**Vernonia amygdalina** Leaf Extract Abates Oxidative Hepatic Damage and Inflammation Associated with Nitrobenzene in Rats

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**Abstract**

Liver diseases have been documented to have great influence on the global burden of mortality and morbidity. This study was conducted to investigate the ability of *Vernonia amygdalina* to protect against hepatic damage and inflammation in nitrobenzene rat model of hepatotoxicity. Thirty male Wistar strain albino rats were used for this study. Rats were exposed to 100 mg/kg body weight (BW) of nitrobenzene via oral administration and treated with 200 and 400 mg/kg BW of methanol leaf extract of *Vernonia amygdalina* (MLVA) and 400 mg/kg BW of Vitamin E for 14 consecutive days. Nitrobenzene significantly (P<0.05) induced hepatic damage with marked serum level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), acid phosphatase (ACP) and alkaline phosphatase (ALP). Furthermore, nitrobenzene mediated oxidative stress and lipid peroxidation with a significant increase in hepatic level of malondialdehyde (MDA), hydrogen peroxide (H₂O₂), with concomitant decrease in level of reduced glutathione (GSH), Catalase (CAT) and Superoxide dismutase (SOD). Similarly, inflammation was observed in nitrobenzene-treated rats with elevated level of nitric oxide (NO) and myeloperoxidase (MPO). However, treatment with the chosen doses of MLVA and Vitamin E significantly reversed all the nitrobenzene-associated hepatic damage, oxidative stress, lipid peroxidation, inflammation and altered antioxidant defence system. Taken together, MLVA exhibited hepatoprotection which may be beneficial for the treatment and management of liver diseases or other related disorders via protecting the structural integrity of the liver, antioxidant, anti-inflammatory mechanisms.

**Keywords:** Hepatotoxicity, oxidative stress, Inflammation, Nitrobenzene, *Vernonia amygdalina*

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**1. Introduction**

Liver diseases have been reported to have huge impact on the global burden of mortality and morbidity (Lozano et al., 2010; Murray et al., 2010). In 2010, Global Burden of Disease (GDB) documented that liver cirrhosis caused more than one million deaths (1,030,800 deaths representing 2.0% of all deaths, 1.4% of all deaths of women and 2.4% of all deaths of men) and 31,027,000 Disability Adjusted Life Years (DALYs) (1.2% of all DALYs, 0.8% of all DALYs for women, 1.6% of all DALYs for men). Systematic analysis of Mokdad et al., (2014) also documented that about 2 million deaths annually are caused by liver diseases. In the study, hepatocellular carcinoma and viral hepatitis caused about 1 million deaths while complications of cirrhosis were responsible for about 1 million deaths globally. The Middle East, Caribbean, Latin America and North Africa are the regions that showed the highest percentage of deaths due to liver related diseases (Briggs, 2003).

Among other factors, exposure to toxic chemicals from industrial and occupational sources has been implicated to play a pivotal role in the aetiology and pathogenesis of liver diseases (Briggs, 2003). Hepatotoxins are toxic foreign compounds capable of damaging the liver structural and physiological functions leading to severe adverse effects on the liver. Nitrobenzene is an example of such hepatotoxics; it is an important chemical material widely applied in national defence and the industries of printing and dyeing, plastics, pesticide and pharmaceutical. Nitrobenzene is considered a dangerous air pollutant and has proven to be an animal carcinogen. According to the 1986 Cancer guide lines, nitrobenzene has been classified as a group B2 chemical, i.e. a likely human carcinogen (Cattley et al., 1994). Intermediates such as nitrosobenzene and phenylhydroxylamine (PH) are produced during metabolism of nitrobenzene. These intermediates have been documented to play a pivotal role in the process of nitrobenzene carcinogenesis (Howard et al., 1983). Following accidental nitrobenzene poisoning in humans, the highest concentration was found in the liver, brain, blood and stomach (International Programme on Chemical Safety, 2003). Akinloye et al (2014) have documented the hepatotoxicity effects of nitrobenzene.

Phytochemicals are naturally occurring compounds regarded as one of the important origins of biologically active natural products (Koksal et al., 2009). The reported biological and pharmacological activities such as anticarcinogenic, anti-inflammatory, and antioxidant, and
antimutagenic activities of these isolated active compounds from plants have awaken research interest towards development of more potent drugs to combat various diseases (Wu et al., 2008; Yang et al., 2006). The bioactive compounds that have been recorded to be present in a number of plants may produce medicinal effects for in treatment of reproductive related disorders.

*Vernonia amygdalina*, commonly known as bitter leaf, is a medicinal plant with several health benefits. The documented phytochemical constituents of various fractions of *Vernonia amygdalina* includes epivernodalol, sesquiterpene lactones, elemanolide (Erasto et al., 2006), edotides (Izevbige et al., 2003), terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones and anthraquinone (Cimanga et al., 2004), saponins and alkaloids (Muraina et al., 2010). These phytochemical constituents have been reported to be responsible for a number of medicinal values of the plant such as antimicrobial activities (Iwu et al., 1999), antioxidant properties (Adesanoye and Farombo, 2010), and anti-inflammatory (Ibrahim et al., 2010). The anti-malarial activity of *Vernonia amygdalina* essentially against *P. falciparum* was reported to be due to the presence of sesquiterpenes lactones compounds which include vernolide, vernodal, hydroxy vernolides and the steroid related constituents, vernoniosid B1 and vernonoid B1 (Magboul et al., 2008).

Therefore, the aim of this study is to investigate the cytotoxic effects of nitrobenzene in the liver of Wistar albino rats via evaluation of liver function tests, oxidative hepatic damage markers histopathological indices and cytoarchitecture of the hepatic cells. Moreover, to investigate the protective effect of methanol leaf extract of *Vernonia amygdalina* against the nitrobenzene-induced hepatic damage. Vitamin E, a standard clinical medicine, is used to compare the protective activities of the extract.

2. Materials and methods

2.1. Chemicals/ Reagents

High purity (> 99.7%) Nitrobenzene was obtained from BDH chemical Poole England. Vitamin E (Alpha Tocopherol) is a product of Embassy pharmaceuticals, Nigeria. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), acid phosphatase (ACP) and alkaline phosphatase (ALP) enzyme diagnostic kits were obtained from Randox. Potassium iodide, Copper sulphate, hydrogen peroxide, KCl, phosphate buffer salts: Na2HPO4 12H2O and 1.19g NaH2PO4 2H2O are of analytical grade and were obtained from Analar BDH Limited, Poole, England. Bovine Serum Albumin (standard), 5, 5’ – dithiobis - (2-nitrobenzoic acid) (Ellman’s reagent), epinephrine, reduced glutathione, 2 – nitro-5-thiobenzoic acid are products of Sigma-Aldrich Co. St Louis, Missouri, USA.

2.2. Collection of Plant Material and Preparation of Extract

Fresh leaves of *Vernonia amygdalina* were collected at the staff quarters in Kings University, Ode-Omu, Osun state, Nigeria. The leaf has been identified at IFE-Herbarium of Botany Department, Obafemi Awolowo University, Ile-Ife with voucher number. The *Vernonia amygdalina* leaves were washed and air-dried at room temperature in the Biochemistry laboratory, Kings University, Nigeria and pulverized using an electric blender. The powdered leaf was defatted in n-hexane using Soxhlet apparatus. Thereafter, methanol extract was prepared by soaking the defatted leaf in 90% methanol for 72 hours. The resulting mixture was then filtered, and the filtrate was concentrated on water bath. The concentrated extract was lyophilized using Bosch freeze drying machine. The full chemical identification and bioactive compounds of *Vernonia amygdalina* have been earlier reported by Oladunmoye et al. (2019) using gas chromatography-mass spectrometry (GC-MS). The technique reveals methyl-2-O-benzyl-d-arabinofuranoside, phytol, hexadecanoic acid, ethyl ester, squalene and 9,12,15 octadecatrienoic acid as the more abundant compounds (>85% abundance) while N-[2-(dimethylamino)-5-pyrimidinyl] benzene sulfonamide, 9,12,15 and octadecatrien-1-ol, p-Menth-(8)-en-9-ol has the less abundance (<12% abundance) compounds.

2.3. Experimental animals

Thirty matured 4-5 months old male Wistar strain albino rats were used in the study. The rats were sourced and raised at the Biochemistry breeding colony of the Biochemistry unit, Department of Chemical Sciences, Kings University, Ode-Omu, Osun state, Nigeria. Animals were kept under ambient standard conditions (25 ± 2 °C and relative humidity of 50 ± 15 %) in stainless steel cages, and metabolic wastes were cleaned twice daily. The rats were allowed to aclimatize to these conditions for fourteen days and were exposed to 12 hrs daylight and darkness cycle, fed with commercially available rat pellet and water *ad libitum*. The experiment was carried out in accordance with current rules and guidelines that have been established for the care of the laboratory animals (NRC, 2011). The rats were randomised into five groups containing six rats each.

**Group A:** received distilled water daily and serve as the Control.

**Group B:** received 100 mg/kg Nitrobenzene orally.

**Group C:** received 100 mg/kg Nitrobenzene and 200 mg/kg *Vernonia amygdalina*

**Group D:** received 100 mg/kg Nitrobenzene and 400 mg/kg *Vernonia amygdalina*

**Group E:** Received 100 mg/kg Nitrobenzene and 400 mg/kg Vitamin E

Treatments were administered to the rats orally for 14 consecutive days.

2.4. Preparation of Serum

The rats were sacrificed 24hrs after the last treatment and blood sample collected into clean, dry centrifuge tube. The blood was left for 10 min at room temperature to clot after which it was centrifuged at 4,000 rpm for 20 min in an MSC (Essex, UK) bench centrifuge. The clear supernatant (serum) was aspirated using a Pasteur pipette into clean, dry sample bottles and then stored at 4 °C for biochemical analyses.

2.5. Preparation of liver homogenates

The livers were immediately excised and blotted to remove blood stains. They were cleansed and rinsed in 1.15% KCl on ice to remove haemoglobin, then weighed. They were then chopped into bits and homogenized in four volumes of the homogenizing buffer (10 mM potassium phosphate buffer, pH 7.4) using a Teflon homogenizer.
The homogenates were centrifuged at 12,500 g for 15 minutes in a cold centrifuge (4 °C) to obtain the post mitochondrial fractions which were collected and used for biochemical analyses.

2.6. Measurement of biochemical markers

AST, ALT, ACP and ALP were measured by following the enzymes kits manufacturer’s instructions (Randox). The protein content of the homogenates was determined using BSA as a standard in the protocol described by Lowry et al. (1951). Nitric oxide (NO) level was assessed by procedure reported by Green et al. (1982). Myeloperoxidase (MPO) activity in the homogenate was quantified following the method of Granell et al. (2003). Superoxide dismutase (SOD) activity was evaluated following the inhibition of adrenaline auto-oxidation in a basic milieu as described by Misra and Fridovich, (1972). Lipid peroxidation was evaluated by monitoring the level of MDA using procedure reported by Varshney and Kale (1990). The reduced GSH content in the brain samples was determined using the protocol reported by Buetler et al. (1963). Catalase (CAT) activity was determined following the protocol documented by Clairborne (1995) using hydrogen peroxide (H₂O₂) as a substrate. Hydrogen peroxide generation was assayed oxidation of ferrous ions and sorbitol colour amplification system using the method of Wolff (1994).

2.7. Histological Examination

The livers were immediately fixed in 10% formalin and embedded in paraffin wax. Fine sections (7–9 mm thickness) of the livers were then dewaxed in xylene, hydrated in decreasing percentage of alcohol and stained with hematoxylin and eosin. The stained sections were observed under a Leitz microscope and their photomicrograph taken at X 100 with a Canon (Meville, NY) Power Shot G2 Digital Camera (Oladele et al., 2017).

2.8. Statistical Analysis

Results obtained were expressed as mean ± standard deviation (mean ± SD) and analysed using one-way analysis of variance (ANOVA) with the aid of SPSS 22.0 computer software package (SPSS Inc; Chicago, U.S.A) to compare the experimental groups followed by Bonferroni’s post-hoc test. Valves at P<0.05 were considered significant.

3. Results

3.1. MLVA enhances hepatic enzymes activities in nitrobenzene-induced hepatotoxicity in rats

Figures 1 and 2 show that rats exposed to 100 mg/kg body weight of nitrobenzene (group B) showed a significant increase (P<0.05) serum concentration levels of ALT, AST, ACP and ALP as compared to the control (group A). This suggests injury to the liver membrane and alteration to liver physiological activities. These altered valves were reverted significantly (P<0.05) toward normal in a dose dependent manner in rats treated with 200 and 400mg/kg body weight of MLVA or vitamin E (group C, D & E respectively).

Figure 1: Effect of MLVA on aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in rats treated with nitrobenzene.

Data are given as mean ± SD of rats per group. n=6. MLVA: methanol leaf extract of Vernonia amygdalina, *: Values differ significantly from group A (control) (P < 0.05). #: Values differ significantly from group B.

Figure 2: Effect of MLVA on acid phosphatase (ACP) and alkaline phosphatase (ALP) activities in rats treated with nitrobenzene.

Data are given as mean ± SD of rats per group. n=6. MLVA: methanol leaf extract of Vernonia amygdalina, *: Values differ significantly from group A (control) (P < 0.05). #: Values differ significantly from group B.

3.2. MLVA inhibits inflammatory activity in liver of rats treated with nitrobenzene

The effects of MLVA on inflammation in liver of the experimental rats was evaluated by measuring MPO activities and NO concentration level. Rats administered nitrobenzene alone demonstrated a marked increase in NO level and MPO activities when compared with the control (Figure 3 and 4). However, treatment with of 200 and 400 mg/kg of MLVA or Vitamin E significantly attenuated both NO and MPO levels in the liver when compared with control.

Figure 3: Effect of MLVA on myeloperoxidase (MPO) activity in rats treated with nitrobenzene.

Data are given as mean ± SD of rats per group. n=6. MLVA: methanol leaf extract of Vernonia amygdalina, *: Values differ significantly from group A (control) (P < 0.05). #: Values differ significantly from group B.

Figure 4: Effect of MLVA on nitric oxide (NO) concentration in liver of rats treated with nitrobenzene.

Data are given as mean ± SD of rats per group. n=6. MLVA: methanol leaf extract of Vernonia amygdalina, *: Values differ significantly from group A (control) (P < 0.05). #: Values differ significantly from group B.
3.3. MLVA suppressed lipid peroxidation and oxidative stress in liver of rats treated with nitrobenzene

Fig. 3 and 4 showed the results of hepatic oxidative stress biomarkers carried out in the experimental rats. There was a marked increase in MDA level (an index of lipid peroxidation) and H$_2$O$_2$ generation in rats administered nitrobenzene only when compared with the control group. Upon treatment with 200 and 400 mg/kg of MLVA or Vitamin E, there was a significant decrease (p < 0.05) in both MDA and H$_2$O$_2$ generation levels in the livers.

3.4. MLVA influenced reduced glutathione level and antioxidant enzymes activities in liver of rats treated with nitrobenzene

Fig. 5-7 show the glutathione level and antioxidant activities of CAT and SOD in the liver of experimental rats. Administration of rat with nitrobenzene alone showed a significant decrease (p < 0.05) in GSH level and decline in activities of CAT and SOD when compared with the control. However, treatment with 200 and 400 mg/kg of MLVA or Vitamin E significantly increased the GSH level and enhanced all the enzymes.
3.5. Ameliorative effects of MLVA on histological alterations in liver sections of nitrobenzene-induced hepatotoxicity in rats

Fig. 8 shows the histological alterations seen with the light microscope in the liver sections of the experimental rats. The cytoarchitecture and morphology of liver of rats from control group appeared normal. However, obvious pathological lesions were observed in the liver sections of nitrobenzene group characterized by a mild loss of liver parenchyma, some mild derangement in the cellular profiles, haemorrhage and presence of inflammatory red cells within and around the central vein including the sinusoids as well as distorted hepatic vessels (red arrows). A, C and E group showed no altered panoramic morphological presentation accompanied by well outlined cellular profile as well as distinct hepatic structures.

4. Discussions

Nitrobenzene is an industrial chemical widely used in the synthesis of aniline and other products such as dyes, analgesics, pesticides, pyroxylin compounds and shoe polishes. However, it has been reported to induce tumour in vital organs (including liver) in experimental animals (Cattley et al., 1994), thus classified as a group B2 toxicants which is likely to be human carcinogen (US EPA, 1996). Vernonia amygdalina is a medicinal plant with potential chemo-preventive activities. This study is designed to investigate the protective ability of methanol leaf extract of Vernonia amygdalina against nitrobenzene-induced toxicities. The efficacy of the extract is compared with vitamin E.

In this study, we demonstrated that exposure of rats to nitrobenzene resulted in a marked increase in the serum levels of ALT, ACP, AST and ALP which is indicative of hepatocellular damage, as previously documented (Oladele and Oyewole, 2017; Oladele et al., 2017; Oyewole et al., 2017). The significant increase in the hepatic markers in the serum of the experimental rats could be as a result of release of these compartmentalized enzymes into the blood circulatory system due to rupture of the membrane and cellular damage caused by nitrobenzene (Oladele et al., 2020a; Essawy et al., 2019). Treatment with graded doses of methanol leaf extract of Vernonia amygdalina or Vitamin E significantly reversed the elevated activities of these enzymes. This effect might be due to the extract’s ability to mitigate free radical mediated oxidative damage in the hepatocytes (Alamoudi, 2019). Also, Vitamin E has been reported to have antioxidant and cyto-protective activities (Oladele et al., 2020c).

Biotransformation of nitrobenzene has been reported to generate free radicals and reactive oxygen species which, in turn, alters the antioxidant system and finally results into oxidative stress and macromolecule damage (Akinloye et al., 2014). In this study, exposure of rats to nitrobenzene caused a marked increase in the level of hepatic hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ can be rapidly decomposed into oxygen and water, and this may produce hydroxyl radicals (•OH) that can initiate lipid peroxidation and cause DNA damage (Sahreen et al., 2011). However, treatment with graded doses of methanol leaf extract of Vernonia amygdalina or Vitamin E significantly mitigated the hydrogen peroxide generation in the liver. This can be attributed to the antioxidant and free radical scavenging effects of the extract.

Lipid peroxidation has been reported to play a critical role in cancer development (carcinogenesis) (Banakar et al., 2004). The process produces some byproducts which are highly toxic to the cells. These toxic byproducts include malondialdehyde (MDA) and 4-hydroxynonenal. They can easily attack cellular targets such as proteins and DNA leading to genetic mutations and ultimately to carcinogenicity (de Zwart et al., 1999). In this study, administration of nitrobenzene into rats caused a significant increase in lipid peroxidation as indicated by marked level of malondialdehyde (MDA) in the liver. However, groups treated with graded doses of methanol leaf extract of Vernonia amygdalina or Vitamin E showed
a significant reduction in level of malondialdehyde when compared to the animals treated with nitrobenzene only. The observed decrease in lipid peroxidation in rats treated with methanol leaf extract of *Vernonia amygdalina* could be due to its ability to scavenge the hydroxyl and peroxyl radicals.

Similarly, nitrobenzene induced inflammation in the liver of the experimental rats with evidence of marked increase in the level of nitric oxide (NO) and myeloperoxidase (MPO) activity. This observation indicates involvement of aggravated inflammatory response in nitrobenzene-induced hepatotoxicity. NO is a toxic defense molecule synthesized by inducible nitric oxide synthase (iNOS) in many cell types involved in immunity and inflammation. Treatment the various doses of methanol leaf extract of *Vernonia amygdalina* or Vitamin E significantly inhibited the inflammatory process in the hepatic cells. This agrees with the previous report that the extract has anti-inflammatory properties.

One of the primary functions of antioxidants and free radical scavenging enzymes such as CAT, and SOD is to protect biological cells against free radical attacks and oxidative stress. This study revealed that administration of nitrobenzene to rats caused a significant decrease in these enzymes activities. The observed reduction in these enzymes activities may have been a result of overwhelming detoxification activities of the enzymes by conjugating with the free radicals/ROS and other toxic by-products to enhance their excretion. However, there was a marked increase in CAT, and SOD in rats treated with graded doses of methanol leaf extract of *Vernonia amygdalina* or Vitamin E. Many scientific reports have proven that one of the protective mechanism of actions of plant extracts is via upregulation of these endogenous antioxidant enzymes (Farombi et al., 2019; Oladele et al., 2020b).

Similarly, a depletion in the GSH level was observed in the nitrobenzene treated rats. This significant decrease in GSH level might have been due to GSH usage by the detoxifying enzyme (GST) and may be responsible for increase in lipid peroxidation (Bansal et al., 2018). Free radical mediated tissue damage can be inhibited or alleviated by ensuring the redox balance to decrease the oxidative stress status. On the other hand, rats treated with *Vernonia amygdalina* or Vitamin E display a marked increase in GSH level. This agrees with previous report of Oladele et al. (2020c) who documented that *Vernonia amygdalina* caused a reversal to decreased GSH level induced by nitrobenzene. This suggests that the protective effect of *Vernonia amygdalina* leaf extract involves the maintenance of antioxidant capacity in preventing the hepatic cells against oxidative damage.

5. Conclusion

This study demonstrated that nitrobenzene induced liver injury which was followed by significant increase in serum activities level of ALT, AST, ACP. Also, inflammation was observed in the liver with increased level of NO and MPO with concomitant increased level of MDA confirming lipid peroxidation. Furthermore, there is a significant decreased in reduced GSH level, CAT and SOD activities indicating oxidative stress in the liver tissue. However, treatment with graded doses of methanol leaf extract of *Vernonia amygdalina* reversed all the nitrobenzene-associated hepatic damage, oxidative stress, lipid peroxidation, inflammation and altered antioxidant defense system. Similarly, histological observations showed that the extract was capable of not only preventing but actually reversing the patho-morphological changes of nitrobenzene-induced liver injury such as changes in fat deposition, mild loss of liver parenchyma, haemorrhage and inflammatory cell infiltration. Taken together, methanol leaf extract of *Vernonia amygdalina* exhibited hepatoprotection which may be beneficial for the treatment and management of liver diseases or other related disorders via protecting the structural integrity of the liver, antioxidant, anti-inflammatory mechanisms.

References


