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ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITY OF *LANNEA BARTERI* METHANOL LEAF EXTRACT

FLORENCE NWAKAEGO MBAOJI[1], AKACHUKWU MARYTHERESA ONWUKA[1,2,*], IKECHUKWU EMMANUEL PETER[1], CYNTHIA CHIOMA MBAOJI[1], KELVIN CHIBUZO UZODINMA[1]

1. Department of Pharmacology & Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Nigeria
2. Department of Infectious Diseases, The University of Melbourne, Vic Australia

ABSTRACT

Lannea barteri is a dioecious plant that is widespread in North and West Africa. It is used in the folkloric treatment of many disease states ranging from epilepsy, diarrhoea, oedema and ulcer etc. This study investigated the antioxidant and hepatoprotective effects of *Lannea barteri* methanol leaf extract (LBME). The *in vitro* models used were α, α- diphenyl-β-picrylhydrazyl (DPPH), reducing power and thiobarbituric acid assay (TBA) while the *in vivo* model, carbon tetrachloride- (CCl_4) induced oxidative hepatic damage in rat was used. The biomarkers assayed in the *in vivo* study were aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), malondialdehyde (MDA), serum total protein, and serum total bilirubin. In the reducing power assay, LBME gave the highest activity (35.04%) than Silymarin (26.88%) at 400 µg/ml. In the DPPH free radical scavenging test, Silymarin gave the highest percentage inhibition (97.37%) than the LBME (91.3%) at 400 µg/ml**.** In the TBA assay, the LBME gave a higher percentage inhibition (31.15%) than Silymarin (24.8 %) at 400 µg/ml. In the *in vivo* assay method, the LBME gave some level of protection to the liver by preserving its enzyme more than Silymarin although not significant. Total serum proteins were preserved while total bilirubin and MDA were reduced when compared to the CCl_4 group. LBME- (100 mg/kg) treated group significantly ($p < 0.05$) decreased ALP when compared to the CCl_4 treated-group. The phytochemical analyses revealed the presence of alkaloids, carbohydrates, fats and oils, flavonoids, glycosides, proteins, tannins, terpenoids, steroids, and resins. Mice receiving LBME (10–5000 mg/kg) orally did not perish nor develop any sign of acute intoxication, indicating that the extract is extremely safe. This study demonstrated that *L. barteri* leaf extract has antioxidant activity and protected the rat liver against damage caused by carbon tetrachloride.

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***Corresponding author:** akachukwu.onwuka@unn.edu.ng; +234-8038357391

INTRODUCTION

Most physiological processes in the body result in the release of free radicals. High level of free radicals tends to cause increased cellular damage and is responsible for oxidative stress that may contribute to cardiovascular and inflammatory diseases, acquired immunodeficiency syndrome (AIDS), neurodegenerative diseases (Alzheimer, Parkinson), diabetes, cancer and ageing [1]. Reactive oxygen species (ROS), a type of free radical, are combated by antioxidants, which also support homoeostasis. Among the mechanisms used by antioxidants are the suppression of ROS production, ROS scavenging activity, reducing power, metal chelation, antioxidant enzyme activity, and the inhibition of oxidative enzymes [2].

Carbon-tetrachloride is a hepatotoxic agent that induces liver damage [3]. The hepatotoxic effects of CCl_4 are largely due to its active metabolites, trichloromethyl radical and peroxy trichloromethyl radical [4, 5]. This lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl_4. AST, ALT, and ALP serum marker enzymes were elevated in this situation [6].

Despite current improvements in the pharmaceutical sector, hepatic disorders remain a world health problem. Herbal medicines are one of the main options for the prevention or treatment of liver disorders because of their multiple beneficial properties such as antioxidant and anti-inflammatory activities [7-9]. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea, and herbs may contribute to shifting the balance toward an adequate antioxidant status [10].

Lannea barteri is one such plant with a putative antioxidant activity. It is a dioecious tree of tropical Africa belonging to the genus Lannea and to the family of Anacardiaceae. The stem and root bark extracts of *Lannea barteri* possess antibacterial, antifungal, radical scavenging, and anticholinesterase inhibitory [1] and anticancer [9] activities. It is used as a traditional remedy for the treatment of diarrhoea, gastritis, rheumatism, sterility, and intestinal helminthiasis [11]. It is also used in a different part of West Africa against oedema, rickets, wounds, scurvy, childhood convulsions, and epilepsy [12, 13].

The present study investigated the antioxidant and hepatoprotective effects of *Lannea barteri* leaf extract.

MATERIALS AND METHODS

Chemicals, Reagents/Solvents

All chemicals used were of analytical grade and include: methanol (Sigma-Aldrich, Germany), distilled water, chloroform, normal saline, hydrogen peroxide, H_2O_2 (40%), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), potassium ferricyanide ($K_3[Fe(CN)_6]$), trichloroacetic acid ($C(Cl_3)COOH$; TCA), ferric chloride ($FeCl_3$), ascorbic acid, α,α-diphenyl-β-dipicryl-hydrazyl (DPPH) radical, thiobarbituric acid, buffer tablets, carbon tetrachloride (CCl_4), liquid paraffin.

Experimental Animals

Male and female adult Wistar albino mice (15–25 g) and rats (150–200 g) were obtained from the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka. The animals were housed in steel cages inside the facility and had unrestricted access to food (livestock pellets) and water. Ethical approval for animal study was granted by the Animal Research Ethics Committee, University of Nigeria, Nsukka with approval number: DOR/UNN/17/0014. Also, the guidelines followed for the animal study were as enunciated by National Code of Conduct for Animal Research Ethics (NCARE).

Collection and Preparation of Plant Material

Fresh leaves of *Lannea barteri* were collected from Benue State, Nigeria. It was identified by a botanist, Mr Alfred Ozioko, of the International Centre for Ethnomedicine and Drug Development (Inter-CEDD) in Nsukka, Nigeria (Voucher number 096). Prior to extraction, the leaves were carefully cut into small pieces, sun-dried, ground, and stored in an airtight container for future use.

Extraction of Plant Material

A continuous soxhlet extractor was used to extract about 1.6 kg of the powdered plant material with about 10 litres of methanol. Extract from *Lannea barteri* (LBME) was made by concentrating the filtrate in a rotary evaporator.

Preliminary Phytochemical Analysis

Standard techniques were used to conduct a phytochemical analysis of the LBME [14, 15].

Acute Toxicity Test

Using the method outlined by Lorke [16], the acute toxicity and lethality (LD_{50}) of the LBME in mice was calculated. The study was conducted in two phases. In the first phase, nine mice were divided into three

groups (n = 3), and orally administered 10, 100, or 1000 mg/kg of LBME (suspended in distilled water), and they were monitored for 24 hours for signs of acute toxicity and demise. No death was reported after 24 h. In the second stage of the test, new mice (n = 1) were given 1600, 2900, and 5000 mg/kg of LBME, respectively, and were monitored for 24 h for signs of acute intoxication and death.

In Vitro Antioxidant Tests

Assay of Ferric Reducing Power

The reducing power was determined by the method described by Athukorala *et al* [17]. Briefly, 1.0 ml LBME of different concentrations (25, 50, 100, 200 and 400 µg/ml) were each mixed with 2.5 ml of 0.2M phosphate buffer (pH 6.6). To each of the mixtures, 2.5 ml of potassium ferricyanide (30 mM) was added and incubated at 50 °C for 20 min. Thereafter, 2.5 ml of 0.6 M trichloroacetic acid (TCA) was added to the reaction mixture and centrifuged for 10 min at 3000 rpm. The upper layer of each solution (2.5 ml) was decanted and mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM). The change in colour was observed and the absorbance of each mixture was measured at 700 nm using a UV-Visible spectrophotometer. The same treatment was used for the different concentrations (25, 50, 100, 200 and 400 µg/ml) of Silymarin (the reference standard), and 3% Tween 80 (the negative control). Tests were performed in triplicates. A higher absorbance of the reaction mixture indicated greater reducing power; the percentage scavenging activity/reducing power was calculated using the relation:

Scavenging activity (%) = $\frac{A1 - A0}{A0} X\ 100$...........1

where: A_0 = the absorbance of negative control
A_1 = the absorbance of test.

Test for Free Radical Scavenging Activity: DPPH Assay

The DPPH radical scavenging activity was measured using previously described methods [18, 19] with some modifications. The reaction mixture (3.0 ml) consisting of 1.0 ml of DPPH in methanol (100 µM), 1.0 ml of methanol and 1.0 ml each of the different concentrations (25, 50, 100, 200 and 400 µg/ml) of LBME or standard was prepared. The reaction mixtures were incubated for 10 min in a dark cupboard, and their absorbance was measured at 517 nm using a UV-Visible spectrophotometer. The positive control (standard) used was Silymarin. The same treatment was given to the normal control that consisted of 1.0 ml of 100 µM DPPH solution in methanol + 2.0 ml of methanol, and the absorbance was determined at 517 nm. Tests were performed in triplicates. A lower absorbance reading of the reaction mixture indicated greater scavenging ability. The scavenging activity (%) was calculated using the formula:

Scavenging % = $\frac{A1 - A0}{A0} X\ 100$......................2

where: A_0 = the absorbance of control and A_1 = the absorbance of test.

Thiobarbituric Acid Method

The thiobarbituric acid method as described by Ottolenghi [20] was used. An amount of 2 ml of 20 % trichloroacetic acid and 2 ml of 0.67% of thiobarbituric acid were added to 1 ml each of different concentrations of the sample and standard (25, 50, 100, 200 and 400 µg/ml). The mixtures were placed in a boiling water bath for 10 minutes. After cooling, the mixtures were centrifuged at 3000 rpm for 20 min. The absorbance values of the supernatant were measured at 532 nm using a UV-visible spectrophotometer.

In Vivo Antioxidant Test

Carbon Tetrachloride-Induced Biologic Oxidation

The model of liver injury induced by carbon tetrachloride was used. After acclimatization for seven days, 36 albino rats of both sexes were divided into six groups at random (n = 6). The test groups (III to V) animals received single oral daily dose of LBME (100, 200, or 400 mg/kg, respectively) for 3 days to protect their liver from the excessive damage from the intended CCl_4 to be administered, whereas the control groups (I and VI) received 3% Tween 80 and Silymarin, respectively. Thereafter, oral treatment of the rats with the extract, Silymarin, and 3% Tween 80 commenced about 24 h later and continued for 8 days after challenging the animals with intraperitoneal (i.p) administration of 1 ml/kg carbon tetrachloride (CCl_4) in liquid paraffin (1:2) which continued on alternate days for 8 days as follows:

Group I: Normal control (3 % Tween; 1 ml/kg/day, i. p).
Group II: CCl_4 control (CCl_4; liquid paraffin (1:2); 1 ml/kg, i.p).
Group III: CCl_4+ LBME (100 mg/kg/day).
Group IV: CCl_4+ LBME (200 mg/kg/day).
Group V: CCl_4+ LBME (400 mg/kg/day).
Group VI: CCl_4+standard drug, Silymarin (25 mg/kg/day; p.o).

Groups II-VI were challenged with CCl_4 in liquid paraffin (1:2) (1.0 ml/kg; i.p) every alternate day for 8 days.

Blood was taken from each rat's retro-orbital plexus on the 9th day and was left to clot for over an hour. The serum portion of the clotted blood was centrifuged at a speed of 3000 rpm at room temperature and the supernatant was used to estimate the liver enzymes ALT, AST, and ALP and other antioxidant parameters.

Statistical Analysis

Results were expressed as mean ± SEM. The data were analyzed using one way ANOVA, followed by Dunnett post-hoc using GraphPad Prism version 6.0 software; $p < 0.05$ was considered statistically significant.

RESULTS

Phytochemical Constituents of LBME

LBME tested positive for alkaloids, glycosides, flavonoids, reducing sugars, carbohydrates, steroids, terpenoids, saponins, tannins, proteins, resins, fats, and oils (Table 1).

Acute Toxicity

In mice that received 10, 100, and 1000 mg/kg of the LBME, no death occurred within the first 24 h. Additionally, none of the second group of mice that received 1600, 2900, and 5000 mg/kg died at the end of the following 24 h. Therefore, after 48 h, mice exposed to the oral LD_{50} of LBME up to 5000 mg/kg did not die nor show symptoms of acute toxicity. Therefore, the LD_{50} is > 5 g/kg.

Ferric Reducing Power of LBME

The LBME and Silymarin elicited concentration-related and significant ferric-reducing power as shown in Fig. 1. At 400 µg/ml LBME and Silymarin gave a percentage reducing the power of 35.04% and 26.88%, respectively placing the LBME with a higher reducing power. Silymarin and LBME gave IC_{50} values of 141 and 1283 µg/ml concentrations respectively.

Effect of LBME on DPPH Free Radical Scavenging Activity

The free radical scavenging activity of LBME were determined using DPPH radical scavenging assay and Silymarin as the standard antioxidant drug. The percentage inhibition values of the LBME are presented in Fig. 2. The LBME and Silymarin elicited significant dose-dependent inhibition of DPPH free radicals. However, Silymarin (400 µg/ml) elicited the highest free radical scavenging activity with 97.37 % inhibitions. Silymarin and LBME gave IC_{50} of 64.93 and 52.84 µg/ml, respectively (Fig. 2).

Effect of LBME on TBA

At 400 µg/ml, LBME and Silymarin gave an inhibition of 31.15 and 24.8% at 532 nm respectively. Silymarin and LBME gave IC_{50} of 524.1 and 676.9 µg/ml respectively. This indicates that Silymarin elicited great antioxidant activity more than LBME (Fig. 3).

Effects of LBME on CCl_4-Induced Oxidation

In this study, a broad range of concentrations of the test samples (LBME and Silymarin) were evaluated. The serum levels of ALT, ALP, AST, total bilirubin, total protein, MDA were measured to determine the protective or ameliorative effect of LBME on liver damage in CCl_4-treated rats. The serum CCl_4-treated rats developed significant ($p < 0.05$) hepatic damage as manifested by a significant elevation in activities of AST, ALP, ALT, total bilirubin, total protein and MDA when compared to the rats treated only with 3% tween (normal control) (Fig. 4-8). LBME at 200 and 400 mg/kg reduced AST and ALT increase induced by CCl_4 although not significant, but 200 mg/kg gave higher reduction (Figs. 4 and 5). Interestingly, LBME at 100 mg/kg significantly ($p < 0.05$) reduced ALP when compared to the CCl_4 -group (Group II) (Fig. 6). For the serum total protein concentration, 200 mg/kg gave a higher reduction (Fig. 7), a trend that was observed in the serum bilirubin assay where there was a significant reduction by 200 mg/kg followed by 400 mg/kg (Fig. 9).

Lipid peroxidation was evaluated by measurement of malondialdehyde (MDA) concentration in the plasma. There was a significant increase in the serum level of MDA in CCl_4-treated rats when compared to the control ($p < 0.05$). Treatment with 200 and 400 mg/kg LBME inhibited the increase in the serum levels of MDA caused by CCl_4 injection in a dose-dependent manner (Fig. 9).

DISCUSSION

The result of this study indicated the antioxidant and hepatoprotective effects of *Lannea barteri* leaf extract against CCl_4-induced hepatic damage in rats. Free radicals are known to be the cause of most degenerative disorders in the body. Antioxidants are free radicals and reactive species suppressants [21]. Antioxidative activity has been proposed to be related to reducing power. At 400 µg/ml, LBME

Table 1: Phytochemical constituents of *Lannea barteri* methanol leaf extract

Phytochemical constituents	LBME
Alkaloids	+
Carbohydrates	+
Fats and Oils	+
Flavonoids	+
Glycosides	+
Proteins	+
Reducing Sugars	+
Saponins	+
Resins	+
Steroids	+
Tannins	+
Terpenoids	+

–= not present; += present; LBME = *Lannea barteri* methanol leaf extract.

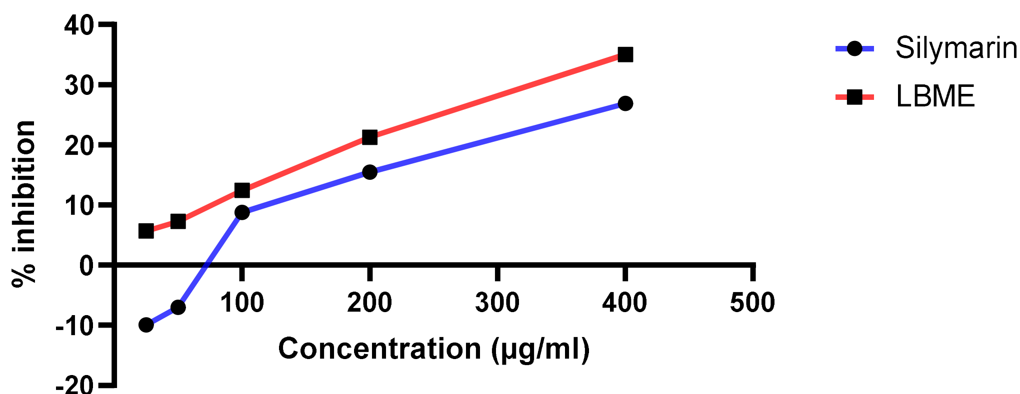

Fig 1: Ferric reducing power activity (%) of LBME

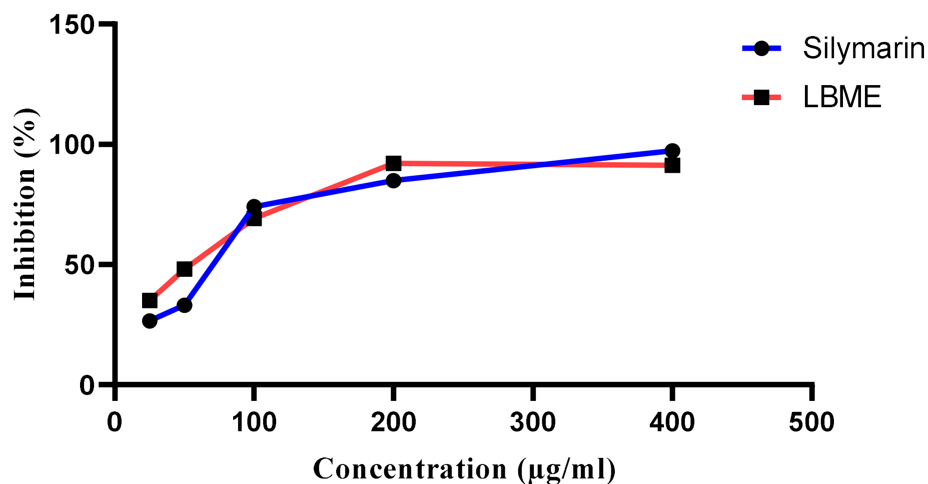

Fig 2: DPPH radical scavenging (%) activity of LBME

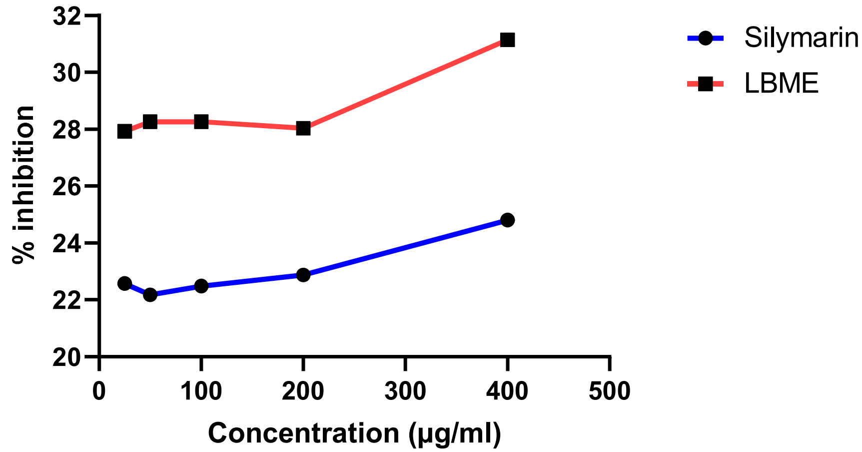

Fig 3: % inhibition of LBME using Thiobarbiturc acid method.

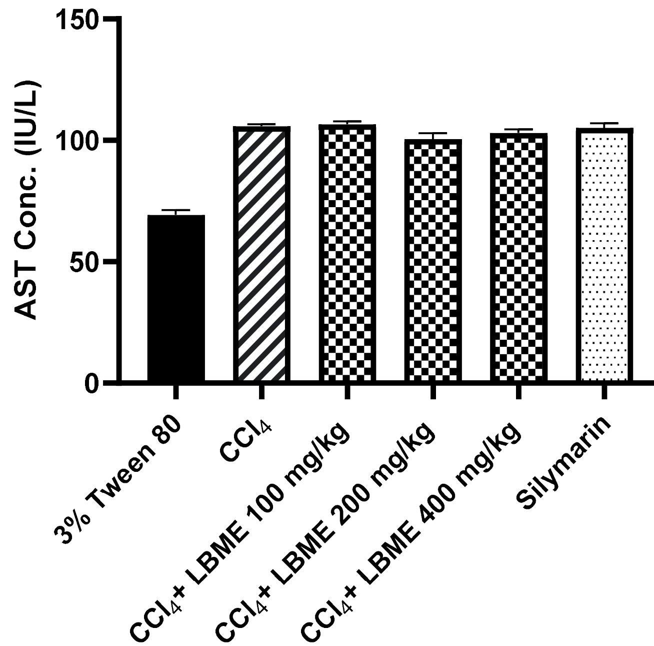

Fig 4. Effect of LBME on serum AST concentration

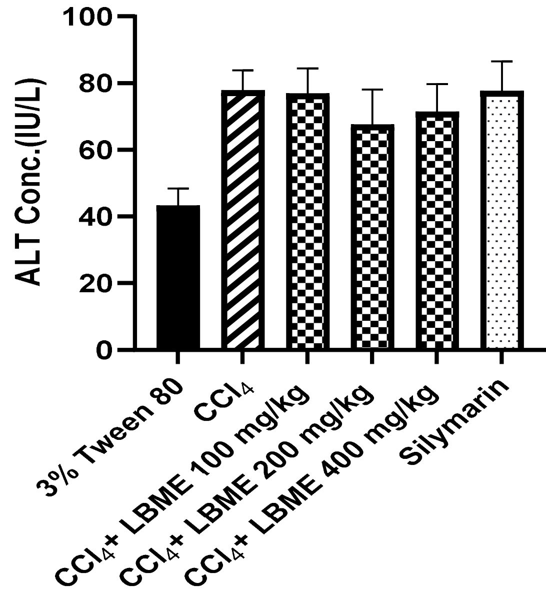

Fig 5: Effect of LBME on serum ALT Concentration

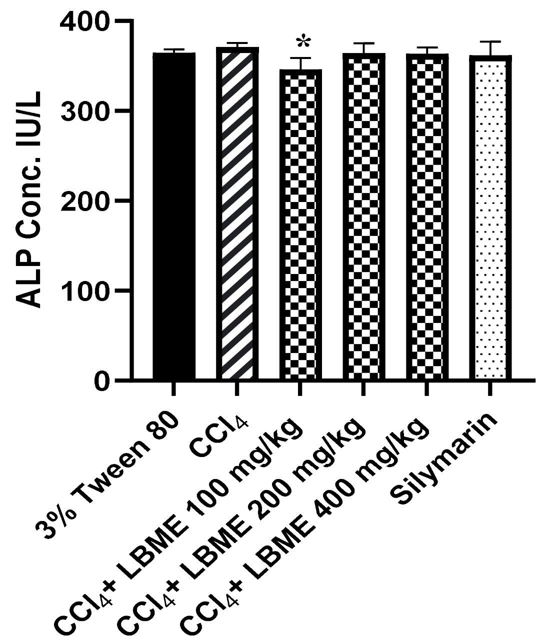

Fig 6: Effect of LBME on serum ALP concentration

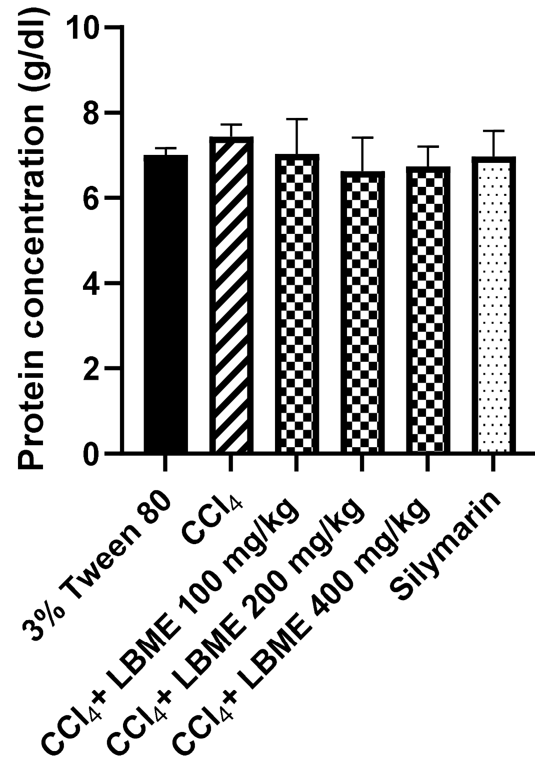

Fig 7: Effect of LBME on serum protein concentration

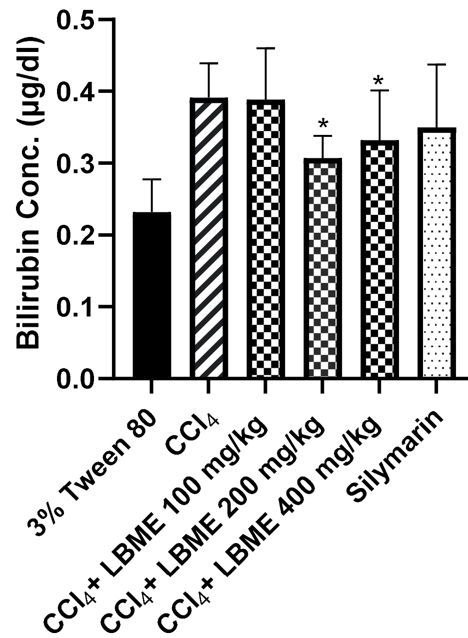

Fig 8: Effect of LBME on serum bilirubin concentration

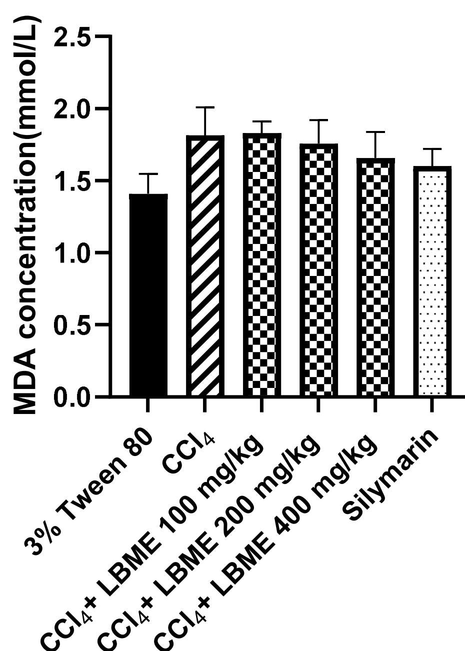

Fig 9: Effect of LBME on serum MDA concentration

significantly ($p < 0.01$) exhibited a percentage ferric reducing power of 35.04% when compared to Silymarin (26.88%). This indicates that LBME has high reducing power activity than Silymarin. DPPH radical scavenging method is widely used to evaluate the free radical scavenging ability of natural antioxidants [22]. In the radical form, the DPPH molecule has an absorbance at 517 nm which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule [23]. The result of DPPH scavenging activity in this study indicates that the plant is a potential antioxidant agent. LBME at 400 µg/ml gave a high percentage scavenging activity of 91.3%. It also showed a concentration-dependent scavenging of DPPH, which may be attributed to its hydrogen-donating ability. Thiobarbituric acid method was used to evaluate the inhibition of the production of carbonyl compounds which are the degraded products from peroxides [20]. LBME which gave higher absorbance values than the Silymarin would give higher protection from the damaging effect of peroxidation of lipids by endoperoxides. This is indication of high antioxidant potency.

CCl_4, a potent hepatotoxic agent, has been used widely to establish an animal model to study liver injury which was characterized by typical centrilobular necrosis and was similar to hepatotoxicity in humans [24, 25]. Administration of CCl_4 generates the free radical $CCl_3^{\cdot}$ which causes hepatic damage due to the activation of the NADPH-Cyt P450 system of the liver endoplasmic reticulum [26] leading to the generation of the more reactive radical, trichloromethyl peroxy ($CCl_3O_2^{\cdot}$) which provokes lipid peroxidation, disruption of Ca^{2+} homeostasis and apoptosis [27]. CCl_4 does not only target the liver but also affects other organs like the lungs, heart, kidney, and brain [25, 28].

In this study, CCl_4-treated rats developed significant hepatic damage as manifested by a significant elevation in concentration of AST, ALT, ALP, and other biochemical parameters when compared to the 3% Tween 80 group. Damaged liver cells release liver-specific enzymes into the bloodstream, such as AST, ALT, and ALP, which are used as biomarkers of liver damage and indicators for evaluating the effectiveness of therapeutic agents [29, 30]. The efficacy of any hepatoprotective drug is dependent on its capacity to either reduce the harmful effect or restore the normal hepatic physiology that has been altered by a hepatotoxin [31].

To assess LBME's ability to protect against CCl_4-induced liver damage, an *in vivo* antioxidant study was conducted. Pre-treatment with Silymarin and LBME decreased CCl_4-induced elevated enzyme level. ALT is a cytosolic enzyme found in its highest concentration in the liver and is more specific to the

liver [32]. LBME reduced the ALT and AST level of CCl_4-induced hepatotoxicity though not significantly ($p > 0.05$).

Increased bilirubin levels are expressed in disease conditions like cirrhosis, hepatitis and hepatobiliary system obstructions (tumours). Elevated total bilirubin levels are also observed in cases of intravascular haemolysis [33]. Treatment with *Lannea barteri* leaf extract (200 and 400 mg/kg) resulted in a significant ($p < 0.05$) reduction in levels of serum total bilirubin of the experimental animals. Administration of CCl_4 leads to an increase in serum total protein. There was no significant difference in the serum total protein concentration between *Lannea barteri* treated rats and CCl_4-treated rats.

Lipid peroxidation was evaluated by measuring the malondialdehyde (MDA) concentration in the plasma. Administration of CCl4 results in the elevation of MDA, a product of lipid peroxidation in the liver [34]. LBME at 400 mg/kg was found to decrease MDA concentration resulting in decrease in lipid peroxidation. Improved biochemical indicators of liver damage were found in all groups treated with extract, demonstrating that the LBME protected the liver from CCl_4-damage.

The phytochemical screening identified the presence of flavonoids, tannins, saponins, terpenoids in the extract of *Lannea barteri*. Flavonoids and tannins may be responsible for the antioxidant action [35]. Flavonoids, tannins, terpenoids, and saponins are all examples of phenolic compounds. The hydroxyl groups in phenolic compounds have been shown to have scavenging ability [36, 37]. Oral administration of the extract (10-5000 mg/kg) to mice did not result in death, indicating a high level of safety.

CONCLUSION

In conclusion, the study's findings show that *Lannea barteri* leaf extract has antioxidant activity and protects rats from carbon tetrachloride-induced liver damage. Based on the findings, *Lannea barteri* plant leaves need to be investigated further to nail down the specific compound that is responsible for the antioxidant activity.

List of Abbreviations

LBME - *Lannea barteri* methanol leaf extract
DPPH- α, α- diphenyl-β-picrylhydrazyl
TBA -thiobarbituric acid assay
CCl4 - carbon tetrachloride
AST -aspartate aminotransferase
ALT -alanine aminotransferase
ALP -alkaline phosphatase
MDA- malondialdehyde
TCA - trichloroacetic acid

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