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Original Research Article

Comparative Hepatoprotective Activity of *Vernonia amygdalina* Leaf Solvent Extracts in Alloxan-Induced Diabetic Rats

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ABSTRACT

This study evaluated the comparative hepatoprotective property of *Vernonia amygdalina* leaf solvent extracts: (aqueous (AQE), ethanol (ETH), methanol (METH), toluene (TOL), and benzene (BENZ) in alloxan-induced diabetic rats. The study consisted of forty Wistar albino rats segregated into eight groups of five rats each (n=5); the negative control (NC), diabetic control (DC), and standard (STD) were designated Group 1, Group 2, and Group 8 respectively. Groups 3-8 designated as AQE, ETH, METH, TOL, BENZ, and STD respectively were induced with 120 $mgKg^{-1}$ body weight alloxan. After induction, hyperglycemic rats were treated with 250 $mgKg^{-1}$ body weight *V. amygdalina* leaf aqueous, ethanol, methanol, toluene, benzene extract, and metformin 200 mg/Kg b. wt. via intubation for 14 days. At the end of the 14 days administration, serum enzyme activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activity, total bilirubin concentration, and histopathology of liver tissues were examined using standard methods. The result indicated a consistent increase in body weight among diabetic rats within the study period; except for the benzene extract treated group. ALT, AST, and ALP activity in the groups receiving aqueous extract, ethanol, toluene, and benzene extract was significantly ($p<0.05$) reduced compared to the diabetic control group. Histopathological examination results indicate a reversal of degeneration of the liver tissues by methanolic and ethanolic extract treatment. The overall results showed that the methanol extract presented stronger protection against diabetes-induced hepatic damage in rats compared to the other solvent extracts.

Keywords: *Vernonia amygdalina*, hepatoprotection, liver function, diabetes milletus, alloxan

Introduction

Diabetes Mellitus (DM) is a heterogeneous syndrome of impaired protein, lipids, and carbohydrate metabolism, attributed to abnormal insulin secretion by β-islets of Langerhans in the pancreas (type I DM), insulin resistance/action (type II DM) or combination of both of these factors.[1,2] β-islets cells destruction/dysfunction results from a combination of genetic factors, environmental factors, and Autoimmune disorders.[3] Diabetes ranks as the most prevalent metabolic disorder among lifestyle diseases; an important predisposing factor associated with the development of macrovascular and microvascular diseases like myocardial infarction and stroke, neuropathy, retinopathy, nephropathy, and delayed wound healing.[4] Diabetes mellitus poses a global disease burden of about 425 million people living with the disease;5 estimates project the figure may increase to over 700 million by 2045.[6]

Diabetes Mellitus alters biomolecular metabolism; resulting in pathological derangement of liver, kidneys, and pancreas function.[7,8]

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The associated abnormalities in carbohydrate metabolism and insulin synthesis elicit hyperglycemia with symptoms such as polyphagia, polydypsia, polyuria, glucosuria, and lethargy.[9] Diabetic mortality and morbidity arise from associated macrovascular degeneration in older people.[10] Several other factors such as hyperlipidemia and antioxidant depletion are implicated in the pathogenesis of diabetes and attendant complications.[11] A variety of therapeutic approaches have been applied, including dieting and the use of oral hypoglycemic agents. Currently, available management options involve monotherapy or a combination of drugs to achieve better glycemic control. The incidence of type 2 diabetes mellitus is widely managed using Metformin, a biguanide antihyperglycaemic agent, which normalizes glycemia.[9] Recently, advocacy for the application of readily available plant products in diabetes management has been on the increase; this is attributable to cost-effectiveness and reduced side effects. Consequently, herbal remedies have been widely researched to provide alternative or complementary drugs or adjuvants to improve diabetes management.[12] *V. amygdalina* is a shrub of the Asteraceae family. It typically thrives in the African tropic; attaining a height of 2–6m at maturity with petiolate small evergreen leaves. Leaf shapes are elliptic with a diameter of about 6 mm. It is widely known as "bitter leaf" due to its astringent bitter taste associated with anti-nutritional factors and phytochemicals.[13] In Nigeria, it is commonly used for its culinary and medicinal benefits and is known by names such as Onugbu, Ewuro, Chusar doki, and Etidot in Igbo, Yoruba, Hausa, and Efik languages.[14,15] *Vernonia amygdalin*a leaf, stem, and bark are locally used in ethno-medicine for the management of fevers, stomach discomfort, diabetes, malaria, hiccups, and as a laxative.[16]

The plant has been studied for its bioactive compounds and pharmacological properties; the works of Farombi and Owoeye,[14] and Ojimelukwe and Amaechi,[17] reported the phytochemicals profile to

include alkaloids, saponin, phenolic acids, flavonoids and sesquiterpenes et.c. Epivernodalol, a sesquiterpene lactone isolated from *V. amygdalina* reportedly possess anticancer activity.[18] The hypoglycemic potency has been earlier reported.[19,20,21] Hypolipidemic, hepatoprotective, antihelminthic, anti-inflammatory, and antioxidant activities are important reported properties of *V. amygdalina* leaf.[20,22-25] The reports of Ejiofor *et al.*,[19,25,26] showed that the methanolic extract of the stem-bark possesses higher antidiabetic and anthelmintic effect than the leaf and root extracts. Differences in the polarity of the solvent are known to affect the number of extractive substances dissolved in the sample extract.[27,28,29] The antidiabetic and hypolipidemic effectiveness of the leaf extract in alloxan-Induced diabetic rats have been reported to be in the order: methanol > ethanol >benzene > aqueous > toluene.[21] These findings prompted us to undertake the present study on the comparative hepatoprotective activity of *Vernonia amygdalina* leaf solvent extracts in alloxan-induced diabetic rats.

Materials and Methods

Chemicals/Reagents
Alloxan monohydrate was obtained from Sigma-Aldrich Mo USA, Ethanol and Methanol (JHD, China), toluene, and Benzene (JHD, China), sodium chloride (Molychem, India), Metformin (Hovid Hong Kong), ALT, AST, ALP, and bilirubin test kits were obtained from Randox Laboratories, United Kingdom. All other chemicals and reagents used in the study were of analytical grade and obtained from reputable sources.

Plant materials
Fresh leaves sample of V. amygdalina Delile was collected in May, 2021 from Anguldi, Zawan Bukuru metropolis, Plateau State, Nigeria. The leaf was authenticated by Mr. O. E. Agyeno of University of Jos, Nigeria, Plant Science and Biotechnology Department, Faculty of Natural Science. The sample was assigned voucher number: JUHN21000359, and deposited at the herbarium unit of the Department.

Experimental animals
Forty (40) healthy male Wistar rats weighing 80-120 g (averaging 8-9 weeks old) were used in this study. They were acquired from Animal friend facility and Veterinary Services, Owerri, Imo State. The acclimation of the rats in steel cages to the animal house of the Biochemistry Department Laboratory, Federal University of Technology, Owerri was for two weeks. They were kept at controlled conditions of temperature (25 ±2ºC), relative humidity (55 ± 5%), and 12hrs daylight throughout the experiment. The rats accessed normal rat chow pellets (Vital Finisher, Nigeria) and portable water ad libitum, but were starved for 12 hr prior to commencement of the experiment. The experimental design and protocol was assessed and approved by the ethics committee of Federal University of Technology, Owerri. Nigeria with approval number BIOSC-BCH-EC-021. Treatment of the animals strictly followed the National Institutes of Health (NIH) guide 1985, Principles of Laboratory Animal Care. Plant use followed the guidelines indicated by the plant variety protection act, 2021.

Preparation of extract
The plant materials were sorted, washed, and shed-dried at room temperature for about 7 days. The sample was pulverized to powder using a mill (Kenwood BL357). Then about 1000g of dry coarsely powdered sample was extracted with polar solvents (methanol, ethanol, and toluene) in a Soxhlet extractor. Similar portion of the ground plant sample was extracted with 2.5 L of benzene and distilled water by maceration for 48 hours with intermittent shaking after every 6 hours and sieved; subsequently filtered with Whatman No 1 filter paper. The filtrates were heated in a water bath at 50^0C and dried in a vacuum desiccator.[30]

Experimental design
Intraperitoneal (I.P) injection of 120 mgKg-1 body weight alloxan monohydrate ($C_4H_4N_2O_5$) in a single dose was used in the induction of diabetes mellitus in the experimental rats, group 1 was designated normal control.[31] The injected rats were allowed for seven (7) days with food and water *ad libitum* and fasted on the 8th day for 12 hours prior to blood glucose determination. Blood glucose level was measured using a Glucometer (Lifescan, Johnson & Johnson, California, USA). Possession of fasting blood glucose concentration > 200 mg/dl was used as inclusion criteria for selecting diabetic animals for the study. The selected rats were further segregated into eight groups comprising of five (5) animals in each group (n=5) and administration regimen are shown in Table 1. All treatments were given orally to experimental rats using a gavage tube in a single dose daily for 14 days, and accessed food and portable water ad libitum.[21]

Measurement of body weight
The rat's body weights were obtained using a weighing balance (Salter, Model 250). Animals were weighed on the 1st, 3rd, 5th, and the last day of the experiment. The weight gain/loss was obtained by subtracting the weight on the first day (initial body weight).[32]

Biochemical analysis
Twelve hours after the last feeding and treatment (overnight fasting), all the rats were humanely sacrificed under light anesthesia using dichloromethane. Blood sample was obtained through a cardiac puncture, placed in plain tubes for 30 minutes to clot, and transferred into a centrifuge tube. The blood was then centrifuged for 10 minutes at 3000 rpm (30 ºC) to facilitate separation; the serum was recovered and stored for biochemical assays.[32]

Assessment of liver function parameters
The parameters aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activities, and bilirubin were assessed as indices of liver function.

Table 1: Grouping and experimental design for alloxan-induced diabetes study

Group	Treatment	Group name
1	1.0ml Distilled water	Normal control (NC)
2	Alloxan (120 mgKg^{-1} b.wt. I.P)	Diabetic control (DC)
3	Alloxan (120 mgKg^{-1} b.wt. I.P) + 250mg kg^{-1} b.wt.) *V. amygdalina* Aqueous extract	AQ
4	Alloxan (120 mgKg^{-1} b.wt. I.P) + 250mg kg^{-1} b.wt.) *V. amygdalina* ethanol extract	ETH
5	Alloxan (120 mgKg^{-1} b.wt. I.P) + 250mg kg^{-1} b.wt.) *V. amygdalina* methanol extract	METH
6	Alloxan (120 mgKg^{-1} b.wt. I.P) + 250mg kg^{-1} b.wt.) *V. amygdalina* toluene extract	TOL
7	Alloxan (120 mgKg^{-1} b.wt. I.P) + 250mg kg^{-1} b.wt.) *V. amygdalina* Benzene extract	BENZ
8	Alloxan (120 mgKg^{-1} b.wt. I.P) + 200 mg kg^{-1} b.wt. Metformin	STD

Serum AST and ALT activities were determined according to the methods of Reitman and Frankel,[33], The test principle is based on ALT and AST catalytic transfer of the amino group from L-alanine to a-ketoglutarate and from L-aspartate to a-ketoglutarate resulting in the formation of pyruvate/L-glutamate and oxaloacetate/L-glutamate pair respectively. ALT and AST activity in the serum sample was measured as 2,4-dinitrophenylhydrazone in an alkaline medium. Serum ALP and bilirubin were assayed by the methods described by Englehardt, [34] and Jendrassik & Grof, [35] respectively. ALP catalyzes the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol with the simultaneous release of a phosphate group. The rate of reduction of p-nitrophenylphosphate measured by determining change in absorbance at 405nm is directly proportional to the ALP activity. Determination of total bilirubin is based on the reaction of diazotized sulphanilic acid with bilirubin in the presence of caffeine, which is measurable spectrophotometrically at 578 nm.

Histopathology

Histopathology of liver tissues of all the groups was assessed for evidence of histopathological changes as described by Abebe and Gebru, [36]. The tissues were fixed with 10% formal saline, and successively dehydrated with ethanol of 30%, 50%, 70%, 90%, and absolute alcohol at intervals of 1hr, 2hrs, and 3hrs respectively. The tissue samples were immersed in xylene and paraffin wax for clearance and embedding; and sectioned to thickness of about 3–6 µm. The staining was done with Hematoxylin and Eosin (H & E), examined and captured under a light microscope fitted with a digital camera (Nikon, ECLIPSE, TS100, Japan).

Statistical analysis

The results are expressed as mean ± standard deviation of five (n=5) determinations; statistical analysis of groups was done using one-way analysis of variance (ANOVA) with a statistical software (Statistical Package for Social Sciences, version 20). The Turkey and Duncan homogeneity of variance test was used to test for statistical significance of means at $p<0.05$

Results and Discussion

The present study examined the hepatoprotective activity of different solvent extracts of *V. amygdalina* in alloxan-induced diabetic rats. Hyperglycemia is mostly caused by insulin resistance. It affects lipid, carbohydrate, and protein metabolism; resulting in non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinomas.[37,38] The diabetogenic property of alloxan is mediated by oxidative stress which causes β-islets cell necrosis and dysfunction. The increase in oxidative stress and abnormal inflammatory response activate pro-apoptotic genes; harm hepatocytes, resulting in diabetes-induced liver damage.[37]

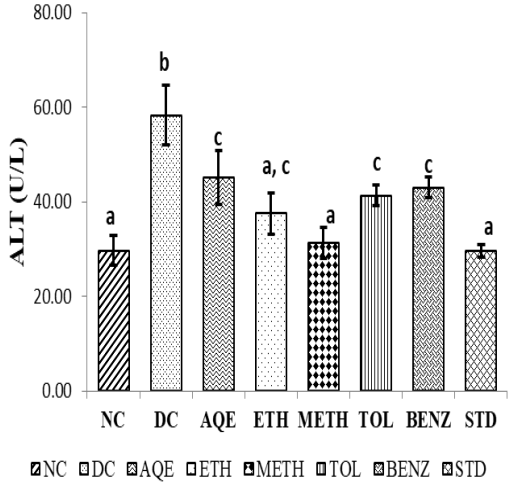

Figure 1: Serum ALT activity

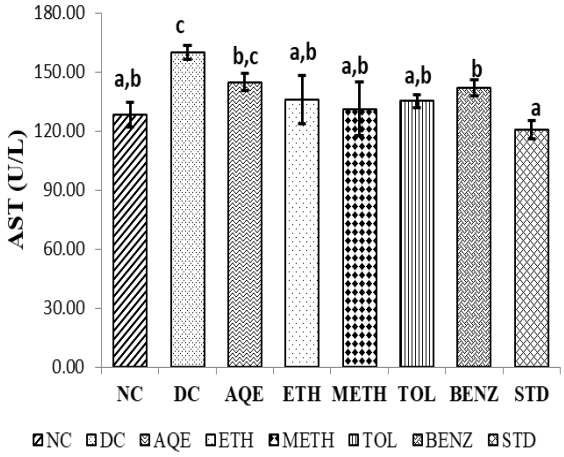

Figure 2: Serum AST activity

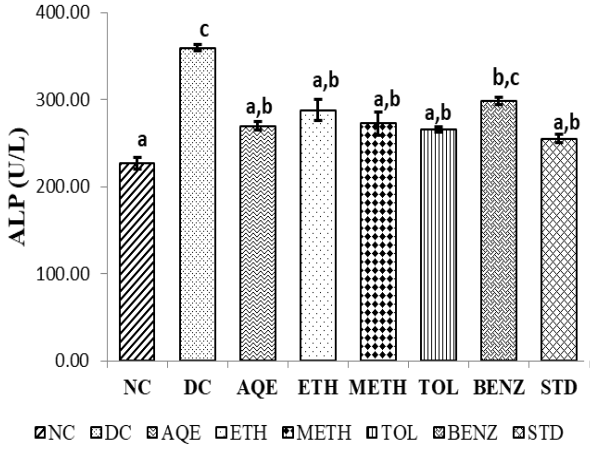

Figure 3: Serum ALP activity

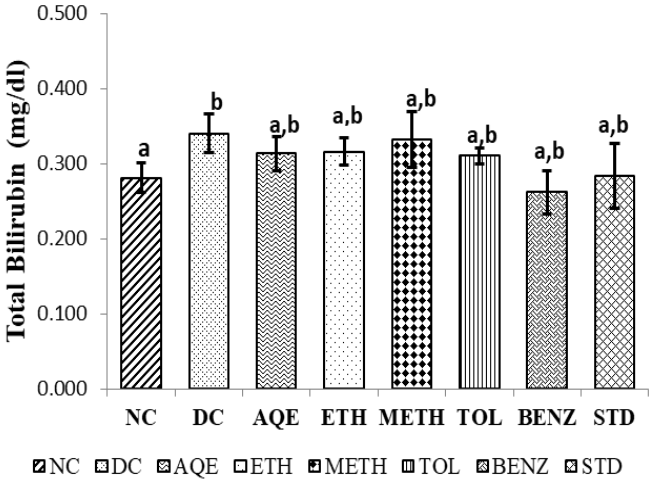

Figure 4: Serum total bilirubin concentration

Figure 1: Effect of *V. amygdalina* leaf solvent extracts (aqueous, ethanol, methanol, toluene, and benzene) treatment on liver function indices in alloxan-induced diabetic rats. Bars bearing different superscripts are significantly different ($p<0.05$).

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Table 2: Effect of *V. amygdalina* leaf aqueous, ethanolic, methanolic, toluene, and benzene extract administration on body weight in alloxan-induced diabetic rats

	Weight Changes (g)							
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 11**	**Day 14**	**Weight gain (%)**
NC	113.60 ± 9.18	122.30 ± 10.91	130.10 ± 9.66	134.00 ± 8.58	139.45 ± 6.96	144.21 ± 15.88	148.1 ± 17.75	30.28^{a}
DC	86.40 ± 10.06	89.50 ± 6.30	100.90 ± 13.56	99.80 ± 11.45	102.43 ± 8.09	108.89 ± 5.44	115.23 ± 9.22	33.36^{a,b}
AQE	54.75 ± 3.59	62.30 ± 22.11	74.25 ± 28.17	103.30 ± 11.15	81.67 ± 7.35	83.6 ± 6.69	84.55 ± 8.0	34.74^{a,b}
ETH	88.80 ± 3.27	89.90 ± 3.21	102.50 ± 3.67	107.80 ± 5.52	110.34 ± 5.52	118.02 ± 7.08	124.12 ± 18.65	39.64^{b}
METH	92.40 ± 10.53	97.70 ± 10.60	97.90 ± 9.76	112.20 ± 12.40	118.23 ± 8.28	121.56 ± 8.51	125.48 ± 11.20	35.80^{a,b}
TOL	116.45 ± 8.73	119.20 ± 9.95	135.45 ± 12.69	140.45 ± 13.89	134.65 ± 6.73	139.89 ± 11.19	143.67 ± 15.25	23.37^{c}
BENZ	151.45 ± 10.40	128.78 ± 13.19	148.20 ± 10.41	149.45 ± 7.93	129.33 ± 6.47	118.33 ± 7.10	116.54 ± 10.14	-4.04^{d}
STD	169.84 ± 13.57	173.10 ± 19.81	179.70 ± 17.42	190.90 ± 19.12	131.33 ± 5.25	136.67 ± 8.20	141.49 ± 9.48	28.81^{c}

Values are mean ± standard deviation of 4 determinations. Difference in superscripts across columns shows statistical significant difference ($p<0.05$)

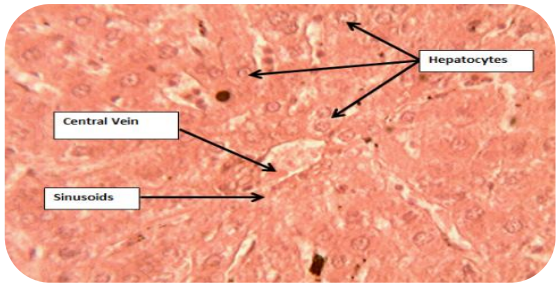

Plate 1: Normal control group (NC)

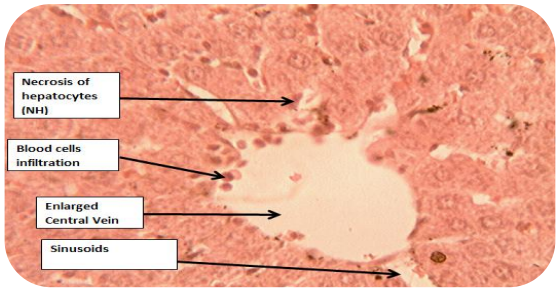

Plate 2: Diabetic control group (DC)

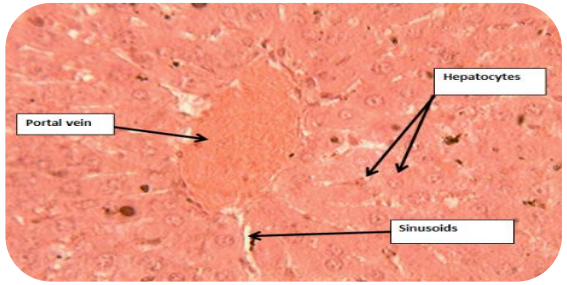

Plate 3: Aqueous extract group (AQE)

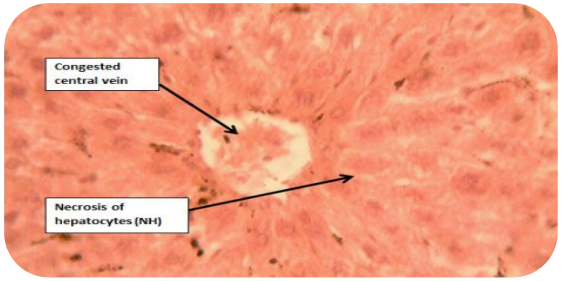

Plate 4: Ethanol extract group (Eth).

Figure 2: Photomicrograph of the liver tissue sections (H & E, x100).

The diseased rats treated with the different solvent extracts of *V. amygdalina* showed a steady increase in body weight within the study period; with the exception of the benzene extract treated group (Table 2). Analysis of the mean body weight changes presenetd in Table 2 indicate that the diabetic control, aqueous, ethanol, and methanol extract treated rats significantly ($p<0.05$) gained weight and matched normal animals. *V. amygdalina* benzene extract caused a drastic decrease in body weight after treatment for 7 days; these changes in the mean body weights of the rats were not normalized in the course of the study duration. A net weight loss of -4.04% was seen in the benzene extract treated group after the 14 days treatment period. The reduction in mean body weight may be ascribed to the cytotoxicity of benzene or phyto-constituents soluble in the solvent. The results obtained on body weight changes corroborate the report of Aldahmash *et al*.,[39] They reported insignificant elevation in body weight of untreated diabetic mice, biotin-treated, and control animals. Body weight loss and dehydration have been linked to diabetes mellitus.[40] The impact of the extract on body weight suggests that the different solvent extract from *V. amygdalina* leaf except for the benzene extract might be beneficial in maintaining the body mass during diabetes progression. Obesity and hyperlipidemia are co-morbidities of diabetes mellitus. The consumption of vegetable-rich diets provides dietary fiber, phenolic antioxidants, and low calories important in combating diabetes mellitus and obesity.[41] The contrast in body weight changes with that reported in the work of Pupim *et al*.,[40] may be linked to differences in the duration of the study.

Earlier studies reported that hyperglycemia and hyperlipidemia characterized alloxan-induced diabetes in rats.[21] In the current study, alloxan-induced diabetes mellitus was accompanied by significant ($p<0.05$) derangement in serum hepatic marker enzymes (Figure 1). Serum ALT, AST, and ALP activity was significantly ($p<0.05$) elevated in the serum of untreated diabetic rats (Figure 1). The increase in this serum marker enzyme activity in untreated diabetic rats may be associated with the destruction of hepatocytes by oxidative stress. Notably, the ethanol and methanol extract of *V. amygdalina* leaf administration caused a reduction in ALT activity; however, only the methanol extract compared to the standard drugs (metformin). The aqueous, toluene, and benzene extracts showed drastically reduced ALT activity compared to the diabetic control group. Similarly, the ethanolic, methanolic, and toluene extract demonstrated the ability to reduce AST and ALP activity. These changes in AST and ALP activity were comparable to that of standard drug treated rats. However, the various studied solvent extracts of *V. amygdalina* leaf did not normalize the altered serum total bilirubin concentration. These results agree with histopathological findings from the liver section of the diabetic and normal rats. The photomicrograph of untreated diabetic rat liver showed sections with an enlarged central vein, infiltrated inflammatory cells, enlarged sinusoids, and necrosis of the hepatocytes (NH) (Figure 2: Plate 2). However, the photomicrograph of diabetic rats liver of group treated with 250mg/Kg b.wt *V. amygdalina* leaf aqueous and ethanol extract (Figure 2: Plate 3 and 4 respectively) showed mild degenerative changes evidenced in congested central veins, areas with mild necrosis of the hepatocytes (NH) with intact sinusoids. While the aqueous extract treated group showed signs of hepatocyte regeneration compared to diabetic control.

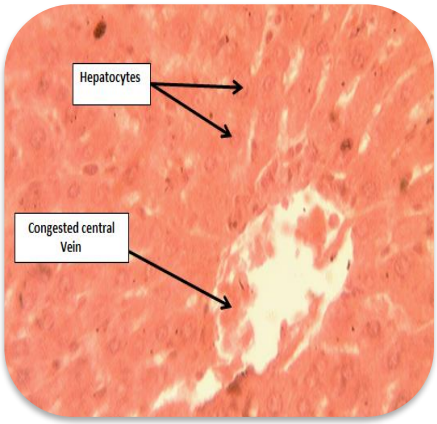

Plate 5: Methanol extract group (METH)

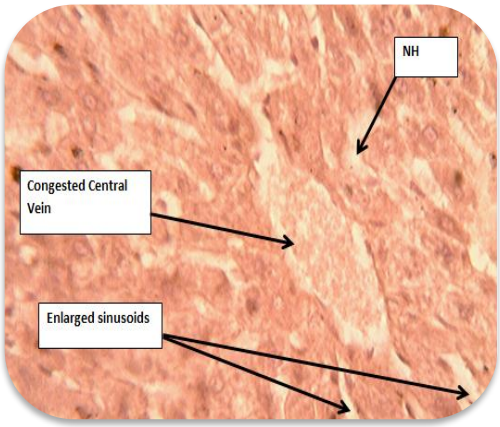

Plate 6: Toluene extract group (Tol)

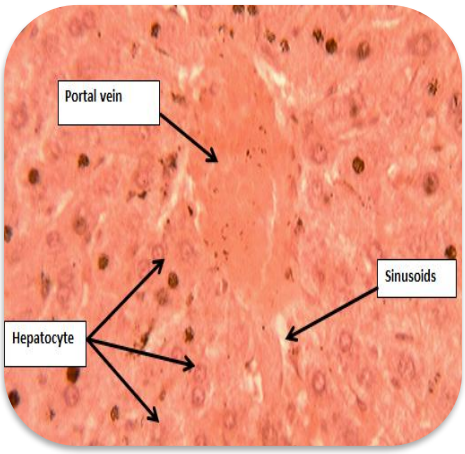

Plate 7: Benzene extract group (BENZ)

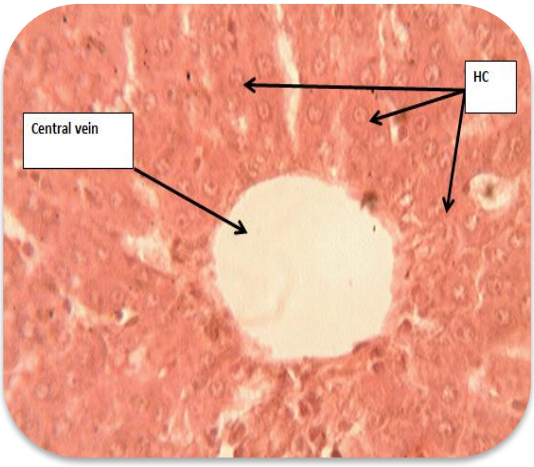

Plate 8: Standard group (STD).

Figure 3: Photomicrograph of the liver tissue sections (H & E, x100).

In addition, the photomicrograph of diabetic rats liver treated daily with 250mgKg-1 b.wt *V. amygdalina* leaf methanol extract showed congested central veins and areas with intact sinusoids. However, the liver tissue showed signs of recovery with the regeneration of hepatocytes when compared to diabetic control (Figure 3: Plate 5). Furthermore, the liver section photomicrograph of diabetic rats treated with 250 mg/Kg b.wt. *V. amygdalina* leaf Toluene and Benzene extract showed a section of the liver with mild degenerative changes, congested central veins, areas with mild necrosis of the hepatocytes (NH), and enlarged sinusoids (Figure 3: Plate 6 and 7 respectively). While photomicrograph of the liver section of our diabetic rats treated daily with 200mg/Kg b.wt metformin showed a section of the liver with apparently normal architecture. The morphological features seen were in line with that of a normal liver, central vein, portal sinusoid, and hepatocytes were evident (Figure 3: Plate 8). The liver section of untreated diabetic rats showed degenerative changes such increased inflammatory cells around the central vein, enlarged sinusoids, and necrosis of the hepatocytes. Notably, the aqueous, ethanolic, and methanolic extracts of *V. amygdalina* showed potency in the regeneration of hepatocytes which is an evidence of liver cell recovery. The regeneration of liver cells was comparable to those of normal liver tissues (Plate 1). Histopathological examinations characterize organ injury patterns and recovery.[42] The liver maintains and manages homeostasis; it is involved in immune regulation and essential pathways for disease recovery. Thus an optimally functioning liver is required for the overall well-being of an individual.[43] Hepatic enzymes are responsible for the catalysis and regulation of DNA replication and metabolism of xenobiotics. Elevated ALT activity accompanied by AST activity are specific marker for hepatic dysfunction.[43] These enzymes leak into circulation from hepatocytes upon injury; thereby causing a rise in their serum activity.[43] In the progression of diabetes, the liver is frequently damaged due to increased oxidative stress and dysregulation of immune function.[45] Increased serum bilirubin concentrations are linked to impaired bilirubin metabolism involving metabolic disturbances in the liver and the manifestation of type II diabetes milletus co-morbidities.[46,47] The hepatoprotective potential of the *V. amygdalina* leaf extract observed in our study was in the order methanol > ethanol > aqueous > toluene > benzene. The higher hepatoprotection of the methanol extracts may be attributed to polarity and soluble phytochemicals extracted by the solvent.[28] The polarity of the extracting solvents relative to water has been ranked as water > methanol > ethanol > benzene > toluene. An equal mixture of water with any of the solvents: methanol, ethanol, and acetone is reportedly optimal for the extraction of phenolics.[28,48] Recent studies have demonstrated the anti-diabetic properties of *V. amygdalina in vitro* and *in vivo*.[21,49] The hepatoprotective potentials seen in our work corroborates with earlier findings in the works of Momoh *et al.*, [50,] Enemali and Stanley, [51] Tokofai *et al.*,[52] reported effective hepatoprotection evidenced in reduced serum liver function enzymes activity and oxidative stress markers.

Conclusion

The present study assessed the hepatoprotective potential of V. amygdalina leaf aqueous, ethanolic, methanolic, toluene, and benzene extract in alloxan-induced diabetic rats. The overall results showed that the different studied solvent extracts have varying degrees of protection against hepatic cell damage. However, the V. amygdalina methanol extract when compared to the other solvent extracts effectively ameliorated diabetes-induced liver damage in rats.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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