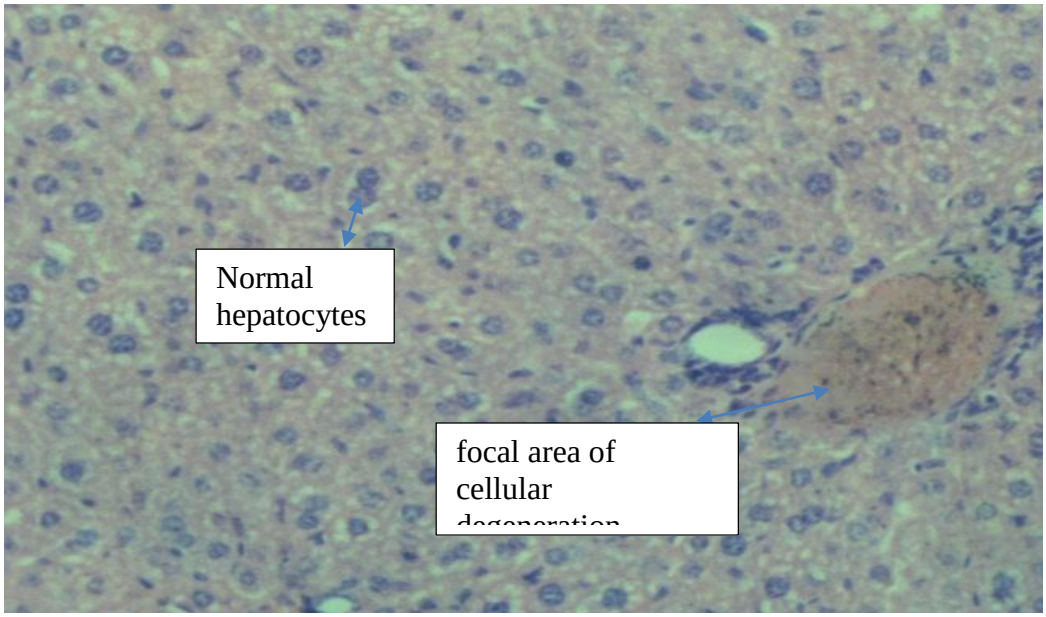

Figure 10: Photomicrograph of liver tissue from the untreated rats showing normal hepatocytes and a focal area of cellular degeneration (CD) and remarkable infiltration of inflammatory exudates with lymphocytic involvement (H&E ×100).

foregoing, the crude extract from *D. bidens* could be assigned class 5 status (LD_{50} > 5,000 mg/kg) which is the lowest toxicity class.

The chemical reagent, 2, 2-diphenyl-1-picrylhydrazil (DPPH) is a valuable dark coloured crystalline powder containing stable free radical molecules used in antioxidant assays. The free radical property of DPPH is neutralized by antioxidants by transferring either electrons or hydrogen atoms as appropriate leading to the generation of a coloured stable diamagnetic compound called diphenylpicrylhydrazine indicated by the colour change of DPPH from purple to yellow [22]. The *in vitro* antioxidant activity assay of the crude extract and fractions of *D. bidens* suggests that the plant has potent, concentration-dependent antioxidant activity comparable with the reference standard, ascorbic acid, used in the study. The concentration-dependent DPPH scavenging activity was in agreement with previous reports [23]. This comparable activity is more in the crude methanol extract, the ethylacetate, and butanol fractions of the plant. The good DPPH scavenging activity of the crude extract and the fractions could be attributed to the presence of flavonoids (phenolics) compounds which was abundant in three fractions - ethylacetate, butanol, and aqueous, and which have been reported to possess high antioxidant potential due to its content of redox-active functional groups in their structures, in addition to their ability to neutralize all types of oxidizing radicals, thereby acting as powerful chain breaking antioxidant [24, 25]. Catalase is an important antioxidant enzyme widely distributed in animal tissues. It catalyzes the decomposition of hydrogen peroxide, a reactive oxygen species (ROS), which is a toxic product of both normal aerobic metabolism and pathogenic ROS production, and therefore protects the system, from highly reactive hydroxyl radicals [26]. Consequently, catalase depletion leads to cellular damage caused by increased absorption of superoxide and hydrogen peroxide. The result of the assay indicates that there was significant ($p<0.05$) increase in the serum catalase levels in a dose-dependent manner. This suggests that the plant possess potent antioxidant activity. Similarly, superoxide dismutase enzyme is one of the most important antioxidant defense enzymes that scavenge superoxide anion by converting it to hydrogen peroxide, thus diminishing the deleterious effect of the radical [27]. From the result, the observed decrease in superoxide dismutase level in the untreated group might be due to the hepatocellular damage caused by oxidizing action of carbon tetrachloride. However, an increase in the superoxide dismutase level following the administration of the fractions implies an efficient protective (antioxidant) property of *D. bidens*. Reactions of reactive oxygen species with polyunsaturated fatty acids of cell membranes lead to lipid peroxidation with the release of malondialdehyde (MDA) [28]. Lipid peroxidation is the oxidative degradation of lipids. Increased lipid peroxidation impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors [29], the low lipid peroxidation in the groups treated with fractions indicated by the low level of MDA further confirms the potent antioxidant ability of *D. bidens*. Elevated levels of serum liver enzymes (AST, ALT, and ALP) in the untreated group indicate hepatotoxicity. However, it could be inferred that the fractions from the crude extract of *D. bidens* possess hepatoprotective property against oxidative stress-induced hepatotoxicity since no remarkable elevation of serum liver enzymes was recorded in the groups treated with the fractions. These submissions were confirmed by the histopathological findings of the liver sections of both the treated and untreated groups. The normal histological features of the groups treated with the fractions confirm that they were actually hepatoprotective compared to the untreated (negative control) group whose liver tissue was characterized by cellular degeneration and remarkable influx of inflammatory exudates.

CONCLUSION

Crude drug extraction using methanol yielded sufficient extracts which contain secondary metabolites that partitioned favourably in ethylacetate. Crude methanol extract of *D. bidens* had good safety profile as evidenced by zero mortality in study animals. The plant showed efficient resolution of *in vitro* and *in vivo* oxidative stress (anti-oxidant activity) by scavenging DPPH, and improving the concentrations of catalase (CAT) and superoxide dismutase (SOD) enzymes as well as reducing lipid peroxidation. The plant did not provoke increased levels of serum liver enzymes (e.g. AST, ALT, and ALP) suggesting its sufficient hepatoprotective property.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Chibueze Ikeh of the Department of Pharmacology and Toxicology for the technical assistance rendered in the study.

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