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Methanol Leaf Extract of *Voacanga Africana* Protects Against Diethylnitrosamine and Carbon Tetrachloride-Induced Hepatotoxicity in Wistar Rats via Antioxidant and Anti-Inflammatory Activities

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Abstract

Background: Hepatotoxicity induced by chemical agents is a common and life-threatening disease that developed from acute or chronic exposure to environmental chemicals. Despite numerous approaches towards the treatment of liver toxicity, no safe and effective therapy exists. This study evaluated the antioxidative and anti-inflammatory effects of methanol leaf extract of *Voacanga africana* (VA) in diethylnitrosamine (DEN) and carbon tetrachloride (CCL_4)-induced hepatotoxicity in rats. The animals were assigned into seven groups: Control, [DEN+CCL_4], [DEN+CCL_4] with VA (100, 200, 400 mg/kg), [DEN+CCL_4] with Sorafenib (SFB), and SFB alone. Rats received DEN (200 mg/kg) once via i.p. and CCL_4 (3 mL/kg) via subcutaneous route once in a week for six weeks. VA was administered orally from the second week until the 15th week. GC-FID analyses of VA revealed active components Voacinol, Voacangine, Amataine, and Ibogaine. [DEN+CCL_4] caused a 26% increase in liver organo-somatic weight, which was reduced by 18%, 20%, and 9% with VA doses of 100, 200, and 400 mg/kg, respectively. [DEN+CCL_4] also significantly increased serum gamma-glutamyl transferase, alanine, and aspartate aminotransferases by 55%, 25%, and 17%, and raised α-fetoprotein and total bilirubin levels by 2.0 and 2.5folds, respectively. Hepatic nitric oxide and myeloperoxidase activities were increased by 52% and 123%, respectively in the intoxicated rats. Additionally, malondialdehyde levels increased by 80% with decrease in glutathione peroxidase, glutathione-s-transferase, catalase and superoxide dismutase by 35%, 34%, 25% and 32%, respectively. Immunohistochemistry showed mild APC and strong Bcl-2 expression, while histology revealed severe hepatic necrosis. VA treatment mitigated oxidative stress, inflammation, and restored liver architecture.

Keywords: Hepatotoxicity, Diethylnitrosamine, Carbon Tetrachloride, *Voacanga Africana*, Antioxidants, Inflammation.

INTRODUCTION

The rising incidence of liver related diseases especially cancer has been one of the major global health challenges [1]. Hepatocellular carcinoma is the most common primary liver cancer that develops from complications of liver diseases such as fibrosis or cirrhosis. This cancer of the liver has been ranked as the fourth leading cause of cancer related-death world-wide [2]. Several risk factors such as Hepatitis B or C infections, alcoholic liver disease, and non-alcoholic liver steatohepatitis have been associated with hepatotoxicity. The HCC has been shown to occur in 80-90% patients with liver cirrhosis [3]. In addition, exposure to aflatoxin B1, *N*-nitrosamine and carbon tetrachloride have also been grouped as major risk factors to hepatotoxicity or HCC development [4].

Diethylnitrosamine (DEN) is a very strong liver carcinogen in rats and an alkylator that interacts directly with DNA, exerting its activity without requiring metabolic activation [5]. Furthermore, exposure to DEN results in the formation of 7-ethylguanine adducts in the liver. These adducts undergo hydroxylation by CYP2E1 enzymes within liver cells, potentially inducing DNA mutations [6]. Consequently, this cascade of events can lead to any of these conditions; fibrosis, cirrhosis and

hepatocellular carcinoma [7]. Also, carbon tetrachloride (CCL_4) is a promoter of the carcinogen since exposure to it has been linked to liver carcinogenesis[8]. In addition to liver toxicity, short-term exposure to carbon tetrachloride through ingestion or inhalation can also lead to systemic toxicity, primarily affecting the central nervous system and kidneys [9]. The metabolism of CCL_4 involves CYP450-mediated activation to form trichloromethyl and trichloromethylperoxy radicals, which can interact with cellular components such as lipids and proteins [8]. According to previous study, chronic exposure to CCL_4 has been linked to liver cell damage [10].

In the past two-decades, liver transplantation and hepatic resection has been the major treatment for advanced HCC with certain level of success[11]. However, seeing a liver donor has been a great challenge and also the poor prognosis following surgical resection is another draw ack. Also, there are limit to availability of effective chemotherapy for the management of hepatotoxicity or even HCC without serious side effects. Therefore, the search for an alternate and novel chemotherapeutic agent from natural sources to mitigate against HCC is warranted. *Voacanga africana*, known locally as 'Ako Dodo' in Yoruba, 'Petepete' in Igbo, and 'Kokiyar' in the Hausa tribe of Nigeria, is a perennial tree that is predominantly found throughout West Africa [12]. This plant belongs to the Apocynaceae family, and extracts from various parts, including the leaves, fruits, and roots, have been widely employed for the treatment of numerous ailments, including malaria, gonorrhea, Alzheimer's disease, and gastric ulcers [13, 14]. Furthermore, previous research has revealed that *Voacanga africana* possesses anti-cancer, anti-inflammatory, and cytotoxic properties[15, 16], particularly through distinct fractions derived from different parts of the plant. Notably, *Voacanga africana* is recognized for its rich content of indole alkaloids, such as voacangine, which is primarily isolated from the root bark. Voacangine is a significant component contributing to the plant's anti-cancer potential by inhibiting angiogenesis[15].

Additionally, Olaleye *et al*., reported the anti-inflammatory and antioxidant potential of the flavonoid fraction extracted from the leaves of *Voacanga africana* through *in vivo* studies [16]. Besides, various phytochemicals, including flavonoids, tannins, phenols, steroids, terpenes, cardiac glycosides, and alkaloids, have been identified in different parts of the plant, including the leaves [17, 18]. The alkaloids of *Voacanga*, including voacamine, voacangine, and vobasine, which have been isolated from the leaves, seeds, trunk, and root bark, consistent with the findings reported by Mbele *et al*., [18]. To date, there is dearth of information on the protective effects of the leaf extract of *Voacanga africana* in rats. Therefore, this study was designed to evaluate the possible ameliorative effects of the methanol leaf extract of *Voacanga africana* on the hepato-renal tissues of rats administered diethylnitrosamine and carbon tetrachloride.

MATERIALS AND METHODS

Chemicals

Diethylnitrosamine, Carbon tetrachloride, reduced glutathione (GSH), hydrogen peroxide (H_2O_2), adrenaline and trichloroacetic acid (TCA) were bought from Sigma Aldrich, while thiobarbituric acid (TBA) and dithionitrobenzoic acid (DTNB) were obtained from British Drug House (BDH). Other reagents used were of analytical grade.

Plant Materials

Freshly harvested *Voacanga africana* (*VA*) leaves were collected around Moniya in Akinyele L.G.A of Oyo State on 13th February, 2023. The leaves were air-dried in the laboratory and ground into a powdery form. 2.6Kg of *VA* leaves was soaked in 12.5 litres of distilled methanol for double extraction in 72hours, filtered and later concentrated using rotary evaporator at 40ºC with a yield of 5.89%.

GC-FID and HPLC Analysis of Methanol Leaf Extracts of *Voacanga Africana* (*VA*)

Gas chromatography (GC) and high performance liquid chromatography (HPLC) analysis of methanol leaf extracts of *Voacanga africana* (*VA*) were carried out according to standard analytical procedure.

Animals and Treatments

A total of fifty-six male Wistar rats weighing 160±20 g obtained from animal breeding centre Faculty of Veterinary Medicine, Ibadan, Nigeria were used for the experiment.The animals were kept in aerated cages under standard maintenance of ambient temperature of 25ºC and fed with standard feed and water with regular changing of beddings.

Experimental Design

The animals were allowed to acclimatize for 2 weeks and then assigned into seven equal groups. Group 1 received corn oil which served as control and group 2 received a combination of [DEN+CCL_4] (a single dose of 200 mg/kg DEN diluted in normal saline through intraperitoneal injection was given the first week whereas CCL_4 diluted in corn oil at 3mL/ kg was administered via subcutaneous route once in a week for six weeks following the initial administration of DEN)[19]. Also, group 3 to 6 intoxicated with [DEN+CCL_4] were treated with 100, 200, 400mg/kg methanol leaf extract of *VA* and Sorafenib (SFB 10mg/kg) respectively while group 7 received SFB (10mg/kg) only starting from the second week after DEN intoxication for the duration of fourteen weeks.

Preparation of Tissue and Serum

Following the last treatment of the rats with the methanol extract of *VA*, the final animal weights were taken and then sacrificed through cervical dislocation.

The liver were harvested, rinsed in cold ice of 1.15% KCl buffer solution and blotted. A lobe from the liver was cut and fixed in bouin's solution for histology while other lobes were homogenized in a 50Mm phosphate buffer of pH 7.4. The homogenate was cold centrifuged to obtain post mitochondrial fraction (PMF) at 10,000g for 15minutes. The post mitochondrial fractions (PMF) obtained were stored at 4°C for biochemical analyses. Also, sera used for enzymes assays were obtained from the blood collected through retro-orbital venous puncture into plain bottles, spun for 15minutes at 3000g after clotting.

BIOCHEMICAL ASSAYS

Serum Alpha-Fetoprotein (AFP) Determination

The serum AFP levels were determined using ELISA kit following the manufacturer's protocols.

Determination of Liver Function Biomarkers

Alanine and aspartate aminotransferases, total bilirubin and Gamma-glutamyl transferase (GGT) were evaluated by the methods described by Reitman and Frankel [20], Mohun and Cook [21], and Jendrassik [22], respectively.

Protein Determination

The levels of protein in the liver PMF and sera were estimated using bovine serum albumin (BSA) as a standard in a procedure described by Lowry *et al*., [23].

Assessment of Lipid Peroxidation

The lipid peroxidation products, especially malondialdehyde produced in the hepatic tissue was estimated by the method of Buege and Aust [24]. In brief, 0.4 mL of liver PMF or serum was reacted with 1.6 mL of Tris-KCl buffer solution containing 0.5 mL 30% TCA. Afterwards, 0.5 mL of 0.75% TBA was added to the mixture and boiled in a hot water bath at 80°C for duration of 45 min. The mixture was allowed to cool and then spun at 3000 rpm for 15 min. The optical density of the supernatant was measured using a spectrophotometer at 532 nm against the blank.

Determination of Catalase Activity

Catalase activity was assessed following the protocols described by Aebi [25]. The reaction mixture comprised 20 µL of phosphate buffer (50 mM, pH 7.0), 10 µL of 19 mM H_2O_2, and 50 µL of the sample. The reaction proceeded for 3 minutes before being halted by adding 20 µL of dichromate in acetic acid solution, followed by heating in a boiling water bath for 10 minutes. After cooling, the absorbance was measured at 570 nm.

Assessment of the Superoxide Dismutase (SOD) Activity

The SOD activity was assessed by observing the autoxidation of adrenaline catalyzed by the superoxide radicals generated by xanthine oxidase, following the procedure described by McCord and Fridovich [26].The reaction mixture consisted of 0.2 mL of the sample and 2.5 mL of carbonate buffer (pH 10.2), equilibrated to room temperature. Then, 0.3 mL of 0.3M adrenaline was added and thoroughly mixed. Absorbance readings were taken at 30-second intervals over 3 minutes.

Assessment of the Glutathione Peroxidase (GPx) Activity

The GPx activity was assessed using the method of Rotruck *et al*., [27]. The reaction mixture comprised 0.5 mL of sodium phosphate buffer solution, 0.1 mL of 10.0 mM sodium azide, 0.2 mL of 4.0 mM reduced GSH, 0.1 mL of 2.5 mM H_2O_2, and 0.5 mL of the sample. This mixture was then adjusted to 2.0 mL with distilled water and incubated in a boiling water bath at 37°C for 3 minutes. Subsequently, 0.5 mL of 10% TCA was added to terminate the reaction, and the entire mixture was centrifuged. The resulting supernatant was utilized to determine the residual GSH content by adding 1.0 mL of Na_2PO_4 (0.3mM) and 0.25 mL of DTNB. The optical density was measured at 412nm to determine GPx activity.

Assessment of the Activity of Glutathione-S-Transferase (GST)

The method of Habig *et al*., [28], were employed for the determination of GST activity using 2-chlorodinitrobenzene as the substrate. The reaction mixture consisted of 10µL of 30mM 2-chlorodinitrobenzene and 1.7 mL of phosphate buffer (100mM, pH 6.5). Following 5-minute pre incubation at 37°C, 20 µL of sample was introduced into the mixture. The optical density at 340nm was monitored for five minutes relative to the blank, and GST activity was calculated using an extinction coefficient of 9.61 mmol−1 cm−1.

Estimation of the Level of Nitric Oxide (NO)

The method described by Palmer *et al*., [29], was used to assay for the levels of NO_3^- and NO_2^- in samples as an indication of NO generation. By utilizing Griess' reaction, nitrite levels were ascertained. For twenty minutes, the reaction mixture consists of 0.5 mL of sample and 0.5 mL of Griess' reagent, which was incubated at room temperature. By comparing the average absorbance of sample to a standard solution sodium nitrite, the nitrite concentration was determined.

Estimation of the Activity of Myeloperoxidase (MPO)

The assessment of MPO activity was conducted using the adapted procedure from Trush *et al*., [30]. Spectrophotometric estimation was employed to measure MPO activity by utilizing *O*-dianisidine in reaction with hydrogen peroxide. The MPO, a lysosomal enzyme, facilitates the oxidation of *O*-dianisidine in the presence of H_2O_2, functioning as an oxidizing agent, resulting in the formation of a brown-colored product with maximum absorption at 470 nm.

Evaluation of Haematological Parameters

Haematological parameters and indices were determined from non-clotted blood samples using standard protocols and according to method described by verma *et al*., [31]. Air-dried thin blood films stained with Giemsa stain were examined microscopically by magnification X100 using immersion oil for differential white blood cell counts.

Red Blood Cells (RBC)

Blood was drawn up to the 0.5 mL mark in the RBC pipette. Thereafter, a diluting solution (consisting of 0.5 g of mercuric chloride, 1 g of sodium chloride, and 5 g of sodium sulphate dissolved in 200 mL of distilled water in a clean beaker) was added. After immediate mixing, the mixture was left to settle for some time. A drop of this diluted sample was then placed in the channel of the Neubauer counting chamber, allowing the cells to settle, and observed under a microscope for counting. The results are presented as the number of cells per cubic millimetre of blood.

White Blood Cells (WBC)

In a sterile test tube, 0.02 mL of blood and 0.38 mL of WBC dilution fluid (containing 1% gentian violet in glacial acetic acid, made up to 109 mL with water) were added and properly mixed. After that, a drop of this diluted sample was added to the channel of the Neubauer counting chamber, enabling the cells to settle before being counted under a microscope. The number of cells per cubic millimetre of blood was reported as the results.

Haemoglobin (Hb)

Drabkin's reagent was well mixed with 0.02 mL of blood. The reagent contained 200 mg of potassium ferricyanide, 50 mg of potassium cyanide, and 1 g of sodium bicarbonate dissolved in water and adjusted to a volume of 1 L. After allowing the diluted blood combination to stand for 15 minutes, its absorbance at 540 nm was measured in comparison to a cyanomethemoglobin standard solution. The findings were given as gram per litre (g/L).

Histology

Liver tissues were examined and cut into small pieces, each not exceeding 4mm thick, before being placed into pre-labeled cassettes. These cassettes were then submerged in 10% formalin for 24 hours to facilitate fixation. Subsequently, an automated tissue processor (Leica TP1020) was employed for further processing. The tissues underwent a series of steps involving immersion in various reagents: stations 1 and 2 contained 10% formalin, stations 3 to 7 involved a gradient of alcohol solutions (70, 80, 90, 95%s, absolute 1, and absolute 11) for dehydration. Stations 8 and 9 contained xylene for clearing, followed by transfer into three wax baths for infiltration. Each processed tissue was embedded in a solid support medium (paraffin wax) using a semi-automatic tissue embedding centre. Molten paraffin wax was poured into metal moulds, embedding and orienting the tissue accordingly; pre-labeled cassettes were positioned atop and transferred to a cold plate for solidification. The resulting tissue blocks were separated from the moulds and trimmed to expose the tissue surface using a rotary microtome set at 6 mm. The surfaces were allowed to cool on ice before sectioning. Approximately 4 micrometers thick, tissue sections were floated on a water bath set at 55°C and transferred onto clean slides. These slides were labeled and placed on a hotplate set at 60°C for 1 hour. Hematoxylin and eosin staining techniques were utilized and the slides were examined by a specialist (Histopathologist.)

Immunohistochemical Analysis

The immunohistochemical staining for BCL-2, APC were done using kits obtained from ABCAM Chemical Inc. and Santa Cruz Biotechnology. The procedure followed the modified approach described by Chakravarthi *et al*., [32]. Briefly, primary antibodies were diluted to 1:100, or as specified by the manufacturer, and allowed to bind to specific antigens. Subsequently, a secondary antibody conjugated with an enzyme was introduced to form antibody-antigen complexes. Under a binocular microscope, the enzyme acted on the substrate in the presence of chromogen and antibody-antigen binding sites, resulting in the formation of coloured deposit. Cells' displaying distinct colours in the cytoplasm, cell membrane, or nuclei, depending on the antigenic locations, was considered positive and compared to external controls. Antigen retrieval was carried out on the sections by heating in a citric acid buffer (pH 6.0) at 100°C for 15 min.

Statistical Analysis

The results were expressed as mean ± standard deviation (SD). Statistical analysis was done using one-way analysis of variance and later by Dunnett's multiple comparisons test carried out by means of GraphPad Prism version 8.3.0 for Mac, GraphPad Software, San Diego, CA (www.graphpad.com). Differences were deemed significant at $p < 0.05$.

RESULTS

Effect of Methanol Leaf Extracts of *Voacanga Africana* on Body Weight of Rats Intoxicated with Diethylnitrosamine and Carbon Tetrachloride

In table 1, a significant ($p<0.05$) decrease in weight of the animals in [DEN+CCL_4] group was observed relative to the control. However, treatment with methanol extracts of *VA* at varying doses 100, 200, and 400mg/kg restored the weight of the animals by 196%, 65% and 142% relative to [DEN+CCL_4], respectively. Moreover, there was a significant ($p<0.05$) increase in the liver organo-somatic weight in [DEN+CCL_4] rats when compared to the control. In contrast, the organo-somatic weight of the liver in [DEN+CCL_4] rats treated with *VA* decreased by 18, 20 and 9% respectively.

Table 1: Effect of methanol leaf extracts of *Voacanga africana* on Body weight of rats intoxicated with DEN+CCL_4

Grouping	Body weight (g) Initial	Final	Weight Diff.	Liver Weight (g)	% OSI
Control	144.50±2.52	189.50±6.26	46.50±7.68	4.95±0.79	2.50±0.05
DEN+CCL_4	175.29±4.04	207.50±13.23	37.33±4.04*	6.53±0.16	3.14±0.08*
DEN+CCL_4+VA(100mg/Kg)	155.40±1.52	256.67±14.47	110.50±2.10**	6.98±0.96	2.56±0.04
DEN+CCL_4+VA(200mg/Kg)	179.20±3.70	240.00±2.83	61.50±3.54**	6.53±0.94	2.52±0.64
DEN+CCL_4+VA(400mg/Kg)	145.20±4.93	236.25±23.96	90.50±23.69**	7.21±0.19	2.87±0.11
DEN+CCL_4+SFB(10mg/Kg)	173.33±4.04	229.00±7.21	55.67±3.79	6.41±0.74	2.71±0.33
SFB Only(10mg/kg)	171.50±3.54	215.50±12.02	44.00±8.49***	7.34±2.02	3.38±0.75

Data are expressed as Mean ± SD; n= 7.
* = P < 0.05 when compared with Control;
** = P < 0.05 when compared with DEN+CCL_4;
*** = P < 0.05 when compared with DEN+CCL_4+VA (100mg/kg, 200mg/kg, 400mg/kg).
DEN= Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

Effect of Methanol Leaf Extracts of *Voacanga Africana* on Haematological Parameters of Rats Intoxicated with Diethylnitrosamine and Carbon Tetrachloride

In table 2, there was an increase in the white blood cells (WBCs) and neutrophils of the animals intoxicated with [DEN+CCL_4] when compared to the control. In contrast, the WBCs and neutrophils of rats treated with methanol extracts of *VA* insignificantly decreased relative to intoxicated group.

Table 2: Effect of methanol leaf extracts of *Voacanga africana* on Haematological parameters in DEN+CCL_4 treated rats

Grouping	PCV(%)	Hb (g/dL)	RBC ($x10^6$µL)	WBC ($x10^3$ µL)	Platelet ($x10^3$/µL)	Lym (%)	Neut (%)
Control	51.67±2.88	16.90±0.42	8.71±0.11	43.75±1.24	132.00±2.83	77.00±1.41	21.67±1.15
DEN+CCL_4	50.25±0.50	16.35±0.07	8.48±0.03	47.00±1.84*	116.67±16.50	73.00±1.4	23.00±1.41
DEN+CCL_4+VA (100mg/Kg)	53.67±3.21	17.10±0.95	8.75±0.06	42.00±0.85**	109.67±11.59	79.00±2.00	16.33±1.15**
DEN+CCL_4+VA (200mg/Kg)	52.33±0.58	16.87±0.29	8.69±0.04	43.00±1.84**	107.67±6.81	77.00±1.00	18.67±0.58**
DEN+CCL_4+VA (400mg/Kg)	46.00±1.00	14.93±0.47	7.35±0.02	45.25±0.95	116.50±4.95	75.00±1.00	21.00±1.73
DEN+CCL_4+SFB (10mg/Kg)	51.67±3.51	16.57±1.14	8.72±0.13	39.75±1.45	126.00±0.00	76.00±1.73	20.00±1.41
SFB Only (10mg/kg)	52.00±2.65	16.73±0.85	8.64±0.06	42.25±1.59	127.00±4.24	76.33±1.53	19.67±0.58

Data are expressed as Mean ± SD; n= 7.
* = P < 0.05 when compared with Control;
** = P < 0.05 when compared with DEN+CCL_4;
*** = P < 0.05 when compared with DEN+CCL4+*VA* (100mg/kg, 200mg/kg, 400mg/kg).
DEN= Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

Effect of Methanol Leaf Extracts of *Voacanga Africana* on Biochemical Indices in Rats Intoxicated With Diethylnitrosamine and Carbon Tetrachloride

Table 3 showed a significant (p<0.05) increase in the level of nitric oxide (NO) in the DEN+CCL_4 treated rats relative to the control. However, the level of NO were decreased in the rats treated with the methanol extracts of *VA* at 100mg/kg, 200mg/kg and 400mg/kg body weight by 35%, 34% and 32%, respectively. In addition, there was a significant (p<0.05) increase in the activities of ALT and AST of [DEN+CCL_4] treated group by 25% and 18% respectively relative to the control. However, the group treated with *VA* (400mg/Kg) had insignificant decreased in ALT and AST. In figure 1, the level of α-fetoprotein (AFP) showed a significant (p< 0.05) increase in [DEN+CCL_4] group as compared to the control. However, a significant decrease was observed in the level of AFP in the groups that received methanol extract *of VA* at 100mg/kg and 200mg/kg by 25% and 23%, respectively. In figure 2, total bilirubin level was significantly (p< 0.05) increase in the [DEN+CCL_4] group and was decreased upon treatment with the methanol extracts of *VA* at 100mg/kg, 200mg/kg and 400mg/kg by 47%, 31% and 50% respectively. Furthermore, methanol extracts of *VA* at 100 and 200mg/kg decreased the activities of gamma glutamyl transferase (GGT) by 15% and 13%, respectively when compared with [DEN+CCL_4] group (figure 2). In figure 3, a significant (p< 0.05) increase in the activities of myeloperoxidase (MPO) was observed in [DEN+CCL_4] group by 55% when compared to the control. However, treatment with extract of *VA* at 100,

200 and 400mg/kg decreased the MPO activities by 60%, 68% and 94%, respectively relative to the toxicant group. Also,, there was a significant ($p< 0.05$) increase in the levels of lipid peroxidation products (LPO) in [DEN+CCL_4] group relative to the control. Conversely, a significant decrease were observed in the levels of LPO in the intoxicated groups that received *VA* at 100 and 200mg/kg by 39% and 45%, respectively as compared to the [DEN+CCL_4] group. Moreover, levels of LPO significantly increased in the intoxicated group treated with SFB when compared intoxicated group.

Table: 3 Effect of methanol leaf extracts of *Voacanga africana* on Nitric Oxide (NO), Alanine and Aspartate Aminotransaminases (ALT and AST) in DEN+CCL_4 treated rats

Grouping	NO (µmol/L)	ALT (U/L)	AST (U/L)
Control	406.50±30.41	373.50±4.95	981.25±37.50
DEN+CCL_4	617.50±48.79*	466.50±3.54*	1126.25±65.41
DEN+CCL_4+VA (100mg/Kg)	401.33±23.03**	472.00±1.41	1087.50±21.21
DEN+CCL_4+VA (200mg/Kg)	402.25±13.82**	462.00±2.83	1178.75±22.41
DEN+CCL_4+VA (400mg/Kg)	420.75±11.27**	446.00±8.49	1133.75±15.61
DEN+CCL_4+SFB (10mg/Kg)	413.67±14.01	459.00±7.21	1068.75±5.30
SFB Only (10mg/kg)	398.00±17.30	492.50±6.36	1127.50±24.75

Data are expressed as Mean ± SD; n= 7.
* = $P < 0.05$ when compared with Control;
** = $P < 0.05$ when compared with DEN+CCL_4;
*** = $P < 0.05$ when compared with DEN+CCL_4+VA (100mg/kg, 200mg/kg, 400mg/kg).
DEN= Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

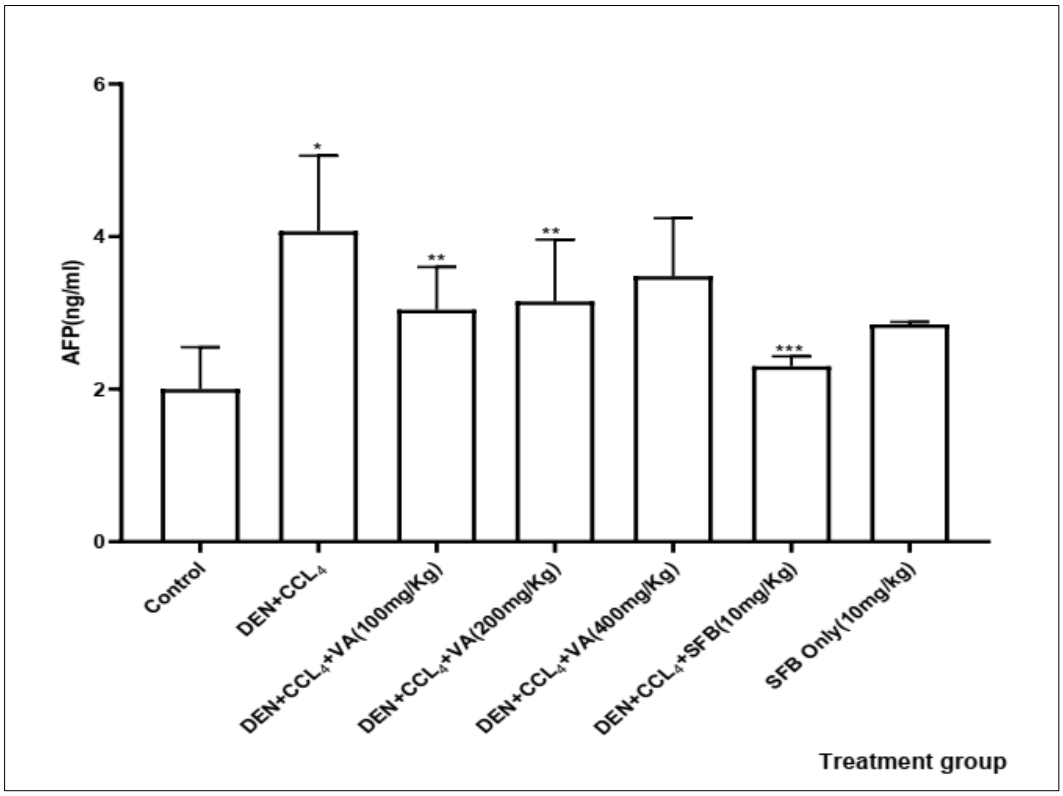

Figure 1: Effect of methanol leaf extracts of *Voacanga Africana* on alpha-fetoprotein (AFP) levels in DEN+CCL_4 intoxicated rats

Data are expressed as Mean ± SD; n= 7.
* = $P < 0.05$ when compared with Control;
** = $P < 0.05$ when compared with DEN+CCL_4;
*** = $P < 0.05$ when compared with DEN+CCL_4+*VA* (100mg/kg, 200mg/kg, 400mg/kg).
DEN = Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

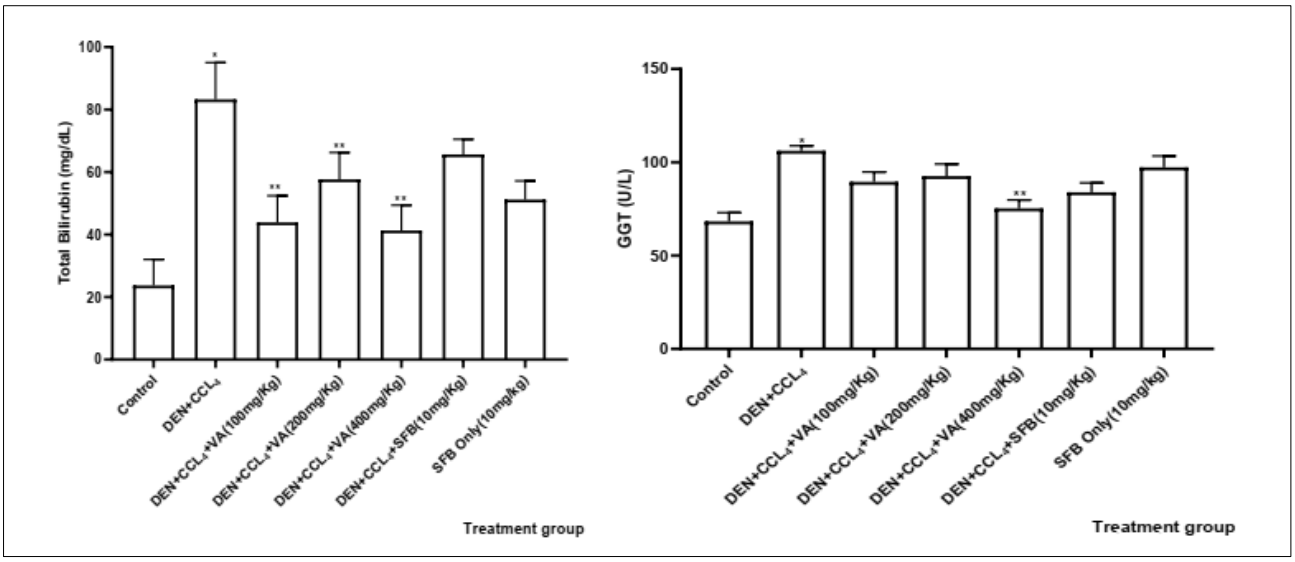

Figure 2: Effect of methanol leaf extracts of *Voacanga Africana* on total bilirubin and gamma glutamyl transferase (GGT) levels in DEN/CCL_4 intoxicated rats

Data are expressed as Mean ± SD; n= 7.
* = P < 0.05 when compared with Control;
** = P < 0.05 when compared with DEN+CCL_4;
*** = P < 0.05 when compared with DEN+CCL_4+VA (100mg/kg, 200mg/kg, 400mg/kg).
DEN = Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

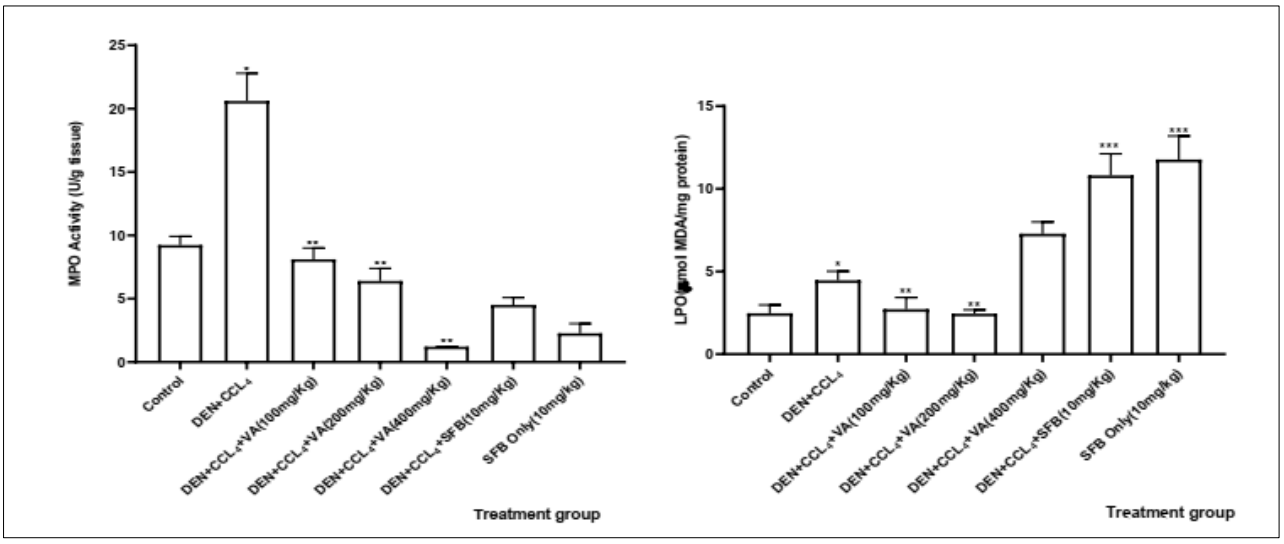

Figure 3: Effect of methanol leaf extracts of *Voacanga africana* on Myeloperoxidase activity (MPO) and lipid peroxidation (LPO) in DEN+CCL_4 intoxicated rats

Data are expressed as Mean ± SD; n= 7.
* = P < 0.05 when compared with Control;
** = P < 0.05 when compared with DEN+CCL_4;
*** = P < 0.05 when compared with DEN+CCL4+*VA* (100mg/kg, 200mg/kg, 400mg/kg).
DEN= Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

GC-FID and HPLC Analysis Showing Active Components of Methanol Leaf Extracts of *Voacanga Africana* (*VA*)

GC-FID and HPLC analysis of *Voacanga africana* **(*VA*)** revealed active components as shown in Table 4 and 5. Some of these constituents include Voacinol,Voacangine, Amataine, Voacafricine, Dregamine, Voacamidine, Yohimbine, Vobtusine, Ibogaine, Voacamine with different peak values.

Table 4: GC- FID Analysis showing the active components of methanol leaf extract of *Voacanga africana*

Components	Retention time	Area	Heights
Solvent	0.350	70559.5930	5675.683
Tabersonine	3.033	520.8780	11.290
Tabernanthin	3.616	217.7095	8.070
Alpha-Tocopherol	4.400	537.9270	37.380
Yohimbine	6.433	150.7225	13.627
Beta-Sitosterol	7.000	125.0050	7.483
Sitosterol	7.466	90.0390	6.597
Vobasine	8.166	430.1140	43.682
Vobtusine	8.433	154.0460	15.490
Voacafrine	9.366	826.5730	28.000
Voacafricine	9.750	1104.8175	112.882
Voaphylline	10.066	209.3330	19.564
Voacryptine	10.933	1354.4535	72.753
Voacinol	11.216	2434.9950	201.703
Voacristine	12.116	1086.1360	67.525
Voacangine	12.600	2195.3300	167.162
Voacamine	13.416	1015.4320	44.631
Ibogaine	13.900	1213.0240	131.445
Ibogamine	14.550	963.6905	65.685
Amataine	15.150	1962.9000	134.693
Rhazine	15.783	559.4200	34.123
Dregamine	16.300	1776.3255	110.470
Voalfolidine	17.033	360.7950	27.463
Voacamidine	17.383	1118.6615	100.487
Voacarpine	18.083	198.4860	9.922
Perakine	18.433	905.2090	81.259
Temposerpine	19.433	696.8190	54.185

Table 5: HPLC Analysis showing the active components of methanol leaf extracts of *Voacanga africana*

Components	Retention time	Area	Heights	External	Units
Tabersonine	1.266	607.5825	24.282	0.0000	%
Tabernanthin	2.516	1444.6620	14.969	168.0827	ppm
Alpha-Tocopherol	4.450	488.6540	8.229	48.8654	ppm
Yohimbine	5.466	280.8930	6.093	0.0000	
Beta-Sitosterol	6.483	113.8690	10.590	0.0000	
Stigmasterol	7.950	87.5880	8.486	0.0000	
Ergosterol	8.416	135.4140	9.808	0.0000	
Voacinol	8.783	95.6260	7.584	0.0000	
Voacristine	9.350	101.4630	4.646	0.0000	
Voacangine	11.050	7385.9650	140.641	0.0000	
Voacamine	12.166	2685.6690	49.932	0.0000	
Ibogaine	13.700	1697.0030	31.675	0.0000	
Ibogamine	17.616	477.4340	6.998	0.0000	
Amataine	19.416	94.2470	6.004	0.0000	
Rhazine	22.083	97.2595	9.140	0.0000	
Dregamine	22.600	90.0200	7.794	0.0000	
Voalfolidine	23.750	91.4480	6.689	0.0000	
Perakine	24.316	118.3560	6.269	0.0000	
Temposerpine	26.533	130.5300	4.091	0.0000	

Effect of Methanol Leaf Extracts of *Voacanga Africana* on Antioxidant Indices in Rats Intoxicated with Diethylnitrosamine and Carbon Tetrachloride

Figure 4 showed a significant ($p<0.05$) decrease in Glutathione peroxidase (GPx) in the [DEN+CCL_4] group when compared to the control. This decrease in the activities of GPx was restored following the administration of the methanol extracts *of VA* at 100mg/kg, 200mg/kg and 400mg/kg. Moreover, there was a significant ($p<0.05$) decrease in Glutathione-S-Transferase (GST) in the [DEN+CCL_4] group relative to the control. However, the activities of GST showed

significant increase when the animals were treated with *VA* at 100mg/kg, 200mg/kg and 400mg/kg by 373%, 168% and 157% respectively. In figure 5, superoxide dismutase (SOD) and catalase activities were significantly decreased by 32% and 25% respectively in the [DEN+CCL_4] group and were insignificantly attenuated upon treatment with varying doses of *VA*.

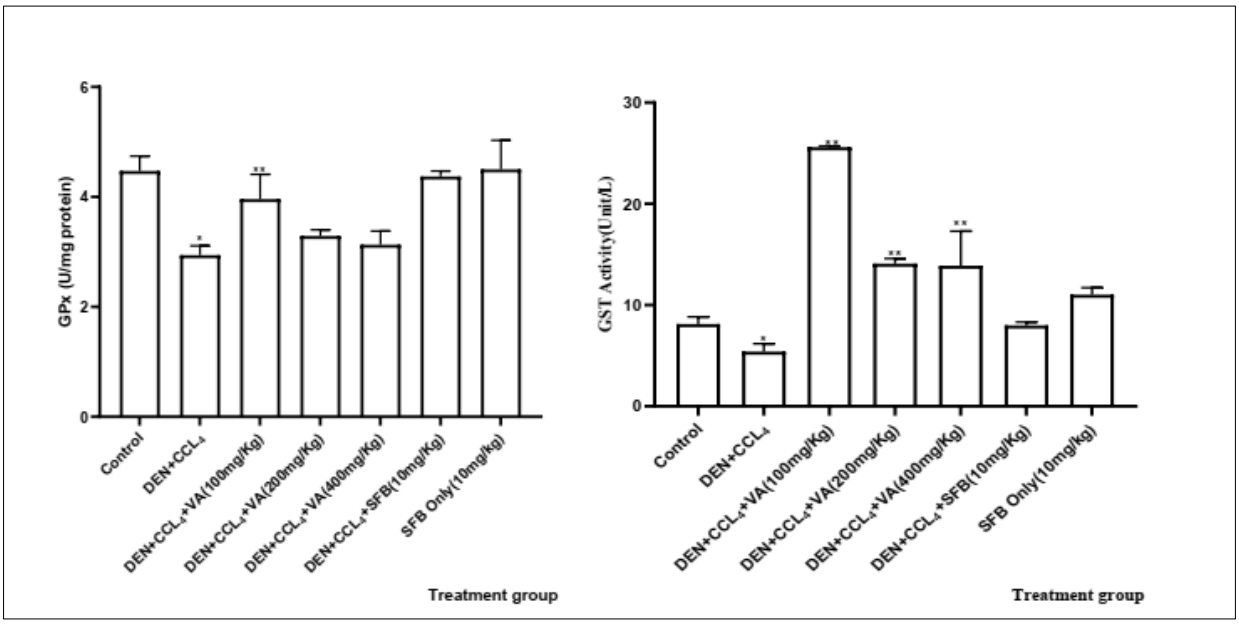

Figure 4: Effect of methanol leaf extracts of *Voacanga africana* on Glutathione peroxidase (GPx) and Glutathione-S-Transferase (GST) in DEN+CCL_4 intoxicated rats

Data are expressed as Mean ± SD; n= 7.
* = P < 0.05 when compared with Control;
** = P < 0.05 when compared with DEN+CCL_4;
*** = P < 0.05 when compared with DEN+CCL_4+*VA* (100mg/kg, 200mg/kg, 400mg/kg).
DEN= Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

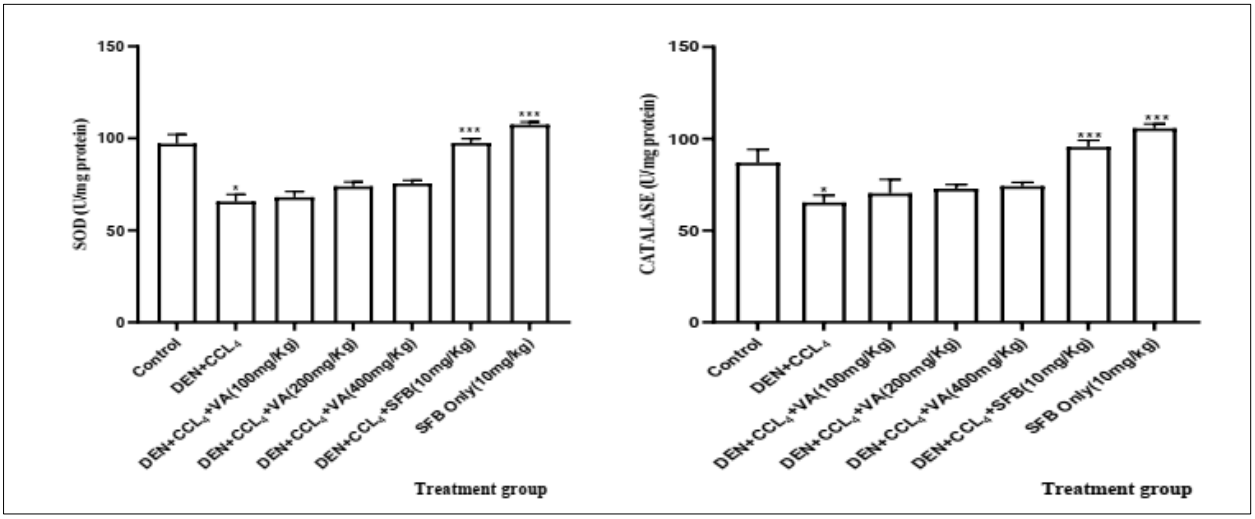

Figure 5: Effect of methanol leaf extract of *Voacanga africana* on Superoxide dismutase (SOD) and Catalase in DEN+CCL_4 intoxicated rats

Data are expressed as Mean ± SD; n= 7.
* = P < 0.05 when compared with Control;
** = P < 0.05 when compared with DEN+CCL_4;
*** = P < 0.05 when compared with DEN+CCL_4+*VA* (100mg/kg, 200mg/kg, 400mg/kg).
DEN= Diethylnitrosamine, CCL_4= Carbon tetrachloride, SFB = Sorafenib (10mg/kg)

Effect of Methanol Leaf Extracts of *Voacanga Africana* on Certain Proteins and Cyto-Architecture of Liver in Rats Intoxicated with Diethylnitrosamine and Carbon Tetrachloride

Histopathological examination of the liver revealed severe necrosis in [DEN+CCL_4] treated rats (figure 6) relative to control. Groups treated with *VA* at 100mg/kg and 200mg/kg showed normal morphology of the liver while group treated with *VA* at 400mg/kg also showed normal morphology but with mild congestion of the portal vein. In Figures 7 and 8, immunohistochemical staining of the hepatic tissues revealed strong expression of BCL-2 in [DEN+CCL_4] rats when compared to control. Treatment with methanol leaf extract of *VA* at 100mg/kg clearly attenuated the expression of BCL-2. However, VA at 200 and 400mg/kg failed to alter the strong expression of BCL-2 by the toxicants. Also, IHC staining of the liver revealed mild expression of APC protein in [DEN+CCL_4] group, but upon treatment with *VA* at the varying doses, there was a moderate expression of APC.

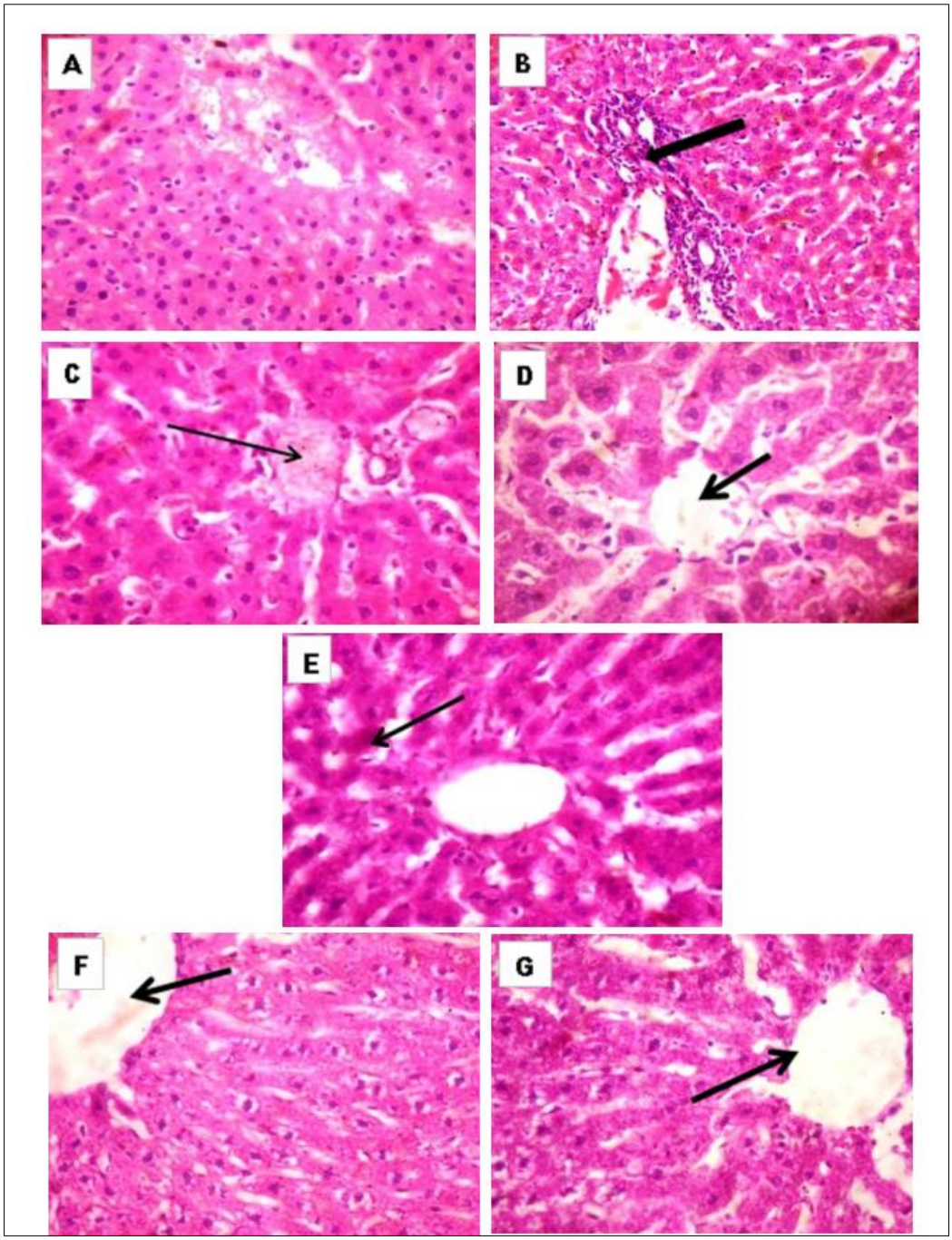

Figure 6: Photomicrographs of liver from rats treated with methanol leaf extract of *Voacanga africana* after intoxication with DEN+CCL4. (H&E) X400

Control (A) showed no visible lesion. (B) DEN + CCL_4 showed severe hepatic necrosis, (C) DEN+CCL_4+*VA* (100mg/kg) showing normal central venules, (D) DEN+CCL_4+VA (200mg/kg) shows normal central venule, hepatocytes show normal morphology,(E) DEN+CCL_4+*VA* (400mg/kg) shows normal central venules ,portal vein show mild congestion,(F) DEN+CCL_4+SFB showing normal central venules, the liver parenchyma shows areas of degenerated hepatocytes exhibiting cytoplasmic vacuolation and balooning of cells, hepatocytes with necrosis are seen. (G) SFB only showing normal central venules and hepatocytes showing mild cytoplasmic vacuolation, sinusoids appear normal

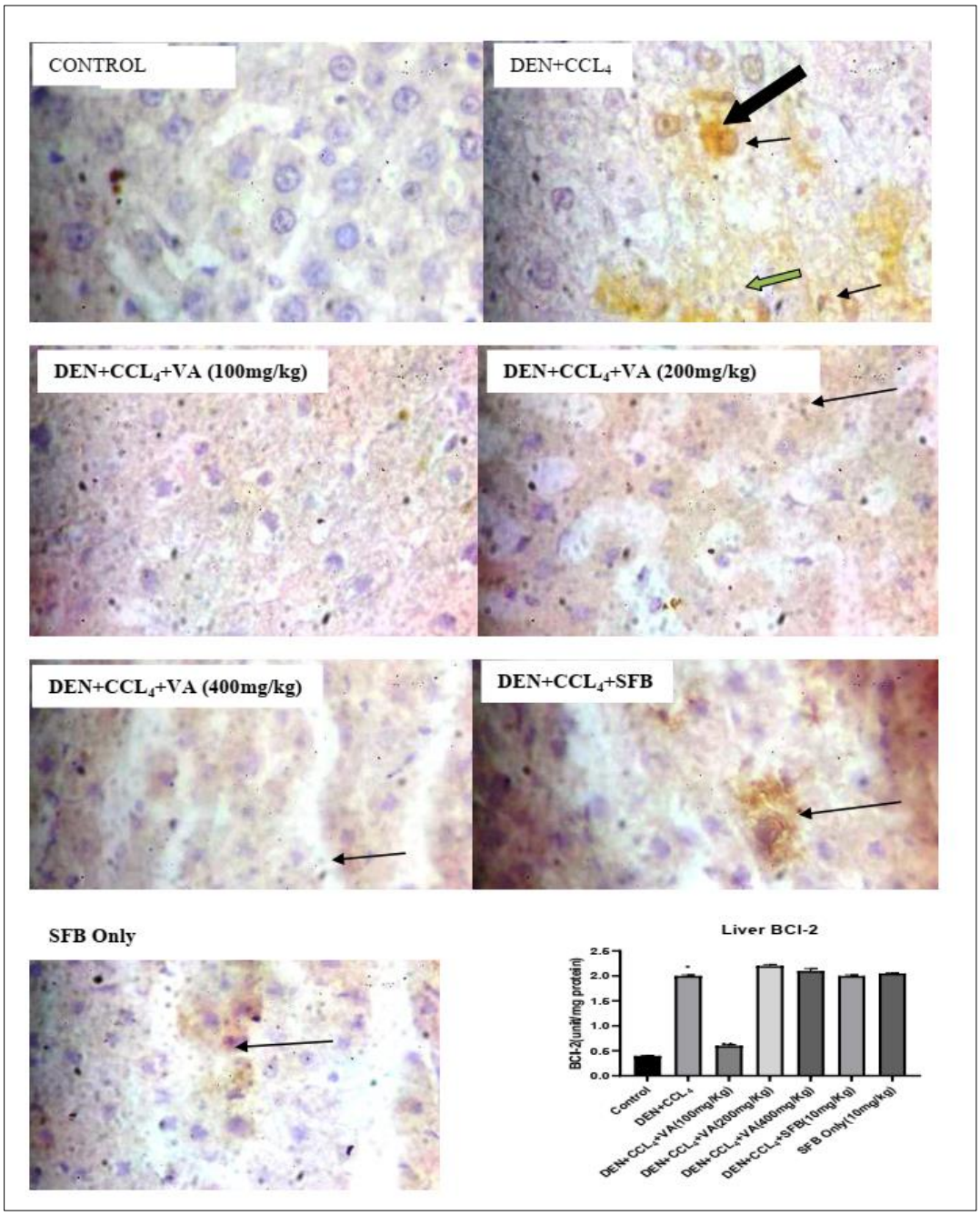

Figure 7: Photomicrographs of Liver from rats treated with methanol leaf extract of *Voacanga africana* after intoxication with DEN+CCL_4 showing expression of BCL-2 protein. X400

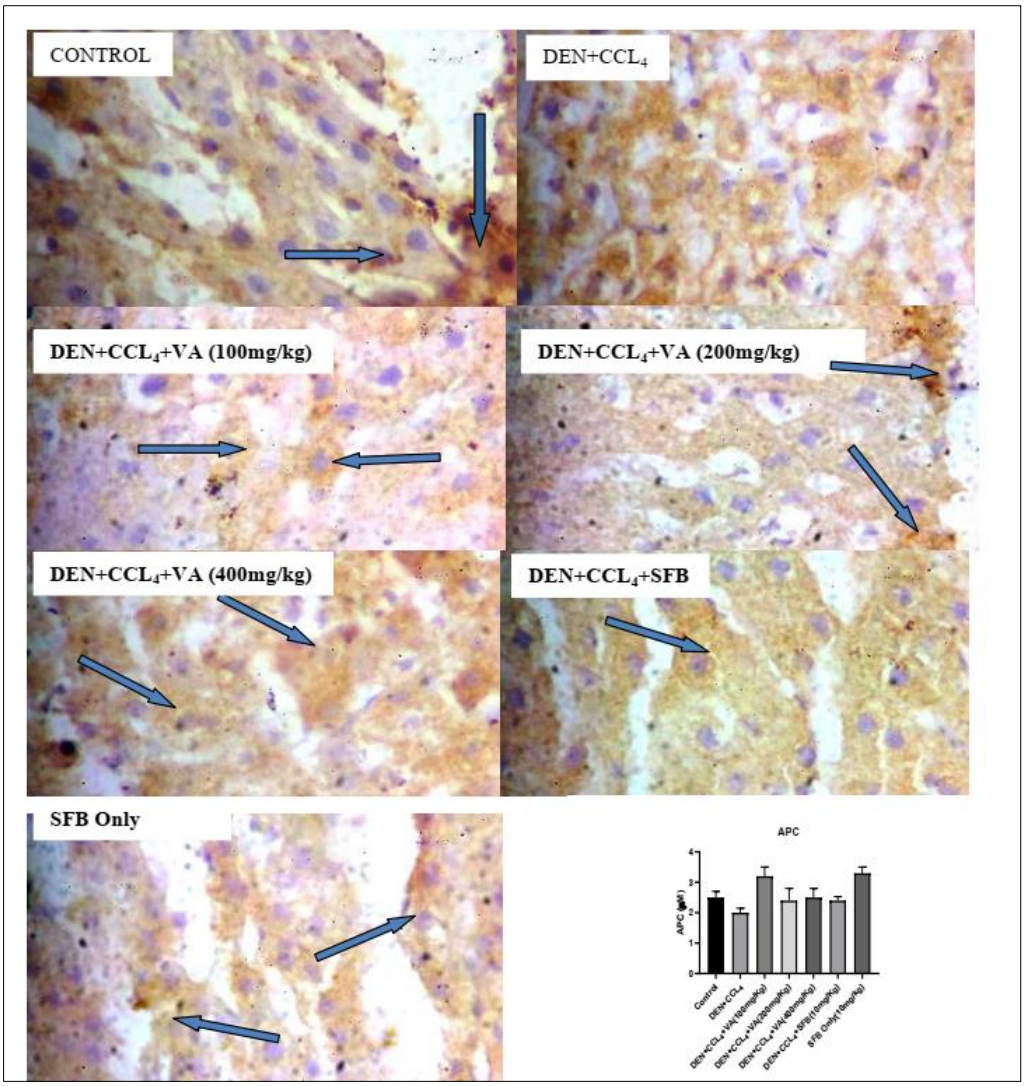

Figure 8: Photomicrographs of liver from rats treated with methanol leaf extract of *Voacanga africana* after intoxication with DEN+CCL_4 showing of Adenomatous Polyposis Coli (APC) protein. X400

DISCUSSION

Exposure to nitrosamine compounds has been documented to pose a risk to both animals and humans, leading to severe liver damage [33]. At low doses; 20mg/kg to 40mg/kg, nitrosamines have been demonstrated to induce hepatic necrosis in rodents and other mammals [34]. Furthermore, a single administration of DEN within the range of 10–90 mg/kg typically exerts an irreversible carcinogenic influence in rodents [34]. Primarily found in various sources including tobacco smoke, water, processed and fried foods, agricultural chemicals, cosmetics, and pharmaceuticals [35], these hepatocarcinogens can predispose humans and animals to hepatic toxicity upon frequent exposure [36]. Such exposure triggers the production of reactive oxygen species (ROS), ultimately paving the way for hepatocellular carcinoma. Diethylnitrosamine requires metabolic activation, facilitated by the liver enzyme; cytochrome CYP2E1 [37]. This process involves hydroxylation and cleavage of the *N*-nitrosamine molecules, resulting in the formation of diazonium ions that alkylate nucleophilic sites including DNA and RNA, thereby initiating carcinogenesis [37].

In recent times, there has been a surge in the application of chemopreventive agents from natural sources largely due to their antioxidant activity and low-toxicity in biological systems [38]. Therefore research into medicinal plants as potent sources of antioxidants, anti-inflammatory, and anticarcinogenic compounds has gained momentum. Extracts obtained from various parts of *Voacanga africana*, including leaves, seeds and roots, have long been utilized in traditional medicine for treating a range of pathological conditions such as malaria, infant convulsions, heart ailments, Alzheimer's disease, and cancer [14]. Carcinogenesis encompasses a multitude of processes, including angiogenesis, cell adhesion, proteolytic degradation, and migration through the extracellular matrix [39]. The prevailing therapeutic strategy in cancer management aims at inhibiting activities associated with reactive oxygen species, tumor

invasion, angiogenesis, and to enhance apoptosis induction [39]. This study explored the potential mitigating effects of methanol extract of *Voacanga africana* leaves against hepatotoxicity in rats administered DEN and CCL_4.

Voacanga plants have been used to manage various conditions, including leprosy, diarrhea, generalized edema, and convulsions in children, as well as to treat orchitis, ectopic testes, and gonorrhoea [38, 39]. Analysis of methanol leaf extracts of Voacanga africana (*VA*) using GC-FID and HPLC in this study identified several components such as Voacinol, amataine, Voacamine, Voacangine, Ibogaine, Vobtusine, and Yohimbine. Previous pharmacological studies on Voacanga extracts and purified alkaloids have highlighted their significant potential as analgesics, stimulants, vasodilators, CNS modulators, antimicrobial agents, antiplasmodial, anti-ulcer, anti-inflammatory, cytotoxic, antitumor, and anti-malarial agents. For instance, amataine from *VA* has demonstrated anti-cancer properties through cytotoxic activity against VERO cells [40]. Voacamine has shown promise in reversing tumor multidrug resistance [41], preventing heart failure, and exhibiting cytotoxic and antimicrobial activity against Gram-positive bacteria [42] . It has also been noted as an inhibitor of EGFR, which shows oncogenic activity in colorectal cancer [43]. Additionally, Voacangine, a key component of *VA*, has been reported to inhibit angiogenesis, a major mechanism in cancer cell proliferation, in both *in vitro* and *in vivo* studies [44] . Voacangine also exhibits anticancer activity against human oral cancer cells [45]. The pharmacological report indicates that Ibogaine plays a role in the central nervous system [46]. Yohimbine, isolated from various plants including *VA*, has been associated with managing erectile and myocardial dysfunction, inflammatory disorders, and cancer [47]. Vobtusine, another alkaloid from *VA*, has shown cardiac depressant effects and demonstrated cytotoxic activity by inhibiting Bcl-2 and Bcl-xl, and upregulating Bax and caspase-3 activities [48].

In this study, the body weight gain in [DEN+CCL_4] intoxicated was significantly reduced relative to control while the organo-somatic weight of liver in [DEN+CCL_4] rats increased. This hepatomegaly contributes to one of the major symptoms of chemically-induced hepatotoxicity [35]. However, treatment with *VA* mitigates this abnormal increase in the liver weight. This observation aligned with the findings of Sawong *et al*., 2022 [49], who reported a significant decrease in progression of liver cancer by lowering the liver to body weight ratio upon administrations of the *Calotropis gigantea* extracts. In this study, DEN and CCL_4 increased the levels WBC and neutrophils. Neutrophils are one of the major types of white blood cells that fight infection [50]. Despite its benefits, excess accumulations are detrimental, and may induce leukocytosis or a high total white blood cell count as seen in this study. However, administration of *Voacanga africana* significantly reduced the levels of these parameters. This report is in line with the findings of Ullah *et al*., 2020 [51] who reported that neutrophils may be a key player in chronic inflammation [52], and may be involved in liver diseases including cirrhosis, fibrosis and liver cancer [53].

Serum parameters such as total alpha fetoprotein, bilirubin, gamma glutamyltransferase (GGT), alanine and aspartate aminotransferases (ALT and AST) were studied in this research. Findings from the study confirmed liver damage in the rats given DEN +CCL_4 on the basis of elevated values of total bilirubin, alpha fetoprotein, ALT and GGT. Upon administration of the *Voacanga africana* at different concentrations, a significant decrease in the levels of the elevated parameters was observed. In addition, inflammatory indices; nitric oxide (NO) and myeloperoxidase (MPO) were significantly increased in the toxicant group which was down-regulated after treatment with different concentrations of the extracts. This indicates that DEN and CCL_4 could induce inflammation in the liver of experimental animals. This outcome is in agreement with the previous study on the flavonoid fraction of *voacanaga africana* leaf extract as anti-inflammatory agent according to Olaleye *et al.*, 2005 [16]. Results from previous studies showed that chronic inflammation is associated with several hepatotoxicants [52], an observation which has been confirmed in this study. Furthermore, α-fetoprotein, a major marker for diagnosing liver damage especially HCC, was elevated in this study which further confirmed liver damage/ diseases [54]. However, upon the administration of varying concentrations (100-400mg/kg) of *VA* extract, the three doses reversed the AFP with 100mg/kg showing most significant reduction relative to 200mg/kg and 400mg/kg. The attenuating effect of the *VA* leaf extract on AFP is in line with the report of Aborehab *et al.*, 2019 [55].

The ability of *VA* to act as free radical scavenger was also explored in this study. Reactive oxygen species (ROS) are a major predisposing factor to numerous pathological conditions such as inflammation, atherosclerosis, neurodegenerative diseases, and cancer [56]. Administration of DEN and CCL_4 induced oxidative stress which caused liver injury by altering main biological molecules such as DNA, proteins and lipids [57]. The ROS has the ability to interact with the polyunsaturated fatty acids of lipid membranes and provoke lipid peroxidation (LPO). In this study, DEN and CCL_4 intoxicated group showed a significant increase in the level of LPO, an observation that agrees with the study of Abdel-Monem *et al*., 2020 [58]. Conversely, administrations of *VA* at 100 and 200mg/kg lowered the levels of LPO significantly, as evidenced with decreased level of malondialdehyde (MDA), one of the end products of LPO. Consequently, increased level of MDA in DEN + CCL_4 group occurred concurrently

with decrease in the activities of antioxidant enzymes, a major line of defense against oxidative stress [59].This observation therefore suggests that one of the mechanisms by which DEN and CCL_4 may induce hepatic damage include and not restricted to induction of oxidative stress pathway via suppression of antioxidant enzymes system. Nonetheless, treatment with methanol *VA* leaf extract at 100mg/kg, 200mg/kg and 400mg/kg ameliorated the increased LPO level by increasing the GPx and GST activities. Although the roles of superoxide dismutase (SOD) in cancer has not been fully elucidated [60], SOD along with catalase are the first line antioxidative defense of the body, while glutathione can directly conjugate and detoxify electrophilic xenobiotics through glutathione s-transferase enzyme system [61]. In addition, glutathione play a role as a reducing agent to provide electrons through glutathione peroxidise so as to cause a reduction of organic peroxide (ROOH) to hydroxyl group (ROH) and water, and thereafter oxidizes to gulathione disulfide (GSSG) which can still become reduced to glutathione (GSH). The ability of *VA* to replenish these antioxidants is indicative of its antioxidative properties.

The expression of anti-apoptotic protein; BCl-2 was monitored in this study, which shows mild expression in the DEN+CCL_4 group relative to control in the cytosol of the hepatocytes clearly indicates that it may promote apoptosis of the liver cells. Administration of the *VA* to the toxicants group showed no expression of BCl-2 at 100mg/kg. The BCl-2, an anti-apoptotic protein play active role in the regulation of apoptosis by inhibiting the activities of caspase-9, 3, 6 and 7, thereby preventing programmed cell death by prolonging the survival time of tumor cells. Findings from this study showed that group treated with *VA* at 100mg/kg down-regulated the BCl-2 expression thereby promoting apoptosis of the damaged hepatocytes. This observation is in line with previous findings by Abdel Moneim 2016 which reported that *Indigofera oblongifolia* leaf extract (IOLE) decreased the expression of Bcl-2 protein on lead acetate (PbAc)-induced hepatotoxicity in adult male Wistar rats [62].

Additionally, Adenomatous polyposis coli (*APC*) protein, a tumor suppressor gene as previously been reported to play a key role in hepatocellular carcinoma was assayed in this study. Furthermore, the *APC* gene has several biological functions, including the regulation of cell cycle, cell migration and adhesion, transcriptional activation, apoptosis, and chromosomal instability [63]. The loss of its functionality by mutation has been implicated in different cancers [64] . The APC gene, is located on chromosome 5q, and is responsible for negatively regulating the b-catenin/Wnt pathway by creating a destruction complex with Axin/Axin2, GSK-3b, and CK1 [46]. In this study, the DEN+CCL_4 administered groups that were later treated with *VA* at 100mg/kg, 200mg/kg and 400mg/kg showed severe, moderate and abundant APC expression, respectively relative to DEN+CCL_4 group. Histological examination showed severe necrosis in the liver of the group treated with DEN+CCL_4. The observation suggests that the administration of the two liver toxicants disrupt the liver tissues as also reported by Salatin *et al.*, 2019 [19]. Interestingly, treatments with *VA* mitigate the severity of damage to the hepatocytes using combination of biochemical and histological indices on the tissue.

CONCLUSION

This present study showed that DEN+CCL_4 induced severe liver damage in male Wistar rats. The metabolism of these carcinogens release of ROS, which resulted into a decline in the activities of antioxidant enzymes, decreased apoptosis, and increased inflammation. However, treatment with *VA* attenuated these adverse conditions by increasing apoptosis, reducing inflammation and restoring the antioxidant status of the rats.

STATEMENTS AND DECLARATIONS

Ethical Approval:

Animals used in this study were treated following the guidelines of the Animal Care and Use Regulatory Committee, University of Ibadan, Nigeria with the approval number (UI-ACUREC/057-0623/27). The sample of *Voacanga africana* was authenticated and deposited at herbarium department, University of Ibadan, Nigeria with voucher specimen number UIH-23226 for future reference.

Competing Interest: Regarding this research, writing, and/or publication of the paper, the authors disclosed that they had no conflicts of interest.

Financial Interest: The authors have nothing to report about their relevant financial or non financial interests.

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Authors Contributions:

Oluwatosin A. Adaramoye conceived and designed the research; Raphael S. Olatoye prepared the materials, collected the data, and conducted the analysis. The manuscript preparation was written by Raphael S. Olatoye while it was reviewed by Oluwatosin A. Adaramoye

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