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Research Article
Effects of Methanol Leaf Extract of *Aristolochia bracteolata* on Diethylnitrosamine Induced Hepatocellular Carcinoma in Rats

I.B. Gabriel, K. Christopher, H.A. Umaru and D. Dahiru

Department of Biochemistry, Modibbo Adama University of Technology, Yola Adamawa State, P.M.B 2076, Nigeria

Abstract

Background and Objective: Diethylnitrosamine (DEN) is a well-known liver carcinogen in rats, forming DNA adducts in the liver and inducing hepatocellular damage. The present study was focused on anticancer and antioxidant effects of methanol leaf extract of *Aristolochia bracteolata* and its fractions against diethylnitrosamine induced hepatocarcinoma in rats. **Materials and Methods:** Hepatocellular damage was induced using diethylnitrosamine orally. Hepatocellular damage was assessed using biochemical parameters of the liver function tests, antioxidant enzymes which are considered as biomarkers in hepatocarcinoma. **Results:** Diethylnitrosamine caused significant hepatocellular damage with liver enzymes (Apatite transaminases, alanine transaminases and alkaline phosphatase activities) of 381±0.84, 125±0.37, 510±0.51 and 293±1.45, 75±1.57, 274±1.71 compared to the control groups at $p<0.05$, respectively. However, treatments with methanol leaf extract of *Aristolochia bracteolata* and its fractions as well as Silymarin significantly reverse the liver damage 284±2.88, 298±4.26 298±1.57 for different doses of the plant extract and fractions and Silymarin 295±0.71, respectively. So also, a significant decrease was observed in total protein, albumin, superoxide dismutase, glutathione reductase, glutathione peroxidase and total antioxidant with a corresponding increase in total bilirubin, interestingly, *Aristolochia bracteolata* significantly increase total protein, albumin, superoxide dismutase, glutathione reductase, glutathione peroxidase and total antioxidant with a decrease in total bilirubin concentration. **Conclusion:** Generally, the of administration of methanol leaf extract of *Aristolochia bracteolata* and its fractions promotes the healing of the liver cells through the anti-proliferative, antioxidant and anti-inflammatory effects of this natural product by maintaining the hepatocellular membrane integrity.

Key words: Antioxidant, *Aristolochia bracteolata*, diethylnitrosamine, silymarin, hepatocarcinoma, necrosis

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Corresponding Author: I.B. Gabriel, Department of Biochemistry, Modibbo Adama University of Technology, Yola, Adamawa State, P.M.B 2076, Nigeria
Tel: (+234) 08138038027

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatocellular carcinoma manifested by cirrhosis (scarring), is a primary risk factor for liver cancer[1]. Hepatocellular carcinoma is one of the most common types of cancer occurring worldwide[2,3]. It is considered as the sixth most common cancer and the second leading cause of cancer deaths around the world affecting both male and female[4]. Hepatocellular carcinoma is divided into two types: Primary and secondary Liver Cancer (LC). The primary LC starts in the liver, it is the most common type of hepatic cancer seen in 80-90% of liver tumors. The secondary LC begins in other organs of the body and spreads to the liver due to metastases of this cancer and creation of the most common cases including colorectal, breast, pancreatic, ovarian and lung cancers[5]. Primary liver tumors include hepatocellular carcinoma, cholangiocarcinoma and sarcoma which account for about 6% of the total cancer burden worldwide especially in Africa and Asia[3,6]. Hepatocellular carcinoma is the most common primary liver disease and is a leading cause of liver disease related deaths in the world. The etiology of hepatocellular carcinoma is multi-factorial, with genetic, environmental, medical and lifestyle factors interacting to produce a given malignancy[7]. Hepatocellular carcinoma has a slow and insidious onset and in most cases they are asymptomatic and diagnosis often prolonged. Access to liver disease treatment in developing countries where the incidence is more prevalent suffers from weaknesses of national health policy and insufficient financing[8]. Diethylnitrosamine (DEN) is a well-known liver carcinogen, forming DNA adducts in the liver and inducing hepatocellular carcinoma without cirrhosis through the development of putative pre-neoplastic enzyme-altered focal lesions[9]. Conventional medications for the treatment of liver damage has high side effects and is often inaccessible to the poor. The use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades[10]. It is therefore necessary to search for alternative drugs for the treatment of liver diseases to replace currently used drugs with severe side effects[8,11]. Plants are seen as an important source of useful medicinal natural products, as well as therapeutic and pharmaceutical novel compounds needed in the discovery of new drugs. Obtaining such drugs from plants will be a great move towards reducing the side effects associated with current liver disease treatment methods as natural and semi-synthetic products are considered safer, has economic advantage and accessibility with minimal side effects than synthetic drugs[12,13]. *Aristolochia bracteolata* (Birthworth Family) is used in traditional medicine as a gastric stimulant and in the treatment of tumors, lung inflammation and dysentery and snake bites[14]. Decoction of the whole plant is given in fever, worms and skin disease and snake bite[15]. This study has focused on alternative sources of curing ailments that are cheap, readily available with and no side effects.

MATERIALS AND METHODS

Plant material, Collection, identification and preparation: The leaf of *Aristolochia bracteolata* was collected from Girei Local Government Area of Adamawa State, Nigeria in the month of September, 2017. The plant was taxonomically identified and authenticated by a Botanist in the Department of Plant Science of Modibbo Adama University of Technology Yola. The leaf of *Aristolochia bracteolata* was washed and shade dried for one week. It was then ground into powder using laboratory mortar and pestle and sieved with 1 mm mesh size and stored in an air tight container until ready for use.

The ground powdered plant material of *Aristolochia bracteolata* (200 g) was extracted with 1200 mL of methanol in an air tight clean flat bottomed flask for 48 hrs at room temperature with occasional stirring and shaking. The methanol extract was first filtered through a fresh cotton plug and then through a Whatman No. 45 filter paper. The filtrate was evaporated to dryness in vacuum by a rotary evaporator at 40°C and the extract kept in a well tight sterile bottle/container under refrigerated conditions until use[16].

Twenty grams of the methanol extract was dissolved in 100 mL of distilled water. The resulting aqueous solution was successively partitioned with 100 mL of chloroform and water. The Chloroform and water fractions were, dried and weighed[17]. The fractions were subjected to *in vitro* antioxidant and phytochemical investigations. So, current study focused on anticancer and antioxidant effects of methanol leaf extract of *Aristolochia bracteolata* and its fractions against diethylnitrosamine induced hepatocarcinoma in rats.

Animals: Male albino rats weighing between (150±20 g) was obtained from National Veterinary Research Institute (NVRI) Plateau State, Nigeria. They were housed in propylene cages and were given standard grower diet (Vital Feeds) and water *ad libitum*. They were maintained under laboratory conditions of temperature (28°C) and 12 hrs. of light and dark cycle for seven days to allow them to acclimatize before the commencement of the experiment. Guide for the care and use

of laboratory animals 2010 of the Institute of Laboratory Animal Research (ILAR) Division on Earth and Life Studies, National Research Council was duly followed.

Experimental design: The rats were divided into nine groups of six rats each base on their weight. Group I served as normal control. DEN was administered orally to groups II, III, IV V, VI, VII, VIII and IX. Group II served as experimental control while group III served as the standard drug control (Silymarin 100 mg kg^{-1} b.wt.). The methanol leaf extract of *Aristolochia bracteolata* was administered to group IV (200 mg kg^{-1} b.wt.) and group V (400 mg kg^{-1} b.wt.) while group VI and VII were given fractions I of the *Aristolochia bracteolata* methanol leaf extract (200 and 400 mg kg^{-1} b.wt., respectively). Group VIII and IX were given fraction II of the *Aristolochia bracteolata* methanol leaf extract (200 and 400 mg kg^{-1} b.wt., respectively). Extract and fractions were administered orally for a period of 14 days.

Biochemical estimation: At the end of the experimental period, rats in all groups were scarified under chloroform as an anesthesia. Whole blood was collected into EDTA anti-coagulated specimen bottles for hematological analysis and some was collected into plane specimen containers for biochemical assays. The specimen collected in plane bottles were centrifuged at 10,000 rpm for 5 min and serum was collected for biochemical assays. About 10 g of liver biopsy was collected into containers containing 10% neutral formalin solution for histological investigations.

Determination of Aspartate Transaminase (AST): The AST activity was determined according to the method of Reitman and Frankel[18] using commercially prepared kits[18]. This enzyme formally known as Glutamate Oxaloacetate Transferase (GOT) will have its level increased in serum if there is an injury in liver, skeletal muscles, kidney, erythrocytes or heart. It can also be increased when liver function is impaired in case of necrosis or cell damage. The enzyme activity is expressed in IU L^{-1}.

Determination of Alanine Transaminase (ALT): This enzyme ALT formally referred to as Glutamate Pyruvate Transaminase (GPT) was also estimated using the method described by Reitman and Frankel[18]. The level increases in serum if there is an injury in liver, skeletal muscles, kidney, erythrocytes or heart. It can also be increased when liver function is impaired in case of necrosis or cell damage.

Alkaline phosphatase: Alkaline phosphatases are a group of enzymes which hydrolysis phosphate at alkaline pH. These enzymes are present in high concentration in liver, bone, intestinal epithelium, kidney tubules and placenta. In adults, the level will be elevated in cholestasis liver disease, it was estimated using the method described by King and King[19].

Total bilirubin: The Bilirubin level in serum was determined using the method of Ou *et al*., 1984. Total serum bilirubin consists of the conjugated and unconjugated form[20].

Total protein: For estimating total protein, 20 micro liter of serum was added to 1mL of biuret reagent. This was mixed thoroughly and incubated for 10 min. After ten min incubation, the blue colour formed was read using a photometer at 640 nm. The level of protein was expressed as g L^{-1} of serum. The total protein concentrations in serum were determined using the methods described by Kroll[21].

Serum albumin: Serum Albumin (ALB) concentration was measured using the method described by Doumas[22]. The measurement of ALB is based on its quantitative binding to the indicator, 3, 3′, 5, 5′-tetrabromo-m cresol sulphonephthalein (Bromocresol Green, BCG).

Glutathione Peroxidase (Gpx): For the determination of Glutathione peroxidase in plasma about 0.02 mL of heparinised blood was treated with 0.1 mL of 5 mM GSH, 0.1 mL of 1. 25 mM H_2O_2, 0.1 mL of 25 mM NaN_3 and phosphate buffer (0.05 mM, pH 7.0) in a total volume of 2.5 mL at 37°C for 10 min. The reaction was stopped by adding 2 mL of 1.65 % HPO32- and the reaction mixture was centrifuge at 1500 rpm for 10 min. About 2 mL of the supernatant was mixed with 2 mL 0.4 M Na_2HPO_4 and 1 mL of 1 mM dithio nitrobenzene (DTNB). The absorbance of the yellow coloured complex was measured at 412 nm after incubation for 10 min at 37°C against distilled water. A sample without the processed blood in the same way was kept as the non-enzymatic reaction. One unit of enzyme activity was defined as decrease in log GSH by 0.001 L min^{-1} after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction and is expressed as U mg^{-1} protein. The result was expressed as U g^{-1} Hb[23].

Total Antioxidant Status (TAS): In total antioxidant status estimation, 800 µL of TAS buffer was added to clean test tubes labeled blank, standard and calibrator. Each test tube of

sample and calibrator contain 50 µL of sample and calibrator, respectively and an initial absorbance at assay wavelength was recorded. TAS chromogen (125 µL) was then added to all three test tubes. This was mixed thoroughly and incubated for 5 min at 37°C. The second absorbance was read at 600 nm[24].

Glutathione Reductase (GR): Glutathione reductase activity was determined by the method of Pippennger[23]. Glutathione reductase was assayed by following the oxidation of NADPH at 340 nm at 37°C. Glutathione reductase activity was expressed as mmol NADPH oxidized/min/mg protein.

Superoxide Dismutase (SOD): In SOD estimation, the procedure that was adopted is that described by Wooliams[55]. The reaction mixture contains, 1.9 mL of phosphate buffer (pH 7.8), 1×10.2 M methionine, 16.8×10.5 M NBT and 1.17×10.6 M riboflavin, with suitably diluted erythrocyte hemolysate in a total volume of 3 mL. Illumination of the solution taken in 10 mL beaker was carried out in an aluminum foil lined box, with a 15 W fluorescent lamp for 10 min. Control without the enzyme source was always included. The absorbance was measured at 560 nm. The values were expressed in U g^{-1} Hb [25,26].

***In vitro* antioxidant activity:** The quantitative antioxidant activity of the methanol extracts and fractions, was determined using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays as described by Sutharsingh *et al.*[27].

Hematological parameter: Electrical impedance method used with Sysmex Hematology analyzer was used in the analysis of complete blood count of the test samples. Parameters studied includes, Hematocrit (HTC), Hemoglobin content, White Blood Cell count (WBC), Red Blood Cell count (RBC) and Platelets count[28].

Histological investigation: The liver biopsies were excised from the experimental animal of each group after collecting the blood sample. The biopsies were fixed in 10% neutral formalin solution. The sections were processed in alcohol xylene series. They were then embedded in paraffin and sections of 0.45 microns were cut using microtome techniques. After microtomy, they were stained with hematoxylin and eosin. The different sections were examined microscopically for the evaluation of histopathological changes[29].

Data analysis: All data were presented as mean value±standard error of mean (SEM). One way ANOVA was used for multiple comparisons of groups followed by Duncan's Multiple Range Test (MRT) for the post-hoc treatment.

RESULTS

The result of phytochemical screening of *Aristolochia bracteolata* methanol leaf extract and fractions was shown in Table 1. From the result in Table 1 cardiac glycosides and free anthraquinones were not detected in methanol extract while poly phenols, steroids and free anthraquinone were absent in aqueous fraction. Terpenoids and free anthraquinone were also not detected in chloroform extract. In addition, both extract and fractions contain alkaloids, flavonoids, tannins, reducing sugars and saponins.

The effect of methanol leaf extract and fractions on liver function parameters in DEN induced hepatocellular damage and normal rats was shown in Table 2. The result showed the activity of ALT is significantly ($p<0.05$) increased in experimental group (125±0.37 IU L^{-1}) when compared to normal rats (75±1.57 IU L^{-1}).

The effect of the extract and fractions on bilirubin, total protein and albumin on DEN induced hepatocellular damage was shown in Table 3. Total protein and albumin decreased significantly in group administered n, n Diethylnitrosamine (51±0.51 and 28±0.45 g L^{-1}) compared to rats in normal control group (68±0.40 and 43±0.84 g L^{-1}) while subsequent treatment with methanol extract, fraction I and fraction II shows a significant ($p<0.05$) increase in the biochemical indices.

Antioxidant enzymes are elevated in tumor conditions as result of increase in free radicals. There were significant ($p<0.05$) decrease in these enzymes SOD (23±0.75 %), GPx (493±1.16 U g^{-1} Hb), TAS (2.19±0.09 mmol L^{-1}) and GR (25±0.68 U L^{-1}) compared to normal control group 46±0.71%, 715±1.77 U g^{-1} Hb, 4.81±0.08 mmol L^{-1} and 45±1.30 U L^{-1}, respectively (Table 4).

The effect of methanol leaf extract and fraction of *Aristolochia bracteolata* on hematological parameters in normal and n, n diethylnitrosamine induced liver damage wasere shown in Table 5. There were significant ($p<0.05$) decreases in hematocrit (HTC) (38±0.13%), Hb (12.67±0.05 g L^{-1}), platelets (348±1.10 cells L^{-1}) and RBC (5.91±0.26 cells L^{-1}) compared to normal control group (57±1.11%, 19.28±0.06 g L^{-1}, 667±2.87 and 9.70±0.13 cells L^{-1}, respectively). However, all these parameters were significantly ($p<0.05$) increased when the rats were treated with methanol leaf extract and fractions of

Table 1: Qualitative phytochemical composition of *Aristolochia bracteolata*, methanol leaf extract and fractions

Phytochemical	Methanol extract	Aqueous fraction	Chloroform fraction
Alkaloids	+	+	+
Flavonoids	+	+	+
Polyphenols	+	+	+
Saponins	+	+	+
Tannins	+	+	+
Terpenoids	+	+	-
Steroids	+	-	+
Reducing sugars	+	+	+
Cardiac glycosides	-	+	+
Phlobatannins	+	+	+
Free anthraquinone	-	-	-

Present: +, Absent: -

Table 2: Effect of methanol extract and fractions of *Aristolochia bracteolata* on liver enzyme in DEN induced hepatocellular carcinoma

Groups	AST (IU L^{-1})	ALT (IU L^{-1})	ALP (IU L^{-1})
Normal control	293±1.45	75±1.57	274±1.71
Exp. control	381±0.84*	125±0.37*	510±0.51*
Std. drug control	295±0.71*^{a}	78±0.73*^{a}	286±5.30*^{a}
Ext. Trt I	301±2.29*^{a}	88±1.66*^{a}	291±3.33*^{a}
Ext. Trt II	298±1.57*^{a}	80±1.30*^{a}	278±2.25*^{ab}
Fr. I Trt III	298±4.26*^{acd}	76±2.63^{abcd}	280±20.91*^{abcd}
Fr. I Trt IV	284±2.88^{abcd}	70±1.98^{abcd}	276±2.11^{abcd}
Fr. II Trt V	342±1.52*^{a}	121±1.71*^{a}	357±6.27*^{a}
Fr II Trt VI	320±1.84*^{a}	94±3.10*^{a}	338±3.48*^{a}

Values are Mean±SEM (n = 6), *Significantly increased (p<0.05) compared to normal control , ^{a}Significantly decreased (p<0.05) compared to negative control, ^{b}Significantly decreased (p<0.05) compared to standard drug control, ^{c}Significantly decreased (p<0.05) compared to Methanol Extract at the same dose, ^{d}Significantly decreased (p<0.05) compared to different fractions at the same dose, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, ME: Methanol extract, FR: Fraction, Trt: Treatment

Table 3: Effect of extract and fractions of *Aristolochia bracteolata* on some non-enzyme biochemical markers of liver disease in DEN induced hepatocellular carcinoma

Group	Bilirubin (umol L^{-1})	Protein (g L^{-1})	Albumin (g L^{-1})
Normal control	3.11±0.03	68±0.01	43±0.84
Exp. control	5.25±0.41*	51±0.51	28±0.45
Std. drug control	3.52±0.11	65±1.44^{a}	42±0.58^{a}
Ext. Trt I	3.68±0.08	64±0.73^{a}	38±0.71^{a}
Ext. Trt II	3.45±0.18	67±1.70^{ab}	43±1.07^{ab}
Fr. I Trt III	3.50±0.28	66±1.10^{acd}	40±0.55^{acd}
Fr. I Trt IV	3.28±0.04	68±2.24^{abd}	45±1.05*^{abcd}
Fr. II Trt V	4.41±0.08*^{bcd}	62±1.69^{a}	35±0.63^{a}
Fr II Trt VI	3.98±0.14*^{bcd}	65±0.51^{a}	40±1.10^{a}

Values are Mean±SEM (n = 6), *Significantly increased (p<0.05) compared to normal control, ^{a}Significantly increased (p<0.05) compared to negative control, ^{b}Significantly increased (p<0.05) compared to standard drug control, ^{c}Significantly increased (p<0.05) compared to methanol extract at the same dose, ^{d}Significantly increased (p<0.05) compared to different fractions at the same dose, ME: Methanol extract, FR: Fraction, Trt: Treatment

Table 4: Effect of methanol extract and fractions of *Aristolochia bracteolata* on antioxidant enzymes in DEN induced hepatocellular carcinoma

Groups	SOD (%)	GR (U L^{-1})	GPx (U g^{-1} Hb.)	TAS (mmol L^{-1})
Normal control	46±0.71	45±1.30	715±1.77	4.81±0.08
Exp. control	23±0.75*	25±0.68*	493±1.16*	2.19±0.09*
Std. drug control	43±0.40*^{a}	38±0.55*^{a}	705±2.48*^{a}	4.57±0.10^{a}
Ext. Trt I	40±0.86*^{a}	38±1.47*^{a}	680±8.63*^{a}	4.00±0.11*^{a}
Ext. Trt II	43±1.00*^{a}	41±2.26*^{ab}	701±1.17*^{a}	4.41±0.07*^{a}
Fr. I Trt III	42±0.86*^{acd}	40±2.35*^{abcd}	710±25.07^{abcd}	4.60±0.17^{acd}
Fr. I Trt IV	45±2.16^{abcd}	44±0.51^{abcd}	720±1.62^{abcd}	4.82±0.11^{abcd}
Fr. II Trt V	34±1.32*^{a}	35±1.66*^{a}	670±3.00*^{a}	3.40±0.16*^{a}
Fr II Trt VI	40±2.52*^{a}	41±2.20*^{ab}	695±1.86*^{a}	3.83±0.22*^{a}

Values are Mean±SEM (n = 6), *Significantly decreased (p<0.05) compared to normal control, ^{a}Significantly increased (p<0.05) compared to negative control, ^{b}Significantly increased (p<0.05) compared to standard drug control, ^{c}Significantly increased (p<0.05) compared to methanol extract at the same dose, ^{d}Significantly increased (p<0.05) compared to different fractions the same dose, SOD: Superoxide dismutase, GTR: Gluthatione reductase, Gpx: Gluthatione peroxidase, TAS: Total antioxidant status, ME: Methanol extract, FR: Fraction, Trt: Treatment

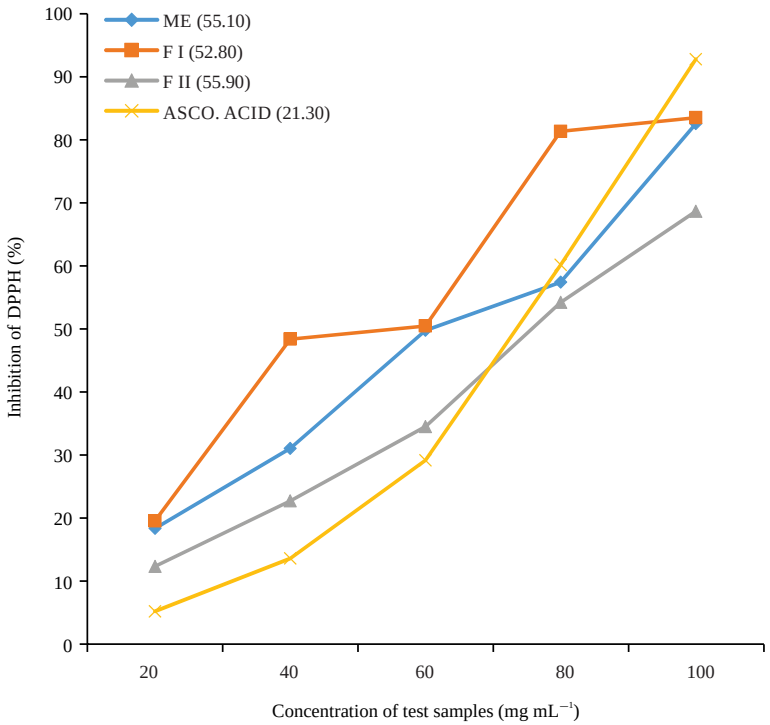

Fig. 1: DPPH radical scavenging activity of *Aristolochia bracteolata* methanol leaf extract and fractions
ME: Methanol extract, FI: Fraction One, FII: Fraction two

Table 5: Effect of methanol extract and fractions of *Aristolochia bracteolata* on hematological parameters in DEN induced hepatocellular carcinoma

Groups	HTC (%)	Hb (g L^{-1})	WBC (cells L^{-1})	RBC (cells L^{-1})	PLT (cells L^{-1})
Normal control	57±0.11	19.28±0.06	11.6±0.05	9.70±0.13	667±2.87
Exp. control	38±0.13*	12.67±0.05*	18.4±0.10	5.91±0.26*	348±1.10*
Std. drug control	56±0.20[a]	18.67±0.10[a]	11.3±0.15	9.52±0.51[a]	668±1.82[a]
Ext. Trt I	52±1.22*[a]	17.34±0.41*[a]	12.7±0.21	8.61±0.14[a]	638±1.32*[a]
Ext. Trt II	54±0.66*[a]	18.02±0.25[a]	12.4±0.19	8.94±0.31[a]	645±5.49*[a]
Fr. I Trt III	55±2.01[acd]	18.33±1.00[acd]	11.8±0.37	9.26±0.14[ad]	652±1.64*[acd]
Fr. I Trt IV	58±1.61[abcd]	19.29±0.24*[a]	11.4±0.11	9.58±0.26[ad]	660±1.59*[ac]
Fr. II Trt V	48±1.08*[a]	16.14±0.36*[a]	13.0±0.27[bd]	8.34±0.12[a]	610±1.068[a]
Fr II Trt VI	51±0.65*[a]	17.03±0.27*[a]	12.8±0.17	8.78±0.30[a]	625±2.10*[a]

Values are Mean±SEM (n = 6), *Significantly decreased (p<0.05) compared to normal control, [a]Significantly increased (p<0.05) compared to negative control, [b]Significantly increased (p<0.05) compared to standard drug control, [c]Significantly increased (p<0.05) compared to Methanol Extract at the same dose, [d]Significantly increased (p<0.05) compared to different fractions at the same dose, HTC: Heamatocrit, Hb: Heamoglobin, WBC: White blood cell, RBC: Red blood cell, PLT: Platelets, M: Methanol extract, FR: Fraction, Trt: Treatment

Aristolochia bracteolata. More so, WBC was found to be significantly (p<0.05) increased in experimental control (18.4±0.10 cells L^{-1}) compared to normal control as presented on the table. However, treatment with methanol leaf extract, fraction I and fraction II significantly ameliorated these changes in hematological parameters, in a dose dependent manner with 200 and 400 mg kg^{-1} b.wt., fraction I, showing the most effect.

The DPPH radical scavenging activity of *Aristolochia bracteolata* methanol leaf extract and fractions was presented in Fig. 1. The DPPH activity was found to be dose dependent in methanol extract and chloroform extract with the highest activity observed at 100 mg mL^{-1}. The IC_{50} for the methanol extract, aqueous fraction and chloroform extract are 55.1, 55.9 and 52.8 mg mL^{-1}, respectively. Similarly, ferric reducing antioxidant power of *Aristolochia bracteolata* methanol leaf extract and its fractions were presented in Fig. 2. Aqueous fraction produced 50 % of antioxidant activity with a concentration of 56.3 mg mL^{-1}.

The photomicrograph of the liver sections was shown in Fig. 3(a-i). There were gross liver cell damages following treatment with DEN as seen in plate A2. Subsequent treatment

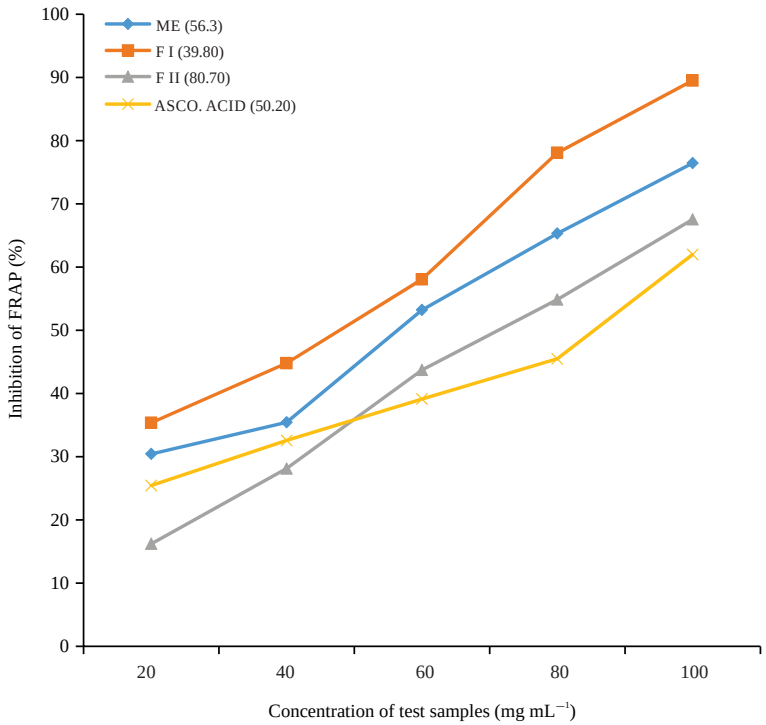

Fig. 2: Ferric reducing antioxidant power of *Aristolochia bracteolata* methanol leaf extract and fractions
ME: Methanol extract, FI: Fraction One, FII: Fraction two

with Silymarin (standard drug) and the methanol leave extract and fractions of *Aristolochia bracteolata* shows less liver cell damages, preserved hepatic architecture, mild fibrosis and focal hyperchromasia and a near normal architecture of the liver (Plate A3 and A9, respectively) compared to normal control animals.

DISCUSSION

Plants were identified as useful sources of natural antioxidants that can protect against oxidative stress and therefore, have a main role to protect against injuries from lipid peroxidation[30]. Antioxidants are substances that protect living cells from the damages caused by free radicals. The antioxidative effect is mainly related to their bioactive compounds such as phenols, flavonoids, tannins, saponins and alkaloids. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which plays an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides[31]. One of the important roles of antioxidants is to inhibit the chain reaction of lipid peroxidation[32]. The presence of significant amount of alkaloids, flavonoids, saponins, tannins and phenols in the methanol leaf extract of *Aristolochia bracteolata* confirmed its use as medicinal plant as reported by Periyasamy and Mahalingam as revealed in Table 1 of this study[31,33].

The use of DPPH radical provides an easy, rapid and convenient method to evaluate antioxidant and radical scavenging potentials of plants products[34]. The antioxidant activity of medicinal plant is mainly related to their bioactive compounds such as phenols and flavonoids. In this study, the methanol extract, aqueous and chloroform fractions of the plant were able to show free radical scavenging activities. In Fig. 1 DPPH radical was used as a stable free radical to determine the antioxidant property of natural compounds and the scavenging of stable radical (DPPH) was considered a valid and easy assay to evaluate scavenging activity of antioxidant[35]. The results of this study indicates that antioxidant activity were found to be significantly ($p<0.05$) higher in aqueous fraction compared to chloroform fraction and methanol extract (Fig. 1). The increased formation of free radicals was associated with the increase in lipid peroxidation. One of the important roles of antioxidants is to inhibit the chain reaction of lipid peroxidation[32].

In FRAP (Fig. 2) method the total antioxidant activity exhibited by the extracts and fractions is revealed. The antioxidant activities has been attributed to the various mechanisms such as prevention of chain initiations, the binding of transition metal ion catalysts, decomposition of

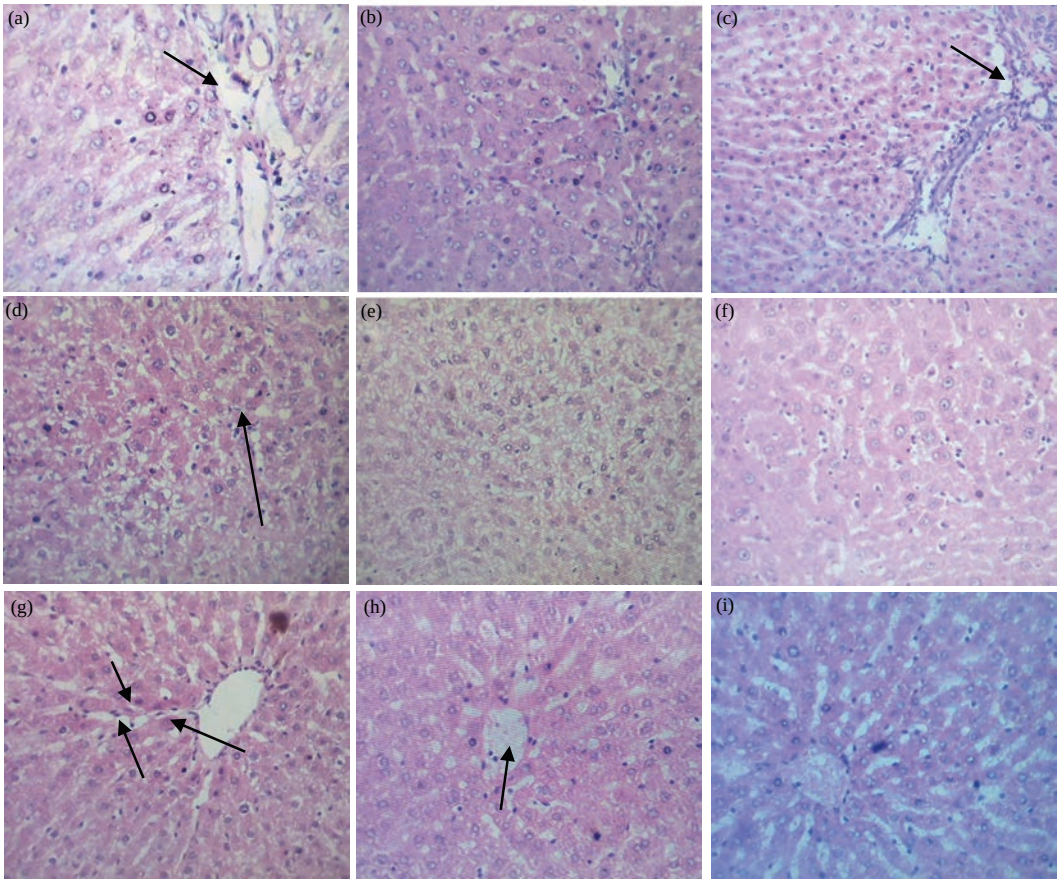

Fig. 3(a-l): Photomicrograph of the liver histological sections shown at 10x magnification

(a) Normal control showed normal architecture of the liver, the portal triad (b) DEN induced and untreated section showed gross liver cell damage, (c) section from group three (3), treated with standard drug showing less liver cell damage, (d) Section of rats treated with 200 mg kg^{-1} b.wt. methanol extract. This section showed marked destruction of hepatocytes and fibrosis, (e) DEN + 400 mg kg^{-1} b.wt. methanol extract showing improved hepatic architecture, (f) This section shows mild fibrosis and focal hyperchromasia, (g) Liver section of rats treated with 400 mg kg^{-1} b.wt. of fraction one (1) showing preserved hepatic architecture (arrow) similar to the control group and (h, i) Shows sections of the liver of rats treated with 200 and 400 mg kg^{-1} b.wt. of fraction II, respectively. There was a near normal architecture compared to normal control animals

peroxides, the prevention of continued hydrogen attraction, the reductive capacity of radical scavenging and the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[36,37]. The findings in this study was in consonance with earlier reports by Evans *et al.*[38,39] on the use of plant as an antioxidant agent[40].

Biochemical marker enzymes are used to screen particularly liver diseases for differential diagnosis, prognosis, monitoring the progress and for assessing the response to therapy[41]. These enzymes are unique and changes in their activities reflect the effect of proliferation of cells with growth potential and its metabolic turnover. The rise in their activities is shown to be a good correlation with the number of transformed cells in liver damage. The result of this shows that administration of DEN in rats led to significant elevation of all liver enzymes markers alanine amino transferase, aspartate amino transferase and alkaline phosphatase as seen in (Table 2). The findings of the present study (Table 2) showed that DEN administration (group two) caused significant liver impairment by causing the plasma levels of cytosolic and mitochondrial enzymes (aspartate transaminases, alanine transaminases and alkaline phosphatase) and bilirubin to significantly increase, compared to the normal control group. This is as a result of tissue damage that leads to leakage of these marker enzymes into the plasma [42]. Hepatocellular injury is assessed by serum increase of aspartate transaminases and alanine transaminases activities. Aspartate transaminases is directly associated with the conversion of amino acids to ketoacids and is increased in the tumor condition[43]. Inflammation or obstruction of the biliary tract (Cholestasis) is indicated by an elevated alkaline phosphatase as revealed in the experimental group[44]. However, oral post treatment of rats

with standard drug Silymarin improves all these liver marker enzymes, similarly, the treatment of rats with the extract of *Aristolochia bracteolata* also shows an improvement. Meanwhile appreciable and significant improvement were observed in rats treated with 200 and 400 mg kg^{-1} b.wt., of both methanol extract and fraction I, respectively in all liver marker enzymes when compared to normal group (Table 2). This was mainly due to the phytochemicals present in these extract and fractions, most especially fraction I (alkaloids, phenols and flavonoids) which may have synergistically scavenged the free radicals generated as a result of DEN administration.

In Table 3, DEN induced hepatocellular carcinoma are characterized by decrease in total protein and albumin similar to the findings of who also observed decrease in serum level of total protein in hepatocellular carcinoma bearing rats as compared to normal rats[45,46]. Bilirubin concentration was increased in DEN administered rats due to the disturbance in the transport function of the hepatocytes as a result of hepatic injury causing the leakage of enzymes from cells as a result of altered permeability of membrane[47]. The observations of the present study is in accordance with earlier reports of Nagaraja *et al.*[45], Arirudran *et al.*[46]. This observed derangement is as a result of hepatotoxicity which leads to hepatocellular damage consequently causing defective protein biosynthesis in the liver. The decrease in albumin is due to the fact that, DEN intoxication causes disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reducing the biosynthesis of protein [46]. In line with this finding, there was a significant decrease in the levels of serum bilirubin and significant increase in total protein and albumin in the liver of rats treated with *Aristolochia bracteolata* methanol extract and fractions. The observed improvements in treated animals is as a result of the plant's ability to scavenge the free hydroxyl and peroxyl radicals which interfere with protein biosynthesis. Decline in the activities of liver antioxidant enzymes (Table 4) such as superoxide dismutase, glutathione reductase, glutathione peroxidase and total antioxidant status shown by the result of this study is in agreement with earlier studies of Patel *et al.*[11] Surendran *et al.*[48]. Superoxide dismutase, glutathione reductase, glutathione peroxidase and total antioxidant status enzymes are important scavengers of superoxide ions and hydrogen peroxide. These enzymes prevent generation of hydroxyl radicals and protect the cellular constituents from oxidative damage. Earlier studies regarding mechanism of DEN induced hepatotoxicity have shown that glutathione peroxidase plays a key role in detoxifying the reactive toxic metabolites of DEN and that liver necrosis begins when antioxidant enzyme stores are markedly depleted[48]. The protective properties of *Aristolochia bracteolata* against DEN induced oxidative stress in rats might be attributed to the antioxidant (alkaloids, flavonoids, tannins, reducing sugars and saponins) present in *Aristolochia bracteolata* which can prevent oxidative changes in composition of the membrane phospholipids, hepatic glutathione depletion and improve the functional markers of liver damage[49].

The significant decrease of hematological indices in DEN treated group (Table 5) have been attributed to DEN toxicity and its direct effect on hematopoietic system as a result of tumor necrosis factor alpha (TNF-α) blunting the physiological effect of erythropoietin and interfering with the ability of the body to store iron[50]. On the other hand, WBC count increased in DEN treated group signifying liver cell damage which has weakened immune system as reported by Gnanaraja[51]. The effect of this fraction (fraction I) as observed on hematological indices may be due to the presence of phenols, flavonoids and alkaloids in the fraction which may inhibit the activity of TNF-α, this could be responsible for the reversal of the damage done to erythropoiesis stimulation site[52].

The photomicrograph (Fig. 3a-i) of DEN induced hepatocellular injury in the liver biopsies indicate hepatocytes that are marked with polymorphs at focal points, cellular hyperplasia and loss of lobular architecture, dilatations of hepatic sinusoids (Plate A2) with kupffer cell hyperplasia. These observations were in close conformity to the findings reported by Youssef *et al.*[53]. Similar findings were reported by Mohammed *et al.*[54], who showed that treatment with DEN leads to vacuolated hepatocytes with fatty change, dilated blood sinusoids, massive portal leucocytic infiltration and disordered arrangement of dysplastic hepatocytes with typical hyper chromatic nuclei. This healing effect is as result of the antioxidant, anti-inflammatory and anti-tumor effects of *A. bracteolata.*

CONCLUSION

Generally, this study has shown the administration of methanol leaf extract of *Aristolochia bracteolata* and its fractions shows healing effect on the liver cells in a dose dependent manner, with significant healing effects seen in aqueous fraction. This occurs possibly through the anti-proliferative, antioxidant and anti-inflammatory effects of this natural product by maintaining the hepatocellular membrane integrity.

SIGNIFICANCE STATEMENT

To our best knowledge, this study is the first of its kind to describe the healing effect of methanol leaf extract of *Aristolochia bracteolata* and its fractions in diethylnitrosamine induced hepatocellular damage. This study shows that the fractions and extract of *Aristolochia bracteolata* can be seen a novel pharmacological and therapeutic agent in hepatocellular damage. However, further study is needed to understand the exact mechanism of action of the fractions and extract of *Aristolochia bracteolata* in this research work.

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