

Hepatoprotective Potentials of the Extract and Fractions of *Brenania brieyi* (De Wild) (Rubiaceae) against Carbon tetrachloride Induced Liver Damage in Rats

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Abstract

Brenania brieyi (De Wild), a plant from the Rubiaceae family, has been traditionally used in treating various illnesses, especially for treating liver disorders. This study aims to verify the hepatoprotective properties of the extract and fractions of *B. brieyi* against carbon tetrachloride-induced liver damage in rats and compare it with the positive control (Silymarin) using the protective model. The plant material was extracted using cold maceration and fractionated with *n*-hexane, ethyl acetate and methanol to get different fractions. Phytochemical constituents were determined using the Gas Chromatography-Flame Ionization Detection (GC-FID) method. Hepatoprotective activity and Histopathology studies were evaluated using standard procedures in determining biochemical parameters such as alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), Acid Phosphatase (ACP), albumin bilirubin, urea, creatinine, and total protein. The extract and fractions were shown to be rich in flavonoids, glycosides, alkaloids, saponins, tannins and steroids. The results demonstrated that 200 mg/kg of the extract significantly ($p < 0.05$) reduced the levels of ALT, AST, and ALP from $(46.60 \pm 2.08, 74.80 \pm 1.59$ and 97.40 ± 1.32 IU/L) to $(23.20 \pm 1.02, 38.40 \pm 0.51$ and 46.40 ± 0.81 IU/L) respectively compared to $(24.20 \pm 0.66, 37.20 \pm 1.15$ and 48.40 ± 0.81 IU/L) reduction obtained from the positive control (Silymarin 25 mg/Kg), indicating its hepatoprotective potentials. At 400 mg/kg the extract significantly reduced ($p < 0.05$) the levels of AST (20.80 ± 0.58), AST (36.80 ± 0.73), and ALP (48.40 ± 0.81) when compared with Silymarin. The study validates the traditional use of *B. brieyi* for treating liver disorders, showing that its extract and fractions have a dose-dependent effect in reducing the levels of ALT, AST, ALP, and other enzymes in carbon tetrachloride-induced liver damage in rats.

Keyword: *Brenania brieyi*, Silymarin, Hepatoprotective potentials, Carbon tetrachloride, liver damage, liver enzymes, biochemical parameters.

Introduction

Liver diseases can result in a decline in liver functions and may ultimately lead to organ failure [1-3]. Non-alcoholic fatty liver disease (NAFLD) is the primary cause of chronic liver disease globally and it is a liver disease where fat builds up in the liver of people who do not drink too much alcohol [4-7]. Other common causes include viral, genetic and immune mediation [8]. The liver plays a central role in the metabolism of drugs and xenobiotics like acetaminophen and environmental chemicals (e.g. carbon tetrachloride), which can be significantly affected by medication/xenobiotic-mediated toxic activity [9]. According to Gupta *et al.* [10], some common risk factors for liver disease include alcohol consumption, obesity, chronic hepatitis B and C infections, non-alcoholic steatohepatitis (NASH), malnutrition, exposure to toxins, and certain tropical infections. Drug-induced hepatotoxicity is common, but its actual frequency is difficult to determine due to underreporting, challenges in detection or diagnosis, and incomplete observation of exposure [11].

Liver function tests or liver enzyme biochemical assay are a group of diagnostic blood tests that measures the levels of various enzymes produced by the liver and can help identify areas of the liver where damage or dysfunction may be occurring [12]. They are enzymes produced by hepatocytes, while these enzymes can also be found in other tissues such as the heart, kidney, and skeletal muscle, their levels are highest in the liver [13]. Alkaline phosphatase is released and its levels can be elevated during cholestasis, bile duct diseases, metastasis, and physiological changes [14]. According to Ducatman *et al.* [15], for adults, the normal range for Aspartate transaminase (AST) levels is less than 40 IU/L [16]. In the liver, unconjugated bilirubin undergoes a process known as conjugation, where it is combined with glucuronic acid to form conjugated bilirubin, the accumulation of bilirubin in the blood leads to hyperbilirubinemia

[17-19]. Albumin interacts with plasma zinc and regulates cellular zinc uptake which plays a significant role and is responsible for controlling the oncotic pressure and volume of blood [20]. Acid phosphatase (ACP) is an enzyme that catalyzes the hydrolysis of phosphate esters under acidic conditions and is present in prostate gland, liver, bones, and red blood cells [21]. When liver cells are damaged, aspartate transaminase and alanine transaminase levels are disproportionately elevated compared to alkaline phosphatase levels, it may indicate hepatocellular injury [6, 22]. Urea represents a significant nitrogenous waste byproduct produced in the liver during protein metabolism [23]. Total protein measurement encompasses the quantification of all protein types present in the bloodstream, encompassing both albumin and globulins. This parameter holds essential significance in the evaluation of nutritional status, liver function, and various medical conditions [24].

According to the Global Burden of Disease Study [25], in 2017, 1.5 billion people worldwide were affected by chronic liver disease (CLD), with the most common causes being non-alcoholic fatty liver disease (NAFLD) at 60%, hepatitis B virus (HBV) at 29%, hepatitis C virus (HCV) at 9%, and alcohol-related liver disease (ALD) at 2%. Cirrhosis and liver cancer, which are the major complications of chronic liver disease (CLD), account for 3.5% of all deaths worldwide, with 1.2 million deaths due to cirrhosis and 790,000 deaths due to liver cancer [26]. Chronic liver disease (CLD) and cirrhosis are major global health concerns, ongoing efforts to address CLD-related morbidity and mortality require accurate contemporary estimates of epidemiology and outcomes [27]. Silymarin, a complex of flavonoids extracted from the milk thistle plant (*Silybum marianum*), has demonstrated hepatoprotective properties through its ability to decrease inflammation and act as an antioxidant, by stimulating the formation of enzymes, increasing the production of bile, reducing inflammation, and soothing mucous membranes, Silymarin can aid in managing liver disorders and protecting against the effects of toxins [28].

B. brieyi is a genus of flowering plants that belongs to the Rubiaceae family. It is commonly found in the evergreen forests of Southern Nigeria and Western Cameroon, and its range extends to Zaïre, Gabon, Cabinda, Central African Republic, Congo, and D.R.Congo [29]. In Abagana town, Njikoka local government area of Anambra State, Nigeria, the plant is known locally as 'mgbunsi' [30]. The fruit is also known to be effective in treating endocrine disorders, such as menopausal symptoms [31]. Several studies have investigated the medicinal properties of *B. brieyi* and have found that its bark and root extract and fractions possess significant anti-inflammatory, antioxidant [32], antimicrobial and antitrypanosomal [33], antimalarial activity [34]. However, there is a lack of scientific proof on the hepatoprotective potentials of *B. brieyi* root extract and fractions. The research aimed to assess and explore the hepatoprotective potentials of *B. brieyi* in promoting liver health and reducing hepatocyte damage.

Materials and Methods

Plant collection and authentication

The fresh roots of *B. brieyi* were obtained from Abagana, a town in Njikoka Local Government Area of Anambra State, Nigeria, in July 2023. The plant was identified and authenticated by Mr. Felix Nwafor, the curator of the herbarium in the Department of Pharmacognosy and Environmental Medicine, University of Nigeria Nsukka (Voucher specimen with the number PCG/UNN/0327). The roots were cleaned before being brought to the laboratory, where they were cut, dried and mechanically ground into powder, the dried powdered root was extracted using standard analytical grade methanol.

Extraction of plant materials

Methanol extract

The powdered root bark of *B. brieyi* plant (1001.4 g) was subjected to cold maceration with 95 % aqueous methanol. The solvent was changed frequently and the extraction was repeated twice, each time for 72 hours with constant agitation. The extract was filtered through a muslin and then through filter paper number No 1 (pore size 11 mm). The filtrate was combined and air dried. The dried methanol extract of *B. brieyi* (MEBB) was stored at 20 °C until further use.

Successive fractionation by liquid-liquid partitioning

To obtain the various fractions, 150 g of the methanol extract of the root of *B. brieyi* was fractionated with *n*-hexane (750 ml), ethyl acetate (1500 ml) and absolute methanol (600 ml) in order of their increasing polarities, using a separating funnel. The fractions obtained were placed in a flask and dried.

Gas chromatography analysis

The determination of phytochemicals and quantification using GC-FID was described by Nwiloh *et al.* [35]. This method was repeated for all the fractions. The phytochemicals were quantified by the ratio of the area and mass of the internal standard to the area of the identified phytochemicals. The concentration of the different phytochemicals was expressed in µg/g.

Acute toxicity test

Acute toxicity (LD₅₀) of the extract of *B. brieyi* was determined according to the Lorke's method with slight modification [36]. Thirteen (13) mice were divided into two stages. In stage one, nine mice were used, they were grouped into three groups of three (3) mice and were administered (10, 100, and 1000 mg/kg) orally of the extract (MEBB) respectively. They were observed for 24 hours for behavioural changes and death. The remaining 4 animals were given 1000, 1600, 2700 and 5000 mg/kg MEBB orally and also observed for 24 hours.

In vivo hepatoprotective studies

Experimental animals

This study involved 45 adult Swiss albino rats, (103-264 g) of either sex. The animals were bred and housed at the animal house of the Nutrition and Dietetics, Faculty of Agriculture, University of Nigeria, Nsukka (UNN). The ethical committee board of the Faculty of

Pharmaceutical Sciences, University of Nigeria Nsukka approved the study with the certification number (FPSRA/UNN/23/0072). The rats were kept in standard laboratory cages for seven days to acclimatize, under natural light-dark cycles of 12 hours each. They had free access to food and water. The rats were handled and treated according to the guidelines for the care and use of laboratory animals under good laboratory practices (NIH publication #85-23, revised in 1985).

Experimental design

The crude extract and fractions of *B. brieyi* were tested for their protective effects on carbon tetrachloride (CCl₄) induced hepatotoxicity in rats using a method described by Japanese Society of Gastroenterology and Japan Society of Hepatology [37] with little modifications. The crude extract was given at three doses (100 mg/kg, 200 mg/kg and 400 mg/kg). *n*-Hexane, ethyl acetate and methanol fraction were administered at 200mg/kg each. Three control groups were used: (i) positive control (induced liver damage and treated with 25 mg/kg of Silymarin), (ii) negative control (induced liver damage but no treatment) and (iii) Normal (No induced liver damage but given 1 ml of the vehicle, normal saline). Treatment with the extract and fractions was done per oral (p.o) daily for 14 days, on Day 3, Day 8 and Day 14, the body weight, and rectal temperature were taken. The survival time was also taken daily during the treatment. At the end of the day 14 after injecting carbon tetrachloride (CCl₄) dissolved in olive oil (CCl₄: Olive oil at the ratio of 1:1) intraperitoneally, blood samples were taken from each rat in all the groups for analysis. The rats were sacrificed, and the liver was harvested from one rat in each group for histological studies.

Grouping of animals

The study used 45 rats, divided into nine groups of five rats each (n=5).

Group 1: Treated with 100 mg/kg of methanol extract of *B. brieyi* (MEBB)

Group 2: Treated with 200 mg/kg of methanol extract of *B. brieyi* (MEBB)

Group 3: Treated with 400 mg/kg of methanol extract of *B. brieyi* (MEBB)

Group 4: Treated with 200 mg/kg of *B. brieyi* *n*-Hexane fraction (BBHF)

Group 5: Treated with 200 mg/kg of *B. brieyi* ethylacetate fraction (BBEF)

Group 6: Treated with 200 mg/kg of *B. brieyi* methanol fraction (BBMF)

Group 7: Positive control (induced liver damage with 0.5 ml of CCl₄: Olive oil at the ratio of 1:1 intraperitoneally and treated with 25 mg/kg of Silymarin (Standard hepatoprotective drug)).

Group 8: Negative control (induced liver damage with 0.5 ml of CCl₄: Olive oil at the ratio of 1:1 intraperitoneally but no treatment)

Group 9: Normal (No induction of liver damage but given 1 ml of the vehicle, normal saline).

Evaluation of study parameters

The degree of prevention of liver damage was assessed: The level of liver enzymes such as Alanine Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP), Acid Phosphatase (ACP), Albumin, Bilirubin, Urea, Creatinine and Total Protein was determined:- Weight of the animal, rectal temperature and survival time were also determined.

Mean survival time

The animals were monitored for 14 days pre-treatment from Day 0 to Day 13. The mean survival time for each group was calculated by:

$$\text{Mean survival time} = \frac{\text{Total survival time in days}}{\text{Total number of animal in a group}} \dots\dots\dots \text{Equation 1}$$

Body weight measurements determination

The body weight of each rat in every group was weighed using the electronic weighing balance on Day 0, Day 8 and Day 14 of the experiment.

Rectal body temperature

Also the rectal temperature of each rat in every group was measured using a digital thermometer on Day 0, Day 8 and Day 14 of the experiment.

Liver function test

Aspartate aminotransferase (AST) activity determination

The activity of aspartate aminotransferase was assayed by the method of Reitman and Frankel [38] as outlined in the Randox kit used. Aspartate aminotransferase activity was described by Burtis and Ashwood [39] and Schmidt [40]. It was measured by monitoring the formation of oxaloacetate hydrazine with 2, 4-dinitrophenylhydrazine. The absorbance was read at a wavelength of 546 nm after 5 minutes. The AST was calculated as follows:

$$\text{AST Activity} = \frac{\text{Absorbance of sample} - \text{Absorbance of standard}}{\text{Slope}} \dots\dots\dots \text{Equation 2}$$

Alanine aminotransferase (ALT) activity

The activities of alanine aminotransferase was assayed by the method of Reitman and Frankel [38] as outlined in Randox kit. The ALT was determined according to Schmidt, [40]. The absorbance were read at wavelength of 546 nm after 5 minutes. The ALT was calculated as follows:

$$\text{ALT Activity} = \frac{\text{Absorbance of sample} - \text{Absorbance of standard}}{\text{Slope}} \dots \text{Equation 3}$$

Alkaline phosphatase (ALP) activity

The activities of alkaline phosphatase was assayed by the method described as Babson *et al.* [41] and Haenseler [42], The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured photo metrically. The absorbance was measured at 630 nm. The alkaline phosphatase present was calculated as follows:

$$\text{Alkaline phosphatase} = \frac{\text{Absorbance of sample} \times \text{value of standard}}{\text{Absorbance of standard}} \dots \text{Equation 4}$$

Assay acid phosphatase (ACP)

The concentration of serum acid phosphatase (ACP) was determined using the method of Tietz [43]. The initial absorbance was read at 405 nm and timer started simultaneously. The absorbance were read again 1, 2, and 3 minutes.

$$\text{Acid phosphatase} = \frac{\text{Change in absorbance/min.} \times 10^6 \times 1.1}{12.9 \times 10^3 \times 1.0 \times 0.1} \dots \text{Equation 5}$$

10⁶ = conversion of moles to millimoles

1.1 = total reaction volume

12.9 x 10³ = molar absorptivity of α-Naphthol-Fast complex at 405 nm

1.0 = light path in cm

0.1 = sample volume (ml)

Determination of Urea

The concentration of serum urea was determined using the method as described by Tietz, [43]. The ammonia is then measured photometrically by Berthelot's reaction. The absorbance was measured at 546 nm.

$$\text{Urea concentration (mg/L)} = \frac{\text{Absorbance of sample} \times \text{standard concentration}}{\text{Absorbance of standard}} \dots \text{Equation 6}$$

Determination of Creatinine

The concentration of serum Creatinine was determined using the method of Tietz [43]. The absorbance was read at 510 nm.

$$\text{Creatinine (mg/L)} = \frac{\text{Absorbance of sample} \times \text{standard concentration}}{\text{Absorbance of standard}} \dots \text{Equation 7}$$

Determination of total protein

The total protein content was determined using the method of Tietz [44]. The absorbance was measured at 500 nm.

$$\text{Protein concentration (g/dl)} = \frac{\text{Absorbance of sample} \times \text{standard concentration}}{\text{Absorbance of standard}} \dots \text{Equation 8}$$

Histopathological examination

This was carried out as described by Bancroft and Gamble, [45]. At the end of the experiment, the liver from the various groups of rats were collected for histopathology after which they were fixed in 10 % formalin in normal saline and dehydrated in ascending grades of ethanol. Thereafter, the tissues were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5 – 6 microns. The sections were deparaffinized in xylene, taken to water and subsequently stained with Hematoxylin and Eosin (H and E) for 15 min. for light microscopy. The prepared slides were examined onto a light microscope using ×4, ×10 and ×40 objective lenses. Photomicrographs of the sections were taken using a Motic TM 5.0 megapixels microscope camera fixed at ×200 magnification.

Statistical Analysis

All the results were reported as Mean ± Standard Error of Mean (SEM) for all groups and analyzed using one-way ANOVA (Analysis of Variance) in Graph pad prism version 10.0. The differences within and between groups were considered significant at p < 0.05.

Results

Rectal temperature, body weight and survival time results

Table 1 provides insights into the effects of these treatments on body weight, which increased across all the treatment groups, survival time, and rectal temperature at various stages of the experiment.

Liver enzyme markers

Table 2 shows the result of all the liver enzyme markers, the treatments gave a good significant reduction at p<0.05 in the liver enzymes except for the values in ACP when compared with the standard/normal range (2.2 – 10.5 IU/L).

Phytoconstituents present using GC-FID

Table 3 - 6 provides a detailed analysis of the various phytochemicals, class of compound and relative abundance present in the extract and fractions of *B. breiji* root; it provides information of the compounds responsible for the therapeutic properties of *B. breiji*.

Table 1: Effect of the *B. breiji* extract and fractions on the rectal temperature, body weight and survival time of the Carbon tetrachloride induced liver damage in rats

Groups	Treatm ent (mg/kg)	Body Weight in (g)			Mean survival time (Days)	Rectal Temperature °C	
		Day 0	Day 8	Day 15		Day 0	Day 8
MEBB	100	191.200±8.108*	204.600±5.732 ^{ab}	203.800±5.731 ^{ab}	15	37.880±0.294	36.600±0.616
MEBB	200	188.000±15.811	189.800±13.991	182.000±12.590	13	37.100±0.447	37.400±0.577
MEBB	400	140.600±5.085	164.400±8.698 ^a	182.400±3.816	15	38.200±0.110	36.600±0.614
BBHF	200	172.400±13.321	185.800±6.094*	206.200±21.037	13	37.440±0.320	37.440±0.287
BBEF	200	180.000±12.300	219.000±16.894	211.800±10.356	15	37.320±0.086	36.640±0.304
BBMF	200	140.600±5.085	164.400±8.698	182.400±3.816	15	38.200±0.110	36.600±0.614
Silymarin	25	148.800±13.124	150.200±6.086	167.000±4.827*	15	37.920±0.156	36.780±0.986
Negative	Nil	178.600±10.366*	231.000±9.772*	234.200±8.188*	14	36.900±0.228	37.160±0.614
Normal	-	116.400±4.545	132.200±1.594	139.200±4.974	15	37.660±0.344	37.840±0.357

Values are expressed as mean ± SEM; n=5 Methanol extract of *Brenania breiji* (MEBB); *Brenania breiji* n-hexane Fraction (BBHF); *Brenania breiji* Ethyl acetate Fraction (BBEF) *Brenania breiji* Methanol Fraction (BBMF). The values are expressed as mean ± SEM (n=5). *P<0.05 treatment versus normal control, ^aP<0.05 extract versus negative control, ^bP<0.05 extract versus standard drug (Silymarin 25 mg/kg).

Table 2: Effect of the *B. breiji* extract and fractions on liver enzymes of carbon tetrachloride-induced liver damage in rats

Group	Treatment (mg/kg)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	ACP (IU/L)	Albumin (g/dl)	Bilirubin (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)	Total Protein (g/dl)
BBCE	100	32.60± 0.872 ^{ab}	47.600± 0.927 ^{ab}	57.000± 0.894 ^{ab}	73.600± 1.631 ^a	5.997± 0.163 ^{ab}	8.132± 0.397 ^a	75.00± 1.897 ^a	4.409± 0.103 ^b	6.146±0.167 ^a b
BBCE	200	23.20± 1.020 ^a	38.400± 0.510 ^a	46.400± 0.812 ^a	62.400± 0.927 ^a	4.553± 0.209 ^a	5.991± 0.145 ^{ab}	73.80± 3.040 ^a	4.338± 0.143 ^b	8.366± 0.105 ^a
BBCE	400	20.80± 0.583 ^a	36.800± 0.735 ^a	46.000± 0.707 ^a	64.200± 1.281 ^a	3.797± 0.264 ^a	5.879± 0.091 ^a	66.80± 1.158 ^a	4.232± 0.112 ^b	8.788± 0.229 ^a
BBHF	200	24.20± 0.663	36.800± 1.158	48.600± 0.510	63.000± 1.414	4.184± 0.305	6.246± 0.108	73.60± 1.327	4.857± 0.319	8.015± 0.412
BBEF	200	25.60± 0.678	36.000± 0.707	46.800± 0.860	65.800± 1.685	4.257± 0.038	6.030± 0.133	76.00± 2.881	4.523± 0.162	8.270± 0.182
BBMF	200	25.80± 0.800	39.400± 1.327	51.800± 1.281	69.400± 0.678	4.654± 0.275	6.471± 0.186	66.00± 0.837	3.487± 0.349	8.106± 0.215
Silymarin	25	24.20± 0.663*	37.200± 1.158*	48.400± 0.812*	63.600± 0.927	4.637± 0.088	6.022± 0.187	67.40± 1.077*	2.968± 0.119	8.864± 0.046
Negative	Nil	46.60± 2.088*	74.800± 1.594*	97.400± 1.327*	102.000± 3.162*	8.050± 0.399*	10.514± 0.321*	83.60± 1.327*	4.995± 0.380*	2.824± 0.257*
Normal	-	18.20± 0.663	34.200± 1.281	42.200± 1.068	59.800± 1.356	3.625± 0.184	5.312± 0.286	57.80± 1.960	3.022± 0.240	8.470± 0.137

Values are expressed as mean ± SEM; n=5, *Brenania breiji* Crude Extract (BBCE), *Brenania breiji* n-hexane Fraction (BBHF); *Brenania breiji* Ethyl acetate Fraction (BBEF) *Brenania breiji* Methanol Fraction (BBMF). Alanine Aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Acid Phosphatase (ACP); The values are expressed as mean ± SEM (n=5). *P<0.05 treatment versus normal control, ^aP<0.05 extract versus negative control, ^bP<0.05 extract versus standard drug (Silymarin, 25mg/kg)

Table 3: Phyto-constituents identified in the GC-FID methanol extract of the root of *B. brieyi*

S/N	R.T	Name of compound	Class of compound	Peak Area	Molecular formular	Molecular weight	Reported Bioactivity
1	4.096	Anthocyanin	Flavonoid	602.105	C ₁₅ H ₁₁ O ⁺	207.0	Antioxidant [46]
2	5.613	Aphyllidine	Alkaloid	592.177	C ₁₅ H ₂₂ N ₂ O	246.35	No activity reported
3	17.033	Aphyllidine	Alkaloid	843.163	C ₁₅ H ₂₂ N ₂ O	246.35	No activity reported
4	28.593	Cardiac glycoside	Glycoside	279.848	C ₂₈ H ₃₂ O ₁₄	780.949	Anticancer [47]
5	1.640	Catechin	Flavonoid	336.190	C ₁₅ H ₁₄ O ₆	290.26	Hepatoprotective [46]
6	9.026	Cyanogenic glycoside	Glycoside	786.357	C ₂₈ H ₃₂ O ₁₄	261.271	Anticancer [47]
7	7.090	Dihydrocytisine	Alkaloid	1376.280	C ₁₁ H ₁₆ N ₂ O	126.14	Smoking cessation treatment [48]
8	43.876	Epihedrine	Alkaloid	401.247	C ₁₀ H ₁₅ NO	165.0	No activity reported
9	30.986	Flavone	Flavonoid	438.357	C ₁₅ H ₁₀ O ₂	222.24	Antimicrobial [46]
10	25.080	Flavonones	Flavonoid	781.331	C ₆ H ₄ OC ₃ H(Ph)O	145.0	Antioxidant [46]
11	0.246	Kaempferol	Flavonoid	782.312	C ₁₅ H ₁₀ O ₆	286.23	Anticancer [46]
12	5.233	Narigenin	Flavonoid	411.183	C ₁₅ H ₁₂ O ₅	272.257	Antidiabetic [46]
13	39.026	Oxalate	Antinutrient	355.527	C ₂ H ₂ O ₄	88.019	Formation of kidney stones [46]
14	3.190	Proanthocyanidin	Flavonoid	596.491	C ₃₁ H ₂₈ O ₁₂	594.5	Anti-stress [46]
15	23.306	Ribalinidine	Alkaloid	291.728	C ₁₅ H ₁₇ NO ₄	275.3	Free radical scavenger [46]
16	44.450	Sapogenin	Saponin	349.075	C ₂₇ H ₄₄ O ₃	416.0	Antibacterial [46]
17	34.116	Sparteine	Alkaloid	570.542	C ₁₅ H ₂₆ N ₂	234.38	Antimalarial [47]
18	1.280	Steroid	Steroid	644.168	C ₁₉ H ₂₈ O ₂	288.4	Antibacterial [48]
19	19.113	Tannin	Tannin	1220.495	C ₁₇ H ₁₆ O ₉	364.0	Antidiarrhea [48]

R.T = Retention Time, GC-FID = Gas Chromatography-flame Ionization Detection

Table 4: Phyto-constituents identified in the GC-FID *n*-hexane fraction of the root of *B. brieyi*

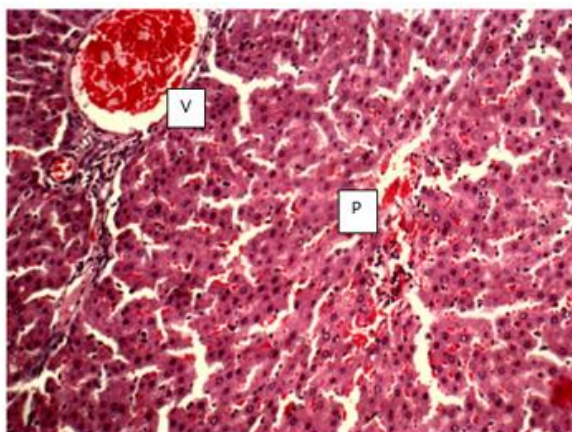
S/N	Type of phytochemicals	Class of phytochemicals	Retention time	Molecular weight (g/mol)	Reported Bioactivity
1.	Kaempferol	Flavonoid	25.563	286.23	Anticancer [46]
2.	Catechin	Flavonoid	36.526	290.26	Hepatoprotective [46]
3.	Proanthocyanin	Flavonoid	0.190	594.50	Anti-stress [46]
4.	Anthocyanin	Flavonoid	3.550	1730.5	Antioxidant[46]
5.	Naringenin	Flavonoid	13.273	272.257	Antidiabetic [46]
6.	Flavonones	Flavonoid	18.950	224.25	Antioxidant [46]
7.	Phytate	Anti-nutrient	28.276	246.35	Antimicrobial [46]
8.	Sparteine	Alkaloid	15.620	234.38	Antimalarial [47]
9.	Lunamarin	Alkaloid	1.583	309.3	Antimicrobial [47]
10.	Epicatechin	Flavonoid	27.910	290.27	Antioxidant[49]
11.	Ribalinidine	Alkaloid	4.400	275.30	Free radical scavenger[49]
12.	Flavan-3-ol	Flavonoid	12.620	226.27	Antidiabetic [50]
13.	Reservatrol	Polyphenol	33.810	228.247	Cardioprotective [51]
14.	Sapogenin	Saponin	42.706	1131.3	Antibacterial[46]
15.	Oxalate	Anti-nutrient	35.650	88.019	Formation of kidney stones [46]
16.	Steroids	Steroid	22.456	228.4	Antibacterial [48]
17.	Cyanogenic glycoside	Glycoside	13.973	261.271	Anticancer [47]
18.	Cardiac glycoside	Glycoside	2.633	780.949	Antiarrhythmia [47]
19.	Rutin	Flavonoid	12.990	126.14	Antioxidant [52]

Table 5: Shows the type, class, structure and bioactivity of phytoconstituents present in the ethyl acetate fraction of *B. brieyi*

S/N	Type of Phytochemicals	Class of Phytochemicals	Retention time	Molecular weight (g/mol)	Reported Bioactivity
1	Kaempferol	Flavonoid	27.536	286.23	Anticancer [10]
2	Epicatechin	Flavonoid	29.860	290.27	Antioxidant [49]
3	Proanthocyanin	Flavonoid	0.210	594.50	Anti-stress [53]
4	Anthocyanin	Flavonoid	7.470	1730.5	Antioxidant [54]
5	Narigenin	Flavonoid	12.970	272.257	Antidiabetic [55]
6	Flavonones	Flavonoid	22.730	224.25	Antioxidant [56]
7	Flavones	Flavonoid	34.600	222.24	Antimicrobial [46]
8	Flavan 3 Ol	Flavonoid	6.016	226.27	Antidiabetic [50]
9	Sparteine	Alkaloid	15.460	234.38	Antimalarial [47]
10	Rutin	Flavonoid	17.963	126.14	Antioxidant [52]
11	Phytate	Anti-nutrient	32.993	246.35	Antioxidant [57]
12	Ribalinidine	Alkaloid	10.366	275.30	Free radical scavenger [58]
13	Epihedrine	Alkaloid	44.170	150.0	No activity reported
14	Naringin	Flavonoid	2.390	580.541	Anti-inflammatory [59]
15	Sapogenin	Saponin	42.276	1131.3	Antibacterial [60]
16	Oxalate	Anti-nutrient	36.876	88.019	Formation of kidney stones [61]
17	Steroids	Steroid	25.650	228.4	Antibacterial [48]
18	Cyanogenic Glycosides	Glycoside	20.313	261.271	Anticancer [47]

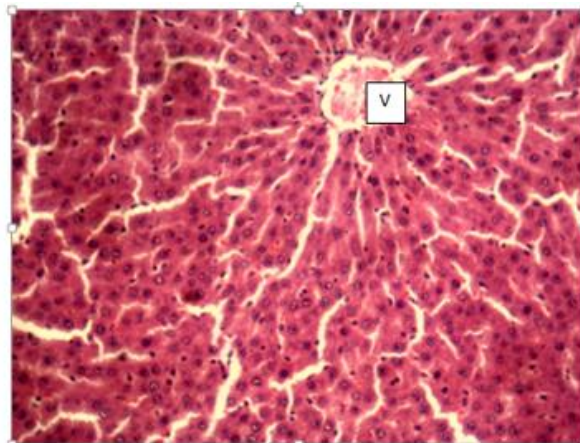
Table 6: Shows the type, class, structure and bioactivity of the phytochemicals present in the methanol fraction of *B. brieyi*

S/N	Type of Phytochemicals	Class of Phytochemicals	Retention Time	Molecular weight (g/mol)	Reported Bioactivity
1	Kaempferol	Flavonoid	28.583	286.23	Anticancer [10]
2	Catechin	Flavonoid	38.320	290.26	Hepatoprotective [62]
3	Proanthocyanidin	Flavonoid	0.096	594.50	Anti-stress [53]
4	Proanthocyanidin	Flavonoid	0.150	594.50	Anti-stress [53]
5	Anthocyanin	Flavonoid	6.893	1730.5	Antioxidant [54]
6	Narigenin	Flavonoid	13.300	272.257	Antidiabetic [63]
7	Flavonones	Flavonoid	22.290	224.25	Antioxidant [56]
8	Flavones	Flavonoid	34.093	222.24	Antimicrobial [64]
9	Sparteine	Alkaloid	15.783	234.38	Antimalarial [47]
10	Resveratrol	Polyphenol	39.583	228.247	Cardio protective [51]
11	Naringin	Flavonoid	2.220	580.541	Anti-inflammatory [59]
12	Ribalinidine	Alkaloid	10.590	275.30	Free radical scavenger [58]
13	Epicatechin	Flavonoid	29.456	290.27	Anti-oxidant [49]
14	Tannins	Tannin	40.933	1701.19	Antidiarrheal [48]
15	Sapogenin	Saponin	42.136	1131.3	Antibacterial [60]
16	Oxalate	Anti-nutrient	397.273	88.019	Formation of kidney stones [61]
17	Steroids	Steroid	26.003	228.4	Antibacterial [48]
18	Cyanogenic Glycosides	Glycoside	19.573	261.271	Anticancer [47]
19	Cardiac Glycosides	Glycoside	3.950	780.949	Antiarrhythmia [47]



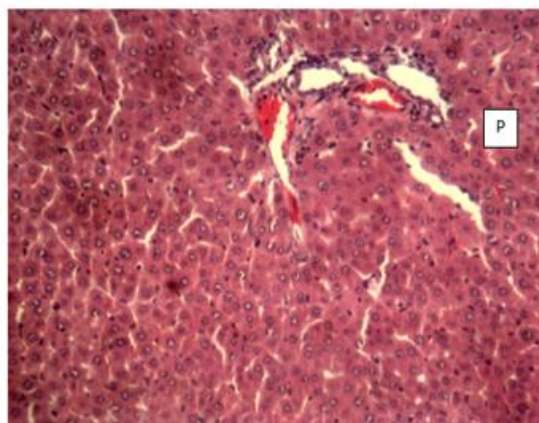
Sections of the **liver** presented in this group showed multifocal areas of hepatocellular necrosis and inflammatory disorder (arrow). Central vein (V); Portal area (P). H&E x200

Figure 1: Histopathology of MEBB treated rat liver (100 mg/kg)



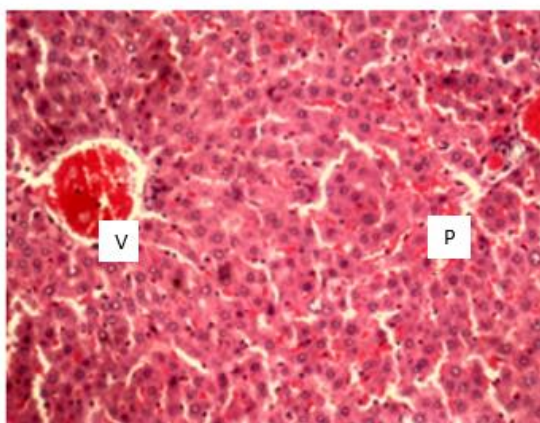
Sections of the **liver** presented in this group showed the normal hepatic histomorphology. Central vein (V). H&E x200

Figure 2: Histopathology of MEBB treated rat liver (200 mg/kg)



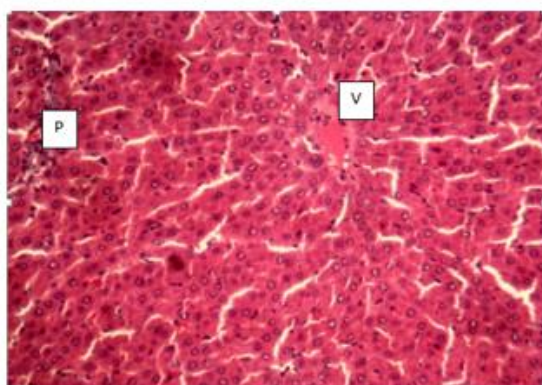
Sections of the **liver** presented in this group showed the normal hepatic histomorphology with little necrosis. Portal area (P). H&E x200

Fig. 3: Histopathology of MEBB treated rat liver (400 mg/kg)



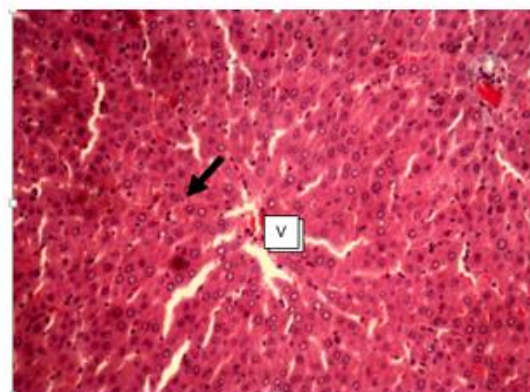
Sections of the **liver** presented in this group showed the normal hepatic histomorphology with necrosis. Central vein (V); Portal area (P). H&E x200

Fig. 4: Histopathology of BBHF treated rat liver (200 mg/kg)



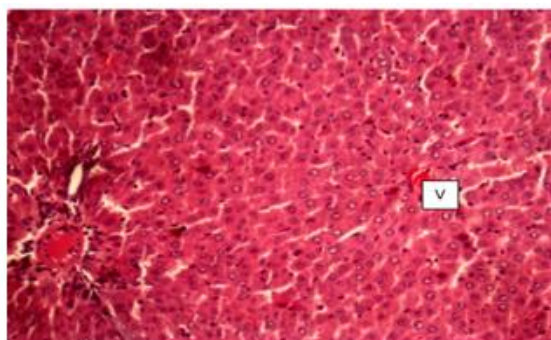
Sections of the liver presented in this group showed the normal hepatic histomorphology. Portal area (P), Central vein (V). H&E

Fig 5: Histopathology of BBEF treated rat liver (200 mg/kg)



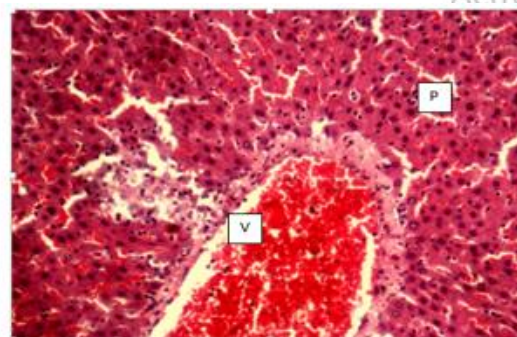
Sections of the liver presented in this group showed the normal hepatic histomorphology. Central vein (V). H&E x200

Fig 6: Histopathology of BBMF treated rat liver (200 mg/kg)



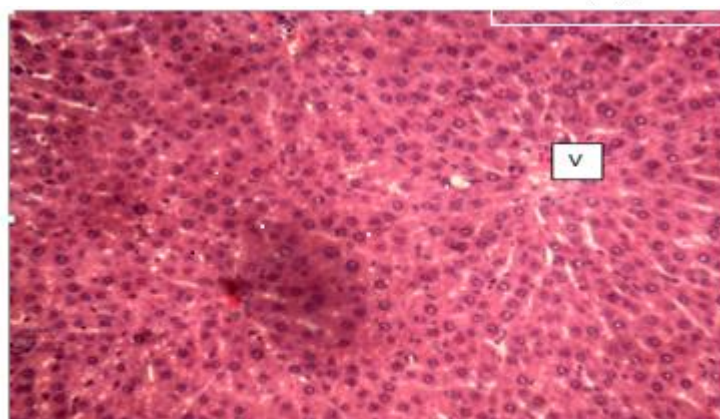
Sections of the liver presented in this group showed mild multifocal areas of hepatocellular necrosis (arrow). Central vein (V). H&E x200

Fig 7: Histopathology of Silymarin treated rat liver (25 mg/kg) (Positive control)



Sections of the liver presented in this group showed multifocal areas of hepatocellular necrosis and inflammatory disorder histomorphology. Portal area (P). Central vein (V). H&E x200

Fig 8: Histopathology of induced but not treated rat liver (Negative control)



Sections of the liver presented in this group showed the normal hepatic histomorphology. Central vein (V). H&E x200

Fig 9: Histopathology of Not induced and untreated rat (Normal control)

DISCUSSION

This study qualitatively and quantitatively evaluated the phyto-constituents of the extract and fractions of *B. brieyi* and investigated its hepatoprotective effects on carbon tetrachloride-induced liver damage in rats using a preventive

model. The extract of the root was found to contain these class of phytochemicals: alkaloids, glycosides, flavonoids, tannins, steroids, saponins and phenols (Table 3.). The GC-FID analysis (Table 3-6) revealed the presence of nineteen (19) different types of phytoconstituents which ranges from a high concentration of different classes of compounds like flavonoids, tannins, glycosides, saponins and alkaloids among the other identified phytoconstituents. Natural polyphenolic compounds, including tannins, flavonoids, and phenols, may contribute to free radical scavenging, metal chelation, and activation of antioxidant systems [33]. These powerful natural antioxidants present in the plant are likely responsible for its hepatoprotective effect and soothing of mucous membrane [65]. The observed pharmacological activity of *B. brieyi* could be attributed to the presence of alkaloids, steroids, phenols, tannins, glycosides and flavonoids found in the root of plant [34]. These secondary metabolites may exert their effects by acting at one or more target sites involved in the physiological process of repairing the hepatocytes in an additive or synergistic manner [47,49]. Catechin and epicatechin a flavonoids, have been reported to have hepatoprotective potential and antioxidant activity [46,49]. Ganeshpurkar *et al.*, [52] reported that rutin a flavonoids have antioxidant activity. These phytoconstituents have the ability to decrease inflammation, soothing mucous membrane and has antioxidant activity. Abundance of flavonoids and alkaloids in both ethyl acetate and methanol fractions of *Brenania brieyi* corresponding to previous studies that prove the anti-inflammatory effects of *B. brieyi* since flavonoids and alkaloids are known to exhibit anti-inflammatory effects [32]. Silymarin (25 mg/kg) was used as a positive control for comparison. The experiment was conducted over 14 days to ensure adequate distribution of the extract and fractions in carbon tetrachloride induced rats and to obtain more reliable results.

According to Lala *et al.*, [12] liver enzyme biochemical assay are a group of diagnostic blood tests that measures the levels of various enzymes produced by the liver and can help identify areas of the liver where damage or dysfunction may be occurring. Carbon tetrachloride is one of the common cause of liver damage, it is associated with significant increase in liver enzyme production leading to increased free radical that causes damage to the hepatocytes [12]. An *in vivo* model was chosen for this study to account for the significant reduction in elevated liver enzymes often experienced in liver damage in rats. The most reliable parameter for assessing the hepatoprotective effects of the extract and fractions is the reduction in the level of liver enzymes. There was a significant decrease difference ($p < 0.05$) between the level of liver enzymes in the negative control and other treated groups.

The administration of 200 and 400 mg/kg *B. brieyi* extract (BBCE) resulted in a higher reduction of liver enzymes (ALT, AST, and ALP) compared to the positive control (Silymarin 25 mg/kg). Specifically, BBCE 200 mg/kg reduced significantly at ($p < 0.05$) for ALT, AST, and ALP from 46.6, 74.80, and 79.40 IU/L to 23.20, 38.40, and 46.40 IU/L respectively but in contrast, the positive control reduced significantly these enzyme levels to 24.2, 37.20, and 48.40 IU/L. Interestingly, the groups treated with BBCE showed a dose-dependent increase in the reduction of liver enzymes. This trend was observed from the group treated with a lower dose of BBCE (100 mg/kg) to the group treated with a higher dose (400 mg/kg). The hepatoprotective effect of the highest dose of BBCE (400 mg/kg) was significantly different ($p < 0.05$) from that of the lowest dose (100 mg/kg) and the positive control (Silymarin 25 mg/kg). This suggests that the extract at 400 mg/kg of *B. brieyi* has a stronger hepatoprotective potential when compared to the controls.

The American College of Gastroenterology (ACG) has established that an abnormal level of alanine aminotransferase (ALT) is greater than 34 IU/L and aspartate aminotransferase (AST) is greater than 40 IU/L. According to Lala *et al.*, [12], elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) above normal range are key indicators of liver damage. From previous literature on the normal range of liver enzymes, we have their normal range to be: ALT (7-34 IU/L), AST (10-40 IU/L), ALP (30-129 IU/L), ACP (2.2 – 10.5 IU/L), Bilirubin (Total bilirubin: 0.2-1.2 mg/dL), Creatinine (0.6-1.2 mg/dL *males) (0.5-1.1 mg/dL* females), Urea (7-20 mg/dL), Albumin (3.5-5.5 g/dL) [12]. The ethyl acetate and methanol fractions at a dose of 200 mg/kg each prevented elevated levels of the enzymes ALT, AST, ACP, ALP, Bilirubin, Creatinine, Urea and Albumin although ethyl acetate fraction showed a slightly higher activity on ALP (46.800 ± 0.860). Acid phosphatases are enzymes that are capable of hydrolyzing phosphate esters in an acidic environment, there was no significant reduction in the levels of acid phosphatase. The positive control gave the highest concentration of total protein while the negative control gave the lowest concentration. The normal showed elevated concentrations of the total protein and the ethyl acetate and methanol fractions showed increasing levels of the total protein. The positive control gave the highest concentration of total protein while the negative control gave the lowest concentration [66]. In this study, the negative control group, which was induced with carbon tetrachloride but not treated, showed elevated levels of all the liver enzymes. This elevation is attributed to the destruction of the hepatocytes, thereby releasing high levels of the liver enzymes.

The histopathology result of the negative group showed multi focal areas of hepatocellular necrosis and inflammatory disorders which are associated with abdominal pains and swelling, fatigue and jaundice which are all symptoms commonly associated with liver damage [67]. The administration of *B. brieyi* extract and fractions resulted in a significant reduction ($p < 0.05$) in the levels of these liver enzymes, thereby reducing the multifocal destruction of the hepatocytes, this demonstrates its potential hepatoprotective effect.

Silymarin, a complex of flavonoids extracted from the milk thistle plant (*Silybum marianum*), has demonstrated hepatoprotective properties through its ability to decrease inflammation and act as an antioxidant [28]. Flavonoids are phytoconstituents which have numerous potentials to stimulate the formation of enzymes, increase the

production of bile, reduce inflammation, and soothing mucous membranes. The oral administration of silymarin extract was effective in preventing hepatocellular damage in a model of acute liver injury [28, 68]. This extract also had a direct impact on the expression of pro-inflammatory and pro-fibrogenic genes in hepatic stellate cells when tested *in vitro* [69]. These mechanisms include blocking and adjusting cell transporters such as p-glycoprotein and estrogenic and nuclear receptors, scavenging free radicals through anti-lipid peroxidation, increasing the level of glutathione in the liver, exerting antioxidant and membrane stabilizing activity, promoting the regeneration of hepatocytes, and reducing inflammation and inhibiting fibrogenesis in the liver [68]. The primary mechanism that ensures hepatoprotection and oxidation appears to be free radical scavenging [69].

CONCLUSION

The extract and fractions of *B. brieyi* root has demonstrated a significant dose-dependent hepatoprotective potential at 200 mg/kg doses by significantly reducing the liver enzymes values thereby decreasing the multifocal hepatocellular necrosis as seen from the histology studies. This finding validates the traditional use of *B. brieyi* in the treatment of liver disorders. This study, therefore, serves as a promising stepping stone towards harnessing the full potential of *B. brieyi* in liver disease management.

Conflict of Interest

The authors have declared no conflict of interest associated with this work.

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Contribution of authors

ICE: Conceptualization of the research work, wrote the manuscript, supervised the animal studies, phytoconstituents studies, OE: participated in writing the literature review, analyzed the results of liver enzymes, and interpreted the histopathology studies, PFU and NJN: supervised the data analysis and the manuscript writing, FIN: contributed in collection of the plant root and literature review, UAC, CEO, and EJU: participated in the *in vivo* studies and phytochemical studies.

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Supplementary data

The chromatograms from the GC-MS analysis of the various samples are available from the corresponding author upon request.

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