



Research article

Antioxidant and hepatoprotective potentials of active fractions of *Lansea barteri* Oliv. (Anarcadiaceae) in ratsFlorence N. Mbaoji^a, Justus Amuche Nweze^{b,*}^a Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, PMB 410001, Enugu State, Nigeria^b Department of Science Laboratory Technology (Microbiology Unit), Faculty of Physical Sciences, University of Nigeria, Nsukka, PMB 410001, Enugu State, Nigeria

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ABSTRACT

Introduction: *Lansea barteri* is used in the folkloric treatment of many disease states ranging from epilepsy, diarrhoea, oedema and ulcers, etc. This study investigated the antioxidant and hepatoprotective potentials of methanol (MFLB), n-hexane (nHFLB) and ethyl acetate (EFLB) leaf fractions of *L. barteri* and identified the active metabolites.

Materials and methods: The *in vitro* models used were 1, 1-diphenyl-1-picrylhydrazyl (DPPH), reducing power and thiobarbituric acid assays while in the *in vivo* model, carbon tetrachloride-induced oxidative liver damage in albino rats was used, and the biomarkers assayed were aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), malondialdehyde (MDA), serum total protein, serum direct and total bilirubin. Also, histopathological examination of the liver, high performance liquid chromatography (HPLC) profiling and liquid chromatography-mass spectroscopy-electrospray ionization (LC-MS-ESI) analysis of the fractions were done.

Results: In the *in vitro* assays, the decreasing order of DPPH free radical scavenging activity of the ascorbic acid and fractions at 400 µg/ml is as follows: ascorbic acid (86.6%), MFLB (52.8%), EFLB (36.6%), and nHFLB (28.1%). The percentage scavenging activity of the samples at 400 µg/ml in the TBA followed this pattern: ascorbic acid (117.1%), MFLB (82.2%), nHFLB (80.0%), and EFLB (46.9%). The ascorbic acid elicited highest reducing power (42.6%), followed by MFLB (22.5%), nHFLB (13.7%), and EFLB (-0.93%). The *in vivo* study showed significant ($p < 0.0001$) reduction in serum AST, ALT, and direct bilirubin with a non-significant reduction in ALP, total bilirubin and MDA, and mild elevation in total protein. Histopathological studies revealed a restorative effect on liver architecture. The phytochemical analyses revealed the presence of resins, terpenoids, flavonoids, carbohydrates, alkaloids, reducing sugar, saponins, tannins and proteins. HPLC-ESI-MS analysis revealed the presence of potentially bioactive compounds in *L. barteri* fractions.

Conclusion: The fractions from *L. barteri* leaf possessed *in vitro* antioxidant and hepatoprotective potentials against CCl₄-hepatic oxidative damage; therefore, proper isolation and characterization of these identified bioactive compounds responsible for the observed effects are ongoing.

1. Introduction

Free radicals are reactive oxygen species (ROS) or nitrogen species (RNOS) which are generally very unstable and react quickly, such as singlet oxygen, hydrogen peroxide, nitric oxide radical, hydroxyl radical, superoxide anion, and various lipid peroxides [1] which can react with and damage the membrane lipids, proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), enzymes and other small molecules [2]. In living cells, numerous ROS formed may be from different reactions, including normal aerobic respiration, activated peroxisomes, polymorphonuclear leukocytes and macrophages, thus, these are the main

endogenous sources of most of the oxidants. The sources of exogenous free radicals are pesticides, tobacco smoke, organic solvents, ionizing radiation, and certain pollutants, etc. [3]. Oxidative stress is a physiological stress on the body caused by the summed effect of free radicals as a result of an imbalance between oxidants and antioxidant systems in the body. The damaging effects of free radicals in cells may lead to ageing, neurodegenerative diseases, renal failure, cataracts, cardiovascular disease, brain dysfunction, diabetes mellitus, cancer, immune system decline, liver diseases, inflammation, and stress among others [3, 4] To prevent these disease conditions and protect the body against free radicals, human body is endowed with special complex and sophisticated

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antioxidant protection systems, that work synergistically and interactively to neutralize free radicals. Therefore, before the cells are attacked by free radicals, antioxidants will stabilize or deactivate them [5]. In nature, there is a dynamic equilibrium between the number of free radicals generated in the body and antioxidants that protect the body against their damaging effects [2, 5]. Unfortunately, in some situations, the physiological amount of antioxidant substances available under normal situation may not be sufficient to neutralize free radicals generated. Consequently, the best option is to enrich our diet with exogenous antioxidants to prevent and protect the body against the deleterious effects of reactive oxygen and nitrogen species [4]. Previous studies have shown that certain plant extracts have good antioxidant activity and thus, may be beneficial in protecting against the oxidative damage of the body [6, 7, 8]. Therefore, in food industry, there is an increased interest in developing preventive medicine with “natural antioxidant” ability from plant materials [9].

Lannea barteri (Oliv.) Engl (Anacardiaceae) is a deciduous tree with a spreading crown. It can grow from 5 to 18 m tall. The pole is usually straight and clear of branches for several meters, it can be up to 40 cm in diameter with thick bark. *L. barteri* can be found in Guinea east to as far as Uganda, Ethiopia, DR Congo, Burundi, Nigeria, Ghana. It is known with the following common names: Fula-fulfulde (Adamawa), Butter-oil tree (English), Babbàn bábàà (Hausa), Akan-asante kuntunkurfi (Ghana) [10, 11]. The plant is used for different folkloric medicinal purposes ranging from ulcers, sores, and leprosy using the bark as a poultice to stomachic, vermifuge, gastric pains, diarrhoea, oedema, paralysis, epilepsy, and madness when the decoction of the bark is drunk; while the root and leaf decoctions are taken to cure hernia and haemorrhoids respectively [10, 11]. Phytochemical studies of petroleum ether extract showed the presence of triterpenes and steroids, while flavonoids, steroid, tannins, saponins, triterpene, and polyphenols were found in both crude chloroform fraction and methanol extract [10, 11]. Pharmacological screening on the plant extracts revealed antioxidant, acetylcholinesterase inhibitory, antibacterial [10], anticonvulsant [11], anti-inflammatory [12], anticancer [13] activities, and haematological properties [14, 15].

2. Results

2.1. Phytochemical constituents of the fractions

The phytochemical analysis showed that the methanol fraction of *L. barteri* (MFLB) tested positive to carbohydrate, alkaloid, reducing sugar, saponins, tannins, flavonoids, resins, and proteins while ethyl acetate fraction of *L. barteri* (EFLB) gave a positive reaction for flavonoids, resins and terpenoids, and n-hexane fraction of *L. barteri* (nHFLB) tested positive to resins, steroids, terpenoids and fats and oils (Table 1).

2.2. Pharmacological tests

2.2.1. Effect of fractions on DPPH radical scavenging assay

The free radical scavenging activities of methanol, n-hexane and ethyl acetate fractions of *L. barteri* were determined using DPPH radical scavenging assay, and ascorbic acid as a standard antioxidant (Figure 1). In this assay, methanol fraction (25 µg/ml) initially showed higher activity (47.4%) than the ascorbic acid (24.8%) but the reverse was the case for the rest of the concentrations tested. At higher concentration (400 µg/ml), ascorbic acid showed higher activity (86.6%) than MFLB (52.8%), EFLB (36.6%) and nHFLB (28.1%).

2.2.2. Effect of fractions on inhibition of lipid peroxidation and reducing power assays

The ability of the fractions to inhibit peroxidation of lipids was evaluated using lipid peroxidation inhibitory assay. As shown in Figure 2, the ascorbic acid showed the highest inhibitory activity at 400 µg/ml with percentage inhibition of 117.1% followed by MFLB (82.2%), nHFLB (80.0%), and EFLB (46.9%).

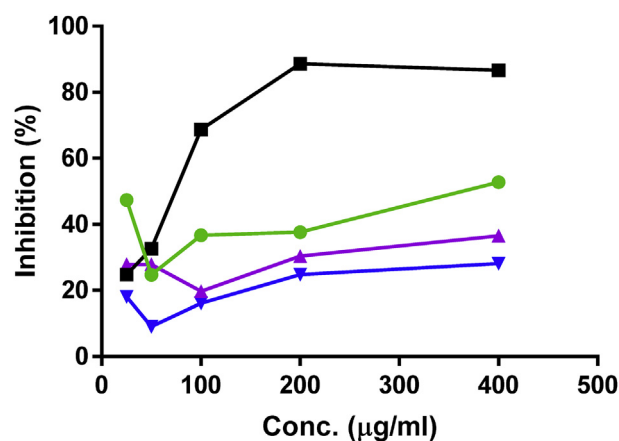


Figure 1. Percent DPPH radical scavenging activities of methanol, n-hexane and ethyl acetate fractions (concentration 1 mg/mL) of *Lannea barteri*. Percentage inhibition versus the concentration of methanol, n-hexane and ethyl acetate fractions of *L. barteri* after an incubation for 30 min, using ascorbic acid as standard antioxidant. ■—Ascorbic acid, ▲—EFLB, ●—MFLB, ▼—nHFLB.

The reducing power of the MFLB, nHFLB and EFLB was determined and the results are shown in Figure 3. Ascorbic acid gave a dose-dependent and the highest percentage (53.2%) reduction of $\text{Fe}_2(\text{SO}_4)_3$ to FeSO_4 at 400 µg/ml followed by nHFLB (33.9%), MFLB (29.8%) and lastly EFLB (15.5%) at the same concentration.

2.2.3. Effect of EFLB and MFLB on serum alanine transferase level (ALT)

The CCl_4 caused a significant increase in serum ALT with a mean value of 507.2 IU/L as against the 7% Tween 80 (65.21 IU/L) and naive (86.006 IU/L)-treated groups (Figure 4). All the pre-treated groups had significant ($p < 0.0001$) protection against the hepatic damage caused by the carbon tetrachloride. The reduction of ALT level in all the treated groups follows this trend starting with the treatment that gave the best reduction: positive control (silymarin) (29.24 IU/L), 400 mg/kg MFLB (48.31 IU/L), 200 mg/kg EFLB (49.90 IU/L), 200 mg/kg MFLB (63.46 IU/L) and 400 mg/kg EFLB (66.76 IU/L) when compared to CCl_4 -treated group (Figure 4).

2.2.4. Effect of EFLB and MFLB on serum aspartate transaminase (AST)

The CCl_4 caused a large increase in serum AST with a mean value of 1195.54 IU/L (Figure 5). The silymarin and naive groups had mean

Table 1. Preliminary qualitative phytochemical constituents of the fractions of *Lannea barteri* extract.

Constituents	Relative abundance		
	nHFLB	EFLB	MFLB
Alkaloids	-	-	+++
Carbohydrates	-	-	+++
Fats and oils	+	-	-
Flavonoids	-	+++	+++
Glycosides	-	-	-
Proteins	-	-	+
Reducing sugars	-	-	++
Resins	+	++	+
Saponins	-	-	++
Steroids	+	-	-
Tannins	-	-	++
Terpenoids	+	+++	-

+= abundant; ++ = moderately abundant; +++ = highly abundant; - = absent; nHFLB = n-hexane fraction of *L. barteri*, EFLB- ethyl acetate fraction of *L. barteri*, MFLB – methanol fraction of *L. barteri*.

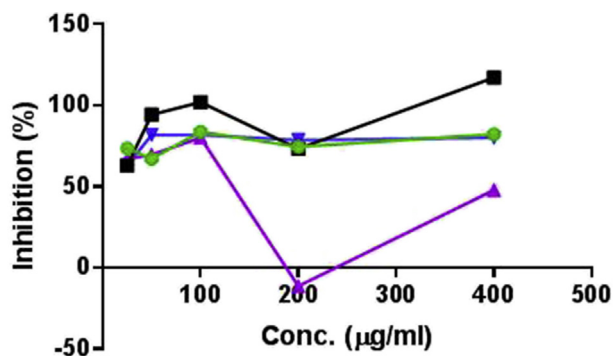


Figure 2. Effect of fractions on lipid peroxidation inhibitory activity. The ability of the fractions to inhibit peroxidation of lipids was determined by plotting percentage inhibition versus the concentration of methanol, n-hexane and ethyl acetate fractions, using ascorbic acid as standard antioxidant. ● MFLB, ■ Ascorbic acid, ▲ EFLB, ■ nHFLB.

serum AST values of 179.28 IU/L and 230.72 IU/L, respectively. The groups that received the fractions had nondose-dependent and significant ($p < 0.0001$) protection of rat's liver against the toxic effect of CCl₄. The EFLB (200 and 400 mg/kg) exerted higher liver protective effect with mean AST values of 220.76 IU/L and 311.38 IU/L, respectively than the MFLB which had mean AST values of 340.26 IU/L and 506.76 IU/L, respectively.

2.2.5. Effect of EFLB and MFLB fractions on serum alkaline phosphatase

The normal control (7% Tween 80), negative control (CCl₄) and the naive groups had smaller mean serum ALP value of 317.34 IU/L, 896.22 IU/L and 525.92 IU/L, respectively (Figure 6). The positive control group had a mean serum ALP value of 696.58 IU/L which was not significantly different from some other groups. The EFLB at 400 mg/kg did not exert any protection and had the highest mean serum ALP level (1628.81 IU/L), which was significantly different from the positive and negative control groups ($p < 0.05$). Other fractions MFLB (200 mg/kg = 679.36 IU/L; 400 mg/kg = 1267 IU/L) and EFLB (200 mg/kg = 1156.8 IU/L) were not significantly different from CCl₄ treated group (Figure 6).

2.2.6. Effect of EFLB and MFLB on serum total bilirubin

There was an increase in serum total bilirubin level in all the groups (Figure 7). The CCl₄-treated group had the highest level of serum total bilirubin (0.628 mg/dl), while the groups that received 7% Tween 80,

silymarin (positive control), and naive groups had serum total bilirubin level of 0.404 mg/dl, 0.392 mg/dl, and 0.192 mg/dl, respectively. The higher concentrations (400 mg/kg) of MFLB and EFLB significantly ($p < 0.05$) protected the rats against the elevated level of serum total bilirubin as caused by CCl₄ (0.628 mg/dl) with their respective serum total bilirubin level of 0.35 and 0.26 mg/dl while at lower concentration (200 mg/kg), they did not significantly reduce the effect as seen from their respective values (0.584 mg/dl) and (0.370 mg/dl).

2.2.7. Effect of fractions on serum direct bilirubin

The CCl₄ significantly increased the mean serum direct bilirubin level in the CCl₄-treated group to 0.144 mg/dl when compared with the values from 7% Tween 80-treated and naive groups which had lower mean values of 0.044 mg/dl and 0.016 mg/dl, respectively (Figure 8). Equally, the MFLB (400 mg/kg), EFLB (400 mg/kg), silymarin, MFLB (200 mg/kg), and EFLB (200 mg/kg) had significant protective effects against liver damage caused by CCl₄ with lower mean serum direct bilirubin values of 0.026, 0.044, 0.06, 0.064, and 0.1 mg/dl, respectively, when compared to CCl₄-treated rats. The protection by the fractions was dose-dependent (Figure 8).

2.2.8. Effect of EFLB and MFLB on serum total protein

The CCl₄-treated group had the lowest mean serum total protein value of 6.64 mg/dl but it was not significantly different from that of the naive group (7.0 mg/dl) (Figure 9). Both MFLB and EFLB (200 and 400 mg/kg), 7% Tween, and silymarin-treated groups, all had restored protein levels (7.36, 7, 7.08, 7, 7.44 and 7.28 mg/dl, respectively) from the sudden drop below the level recorded in the naive control group as caused by CCl₄ (Figure 9), although these values from all the groups including the CCl₄-treated group are on the normal range of values (5.6–7.6 mg/dl) for serum total protein in rat.

2.2.9. Effect of EFLB and MFLB on serum malondialdehyde (MDA)

The CCl₄-treated group had the highest mean serum MDA level of 0.0787 AU/ml while the 7% Tween 80 and naive groups had the lowest mean serum MDA levels of 0.0450 and 0.0505 AU/ml, respectively (Figure 10). Although not significantly different from naive and normal groups, the silymarin, MFLB and EFLB (200 and 400 mg/kg) treated-groups in a dose-dependent manner (for the fractions) reduced the elevated MDA level caused by CCl₄ with mean serum MDA levels of 0.0770, 0.0755, 0.0682, 0.0676 and 0.0673 AU/ml, respectively (Figure 10).

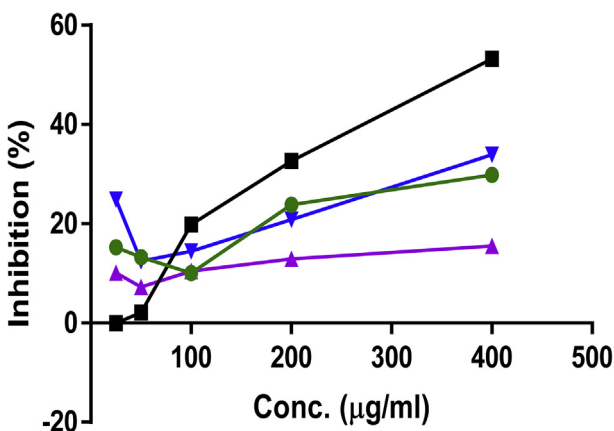


Figure 3. Reducing power assay of different fractions of *Lananea barteri*. The ability of the fractions from different extracts of *Lananea barteri* to reduce Fe₂(SO₄)₃ to FeSO₄, using ascorbic acid as standard antioxidant, were expressed as percentage reduction at 700 nm. ● MFLB, ■ Ascorbic acid, ▲ EFLB, ■ nHFLB.

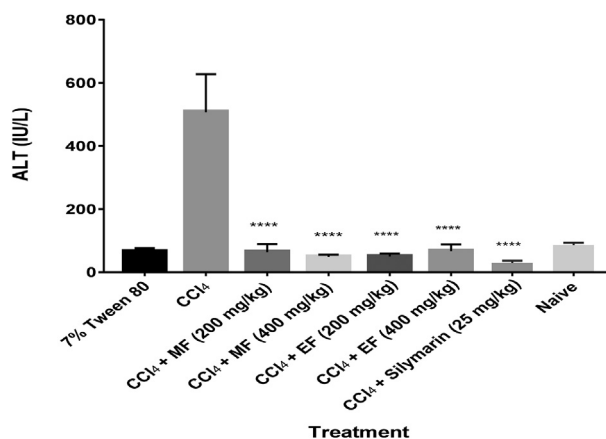


Figure 4. Effect of methanol and ethyl acetate fractions on serum alanine transferase level. n = 5, **** $p < 0.0001$ is significant when compared with CCl₄-treated group. MFLB – methanol fraction of *Lananea barteri*; EFLB - ethyl acetate fraction of *Lananea barteri*.

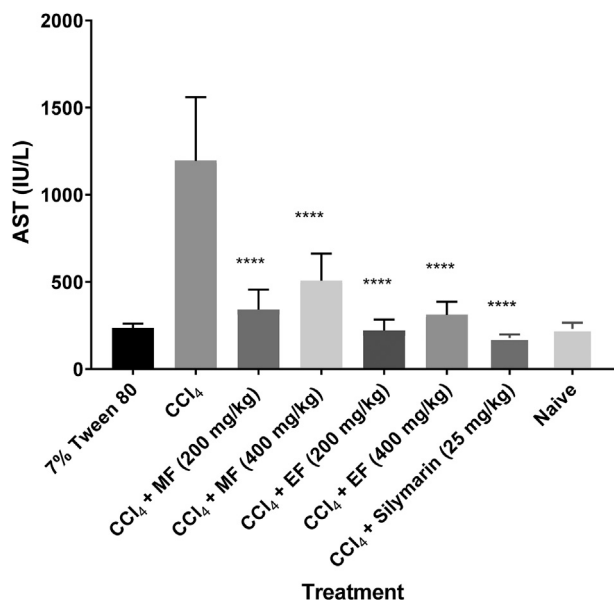


Figure 5. Effect of fractions on serum aspartate transaminase (AST). ‘*’ Represents the degree of significant difference existing between a group and the CCl₄ group, *****p* < 0.0001; MFLB – methanol fraction of *Lannea barteri*; EFLB - ethyl acetate fraction of *Lannea barteri*.

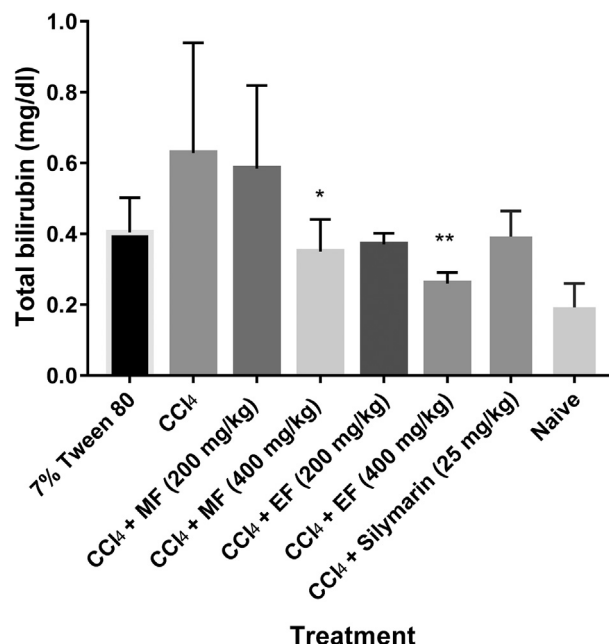


Figure 7. Effect of EFLB and MFLB on serum total bilirubin. **p* < 0.05, ***p* < 0.01 significant when compared to the CCl₄-treated group. MFLB – methanol fraction of *Lannea barteri*; EFLB - ethyl acetate fraction of *Lannea barteri*.

2.2.10. Effect of the fractions on the histology of the liver of CCl₄-treated rats

The liver sections isolated from the animals treated with 7% Tween and naive groups showed normal hepatic histo-architecture. These sections showed normal hepatic lobules consisting of normal hepatocytes which are arranged in interconnecting cords; radiating towards the periphery of the lobules where it joins with the constituent of the portal triads (hepatic artery, hepatic vein and bile duct) (Figure 11: 1 and 2). However, the liver sections collected from the animals that received CCl₄, silymarin, 200 and 400 mg/kg ethyl acetate and methanol fractions showed severe histopathological lesions primarily consisting of

centrilobular and mid-zonal degeneration and necrosis of the hepatocytes with random aggregations of inflammatory mononuclear phagocytes (Figure 11: 3–8). The affected hepatocytes were variably swollen; showing numerous tiny clear cytoplasmic vacuoles (micro-vesicular steatosis) or large clear cytoplasm vacuole with pyknotic eccentric nuclei (macro-vesicular steatosis). The severity of liver damage caused by the CCl₄ was quite alarming in the group that received CCl₄ alone while the groups that received the fractions and silymarin had milder damage showing some level of liver protection from the hepatotoxic effect of CCl₄ although, MFLB gave better protection than the EFLB. This was obvious from the extent of reduction in the level of liver enzymes as caused by these fractions in comparison with the CCl₄-treated groups’ liver enzyme levels.

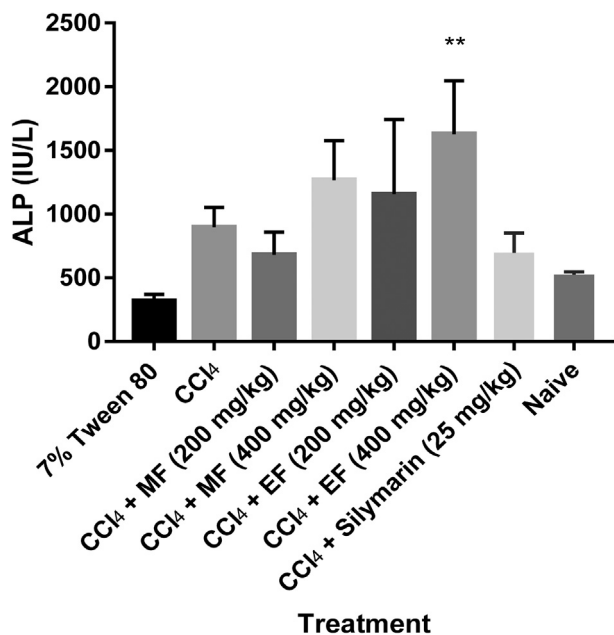


Figure 6. Effect of EFLB and MFLB on serum alkaline phosphatase. ***p* < 0.01 significant when compared to the CCl₄-treated group; MFLB – methanol fraction of *Lannea barteri*; EFLB - ethyl acetate fraction of *Lannea barteri*.

2.3. HPLC-UV and ESI-MS analysis

Using HPLC-ESI-MS, the potentially bioactive compounds in *Lannea barteri* fractions were analyzed, leading to the identification of some metabolites. The chromatography, UV and ESI-MS data of marker compounds identified from the fractions are as follows: Myricetin-3-O-rhamnoside, Quercetin-3, 7, 3', 4'-tetramethyl, Quercetin-3-O-galactoside (Hyperside), Quercetin-3-O-rhamnoside (Quercetrin), Kaempferol-3-O-rhamnoside, Quercetin-3-O-arabinofuranoside, fatty acid derivatives and pigments (Figure 12; Table 2).

3. Discussion

This study aimed to determine the antioxidant and hepatoprotective activities of the methanol, n-hexane and ethyl acetate fractions of leaf of *Lannea barteri* using *in vitro* and *in vivo* antioxidant models. The phytochemical analysis of the fractions showed that the fractions are rich in phytoconstituents. The MFLB which exhibited the greatest *in vitro* and *in vivo* antioxidant effect possessed a good number of these phytochemicals (alkaloids, flavonoids, carbohydrates, proteins and tannins among others) more than the other fractions which are also highly rich in terpenoids. Several reports have documented the antioxidant activity of flavonoids in *Auricularia auricular* [16], *Ligustrum vulgare* [17] and *thymus*

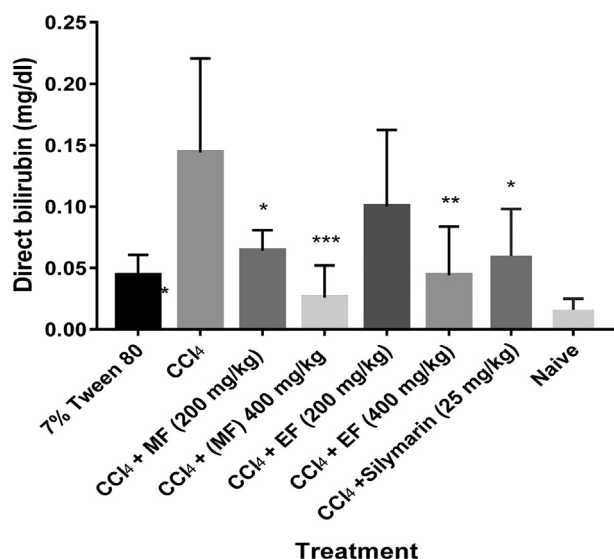


Figure 8. Effect of EFLB and MFLB on serum direct bilirubin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to CCl₄ group. MFLB – methanol fraction of *Lannea barteri*; EFLB - ethyl acetate fraction of *Lannea barteri*.

vulgaris [18]; tannins in *Terminalia chebula* [19] and terpenoids in wine [20]. A study conducted in Abidjan, Côte d'Ivoire showed the presence of similar chemical constituents (such as flavonoids and polyterpenes) in ethyl acetate extract of stem and root of *L. barteri*, which, in contrast to our study, recorded absence of alkaloids and flavonoids in the methanol extract [10].

The radical scavenging activity of antioxidant compounds is usually determined using DPPH, which is reduced in methanol solution in the presence of hydrogen-donating antioxidants due to formation of the non-radical form DPPH-H [5]. As the free electron of the radical gets paired off due to the availability of a hydrogen donor, for instance, a free radical-scavenging antioxidant, the absorption strength is reduced, and the resulting discoloration (from purple to yellow) is stoichiometric with regards to the number of electrons captured [21]. In this study, the MFLB produced a maximum percentage inhibition (52.8%) of DPPH radical more than the EFLB (36.6%) and nHFLB (28.2%). A previous study reported higher percentage inhibition (83.64%) of DPPH radical by methanol extract of stem bark from *L. barteri* [10]. This implies that the

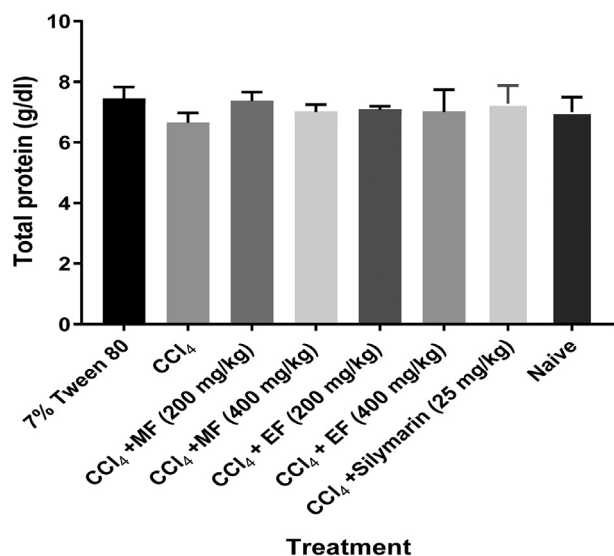


Figure 9. Effect of EFLB and MFLB on serum total protein. MFLB – methanol fraction of *Lannea barteri*; EFLB - ethyl acetate fraction of *Lannea barteri*.

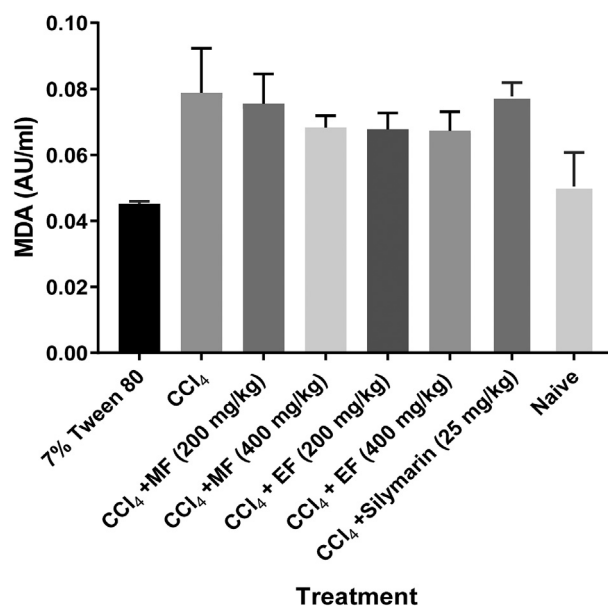


Figure 10. Effect of EFLB and MFLB on serum malondialdehyde (MDA). MFLB – methanol fraction of *Lannea barteri*; EFLB - ethyl acetate fraction of *Lannea barteri*.

fractions possessed radical scavenging activity and are capable of inhibiting and mopping up free radicals in the system and consequently reduce damage to cell membranes and death.

The ability of the plant extracts to inhibit lipid peroxidation caused by TBA is a measure of their antioxidant activity [15]. The MFLB exhibited the strongest antioxidant activity, followed by nHFLB and EFLB towards the inhibition of lipid peroxidation at a higher dose. This was shown by their low absorbance and high percentage inhibition observed in the assay. Lipid peroxidation is commonly considered as the central molecular mechanism involved in the oxidative damage to cell structures (impaired cellular functioning, even cell rupture, and loss of membrane elasticity and fluidity) and in the toxicity process that leads to cell death [22].

In the reducing power assay, an increase in absorbance indicates an increase in antioxidant property. The MFLB had the highest reduction capacity followed by nHFLB and EFLB in this order. This signifies that the fractions can convert the oxidized form of Fe³⁺ to reduced form of Fe²⁺ which is an indication of antioxidant activity. Also, the reducing capacity is commonly associated with the availability of reductants and the antioxidant action of a reductant is based on its ability to break the free radical chain by donating a hydrogen atom [21].

In the experimental study of liver disorders, one of most the hepatotoxic agents commonly used is carbon tetrachloride (CCl₄), which exerts its hepatotoxic effect by using activated radicals (trichloromethyl radical) to covalently bind to the macromolecules leading to induction of peroxidative degradation of membrane lipids of endoplasmic reticulum, which is rich in polyunsaturated fatty acids (PUFA). CCl₄ does not only target the liver, but it also affects several organs of the body such as lungs, hearts, testes, kidney, and brain [23, 24]. The efficacy of any hepatoprotective drug is determined by its capability of either maintaining the normal hepatic physiological mechanism which has been imbalanced by a hepatotoxin or protecting the liver from or reducing the harmful effects of the hepatotoxin [25]. The remarkable elevation in the rat's bilirubin and marker enzymes (AST, ALT and ALP) in CCl₄ administered rats in this study is a substantiation of previous reports on the hepatotoxicity of CCl₄. The increased levels of these biochemical parameters are an indication of alterations in the hepatic structural integrity [8, 23, 26]. An increase in the serum levels of ALT is particularly an indication of liver damage. Once the cellular membrane is damaged, these enzymes

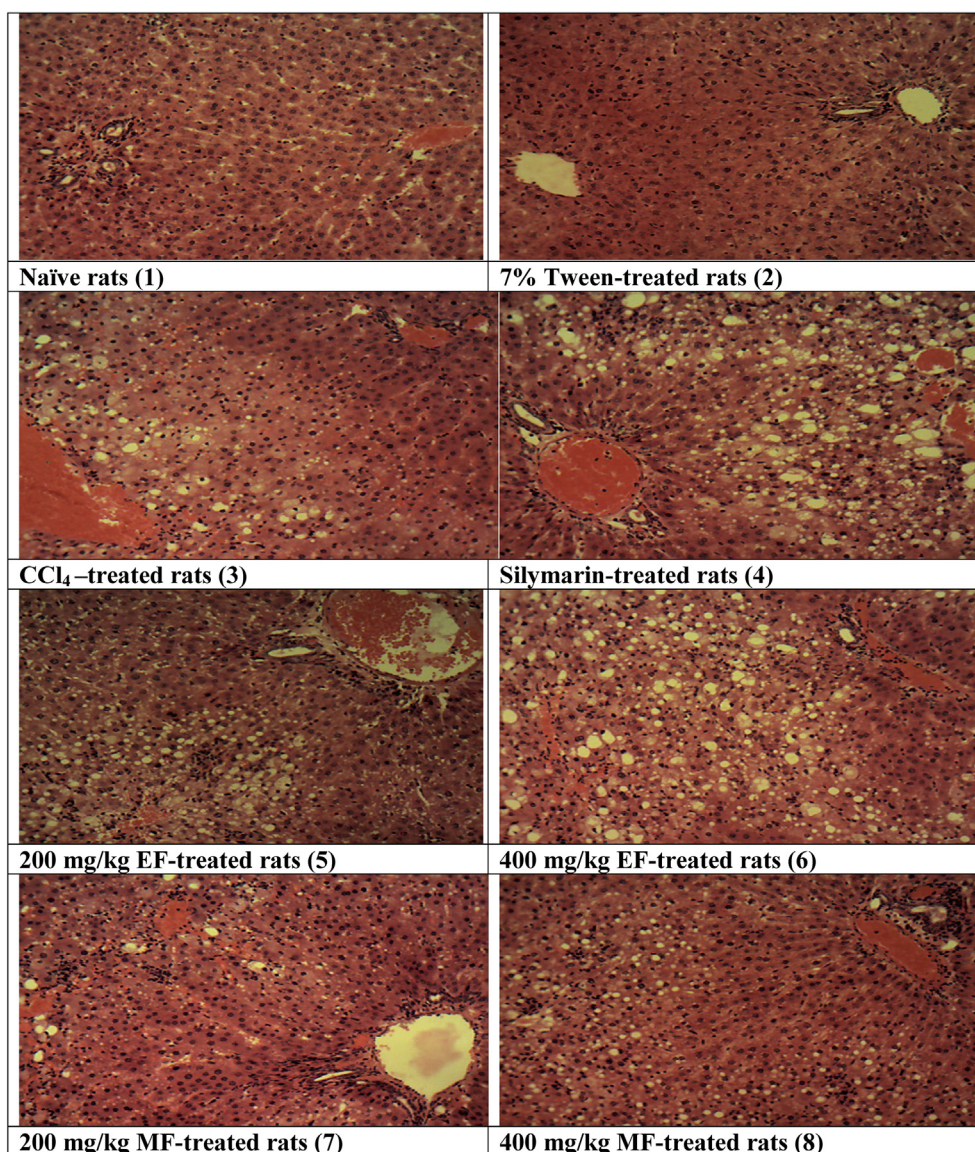


Figure 11. Histopathology of liver of rats treated with EFLB, MFLB, silymarin and controls (7% Tween 80, naive and CCl₄). Normal hepatic histo-architecture with normal hepatic lobules consisting of normal hepatocytes which are arranged in inter-connecting cords, radiating towards the periphery of the lobules where it meets with the components of the portal triads (hepatic artery, central vein (V), portal triad (P), hepatic cord (arrow) (1 and 2); central vein (V) degenerate and necrotic hepatocytes (arrow) portal triad (P) (3); central vein (V) degenerate and necrotic hepatocytes (black arrow), portal triad (P) (4); central vein (V), degenerate and necrotic hepatocytes (black arrow), portal triad (P), inflammatory cellular aggregates (white arrow) (5); central vein (V), degenerate and necrotic hepatocytes involving the 3 zones in the hepatic lobule (black arrow), components of the portal triad [hepatic artery – HA, bile duct - BD] (6); Central vein (V), degenerate and necrotic hepatocytes (black arrow) portal triad (P) (7); Central vein (V), degenerate and necrotic hepatocytes (black arrow), portal triad (P) (8). EF – ethyl acetate fraction of *Lannea barteri*, MF – methanol fraction of *Lannea barteri*, H and E x160. H - hematoxylin, E-Eosin.

(originally present in the cell cytoplasm) are leaked into the circulation [26]. However, the MFLB and EFLB markedly reduced the level of these enzymes markers (AST and ALT), and this aligns with the reports that serum levels of transaminase return to normal due to the healing of hepatic parenchyma and the regeneration of hepatocytes [25]. Also, ALP is found in the proximal convoluted tubule of the kidney, liver, bone, placenta and mucosal epithelia of the small intestine. It performs lipid transportation in the intestine and calcification in the bone. Some conditions such as acute cirrhosis, congestive cardiac failure, hepatitis, tumors, hepatic and bony metastasis can maintain or moderately increase or elevate the serum ALP [27]. In this study, a-dose-dependent increase in the level of ALP was observed in the groups pre-treated with the leaf fractions while silymarin reduced the level of ALP with respect to the negative control. The activity of ALP is linked to the functioning of hepatocytes. So, when the increased ALP activity is suppressed, it points to a reduction in biliary dysfunction in rat's liver during subacute hepatic injury with CCl₄. Therefore, it can be inferred from this study that higher concentration of MFLB and both low and high concentrations EFLB have no effect on biliary dysfunction and had a significant elevation in the levels of the enzyme, unlike silymarin which stabilized biliary dysfunction. Although this is the first report on these, the report of Oriakhi et al [15] is similar with the findings of this study due to the decreased activity of AST, ALT, and increased content of total protein and albumin levels in

CCl₄-induced hepatotoxicity when treated with aqueous extract of medicinal plant.

Evidence has shown that an increase in bilirubin concentration in the tissue or serum may be as a result of an obstruction in the excretion of bile [26]. Therefore, increased levels of bilirubin observed in this study in a group administered with CCl₄ only could be as a result of liver damage. The decrease in bilirubin levels in rats pre-treated with the fractions is a sign of reversal of liver damage [25]. Bilirubin is a catabolic product of haemoglobin produced within the reticuloendothelial system and released in an unconjugated form (indirect bilirubin) which enters into the liver, converted to conjugated form (direct bilirubin), bilirubin mono and diglucuronides by the enzyme UDP-glucuronyltransferase [28,29]. In this study, direct bilirubin (conjugated form) level increased on the administration of CCl₄ but was decreased by the fractions in a dose-dependent manner.

Administration of CCl₄ leads to a decrease in total protein. The albumin and total protein diminution induced by CCl₄ is a further indication of liver damage [28, 29]. There are reports on the hepatoprotective and antioxidant effects of plant extracts against CCl₄-induced hepatotoxicity in rats, a reduced level of total protein was observed in the CCl₄ administered group which was reversed on the administration of alcohol extract of the plant [26, 28, 29, 30]. The present study showed that the fractions reversed the decreased level of total protein caused by CCl₄,

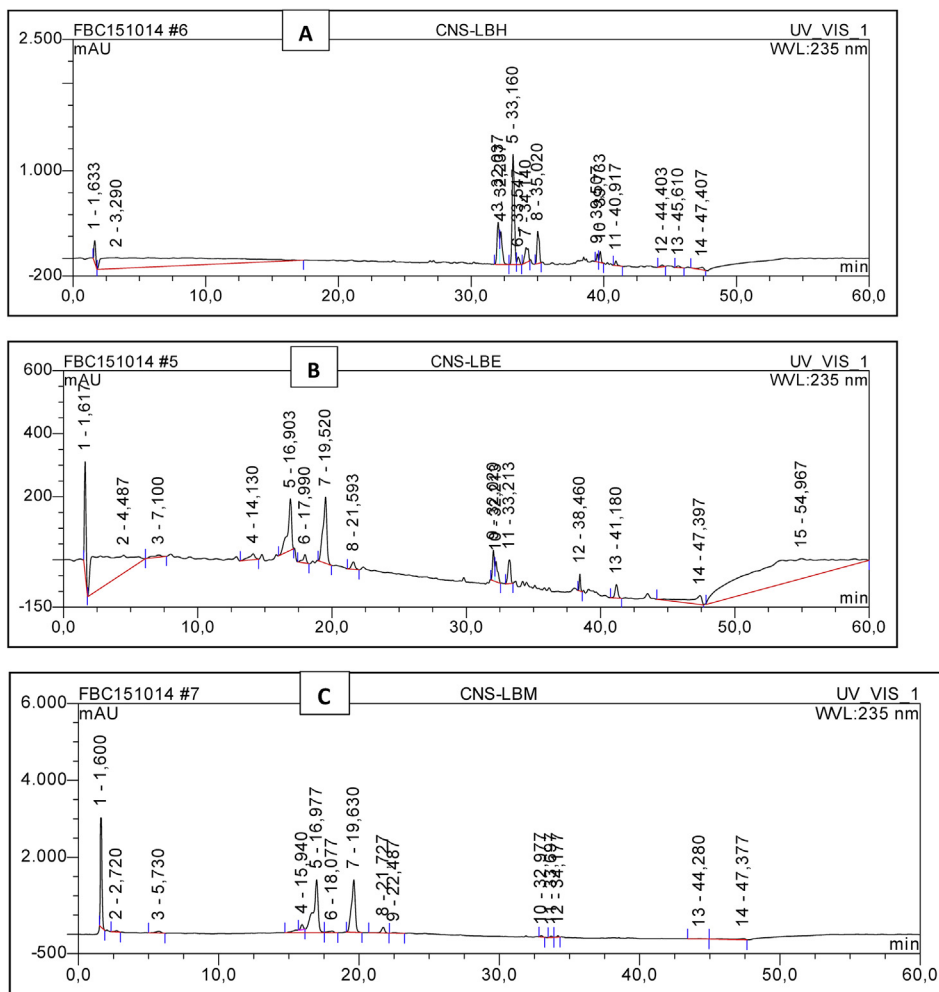


Figure 12. HPLC chromatogram of (A) nHFLB, (B) EFLB and (C) MFLB. The identity of compounds are shown in Table 2.

Table 2. Chromatography, UV and ESI-MS data of marker compounds.

Peak No.	Peak label	t _r (min)	UV (PDA) λ_{max} (nm)	[M + H] ⁺ (m/z)	Fragment ions (+)	[M-H] ⁻ (m/z)	Fragment ions (-)	Molar mass (g/mol)	Compound
From nHFLB									
4	1		234.9						Fatty acid derivative
	2		235.2						Fatty acid derivative
From EFLB									
4	1	15.80	260.4, 359.9	465.1	319.4	463.4	316.4, 317.4	464	Myricetin-3-O-rhamnoside
5	2	16.88	261.2, 252.4						Quercetin-3,7,3',4'-tetramethyl
6	3	18.05	257.5, 355.9	464.8	303.2	463.3	300.4	464	Quercetin-3-O-galactoside (Hyperside)
7	4	19.52	257.5, 350.3	448.8	303.2	447.4	300.4, 301.6	448	Quercetin-3-O-rhamnoside (Quercetrin)
8	5	21.59	264.9, 343.6						Kaempferol-3-O-rhamnoside
	6	32.02	234.1						Fatty acid derivative
	7	32.21	234.7						Fatty acid derivative
	8	33.21	233.7						Fatty acid derivative
	9	38.46	226.3, 408.1						Pigment
	10	41.18	329.8, 377.0, 407.3						Pigment
From MFLB									
4	1	15.94	259.3, 359.7	480.4	303.2	479.7	301.1	480	Myricetin-3-O-galactoside (Hyperside)
5	2	16.98	265, 356	434.4	303.2	433.4	301.1	434	Quercetin-3-O-arabinofuranoside
6	3	18.08	257.7, 355.9	464.8	303.2	463.3	300.4	464	Quercetin-3-O-galactoside
7	4	19.63	259, 355.5	448.8	303.2	447.4	300.4, 301.6	448	Quercetin-3-O-rhamnoside (Quercetrin)
8	5	21.73	264.9, 343.6					432.38	Kaempferol-3-O-rhamnoside

nHFLB = n hexane fraction of *Lannea barteri*, EFLB = ethyl acetate fraction of *Lannea barteri*, and MFLB = methanol fraction of *Lannea barteri*.

although these changes were significantly not different from the CCl₄-treated group and are still on the normal range. The total protein is made up of albumin and globulin. The globulins bind to haemoglobin, which transports metals (such as iron) in the blood and help defend against infections. Albumin maintains the water balance in plasma and serum, and store as well as transport a wide variety of ligands, including calcium, fatty acids, hormones (e.g. thyroxine) and bilirubin. Also, albumin is an endogenous source of amino acid [28, 30]. Decreased level of total protein could lead to a decrease in these functions; hence, the fractions are capable of protecting the serum total protein in the body, ensuring their continuous functions.

Malondialdehyde (MDA), a product of lipid peroxidation in the liver of rats, has also been reported to be elevated by the administration of CCl₄. A rise in MDA level is ascribed to increased lipid peroxidation, which leads to the damage of tissues and failure of antioxidant defence mechanisms to stop excessive free radical formation [24]. The result from this study showed that the fractions slightly decreased the elevated MDA levels with respect to the CCl₄-treated group and this implies that they can inhibit lipid peroxidation which consequently leads to protection of biological membranes.

The chromatographic separation of the fractions showed that these compounds - Myricetin-3-O-rhamnoside, Quercetin-3, 7, 3', 4'-tetramethyl, Quercetin-3-O-galactoside (Hysperoside), Quercetin-3-O-rhamnoside (Quercetrin), Kaempferol-3-O-rhamnoside, Quercetin-3-O-arabinofuranoside, fatty acid derivatives and pigments identified from the fractions either in combination or singly may be accountable for the antioxidant and hepatoprotective effects possessed by the various fractions. Several reports have shown that the antioxidant and hepatoprotective activities of similar compounds from other plant species as found in these fractions have been investigated. Myricetin-3-O-rhamnoside isolated from *Myrtus communis* and myricitrin, a metabolite from myricetin-3-O-rhamnoside isolated from the bark of *Myrica esculenta* (Myricaceae), leaves of *Myrica gale* and *Chrysobalanus icaco*, have been shown to possess antioxidant activity against DPPH radical oxidant activity [31, 32] while Myricetin 3-O-galactoside isolated from *Myrtus communis* was found to inhibit lipid peroxidation [32]. There is also a report that equally demonstrated that Quercetin-3-O-arabinofuranoside and Quercetin-3-O-rhamnoside isolated from *Leucaena leucocephala* inhibited strongly the *in vitro* oxidant effect of DPPH radical [33].

4. Conclusion

In conclusion, results from the study showed that the methanol, n-hexane and ethyl acetate fractions of *Lannea barteri* leaf had some levels of *in vitro* antioxidant and hepatoprotective activities against CCl₄-induced hepatic damage in rats. The hepatoprotective effects may at least in part be related to the antioxidant properties of the fractions. Further studies to isolate and characterize the compound(s) responsible for the antioxidant and hepatoprotective activities and possibly elucidate the mechanism of action are in the pipeline.

5. Materials and methods

5.1. Animals

Forty – eight albino rats of both sexes (170–220 g) were used for the study. They were purchased from the Laboratory Animal Facility, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The rats were kept in standard laboratory conditions and were fed with livestock pellet and water *ad libitum*. All animal experiments were conducted according to the institutional principles on the use of laboratory animals and in compliance with the international guidelines for care and use of laboratory animals (Pub. No.85-23, revised 1985). Ethical clearance was obtained from the Animal Research Ethics Committee, University of Nigeria, Nsukka before conducting this research (Reference number DOR/UNN/17/0014).

5.2. Chemicals, solvents, reagents and drugs

These include methanol (Sigma - Aldrich, Germany), Tween 80 (BBI Life Sciences, China), carbon tetrachloride (CCl₄) (Thermo Fisher Scientific, MA, USA), liquid paraffin (Spectrum Chemical Mfg. Corp., USA), distilled water (Department of Pure and Industrial Chemistry Lab, University of Nigeria, Nsukka), ascorbic acid (Sigma – Aldrich, Germany), thiobarbituric acid, trichloroacetic acid, 10% egg homogenate, 1,1 - diphenyl -2-picrylhydrazine (DPPH), ferrous chloride, potassium ferricyanide [K₃Fe(CN)₆], and phosphate buffer at pH 6.6. Silymarin (Microlabs, India) and ascorbic acid (BBI Life Sciences, China). All chemicals used were of analytical grade.

5.3. Equipment and instruments

Electronic weighing balance, spatula, beakers, measuring cylinder, test tubes, syringes and needles, digital pH meter, centrifuge tubes, incubator, grinder (Lab mil, serial no 4745, Christy and Norris Ltd, England), fractionating column, soxhlet extractor, UV-Visible spectrophotometer (Easy-Way Medical England 752W, England), centrifuge, plastic containers, aluminium foils/sheet, stopwatch, animal cages, rotary evaporator (R210 Buchi Rotavapor, Buchi Labortechnik AG, Switzerland).

5.4. Collection and preparation of plant materials

Fresh leaves of *L. barteri* were obtained from the forest in Benue State, Nigeria in June and July. Mr Alfred Ozioko of the International Centre for Ethnomedicine and Drug Development (Inter-CEDD), Nsukka, Nigeria helped with the identification and authentication of the plant (Voucher number 096). The leaves were carefully removed from the stalk and were dried under shade. The dried leaves were pulverized using a grinder (Lab mill, serial No. 4745, Christy and Norris Ltd, England) into powder, weighed and kept for future use in an airtight container.

5.5. Extraction of plant material and fractionation of the methanol extract

The powdered material (1100 g) was extracted using about 5 L of methanol by cold maceration method. The extract was separated from the marc by filtration using Whatman Number 4 filter paper. The extracted material was evaporated using a rotary evaporator to obtain dried methanol extract (ME) which was weighed and then fractionated with the aid of a fractionating column starting with less polar solvent to more polar solvent. The fractionation yielded n-hexane (nHFLB), ethyl acetate (EFLB) and methanol (MFLB) fractions of *Lannea barteri*.

5.6. Phytochemical analysis of fractions of the leaf extract of *L. barteri*

The test carried out was based on standard procedure [34].

5.6.1. Pharmacological tests

5.6.1.1. *In vitro* antioxidant assay

5.6.1.1.1. 1, 1 -Diphenyl -2-picrylhydrazyl radical scavenging assay protocol. The DPPH radical scavenging activity of the fractions was determined using 1.0 ml of 0.135 mM DPPH solution in methanol and 1.0 ml of plant fractions (0.025–0.5 mg) prepared in methanol, and standard drug (silymarin). It was left in the dark at room temperature for 30 min after the reaction mixture was vortexed thoroughly. The control used was 7% Tween 80. The absorbance of the mixture was measured using a spectrophotometer at 517 nm [35]. The equation below was used to calculate the ability of the plant fractions to scavenge DPPH radical in percentage.

$$\text{DPPH radical scavenging activity} = \left\{ \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{(\text{Abs}_{\text{control}})} \right\} \times 100$$

Where Abs_{control} is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + fraction or standard.

5.6.1.2. In vitro inhibition of lipid peroxidation assay. The lipid peroxidation assay was determined by boiling for 10 min the mixture of 1ml of fraction dissolved in 7% Tween 80, 0.67% of thiobarbituric acid and 20% trichloroacetic acid, followed by centrifugation after cooling at 3000 rpm for 20 min. Using ascorbic acid as a reference standard and 7% Tween 80 as a control , the absorbance activity of the supernatant was measured at 532 nm [36] and the inhibition of lipid peroxidation in percentage was calculated by using the relation below:

$$\% \text{ in vitro inhibition of lipid peroxidation} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \times 100$$

Where Abs_{control} is the absorbance of 7% Tween 80; Abs_{sample} is the absorbance of fraction or standard.

5.6.2. Reducing power assay

The reducing power of the fractions was evaluated according to the method of Yen and Chen [37]. A volume of 1.0 ml of the fraction prepared in 7% Tween 80 and ascorbic acid (0–5.0 mg/ml) were mixed individually to the mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide [K₃Fe(CN)₆] (1% w/v). The resulting mixture was incubated at 50 °C for 20 min, followed by the addition of 2.5 ml of trichloroacetic acid (10% w/v), which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of ferrous chloride (0.1%, w/v). Tween 80 (7%) served as a control. The absorbance was measured at 700 nm against a blank sample. Increased absorbance of the reaction mixture indicated a higher reducing power of the fraction. The reducing power (%) was calculated using the relationship:

$$\text{Reducing power (\%)} = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}) / \text{Abs}_{\text{sample}} \times 100$$

Where Abs_{control} is the absorbance of 7% Tween 80; Abs_{sample} is the absorbance of fraction or standard.

5.6.2.1. In - vivo antioxidant assay

5.6.2.1.1. Carbon tetrachloride-induced biologic oxidation assay. After 7 days of acclimatization, 48 rats of both sexes were divided into 8 groups of 6 animals per group. On the first day of treatment, the animals in the test groups (III - VI) were given 200 mg/kg and 400 mg/kg doses of EFLB and MFLB respectively, to protect their liver from the intended excessive damage induced by the carbon tetrachloride. Twenty-four hours later, normal treatment commenced for the next 7 days after challenging the animals with intraperitoneal administration of carbon tetrachloride, according to body weight as follows:

Group I: Normal control (7% Tween 80; 2 ml/kg/day, p.o).

Group II: Negative control (CCl₄: liquid paraffin (1:2); 1 ml/kg/day, i.p).

Group III: CCl₄ + EFLB (200 mg/kg/day; p.o + CCl₄: liquid paraffin (1:2); 1 ml/kg/day, i.p).

Group IV: CCl₄ + EFLB (400 mg/kg/day; p.o + CCl₄: liquid paraffin (1:2); 1 ml/kg/day, i.p).

Group V: CCl₄ + MFLB (200 mg/kg/day; p.o + CCl₄: liquid paraffin (1:2); 1 ml/kg/day, i.p).

Group VI: CCl₄ + MFLB (400 mg/kg/day; p.o + CCl₄: liquid paraffin (1:2); 1 ml/kg/day, i.p).

Group VII: CCl₄ + silymarin (25 mg/kg/day; p.o + CCl₄: liquid paraffin (1:2); 1 ml/kg/day, i.p).

Group VIII: Naive group (received nothing).

After 24 h of the last treatment, blood was collected from the retro-orbital plexus of each of the experimental rats. The blood samples were allowed to clot for more than an hour and then the serum was separated by centrifugation at 3000 rpm at room temperature. The serum was used

for the following biochemical analysis: ALT, AST [38], ALP [28], serum-free (total) and conjugated (direct) bilirubin [39], serum total protein [40] and malondialdehyde (MDA) [29].

5.7. Histopathological examination of the rat liver

5.7.1. Tissue preparation

In the end, surviving experimental animals were euthanised and histopathological examination of their liver sections was carried out. A phosphate-buffered formalin (10%) was used to fix the tissues, and subsequently trimmed, dehydrated in 4 grades of alcohol (90%, 80%, 70% and absolute alcohol), cleared in 3 different xylene immersions and embedded in molten wax. The solidified tissue blocks were cut into 5µm thick tissue sections, floated in a water bath and incubated for 30 min at 60°C. Three different immersions in xylene and 3 grades of alcohol (90%, 80% and 70%) were respectively used to clear and rehydrate the tissues and then stained with hematoxylin for 15 min. Using ammonium hydroxide, blueing was done, followed by differentiation in 1% acid alcohol before counterstaining with eosin. Permanent mounts were made on degreased glass slides using a mountant - distyrene plasticizer xylene (DPX) [41].

5.7.2. Slide examination and photomicrograph

The prepared slides were examined with a compound light microscope using x4, x10 and x40 objective lenses. The photomicrographs were taken using a megapixels microscope camera (Motic™ 90) at x160 magnifications.

5.7.3. High performance liquid chromatographic (HPLC) separation

The fractions were subjected to reversed-phase HPLC separation using a solvent mixture containing 80% phosphate buffer and 20% acetonitrile which was allowed to run for 10 min. The column was allowed to run through with different solvent combination ratios until 20% phosphate buffer and 80 % acetonitrile which was held for 30 min before the system rolled back to original solvent combination ratio and allowed to equilibrate for 20 min before the next injection. The period of a run for each fraction injection was 60 min at a monitoring wavelength of 235 nm while maintaining the temperature at 30°C (to avoid alteration in the retention time). The fractions were diluted 1000 fold from 50 µg/ml to 50 ng/ml before loading 40 µl into the autosampler maintained at about -4 °C. The chromatographic spectrum of each fraction was visualized on the monitor and labelled accordingly.

5.8. Statistical analysis

The data were analyzed by one-way ANOVA and subjected to Dunnett post hoc test using Graph Pad Prism version 7.03 software and the results were considered significant at $p < 0.05$ and were expressed as means \pm SEM.

Declarations

Author contribution statement

Florence N. Mbaaji, Justus Amuche Nweze: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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