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Hepatoprotective Effects of *Calotropis procera* (Ait.) R. Br Root Bark Extracts against Diethylnitrosamine Induced Liver Injury in Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author ENS performed the extraction as well as the statistical analysis. Authors ENS, JYN, DP and TKT took part in carrying out the anti-hepatotoxic and phytochemical studies. Authors NO, GGO, AH and AT supervised the works. Finally, the drafting of the manuscript was carried out by authors ENS, NO, AH, GGO and AT. All authors read and approved the final manuscript.

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ABSTRACT

Background: Diethylnitrosamine (DEN) is a hepatotoxin whose metabolic activation by liver cytochromes P450 is responsible for the necrosis, mutagenicity and carcinogenicity of liver cells. The purpose of this study was to evaluate the protective effects of *Calotropis procera* roots bark against DEN induced hepatocellular damage in rats.
Material and Methods: Hepatoprotective activity of the ethanolic extract of *Calotropis procera* root

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bark were evaluated by induction of liver injury with DEN in Wistar male rats distributed in six groups of six. Serum hepatic markers, alanine amino transferase (ALAT), aspartate amino transferase (ASAT), alkaline phosphatase (ALP), total protein and albumin were evaluated and the enzymes antioxidant activities, superoxide dismutase (SOD) and catalase, as well as the level of malonedialdehyde (MDA) were determined in the liver homogenate. Histological analysis was carried out on sections of rat livers. Phytoconstituents have also been studied.

Results: Pretreatment of rats with the extract showed a significant decrease in ALAT, ASAT and ALP while there was an increase in total protein and albumin compared to rats treated only with DEN. It also showed a significant increase in SOD and catalase and a decrease in MDA levels suggesting the hepatoprotective effect of the extract. Observation of liver sections confirmed the results of the biochemical parameters which would attest that the extract is hepatoprotective. Phytoconstituents such as sterols, triterpenes and phenolic compounds have been demonstrated.

Conclusion: Ethanolic extract of *Calotropis procera* roots bark has shown hepatoprotective effects that could be due to its content in sterols and triterpenic and phenolic compounds.

Keywords: Calotropis procera; diethylnitrosamine; liver injury; hepatoprotective effect.

1. INTRODUCTION

Liver is an organ known for its various and important functions within the body. It allows the detoxification of drugs and toxic chemicals of natural or synthetic origin ingested by animals or humans [1]. Most of the toxic chemicals (xenobiotics) ingested through the gastrointestinal tract are absorbed by liver cells where they are converted to less toxic substances through enzymatic liver systems for excretion. However, some toxic substances are converted into active metabolites causing hepatocelluar damage. Hepatic viruses, aflatoxins, xenobiotics, alcohol and certain drugs are the essential etiological factors of hepatocellular damage [2]. Diethylnitrosamine (DEN) is hepatotoxin used into experimental animal models to induce hepatocellular damage. Diethylnitrosamine is widely distributed in food products such as cheese, soybean, fish, cured meat, alcoholic beverages as well as in ground water having a high level of nitrates [3,4]. It is also produced from the metabolism of certain drugs [5]. Diethylnitrosamine undergoes biotransformation in the liver by mixed function cytochrome P450 dependent mono-oxidase systems and its metabolic activation is responsible for the onset of its toxic effects [6,7]. DEN activation results in increased production of reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) causing oxidative stress [8]. This oxidative stress is responsible to necrosis, cytotoxicity, mutagenicity and carcinogenicity of liver cells [9]. Hepatocellular damage caused by DEN, carbon tetrachloride (CCl_4), thioacetamide, afflatoxin B_1, alcohol or hepatic viruses such as hepatitis B and C viruses usually results in oxidation of lipids or proteins of the hepatocyte membranes causing increased transaminases (alanine aminotrasferase, aspartate aminotransferase), alkaline phosphatase, bilirubin, creatinin and decreased total protein, albumin in serum; Its cause a decrease liver antioxidant enzyme activities, superoxide dismutase, catalase, glutathione peroxidase and an increase of lipid peroxidation in liver [2].

World Health Organization estimates that more than 80% of the world's population use medicinal plants for treatment [10]. In Burkina Faso, nearly 60-79% of the population uses medicinal plants for their primary health problems [11]. In order to provide a justification for the use of medicinal plants in the treatment of liver diseases and possibly to set up a phytomedicine, an ethnobotanical survey on plants traditionally used in the treatment of liver diseases in Burkina Faso has been carried out. Following this investigation, *Calotropis procera* (Ait.) R.Br (Apocynaceae) was selected for this study. The plant is used traditionally in Burkina Faso in infections treatment, respiratory and cutaneous disorders, mental disorders, epilepsy, witchcraft, hallucinations, loss of consciousness [12,13]. In India, the various organs of the plant are used traditionally to treat various diseases including asthma, leprosy, tumor, bronchitis, ulcer, liver and spleen problems [14]. The purpose of this study was to evaluate the hepatoprotective and antioxidant properties of *Calotropis procera* root bark against diethylnitrosamine-induced liver inflammation in Wistar rats.

2. MATERIALS AND METHODS

2.1 Plant Material

Calotropis procera (Ait.) R.Br roots bark were harvested in Ouagadougou (Burkina Faso) in 2015 using GPS coordinates (12 ° 25'28.2"N; 1 ° 28'0.06"W). They were dried away from the sun and then reduced to powder by a blade mill (Gladiator Est., 1931 Type BN 1 Mach 40461 1083). The authentication of the species was made by Professor Amadé Ouédraogo, botanist at Joseph Ki-Zerbo University. A herbarium of the species has been deposited at the herbarium of the UFR/SVT (Joseph Ki-Zerbo University) under the identification code ID-17033.

2.2 Chemical Reagent

Monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), sodium chloride (NaCl), hydrogen peroxide (H_2O_2), iron dichloride ($FeCl_2$), sodium hydroxide (NaOH) , trichloroacetic acid (TCA), thiobarbituric acid (TBA), sylimarin, sodium carbonate (Na_2CO_3), diethylnitrosamine (DEN), ethanol, epinephrine were obtained from Sigma-Aldrich. They were all of analytical grade.

2.3 Preparation of Extract

To vegetable powder was added ethanol (96%) at ratio of 1:10 (mass/volume) and then the mixture was placed on mechanical stirring for 24 hours at room temperature. The mixture was filtered and the resulting extract was concentrated in a rotavapor and then dries in an oven at 40°C for 48 hours before being stored in a refrigerator at 4°C.

2.4 Phytochemical Investigation

The phytochemical screening consisted of the detection of the main phytoconstituents present in the ethanolic extract of *C. procera* [15]. The following phytoconstituents were investigated:

- Sterols and triterpenes (Liebermann-Burchard reaction)
- Cardenolides (Valger reaction)
- Anthracenosides (Bornträger)
- Coumarins and derivatives (Feigl reaction)
- Flavonoids (Shibata or cyanidin test)
- Anthocyanosides (Bates and Smith reaction)
- Tannins/phenol acid ($FeCl_3$ test)
- Saponosides (Foam index)
- Alkaloids (Dragendorff, Mayer)
- Reducing compounds (Fehling reaction)

2.5 Hepatoprotective Study

2.5.1 Experimental animals

Male and female mice (NMRI strain) with an average weight of 27 ± 2 g and male rats (wistar strain) with an average weight of 180 ± 20g were acquired from the pep shop of MEPHATRA/PH and from the pep shop of the UFR/SVT of the University Joseph Ki-Zerbo for this study.

The rats were placed in groups of six in cages and were acclimated two weeks before the start of the experiment. During acclimation period the animals were fed pellets and water and then kept under the temperature conditions at 23°C ± 2°C, humidity at 60% ± 10% and the light/dark cycle 12h/12h. The dark phase of this cycle began at 12 p.m. and the different experiments always took place from 1 p.m. to 6 p.m., due to the nocturnal activity of the animal (active phase).

2.5.2 Acute toxicity study

Male and female mice were used to evaluate the acute toxicity of extracts according to the method described by the OECD in guideline 423 [16]. The mice were randomized into 2 groups of 3. The mice received ethanolic extract at a single dose of 2000 mg/kg body weight and the control group received distilled water (0.1 mL/g body weight). The observations were made at 30 min, 1 h, 2 h, 24 h, 48 h, 72 h and 14 days after the oral administration of the extracts.

2.5.3 DEN-induced hepatotoxicity

Group I (normal control) and group II (DEN control) received daily oral distilled water for 6 days. Group III (silymarin control) received daily oral 100 mg/kg body weight of silymarin for 6 days. The test groups (IV, V and VI) received daily ethanolic extract at different doses (respectively 50, 100, 200 mg/kg body weight dissolved in distilled water) for 6 days. On day 7, all groups except group I received intraperitoneal DEN at a dose of 200 mg/kg body weight and 24h after DEN administration, all the rats are sacrificed, the blood and liver are collected. The blood is obtained by cardiac puncture in each rat previously anesthetized with ketamine (150 mg/kg body weight).

2.5.3.1 Biochemical parameters

The blood was centrifuged at 3000 g for 15min then the serum was collected to evaluate various biochemical parameters, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), total protein and albumin through kits (LABKIT) according to the manufacturer's instructions.

2.5.3.2 Antioxidant Enzymes and lipid peroxidation inhibition activities

The liver removed is ground to a ratio of 10% in 0.1 M tris buffer (pH 7.4). The homogenate obtained was centrifuged at 12000 g for 15 min and then the supernatant was removed to evaluate enzymatic and non-enzymatic antioxidant activities. In this study, we evaluated the antioxidant enzymes activities of superoxide dismutase (SOD) and catalase as well as the lipid peroxidation inhibition activity by the quantification of malonedialdehyde (MDA).

2.5.3.3 Activity of superoxide dismutase

The activity of SOD was determined by the standard method disclosed by Misraand Fridovich [17]. It is based on inhibition of the transition from epinephrine to adrenochrone by the enzyme. The reaction mixture comprises 0.5 mL of liver homogenate supernatant, 0.5 mL of distilled water to dilute the sample, 0.25 mL of ice cold ethanol and 0.15 mL of chloroform was added to precipitate the reaction mixture. The reaction mixture is stirred well for about 5 minutes at 4 ° C, then centrifuged. The adrenochrone produced in the reaction mixture contains 0.2 mL of EDTA (0.6 mM), 0.4 mL of Na_2CO_3 (0.25 M) and 0.2 mL of epinephrine (3 mM), the volume final was adjusted to 2 mL with distilled water (0.3 mL) then absorbance readings were measured at 420 nm in a UV Visible spectrometer. The transition from epinephrine to adrenochrome was determined by the addition of the required amount of enzyme to assess enzyme activity expressed in units / min / mg of protein.

2.5.3.4 Activity of catalase

The activity of catalase was determined using the standard method given by Beers and Siezer [18]; Degradation of H_2O_2 by addition of the enzyme is followed by absorption of light. The absorption of the peroxide solution in the UV region is determined. The reaction mixture comprises 1.9 ml of phosphate buffer (0.05 M, PH 7), 1.0 ml of H_2O_2 substrate (30 mM) and 0.1 ml of liver homogenate supernatant. Activity was measured as a change in optical activity at a density of 240 nm at oneminute intervals for approximately 3 minutes. Catalase activity was expressed in terms of µmol H_2O_2 consumed / min / mg protein.

2.5.3.5 Activity of lipid peroxidation inhibiton

This activity was determined by the 2-thiobarbituric acid method [19]. This method colorimetrically measures the reaction product of thiobarbituric acid with malondialdehyde (MDA), a by-product of oxidized lipids (red complex). The reaction mixture consisted of 1.0 mL of liver homogenate (10%), 200 µL of PBS buffer, 50 µL of $FeCl_2$ (0.5 mM) and 50 µL of H_2O_2 (0.5 mM). The mixture was then incubated at 37°C for 60 minutes, then 1 mL of trichloroacetic acid (TCA) (15%) and 1 mL of 2-thiobarbituric acid (TBA) (0.67%) were added and the mixture is heated in a water bath at 100 ° C for 15 minutes. Absorbances were read at 532 nm using the spectrophotometer. The inhibition of lipid peroxidation is measured by quantifying the malonedialdehyde formed, which is expressed in µmol MDA/mg of protein.

2.5.4 Histopathological analysis of liver

The liver of each treated animal was removed, weighed and used for histological analysis in the anatomo-pathology laboratory of CHU/Sourou Sanou in Bobo Dioulasso. Liver sections (approximately 0.2 × 0.2 cm) made with the rotary microtome (Leitz 1512) were fixed with 10% formalin for 24 hours and then placed in a paraffin bath. The liver sections were then stained with hematolun-eosin. Finally, the labeled liver slices were subjected to microscopic examination for histological analysis.

2.6 Statistical Analysis

The data were expressed as mean ± SD and they analysed using the one-way analysis of variance (ANOVA), and the differences between the groups were determined using the method of Turkey post hoc test as provided by the Graph Pad Prism version 5.0 Instat software package. P-values less than 0. 05 were considered to be significant.

3. RESULTS

3.1 Phytochemical Investigation

Table 1 shows the results of the test for the detection of secondary metabolites in the ethanolic extract. The test made it possible to demonstrate triterpenes and sterols as well as their glycosilated forms, alkaloids, saponosides, cardiotonic cardenolics, reducing compounds, coumarins and derivatives. The extract contains traces of flavonoids, tannins and phenol acids.

3.2 Hepatoprotective activity

3.2.1 Oral acute toxicity study

The administration of the single dose extract of 2000 mg/kg body weigh showed no evidence of toxicity or death (Table 2). The LD50 of the extract is estimated to be greater than 5000 mg/kg body weight according to the Globally Harmonized Classification System (GHS).

3.2.2 Biochemical parameters

3.2.2.1 ALAT, ASAT and ALP activities

Serum hepatic markers activities of ALAT, ASAT and ALP are shown in Fig. 1. These markers are significantly elevated in DEN control group compared to the normal control group ($P < 0.05$). Pretreatment of the Group III, V, VI respectively with silymarin and the extract at doses 100 and 200 mg/kg body weight for 6 days showed a significant decreased of the serum markers compared to the control group DEN ($P < 0.05$). The ethanolic extract at a dose of 200 mg/kg body weight showed the best liver cell protection activities with ALAT (35 ± 6.5 IU), ASAT (72.33 ± 7.5 IU), ALP (34.5 ± 6.5 IU).

3.2.2.2 Total protein and albumin essay

Total protein and albumin levels in the serum are shown in the figure 2. These results reveal a decrease in the levels of total proteins (49.5 ± 4.5 g/L) and albumin (22.33 ± 4.5 g/L) for the DEN control group compared to those in the normal control group respectively 63 ± 5.56 g/L; 30.33 ± 0.57 g/L ($P < 0.05$). The groups pretreated with sylimarin and the extract at different doses (50, 100 and 200 mg/kg PC) showed an increase in the total protein and albumin levels compared to the DEN control group ($P < 0.05$). The pre-treated group at 200 mg / kg showed the highest levels of total protein (65.25 ± 5.73 g/L) and albumin (38 ± 3.16 g/L).

3.2.2.3 Antioxidant Enzymes and Malondialdehyde activities

Fig. 3 illustrates the antioxidant enzymes SOD and catalase activities as well as MDA activity in experimental rat models. DEN control group showed that the administration of DEN to rats leads to an increase in the level of MDA (140.94 ± 8.97 µmol MDA/10 mg protein) and a decrease in the antioxidant enzymes activity, SOD (101.49 ± 17, 32 nmol SOD/mg protein) and Catalase (12.83 ± 2.88 nmol H_2O_2/min/mg protein) compared to the normal control group with respective values of 57.01 ± 6.53 µmol MDA/10 mg protein ; 173.32 ± 29.22 nmol SOD/mg protein; 47.07 ± 4.72 nmol H_2O_2/min/mg protein. Pretreated groups with silymarin and the extract at doses of 100 and 200 mg/kg body weight showed a rise in SOD activity compared to the DEN control group ($P < 0.001$). Pretreated group at 200 mg/kg body weigh revealed the best SOD activity with a significantly higher value (176.91 ± 34.63 nmol SOD/mg protein) than that of silymarin (138.49 ± 17.86 nmol SOD/mg protein) ($P < 0.001$). Catalase activity revealed that pretreated groups with sylimarin (38.94 ± 5.88 nmol H_2O_2/ min/mg protein) and the extract at a dose of 200 mg/kg body weight (44.37 ± 8.01 nmol H_2O_2/min /mg protein) showed a significant increase compared to the DEN control group, 12.83 ± 2.88 nmol H_2O_2/min/mg protein ($P < 0.01$). Pretreated group with sylimarin and those pretreated with the extract at different doses showed a significantly lower level of MDA compared to the DEN control group ($P < 0.001$). Pretreated group with the extract at the dose of 200 mg / kg body weight showed the best rate of MDA with a value of 66.26 ± 9.08 µmol MDA/10 mg protein.

3.2.3 Histopathological study of liver sections

Histopathological study of the livers in the DEN control group shows the presence of hepatocyte necrosis, inflammatory infiltrates lymphocytic intralobular and vascular congestion which testify to the hepatotoxic effect of DEN (Fig. 4b). However, histopathological study of livers sections pretreated with the ethanolic extract shows an interesting reduction in hepatocyte necrosis, inflammatory infiltrates lymphocytic intralobular which are increasingly reduced when the dose of the extract increases compared to the control group DEN (Figs. 4 d,e,f).

Table 1. Results of the characterization test

Chemical groups	Ethanolic extract
Triterpenes and sterols	+
Cardenolides	+
Anthracenosides	-
Coumarins and derivatives	+
Alkaloids	+
Reducing compounds	+
Flavonoids	+/-
Tannins/ phenols acid	+/-
saponosides	+
Anthocyanosides	-

+ : Presence, - : Absence, +/- : Traces

Table 2. Oral acute toxicity study of the plant extract

Plant	Dose	Mice used	Percent of dead						
			30 min	1h	2h	24h	48h	72h	14 days
C. procera	2000 mg/kg body weigh	Females (6)	00	00	00	00	00	00	00
		Males (6)	00	00	00	00	00	00	00

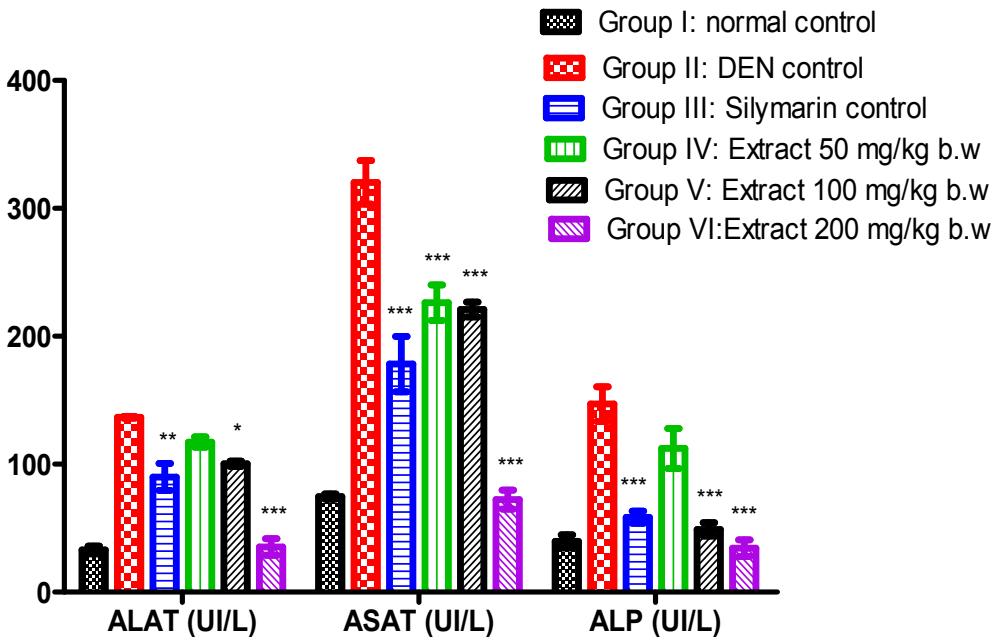

Fig. 1. Effect of ethanolic extract of *Calotropis procera* on serum levels, Alanine aminotransferase (ALAT), Aspartate aminotransferase (ASAT), Alkaline phosphatase (ALP). p> 0.05: the difference is not significant; 0.05 > p > 0.01: the difference is significant *; 0.05 > p > 0.001: the difference is highly significant **; p < 0.001: the difference is very highly significant *. Compared with the DEN control**

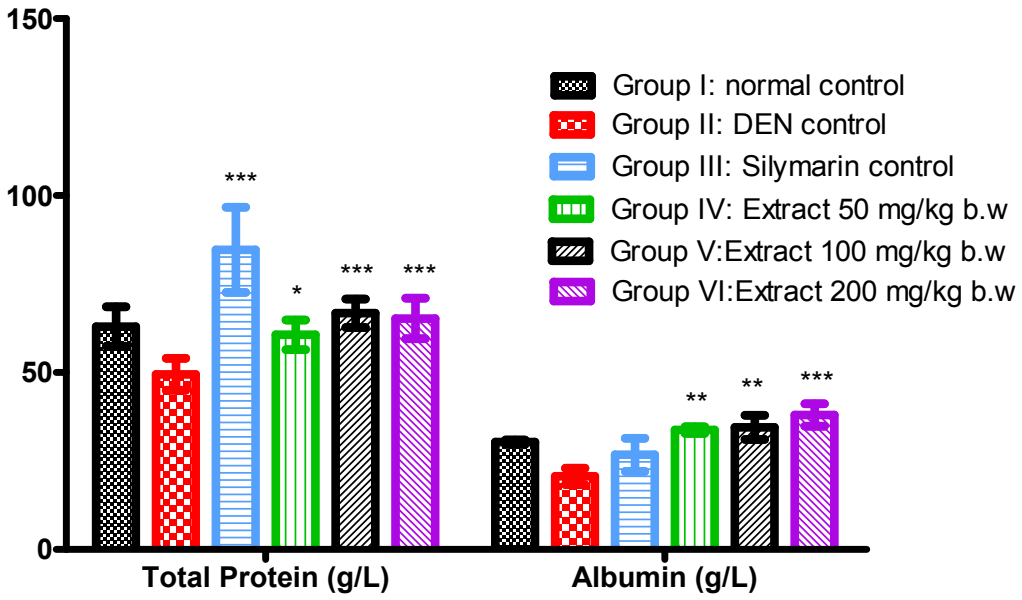

Fig. 2. Effect of extract of *Calotropis procera* on biochemical parameters levels in serum (Total protein, albumin). p> 0.05: the difference is not significant; 0.05 > p > 0.01: the difference is significant *; 0.05 > p > 0.001: the difference is highly significant **; p < 0.001: the difference is very highly significant *. Compared with the DEN control**

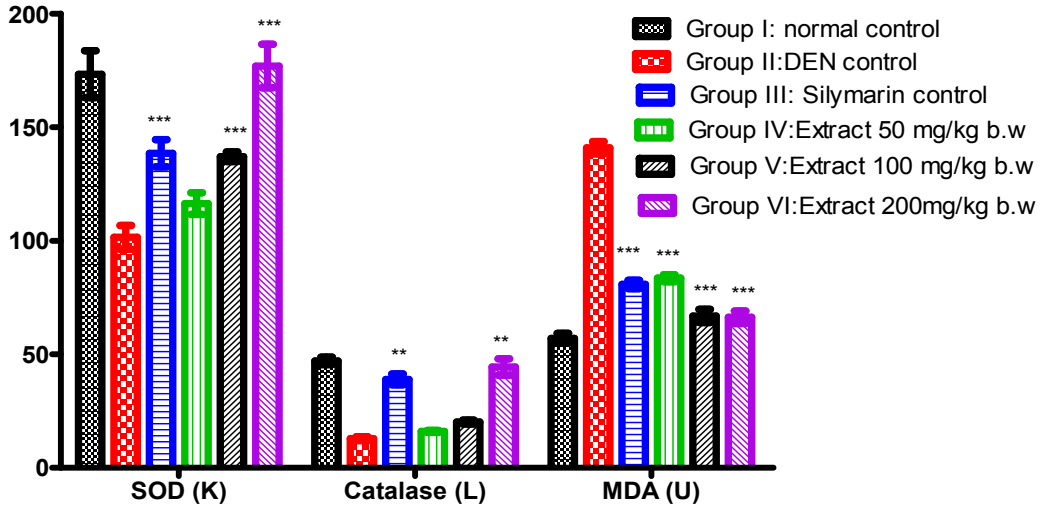

Fig. 3. Effect of extract of *Calotropis procera on* Superoxide dismutase (SOD), Catalase, Malonedialdehyde (MDA). K = nmol SOD/mg protein; L = nmol H_2O_2/min/mg protein; U = µmol MDA/10 mg protein.p> 0.05: the difference is not significant; 0.05 > p > 0.001: the difference is highly significant **; p < 0.001: the difference is very highly significant *. Compared with the DEN control**

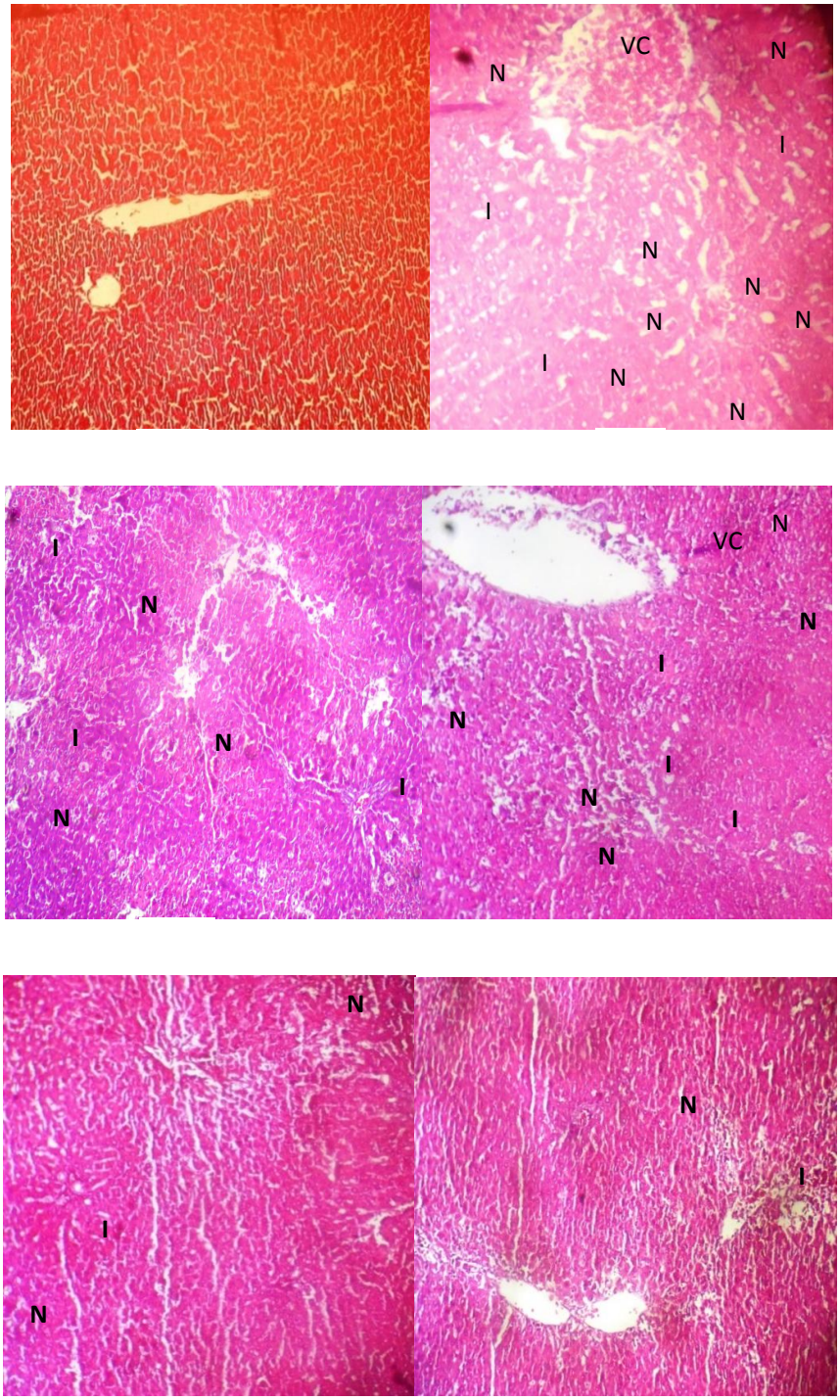

Fig. 4. Histopathological of liver sections pretreated with ethanolic extract at different doses (stained with haematein eosin, seen at magnification 10): (a) group I (normal control), normal liver tissue; (b) group II (DEN control); (c) group III (Sylimarin control); (d) group IV (Extract, 50 mg/mg b.w); (e) group V (Extract, 100 mg/kg b.w); (f) group VI (Extract, 200 mg/kg b.w). N: hepatocyte necrosis; I: Inflammatory infiltrates lymphocytic intralobular; VC: vascular congestion

4. DISCUSSION

Alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase are enzymes present at high concentrations in liver cells. During necrosis or inflammation of liver cells, these enzymes are released into the bloodstream, increasing their levels in the plasma [20]. ALAT, ASAT and ALP are important biomarkers used in the diagnosis of liver diseases [21]. The administration of DEN in rats led to an increase in the level of these biomarkers [22]. The increase in the level of these biomarkers in the serum would imply a necrosis of the hepatocytes caused by the free radicals produced in excess during the metabolism of the DEN. Pretreatments with silymarin and the extract, on the other hand, showed a decrease in the level of these enzymes in the serum compared with the control DEN, suggesting that the extract would have a protective activity against the free radical-induced hepatocyte lysis during hepatotoxicity of DEN. This activity could be due to the triterpenoids and phenolic compounds it contains; But also to sterols that it would contain [23]. Previous studies have reported that triterpenes have hepatoprotective activity against induced liver inflammation in mice [24].

Hepatocellular damage is accompanied by a decrease in plasma protein synthesis in both humans and animals [25]. DEN administration alone to rats resulted in a decrease in total protein and albumin synthesis in serum demonstrating that both of these parameters could be considered useful markers in hepatocyte dysfunction during hepatotoxicity. On the other hand, pretreatments of rats with silymarin and the extract showed a considerable increase in total protein and albumin. This suggests that the extract would promote plasma protein synthesis and thus play an important role in hepatoprotective activity by facilitating the hepatocytes proliferative process [26].

SOD, catalase and glutathione peroxidase are endogenous defense enzymes of the body against reactive oxygen species [9]. SOD converts the highly reactive radical of superoxide into hydrogen peroxide (H_2O_2) which is alternatively metabolized by catalase or peroxidase glutathion to give H_2O and O_2, thus protecting the cell against oxidative damage that could have caused hydrogen peroxide (H_2O_2) and hydroxyl radical [27]. SOD and catalase activities significantly increased in pretreatment groups with sylimarin and the extract at different doses, thus demonstrating their antioxidant capacity. This would also imply that the extract has the ability to maintain SOD and catalase hepatic activities in DEN-induced hepatotoxicity [26]. Triterpenes, sterols and phenolic compounds could be responsible for this activity.

Peroxidative degradation of membrane lipids by reactive oxygen species promotes the formation of malonedialdehyde (MDA) which is an important marker in hepatotoxicity. DEN administration to rats resulted in increased MDA levels in the liver as demonstrated by the DEN control group [28]. However, the pretreatment of the rats with the extract resulted in a significant reduction in the MDA level compared to the rats in the DEN control group. This would demonstrate that the extract has a protective capacity of the structure and function of the hepatocyte membrane. The compounds of the extract would help to significant modulation of DEN metabolism by decreasing the cytochrome P450 activity or neutralize the free radicals formed during DEN hepatotoxicity [28]. Phenolic compounds are known for their hepatoprotective activities [29].

These results obtained were confirmed by the histopathological study carried out on the livers pretreated with the ethanolic extract. They corroborate those of other authors, who have shown that of *Calotropis procera* root barks collected in India, have hepatoprotective properties [30, 23] but are contrary to those of other studies carried out in Nigeria by Dahiru et al. [31] who reported that the ethanolic extract of *Calotropis procera* root bark did not protect the liver or the kidney from the toxic effect of CCl4. The synthesis of secondary metabolites by the plant which is linked to climatic and edaphic factors could explain this difference in results.

5. CONCLUSION

This study demonstrated that the ethanolic extract of *Calotropis procera* root bark possess hepatoprotective effects *in vivo* against diethylnitrosamine-induced hepatotoxicity. The triterpene, sterols and phenolic compounds that the extract contains may be responsible for its hepatoprotective effect. These results could justify the

traditional use of the plant in the treatment of liver diseases.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experiments carried out on experimental animal models have been made in strict compliance with the instructions of the Animal Institutional Ethics Committee (2010/63/EU) on the protection of animals used for scientific purposes. Ethical approval code: 2010/63/EU, date of approval: October 20, 2010.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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