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Original Research Article

HPLC Analysis and Protective Effect of Fractions from Ethanolic Extract of *Calotropis procera* (Ait.) R. Br Root Bark Against Diethylnitrosamine Induced-Hepatic Damage in Wistar Rats

Ernest N. Sombié[1]*, Donzèo G. Somda[3], Soumaila Konaté[4], Ollo Youl[1,4], Tata K. Traoré[3], Jotham Y-P. N'do[1], Wendkouni L.M.E. Belem-Kabré[1], Geoffroy G. Ouédraogo[2,3], Noufou Ouédraogo[2,3], Adama Hilou[1]

[1]*Laboratory of Biochemistry and Applied Chemistry, Department of Biochemistry-Microbiology, Faculty of Science and Technology, Joseph Ki-Zerbo University, Ouagadougou, Burkina Faso*
[2]*Department of Medicine and Traditional Pharmacopoeia/Pharmacy, Institute for Research in Health Sciences, National Center for Scientific and Technological Research, Ouagadougou Burkina Faso*
[3]*Drug Development Laboratory, Department of Pharmacology and Toxicology, Faculty of Health Sciences, Joseph Ki-Zerbo University, Ouagadougou, Burkina Faso*
[4]*National Agency for Environmental, Food, Occupational and Health Product Safety, Ouagadougou, Burkina Faso*

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ABSTRACT

Calotropis procera (Ait.) R.Br (Apocynaceae) is a medicinal plant known for its multiple therapeutic virtues including the management of liver diseases in Burkina Faso. This study aimed to evaluate the hepato-protective properties of the fractions of the ethanol extract of *Calotropis procera* roots barks while identifying chemical compounds in these extracts. Sterols, triterpene and phenolic compounds were sought and quantified in the fractions of the ethanol extract of *Calotropis procera* root bark by HPLC-DAD. The anti-hepatotoxic activity of fractions was evaluated using diethylnitrosamine (DEN) to induce acute liver inflammation in male Wistar rats. Serum biomarkers, alanine transaminase (ALAT), aspartate transaminase (ASAT), alkaline phosphatase (ALP), total proteins and Albumin were evaluated and the activities of antioxidant enzymes (Superoxide dismutase and catalase) as well as the level of malondialdehyde (MDA) were determined in the liver homogenate. The histopathological study of sections of livers pretreated with the fractions was also carried out. The evaluation of the anti-hepatotoxic activity showed that the n-butanol fraction and residual aqueous fraction had best effects on serum biomarkers (ALAT, ASAT, ALP) as well as on the activities of the antioxidant enzymes Superoxide dismutase and catalase and on hepatic tissue. Gallic acid, an antioxidant, anti-hepatotoxic, antifibrotic compound was identified in significant quantities in these two fractions at the respective contents of 17.92 ± 0.26 µg/mg and 18.76 ± 0.27 µg/mg of dry extract, suggesting that this compound would participates in the hepatoprotective properties of these extracts.

Keywords: Liver Diseases, *Calotropis procera*, diethylnitrosamine, phytochemical molecules, anti-hepatotoxic activities

Introduction

Hepatitis is an inflammation of the liver that can be of viral, alcoholic, toxic or immune origin. Liver inflammation can be acute or chronic or even progress to fibrosis, cirrhosis and liver cancer. Viral hepatitis is the most common. Hepatitis B is very prevalent in developing regions and those with large populations, such as Southeast Asia, China, sub-Saharan Africa, and the Amazon basin, where at least 8% of the population is a chronic carrier of HBV.[1] According to the WHO, about 19 million people are infected with chronic hepatitis C in Africa.[2] In Burkina Faso, the prevalence of hepatitis B is estimated at around 9.1% and that of hepatitis C at around 3.6%, classifying Burkina Faso as highly endemic for hepatitis B and low-intermediate for hepatitis C.[3]

*Corresponding author. E mail: ernestsombie@yahoo.fr
Tel: +226 70015626

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Furthermore, aflatoxins, particularly aflatoxin B_1 produced by microscopic fungi (*Aspergillus flavus* or *A. parasiticus*) which develop on food crops following poor drying and storage conditions, constitute potential factors for developing liver cancer in humans.[4] Excessive consumption of alcohol and tobacco are also important risk factors for hepatitis and liver cancer. Indeed, acute and/or chronic inflammation of the liver is accompanied by increased production of reactive oxygen species, activation, and proliferation of macrophages (kupffer cells) which promote the production of pro-inflammatory cytokines (IL-6, TNF-α, IL-8) and profibrotic cytokines (TGF-β), which stimulate hepatocyte lysis. This lysis causes the release of transaminases (ALAT and ASAT) and alkaline phosphatase (ALP), the levels of which increase in the plasma. It also causes an increase in the levels of bilirubin, gamma-glutamyltransferase (GGT), and a decrease in total protein and serum albumin, which are important biomarkers used in diagnosing liver disease.[5] Notable progress has been made in the treatment of hepatitis. As a preventive measure, a vaccine was introduced in 1991 to prevent and limit the spread of HBV, with effectiveness estimated at 95%. For curative treatments, several drugs are offered alone or in combination for over 12 to 24 weeks. However, these treatments remain expensive for poor populations and often have limited efficacy due to poor tolerance of side effects.[6,7] These treatment limitations give considerable interest to the search for natural plant substances.[8] Traditional medicine remains, until today, the main recourse of a large majority of populations to solve their health

problems, not only because it constitutes an important element of the cultural heritage but also for the limited financial means faced with conventional products.[9] In Burkina Faso, about 60 to 79% of the population uses medicinal plants for their primary health problems according to the direction of traditional medicine and pharmacopeia.[10] *Calotropis procera* (Ait.) R.Br (Apocynaceae) is a medicinal plant known for its multiple therapeutic virtues. In Burkina Faso, it is used to manage many diseases, including liver diseases.[11]
In our recent studies, the ethanol extract of *C. procera* root bark showed good protection of hepatocytes against the toxic effect of diethylnitrosamine.[12] In order to better explain the mechanism of action and to know the chemical constituents of this extract, it was necessary to evaluate the anti-hepatotoxic activity of its fractions while identifying phytochemical molecules which could be responsible for these hepatoprotective properties.

Material and Methods

Plant collection
Calotropis procera root barks were harvested in Ouagadougou (12°25'28.2"N; 1°28'0.06"W) in August 2019. They were shade-dried and pulverized into a fine powder using a blade mill (Gladiator Est. 1931 Type BN 1 Mach. 40461 1083). The species has been authenticated by the herbarium of the UFR/SVT of Joseph Ki-Zerbo University under the identification code ID-17033.

Chemical reagent
Ethanol, methanol, acetonitrile, n-hexane, dichloromethane, ethyl acetate, n-butanol, diethylnitrosamine (DEN), ursolic acid, β-sitosterol, stigmasterol, campesterol, rutin, quercetin, kaempferol, gallic acid, vanillic acid, ellagic acid, cafeic acid and sinapic acid were obtained from Sigma-Aldrich. They were all of the analytical grades. Diagnostic kits for estimation of serum alanine transaminase (ALAT), aspartate transaminase (ASAT), alkaline phosphatase (ALP), Total protein, Albumin, were obtained from SpinReact, Spain.

Preparation of plant extracts
Calotropis procera root barks powder (50 g) were macerated with 500 ml of 96% ethanol. The mixture was subjected to mechanical stirring for 24 h at ambient laboratory temperature. The mixture was then filtered, and the extract obtained was concentrated using a rotary evaporator equipped at reduced pressure and then concentrated to dryness in an oven at 40°C for 48 hours before being stored in the refrigerator at 4°C.
The ethanol extract obtained was fractionated using a series of solvents with increasing polarity, n-hexane, dichloromethane, ethyl acetate, and n-butanol. The crude ethanolic extract was initially dissolved in distilled water. This solution was then subjected to a liquid-liquid partition (v/v) 100 mL/100 mL, successively with n-hexane, dichloromethane, ethyl acetate, and n-butanol, using a separatory funnel to obtain the different fractions, respectively. The aqueous phase resulting from the fractionation with the different solvents constituted the residual aqueous fraction. Fractionation was repeated twice with each solvent. The solvents were removed under reduced pressure, then the concentrates were dried in an oven at 40°C.

Animals
Wistar male rats with an average weight of 180 ± 20g were obtained from the MEPHATRA/PH (IRSS) animal facility for this study. They were placed in groups in cages and then acclimatized for two weeks before the start of the experiment. During the acclimatization period, the animals were fed pellets and water and then kept under the conditions of temperature at 23°C ± 2°C, humidity at 60% ± 10%, and light/dark cycle at 12h /12h.

Ethical approval
The experiment was carried out in strict compliance with the instructions of the Institutional animal ethics committee of Joseph Ki-Zerbo University. To this end, approval was obtained from this committee for experimentation on animals under the approval number: CEEA-UJKZ/2020-05

Experimental design
Wistar male rats were randomized into eight (8) groups of six (6) rats each. During the first six days, the animals were treated as follows:
Group I (Normal control) received daily distilled water orally.
Group II (DEN control) received daily distilled water orally.
Group III (Silymarin Control) received daily silymarin orally at a single dose of 50 mg/kg of body weight.
Test groups (IV, V, VI, VII, and VIII) received the different fractions daily, respectively the n-hexane fraction, dichloromethane fraction, ethyl acetate fraction, n-butanol fraction, residual aqueous fraction, all at a single dose of 100 mg/kg body weight orally.
On day 7, all groups except group I (Normal control) receive diethylnitrosamine (DEN) intraperitoneally at a single dose of 200 mg/kg body weight to induce acute liver inflammation. All the rats were left fasting, and 24 h later, they were sacrificed. The blood and the liver were removed. Blood was obtained by cardiac puncture after using ketamine (150mg/kg body weight) as an anesthetic.

Biochemical parameters
The blood collected was centrifuged at 3000g for 15 min then the serum was collected to evaluate the various biochemical parameters, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), total proteins and Albumin through kits. Determining these parameters made it possible to assess the hepatoprotective effect of the extracts.

Antioxidant activities *in vivo*
Part of the sampled liver was ground to a proportion of 10% in PBS buffer. The homogenate obtained was centrifuged at 12000g for 15 min then the supernatant was used to evaluate the enzymatic and non-enzymatic antioxidant activities, including superoxide dismutase (SOD),[13] catalase,[14] malondialdehyde (MDA)[15].

Hepatic histopathological analysis
The other part of the liver was preserved in 10% formalin buffer for histological analysis in the anatomy-pathology laboratory of the CHU/Yalgado Ouédraogo in Ouagadougou. These livers were dehydrated in graduated ethanol solution (50-100%) and cleaned in a solution of xylene. They were then fixed in paraffin. Liver sections approximately 4 µm thickness were made with the rotary microtome (Leitz 1512) from the paraffin-embedded block. Liver sections were subsequently stained with hematolin-eosin. Finally, the labeled liver slices were subjected to microscopic examination for histological analysis.[16]

Phytochemical screening
The phytochemical screening consisted of detecting the main phytoconstituents present in the fractions of ethanol extract of *C. procera*.[17] The following phytoconstituents were investigated:
- Sterols and triterpenes (Liebermann-Burchard reaction)
- Reducing compounds (Fehling reaction)
- Flavonoids (Shibata or cyanidin test)
- Tannins/phenol acid ($FeCl_3$ test)
- Saponosides (Foam index)

High-performance liquid chromatography–diode array detection (HPLC-DAD) analysis
The HPLC-DAD method was used to analyze the fractions of the ethanol extract of *Calotropis procera* root bark to identify and quantify triterpenes and sterols. The analysis was performed in isocratic mode using a reverse phase column C8 (4.6 mm × 150 mm) packed with 5 µm diameter particles.[18] The mobile phase consisted of methanol, acetonitrile, and water (70:25:5, v/v/v) containing 0.5% acetic acid. The mobile phase was filtered through a 0.45 µm membrane filter and degassed by an ultrasonic bath before use. Four standards were used, including one triterpene (ursolic acid) and three sterols (β-sitosterol, stigmasterol, campesterol). Each standard was dissolved in the mobile phase at an initial concentration of 400 µg/mL then a serial dilution was

carried out up to 6.25 µg/mL in 1.5 mL vials, and the calibration curve for each standard is produced. The ethanol extract's n-hexane fraction and dichloromethane fraction were also dissolved in the mobile phase. The standard solutions and the extracts were also filtered through a 0.45 µm membrane filter. The compounds were detected at different wavelengths, 220 nm for ursolic acid and 250 nm for the other standards. The injection rate was 1 mL/minute, and the injection volume was 50 µL.

HPLC-DAD analysis for the identification and quantification of phenolic compounds in the fractions of the ethanol extract of *C. procera* roots barks was carried out under isocratic conditions using a reverse phase column C18 (4.6 mm × 250 mm) packed with 5 µm diameter particles. The mobile phase consisted of methanol, acetonitrile, and water (40:15:45, v/v/v) containing 1% acetic acid.[19] The mobile phase was filtered through a 0.45 µm membrane filter and then ultrasonically degassed before use. Eight standards, including three flavonoids (rutin, quercetin, and kaempferol) and five phenol acids (gallic acid, vanillic acid, ellagic acid, cafeic acid, and sinapic acid) were used. Each standard was dissolved in the mobile phase at an initial concentration of 400 µg/mL, then a serial dilution was carried out up to 6.25 µg/mL in 1.5 mL vials, and the calibration curve for each standard was produced. The residual aqueous fraction, n-butanol fraction, and ethyl acetate fraction were also dissolved in the mobile phase. The standard solutions and the extracts were also filtered through a 0.45 µm membrane filter. The compounds were detected through the wavelengths of maximum absorption, 365 nm for the three flavonoids, 327 nm for cafeic acid, sinapic acid, and 271 nm for gallic acid, ellagic and vanillic acid. The injection rate was 0.5 mL/minute, and the injection volume was 10 µL.

Identification and quantification of compounds

All the chromatographic operations were carried out at room temperature and in triplicate. The chromatographic peaks of the extracts were confirmed by comparing their retention time and their UV spectra with those of the standards. The quantification of the compounds was made by integration of the peaks against the standard curves. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the intercept values of the calibration curve (σ) and the slope (S) using three independent analytical curves, following the guidelines of the ICH and the following equations were used: LOD = 3.3 σ /S, LOQ = 10 σ /S.[20]

Statistical analysis

All results were expressed as the mean ± standard deviation. The Graph Pad Prism software (version 5.0) was used for the analysis with Anova one way followed by the Bonferroni test to measure the degree of statistical significance of the results. A significant difference was considered at $p<0.05$.

Results and Discussion

Hepatoprotective activity

The groups pretreated with the fractions of the extract showed a significant decrease in the serum markers ALAT, ASAT and PAL compared to the control group DEN ($P<0.01$) (Table 1). The n-butanol fraction and residual aqueous fraction revealed the best ALT levels, with 74.33 ± 21.82 IU and 97.75 ± 29.98 IU, respectively. The aqueous, n-butanol and n-hexane fractions showed the best AST and PAL levels with, respectively, 183 ± 11.36 IU; 202.25 ± 37.89 IU; 185.75 ± 13.81 for AST and 71 ± 15.39 IU; 78.66 ± 17.01 IU; 108.33 ± 9.07 IU for PAL. The results of the evaluation of total proteins and albumin (Table 1) show no significant difference in the level of total proteins and albumin between the groups pretreated with the fractions of the ethanol extract of *C. procera* roots barks and the one having received only DEN. Table 2 shows the activities of the antioxidant enzymes and the inhibition of lipid peroxidation. These results show a significant increase in the activities of SOD and catalase for the groups pretreated with the different fractions of the ethanol extract of *C. procera* roots barks compared to those of the DEN control group ($P < 0.05$). The residual aqueous fraction showed the best SOD activity (331.04 ± 95.57 nmol SOD/mg protein). The pretreatment of the rats with the different fractions leads to a statistically significant drop in the MDA level compared to the rats of the DEN control group ($P < 0.05$). The DCM fraction (26.29 ± 5.57 µmol MDA/10 mg protein), n-butanol fraction (27.92 ± 7.35 µmol MDA/10 mg protein), and ethyl acetate fraction (29.20 ± 3.37 µmol MDA/10 mg protein) showed the best MDA levels. The hepatic histopathological analysis carried out on sections of livers treated only with DEN shows the presence of numerous hepatocyte necrosis, intralobular lymphocyte inflammatory infiltrates, vascular congestion, and fatty liver, which testifies to the toxic effect of DEN on the liver and onset of acute inflammation of the liver (Figure 1). Livers sections pretreated with the different fractions showed an improved appearance of hepatic tissue with an interesting reduction in hepatocyte necrosis, inflammatory intralobular lymphocyte infiltrates, fatty liver, and an absence of vascular congestion (except livers sections pretreated with the hexane fraction) compared to liver sections from the DEN control group (Figure 1). The n-butanol and residual aqueous fractions showed better protection of hepatic tissue with markedly improved aspects than that pretreated with silymarin used as a reference substance.

Acute liver inflammation caused by diethylnitrosamine in rats leads to hepatocyte necrosis characterized by an increase in serum biochemical markers such as ALT, AST, PAL, bilirubin, total proteins, albumins, and a decrease in antioxidant enzyme activities SOD, catalase of even a drop in MDA levels in the liver.[21] The pretreatment of rats with the different fractions leads to the protection of hepatocytes against cell lysis induced by diethylnitrosamine through a significant decrease in the biochemical markers ALAT, ASAT, and PAL compared to rats that received only diethylnitrosamine.[22,] Unlike fractions, the crude extract has interesting effects on the production of total proteins;[12] this suggests that the compounds of the crude ethanolic extract act in synergy on this parameter. The activities of the antioxidant enzymes SOD and catalase increase significantly in rats pretreated with different fractions compared to those receiving only DEN, suggesting their capacities to modulate the expression of antioxidant defense systems and protect liver cells against oxidative stress induced during DEN hepatotoxicity.[23]

Table 1: Effect of the fractions of ethanolic extract of *C. procera* on liver serum markers profile

	ALAT (IU/L)	ASAT (IU/L)	ALP (IU/L)	Total Protein (g/L)	Albumin (g/L)
Normal Control	37.14 ± 5.75	121.00 ± 7.19	127.25 ± 13.60	67.55 ± 0.87	39.25 ± 4.45
DEN Control	272.67 ± 22.00	328.00 ± 18.52	285.50 ± 47.03	60.48 ± 5.24	34.34 ± 2.52
Silymarin + DEN	114.25 ± 32.95***	194.60 ± 30.83***	198.75 ± 49.82***	71.51 ± 5.12**	38.70 ± 4.13
n-hexane fraction + DEN	145.80 ± 29.27***	185.75 ± 13.82***	108.33 ± 9.07***	55.17 ± 6.04	36.42 ± 3.60
DCM fraction + DEN	199.67 ± 35.30***	236.33 ± 14.84***	114.50 ± 39.81***	64.72 ± 6.80	37.90 ± 2.68
Ethyl acetate fraction + DEN	195.33 ± 9.07***	283.67 ± 35.64***	155.00 ± 45.26***	60.90 ± 2.74	38.17 ± 3.17
n-butanol fraction + DEN	74.33 ± 21.82***	202.25 ± 37.90***	78.67 ± 17.01***	65.40 ± 3.47	36.30 ± 1.93
Aqueous residual fraction + DEN	91.80 ± 29.17***	183.00 ± 11.36***	71.00 ± 15.39***	64.02 ± 5.42	37.80 ± 1.68

The values are mean ± standard deviation (n = 6). Anova one way, Bonferroni test: *** $P<0.001$, a very significant difference compared with the DEN control; ** $P<0.01$, a highly significant difference compared with DEN control; * $P < 0.05$, a significant difference compared with DEN control.

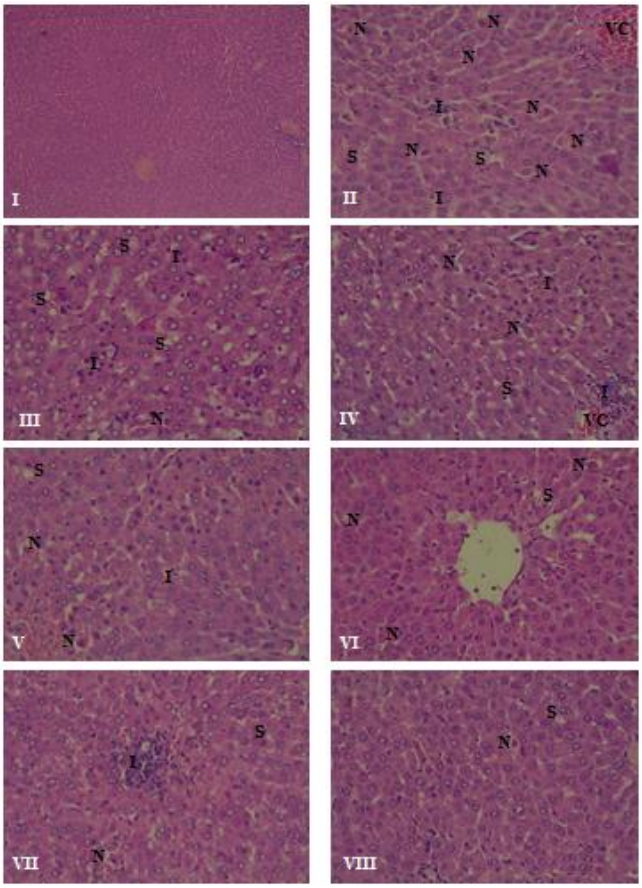

Figure 1: Histopathological of liver sections pretreated with different fractions of ethanolic extract (stained with hematein eosin, all seen at 40 magnification except I, which is seen at ten magnification): I (Normal control), normal liver tissue; II (DEN control); III (Silymarin + DEN); IV (n-hexane fraction + DEN); V (DCM fraction + DEN); VI (Ethyl Acetate fraction + DEN); VII (n-butanol fraction + DEN); VIII (Aqueous residual fraction + DEN). N: hepatocyte necrosis; I: Inflammatory infiltrates lymphocytic intralobular; VC: vascular congestion; S: fatty liver

The different fractions also lead to a significant drop in the level of MDA in the liver, suggesting their ability to reduce the lipid peroxidation of hepatocyte membranes, which increases with the toxic effect of DEN.[23] Phytochemical screening shows that all fractions contain triterpenes, sterols, saponosides, and reducing compounds (Table 3). The butanol fraction and residual aqueous fraction also contain flavonoids and phenolic acids (Table 3). The most active fractions, the n-butanol fraction and residual aqueous fraction, which are also the richest in flavonoids,[24] showed anti-hepatoxic activities against DEN, which could be essentially due to known phenolic compounds for their hepatoprotective properties,[21] but also to the steroid and triterpene compounds present in these extracts,[12] and which are also known for their liver-protective capacities.[25] These results are confirmed by the histopathological study, which also shows an improved tissue architecture in sections of livers pretreated with the different fractions. The livers pretreated with the n-butanol and residual aqueous fraction showed improved liver tissue aspects. The n-hexane fraction and dichloromethane fraction, which are rich in triterpene compounds,[24] showed interesting anti-hepatotoxic potentials against the toxic effect of DEN, suggesting that triterpenes would have hepatoprotective potentials.[26] The n-hexane fraction and ethyl acetate fraction of the methanolic extract of *C. procera* root bark harvested in India showed significant hepatoprotective activities against CCl4-induced hepatotoxicity in mice.[27]

Identification and quantification of compounds by HPLC-DAD

HPLC chromatograms of fractions were recorded at different wavelengths and at different retention times (Table 4). The LOD was defined as the lowest concentration level that can be detected, and the LOQ was defined as the lowest concentration level that can be quantified. LOD values vary between 0.0998-2.8118 µg/mL, and LOQ values vary between 0.3025-8.5207 µg/mL for triterpenes and sterols (Table 4). For phenolic compounds, LOD values vary between 0.6837-5.5399 µg/mL, and LOQ values vary between 2.07187-16.7876 µg/mL (Table 4). The correlation coefficients of the standard curves of all the references used vary between 0.9914 and 0.9999 in a concentration range of 6.25 µg/mL to 400 µg/mL (Table 4*)*. All detailed analysis performance characteristics are given in Table 4.

Ursolic acid, stigmasterol, campesterol, and β-sitosterol were identified in the n-hexane and dichloromethane fractions (Figure 2). The n-hexane fraction and dichloromethane fraction showed β-sitosterol contents interesting with respectively 547.46 ± 26.51 µg/mg of dry extracts, 71.21 ± 10.08 µg/mg of dry extract, and stigmasterol with respectively 246.59 ± 10.22 µg/mg of dried extract; 87.46 ± 11.99 µg/mg of dry extracts. Ursolic acid and campesterol have been identified in small quantities (Table 5). Triterpenes, including oleanolic acid, ursolic acid, betulinic acid, 3-epimaslinic acid, alphitolic acid, and euscaphic acid, showed hepatoprotective properties against CCl_4-induced hepatotoxicity in female rats.[28] Oleanolic acid, ursolic acid, stigmasterol, and β-sitosterol isolated from *Aralia racemosa* each showed interesting hepatoprotective activities against paracetamol-induced hepatotoxicity in rats.[29] Studies have also shown that ursolic acid alone or combined with oleanolic acid significantly lower transaminase and alkaline phosphatase levels, thus demonstrating their hepatoprotective potential against CCl_4 hepatoxicity in animals.[29,30] β-sitosterol, which is quantitative in the n-hexane fraction and dichloromethane fraction according to the qualitative and quantitative analysis of HPLC-DAD showed good protection of the hepatobiliary function against the toxic effect of CCl_4 in rats.[31]

Table 2: Effect of the fractions of ethanolic extract of *C. procera* on liver antioxidant activities profile

	SOD (nmol SOD/mg proteins)	**Catalase (nmol H_2O_2/min/mg proteins)**	**MDA (µmol MDA/10 mg proteins)**
Normal Control	438.10 ± 59.66	48.89 ± 6.52	23.84 ± 6.61
DEN Control	79.11 ± 24.34	19.77 ± 4.63	77.13 ± 20.56
Silymarin Control	169.21 ± 27.84***	56.99 ± 15.51***	49.09 ± 6.15***
n-hexane fraction + DEN	151.71 ± 27.59***	52.72 ± 11.01***	46.06 ± 5.71***
DCM fraction + DEN	253.33 ± 46.26***	51.97 ± 9.61***	26.29 ± 5.57***
Ethyl acetate fraction + DEN	198.46 ± 35.57***	68.30 ± 7.03***	29.20 ± 3.37***
n-butanol fraction + DEN	253.33 ± 7.64***	48.71 ± 6.43***	27.92 ± 7.35***
Aqueous residual fraction + DEN	331.04 ± 95.57***	59.81 ± 13.60***	40.44 ± 3.16***

The values are mean ± standard deviation (n = 6). Anova one way, Bonferroni test: *** P<0.001, a highly significant difference compared with the DEN control

β-sitosterol also prevents the accumulation of collagen in mice, thus suggesting its antifibrotic effect against liver fibrosis induced in mice by dimethylnitrosamine.[32] Stigmasterol and β-sitosterol significantly attenuate long-term non-alcoholic fatty liver disease in mice through a decrease in hepatic cholesterol levels, triacylglycerols with polyunsaturated fatty acids and alterations in hepatic fatty acids free.[33] In the n-hexane fraction of the methanolic extract of the root barks of *C. procera*, stigmasterol has been identified, which has shown anticancer properties, particularly against prostate tumor cells.[34] Stigmasterol possesses potent apoptosis-inducing effects in hepatocarcinoma cells (HepG2) through up-regulation of pro-apoptotic gene expressions (Bax, p53) while down-regulating anti-apoptotic genes (Bcl-2).[35] The n-hexane extract of *Acrocarpus fraxinifolius* Arn. (Leguminosae) which is rich in triterpenes like lupeol and sterols like tocopherol; campesterol showed hepatoprotective activity against paracetamol-induced hepatotoxicity in rats.[36]

The HPLC chromatograms show that only gallic acid was identified in the residual aqueous fraction and n-butanol fraction (Figure 3) at the respective concentrations of 18.76 ± 0.27 µg/mg of dry extract and 17.92 ± 0 .26 µg/mg of dry extract. Vanillic acid was identified in the ethyl acetate fraction (Figure 3) at a low concentration (0.61 ± 0.01 µg/mg of dry extract). Ellagic and sinapic acid were also identified in the ethyl acetate fractions (Figure 3) in small quantities with respective contents of 1.59 ± 0.004 and 1.24 ± 0.008 µg/mg of dry extract. None of the flavonoid references were identified in the extracts. Ellagic acid attenuates the hepatotoxicity induced by thioacetamide thanks to its antioxidant, metal-chelating capacity and anti-inflammatory properties.[37] Sinapic acid also showed anti-fibrotic properties of the liver against thioacetamide-induced liver fibrosis.[38] Gallic acid, which was identified in the n-butanol fraction and residual aqueous fraction by HPLC-DAD, is known for its antioxidant power and effective anti-inflammatory properties.[39] Studies have shown that gallic acid significantly lowers the level of hepatic parameters ALT, AST, PAL, GGT, total and conjugated bilirubin, and the level of MDA and allows restoration of the endogenous antioxidant system in rats/mice poisoned with diethylnitrosamine,[40] and paracetamol.[41] It also has an antifibrotic effect which results in a significant drop in COX-2 levels leading to a significant decrease in collagen levels and/or restoration of liver tissue in rats poisoned with diethylnitrosamine.[40]

These compounds identified in the different extracts could act either by preventing the activation of DEN through the inhibition of the expression of Cytochrome P450 2E1 or by neutralizing the reactive oxygen species produced during the hepatotoxicity induced by DEN,[42] or by increasing the expression of antioxidant defense systems.[43,44]

Conclusion

All fractions showed interesting anti-hepatotoxic activities. The butanol fraction and residual aqueous fraction showed the best activities both in terms of biochemical parameters and in terms of histopathological study. These results could be due to phenolic compounds, including gallic acid, identified in these two fractions and known for its hepato-protective properties

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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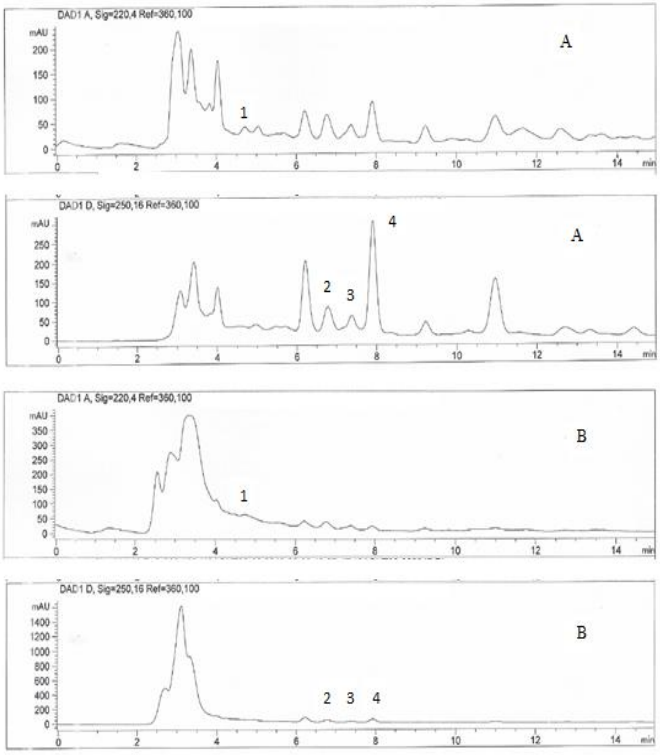

Figure 2: HPLC chromatogram of hexane and dichloromethane fraction of ethanolic extract of *C. procera* roots bark; A: Hexane fraction; B: Dichloromethane fraction; 1: Ursolic acid; 2: Stigmasterol; 3: Campesterol; 4: β-sitosterol

Table 3: Phytochemical screening of the fractions of ethanolic extract of *C. procera*

Chemical group	Hexane fraction	DCM fraction	Ethyl acetate fraction	Butanol fraction	Residual aqueous fraction
Triterpenes and sterols	+	+	+	+	+
Reducing compounds	+	+	+	+	+
Flavonoids	-	-	+	+	+
Tannins/ phenols acid	-	+	+	+	+
Saponosides	+	+	+	+	+

+ : Presence, - : Absence

Table 4: Calibration curves, LOD, and LOQ data of the compounds studied by HPLC

Standards	Retention time Rt (min)	Right equation	Correlation coefficient R^2	LOD/ µg mL^{-1}	LOQ/ µg mL^{-1}
Ursolic acid	4.862	2.82X – 33.90	0.9929	1.82917894	5.54296647
Stigmasterol	6.830	0.36X – 0.60	0.9968	0.28004629	0.84862513
Campesterol	7.262	2.85X – 0.74	0.9982	0.01768713	0.05359738
β-sitosterol	7.817	0.63X – 0.017	0.9990	0.00400066	0.01212322
Gallic acid	5.631	30.85X + 59.45	0.9999	0.08906752	0.26990158
Vanillic acid	6.982	87.60X + 157.87	0.9997	0.06484743	0.19650736
Ellagic acid	8.322	33.10X – 356.59	0.9937	0.19391773	0.58762947
Cafeic acid	6.622	35.49X + 139.67	0.9989	0.15215688	0.46108146
Sinapic acid	7.789	32.50X – 229.64	0.9954	0.11278050	0.34175910
Rutin	6.869	5.23X + 19.49	0.9977	0.49531884	1.50096617
Quercetin	12.108	16.46X + 17.63	0.9992	0.19411286	0.58822078
Kaempferol	17.073	14.91X + 69.07	0.9976	0.27347218	0.82870357

Table 5: Quantification of identified triterpene and steroid compounds in fractions

Fractions	Standards			
	Ursolic acid (µg/mg of the dry fraction)	Stigmasterol (µg/mg of the dry fraction)	Campesterol (µg/mg of the dry fraction)	β-sitosterol (µg/mg of the dry fraction)
Hexane fraction	5.23 ± 0.33	246.59 ± 10.22	12.75 ± 2.48	547.46 ± 26.51
Dichloromethane fraction	5.07 ± 0.04	87.46 ± 11.99	5.73 ± 1.13	71.21 ± 10.08

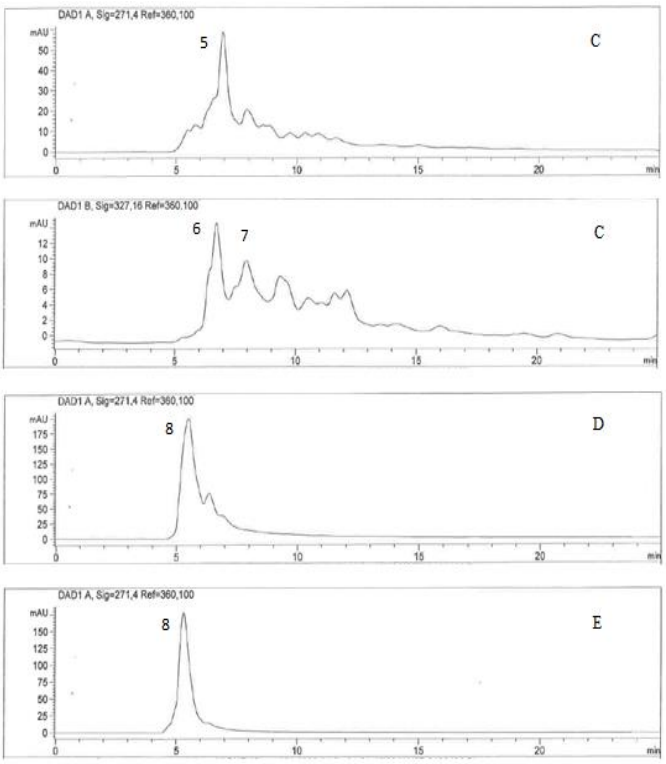

Figure 3: HPLC chromatogram of hexane and dichloromethane fraction of ethanolic extract of *C. procera* roots bark. C: ethyl acetate fraction; D: butanol fraction; E: residual aqueous fraction; 5: vanillic acid; 6: cafeic acid; 7: sinapic acid; 8: gallic acid

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