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Evaluation of *In-vivo* and *In-vitro* Antioxidant Activities of Methanol Extract of *Salacia lehmbachii* Loes Leaf

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Authors' contributions

This work was carried out in collaboration between all authors. Author BASL designed the study and supervised the work, author WNO performed the experiments and wrote the first draft of the manuscript, authors NPW and JH did extensive literature review and performed the statistical analysis. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This study was carried out to evaluate the *in-vitro* and *in-vivo* antioxidant activities of methanol extract of *Salacia lehmbachii* leaf (SLLE).
Place and Duration of Study: Department of Pharmacology, University of Calabar, NIGERIA, between October, 2014 and December, 2014.
Methodology: Ability to scavenge 2,2–diphenylpicryl hydrazyl (DPPH) radicals as well as chelate divalent ferrous ions served as paradigms for *in-vitro* methods. On the other hand, cross-clamping of both hepatic artery and hepatic portal veins for 60 min and reperfusion for the next 60 min in rats produces oxidative stress with consequent lipid peroxidation against which the activity of the extract was tested.
Results: SLLE showed considerable potency *in-vitro* in the brine shrimp assay with an IC_{50} of 4.66 µg/ml. At very low concentrations (<10 µg/ml), *SLLE* showed superior activity over that of Vitamin C

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in the DPPH assay with IC_{50} of 4.9 µg/ml and 9.6 µg/ml for SLLE and Vit C respectively. At higher concentrations (10-1000 µg/ml), the antioxidant activity was found to be very weak indeed. The FIC assay showed a dose-dependent and significant (*P*<.01) response for both the SLLE (2.199±19.29) and the standard ethylenediaminetetraacetic acid (EDTA) (81.94±5.022); while EDTA showed ability to prevent Fenton-type reaction, SLLE showed a lack of this ability and even a possible enhancement of Fenton-type reactivity. When given at a dose of 100 mg/kg, SLLE also produced significant (*P*<.05) protective activity against hepatic lipid peroxidation in ischaemic / reperfussion injury in rats.

Conclusion: The antioxidant activity of the extract was found to be superior to that of Vit C at low concentrations in the DPPH while not so remarkable at higher concentration and FIC ability. Significant activity in lowering MDA in ischemic-reperfusion model in rats seen at the highest dose (100 mg/kg) (*P*<.05) demonstrated its tissue protective potential.

Keywords: Salacia lehmbachii; antioxidant; DPPH scavenging; ferrous ion chelation; malondialdehyde; methanol extract.

1. INTRODUCTION

The enhanced understanding and the current state-of-the-art in free radicals biology and reactive oxygen species (ROS) has resulted in better management of diseases related to age [1] and degenerative conditions such as cancer, cataracts, cardiovascular diseases, immune system decline and brain dysfunction [2]. Free radicals have been implicated as major contributors to these and various other pathological conditions [2]. Agents that exhibit antioxidant characteristics are therefore capable of potential benefit in these conditions and such antioxidants may exert their effect on biological systems by different mechanisms including, but not limited to, electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation [3].

Plants of the *salacia* genus have been reported to possess antioxidant properties- particularly those that have been used traditionally for the management of diabetes mellitus such as *S. Oblonga* [4]. Other known species of this genus that have shown remarkable antioxidant effect are *S. reticulata*, and *S. chinensis* [5].

Salacia lehmbachii Loes belongs to the plant family Celestraceae and is a plant of west tropical African origin which is also found in the southern part of Nigeria such as Akwa Ibom State and in the Oban hills of Cross River State of Nigeria as well as in Cameroon and the south west province of Bakassi forest reserve. The plant belongs to the Family Celastraceae although little is known about its full biological profile, its use in the treatment of malaria infection locally and other possible biological effects of both the leaf and root bark are currently under investigation.

Because there is no documented report of scientific studies on *S. lehmbachii* regarding the antioxidant activity of the leaves, this study was therefore aimed at investigating the potential antioxidant characteristics of the leaves of this plant by evaluating its ability to modulate the effect of oxidative stress both *in-vivo* and *in-vitro.*

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

2,2–diphenylpicryl hydrazyl (DPPH), Vitamin C (Vit C), ferrous sulphate ($FeSO_4$), 1,10-phenanthroline, ethylenediaminetetraacetic acid (EDTA), 2,4-diphenylhydrazine, thiobarbituric acid (TBA), and sodium dodecyl sulphate (SDS) were sourced from Sigma Chemical Company Inc., St. Louis, MO, USA. n-hexane, chloroform, methanol, and acetic acid were all of analytical grade and purchased from a reputable chemical store in Calabar, Nigeria.

2.2 Plant Materials

The leaves of *S. lehmbachii* were obtained from the forest region in Akwa Ibom State of Nigeria. The plant material was harvested in the morning and processed for extraction within 24 hours of harvesting. The plant was identified by Mr Abe Noa in Cameroon National Herbarium (CNH), Yaounde, with Voucher No. 40730/SRF/CAM.

2.3 Animals

A total of 24 healthy Sprague-Dawley albino rats of both sexes with an average weight of 100-150 g were used in the study. They were sourced from the animal house, Department of Pharmacology, University of Calabar, Cross River State. The animals were maintained under

laboratory condition for two weeks in order to acclimatize.

2.4 Extraction Procedure

The leaves of *S. lehmbachii* were air-dried at room temperature for 2 days, oven-dried at 40°C to dryness and ground to uniform powder using a grinding machine. 50 g of the finely ground powder was soaked with 500 ml of n-Hexane and left for 24 hours after which the solution was filtered through a Whatmann filter paper No 42 (125 mm). The marc from the preceding was further soaked in chloroform for 24 hours, filtered, and soaked finally in methanol for 24 hours and filtered. All the above filtrates were evaporated to dryness in a laboratory oven at 40°C [6] to obtain the n-hexane, chloroform and methanol extracts respectively.

2.5 Brine Shrimp Toxicity Testing

The toxicity potential of the methanolic leaf extracts of *S. lehmbachii* (SLLE) was evaluated using the brine shrimp assay procedure according to Meyer et al. [7]. In brief, the brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water (9.5 g NaCl in 250 ml of distilled water). After 24 hours, the hatched nauplii (brine shrimp larvae) were allowed to stand for 1 hour. The extract was dissolved in the saline solution to varying concentrations vis 0, 5, 10, 20, 40, 50, 60, 80, and 100 µg/ml. Sea water without extract was used as the negative control. Fifteen (15) nauplii were withdrawn through glass capillary and placed in each vial containing 4.5ml of brine solution. A volume of 0.5 ml of the plant extract was added to 4.5 ml of the brine solution and maintained at room temperature for 24 hours under light. The dead larvae were counted after 24 hours and percent mortality for each level of exposure was calculated as follows:

$$\%\,Mortality = \frac{No\ of\ dead\ brine\ shrimps}{Total\ no\ of\ brine\ shrimps} x\ 100$$

Equation 1

The mortality data was fitted to a nonlinear equation of the inverse exponent type from which the LC_{50} was determined. The equation employed is as follows:

$$Y = A * (1-Exp(-C*X)) + B$$

Equation 2

2.6 *In-vitro* Antioxidant Assay

The plant extract was analyzed for its free radical scavenging activity using two different methods; the DPPH method of Brand-Williams et al. [8] and modified by Sanchez-Moreno et al. [9] and the ferrous-ion chelating method [10] with modifications.

2.6.1 DPPH free radical scavenging activity

Low concentrations (2 µg/ml-10 µg/ml) as well as high concentrations (12.5 µg/ml-1000 µg/ml) of SLLE and Vit C were prepared for the study. Fresh solution of DPPH (152 µM) was prepared, wrapped in aluminum foil and kept in the dark to prevent autoxidation. To 1 ml each of the SLLE and Vit C solutions was added 1 ml of the DPPH solution, the mixture was shaken vigorously and allowed to stand in the dark for 1 hour and subsequently its absorbance was measured spectrophotometrically at 517 nm. A blank solution containing only the solvent methanol without the SLLE or Vit C was subjected to the same DPPH treatment. All determinations were performed in triplicate and the radical scavenging activities of the test and standard samples expressed as percentage of inhibition were calculated according to the following equation;

$$\%\,Inhibition\ of\ DPPH\ Activity = \frac{[A_b - A_a]}{A_b} x\ 100$$

Equation 3

Where; A_b-absorbance of the blank (solution without the extract or standard); A_a-absorbance of the SLLE or Vit C solution.

2.6.2 Ferrous-ion chelating assay

Concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml) of SLLE and EDTA were prepared for the study. To 1 ml each of these solutions, 3.5 ml of methanol, 0.1 ml of $FeSO_4$ (2 mM) solution and 0.2 ml of 1, 10 phenanthroline (5mM) were added sequentially. The resulting mixtures were allowed to incubate for 10 min at room temperature and the absorbance was read at 562 nm using a spectrophotometer (SpectroVis Plus, Vernier International, 5026 Calle Minorga, Sarasota, FL.,34242 U.S.A). A blank solution containing the test samples without the phenanthroline reagent was subjected to the treatments with the same reagents as above. All determinations were performed in triplicate and

the percentage ferrous ion chelating ability was calculated using the following formula:

$$\%\ Ferrous\ ion - chelating\ Activity = [1 - (Ab_s/Ab_c)]\ x\ 100$$

Equation 4

Where Ab_s = Absorbance value of the test sample. Ab_c = Absorbance value of the control.

2.7 Effects of SLLE on Malondialdehyde Levels in Hepatic Ischaemic / Reperfusion Injury in Rats

Twenty four albino rats were randomly allocated to four groups of six rats each as follows: Group I, control (0.5 ml/kg) Phosphate buffered saline {PBS}); Group II, Vit C (100 mg/kg); Group III, SLLE (50 mg/kg); Group IV, SLLE (100 mg/kg). Treatments were administered to the animals daily for 10 days and on the 10th day they were fasted for 12 hrs and anesthetized intraperitoneally with 20% w/v urethane (0.6 ml/100 g). Following anesthesia, warm ischemic injury was induced in their livers by cross clamping the portal vein and hepatic artery for 60 minutes, followed by reperfusion/reflow for another 60 minutes. The liver tissues were rapidly excised and rinsed in ice cold saline.

A 10% w/v homogenate of the liver was prepared using PBS and to 0.1 ml of the homogenate, 1.0 M acetic acid-sodium acetate buffer, pH 4.0 and 1.5 ml of TBA reagent (0.5 g of TBA and 0.3 g of SDS in 100 ml PBS) were added sequentially. The tube was capped in a glass bead and the mixture was heated for 15 min in a boiling water bath, cooled in ice water and 1ml of glacial acetic acid followed by 2 ml of chloroform were added. The mixture was shaken and centrifuged. The optical density of the supernatant was determined at 532 nm using a 1 cm cuvette. The final volume was ca. 4.2 ml, while a reagent blank was run simultaneously. The concentration of the malondialdehyde (MDA) was computed using the molar extinction coefficient was 1.56 x 10^5 in the following equation:

$$C = A/[e \times b] \qquad \text{Equation 5}$$

Where e = molar extinction co-efficient (1.56×10^5 $m^{-1}cm^{-1}$); b = pathway (width of the cuvette, 1 cm); c = concentration of the MDA in the sample and A = Absorbance of the sample read from the spectrophotometer.

2.8 Statistical Analysis

Data obtained from the experiments were analyzed by ANOVA using Statistical Package for Social Sciences (SPSS) software for windows and post-hoc testing was performed for inter-group comparison using Tukey's multiple comparison. Where applicable students t-test was used to compare significant differences between treated and control groups at discreet dose levels. All data were expressed as mean±standard error of mean (SEM). The values of P<.05 were considered significant.

3. RESULTS

3.1 Brine Shrimp Toxicity Assay

Lethality of the SLLE was determined by fitting a nonlinear regression curve to the mortality data obtained from the assay as shown in Fig. 1. The parameter values calculated for the inverse exponent equation were 69.99, 25.92 and 0.091 for parameters A, B and C respectively from Equation 2 above. The LC_{50} was then calculated to be 4.66 µg/ml.

3.2 Dpph Stable Radical Scavenging Activity of SLLE

The stable radical inhibitory activities of SLLE and Vit C are presented below (Fig. 2). At low concentrations (<10 µg/mL), The mean percentage inhibition of the SLLE (39±8.8) when compared to that of Vit C. (22±7.3) was found not to be significant after ANOVA. When t-test was carried out across all concentrations below 10 microgram/mL however, some significant difference was seen at some concentrations. At higher concentrations (10-500 µg/mL) also, mean for SLLE (22±6.4), though higher than that for Vit C (10±7.7) was not significantly different. As shown, the SLLE exhibited a much more superior antioxidant effect when compared with Vit C at these two concentration ranges. When comparison was made between SLLE and Vit C at individual concentrations however, it was found that SLLE showed significant difference to Vit C at some concentrations (Figs. 2a. and 2b).

3.3 Ferrous-Ion Chelating Activity of SLLE and EDTA

Table 1. shows the relative ability of SLLE to chelate ferrous ion (Fe^{2+}) compared to EDTA. The mean percentage inhibition of the SLLE (2.199±19.29) when compared to that of EDTA (81.94±5.022) was found to be significant (P<.01). Results generally showed that while

EDTA was dose-dependently inhibiting Fe^{2+}, SLLE was showing a reverse activity such that the activity was declining from a positive value at 50µg/mL to a negative value at 200 µg/mL. At the lowest dose employed in this study (50 µg/mL), the extract showed a chelating activity of 51.39±9.7 while the standard (EDTA) showed chelating activity of 70.37±11.26.

Table 1. Ferrous-Ion chelating activity of SLLE and EDTA

Concentration (µg/ml)	Ferrous Ion chelating activity (%)	
	SLE	EDTA
50	51.39±9.7	70.37±11.26
100	3.70±6.07	91.67±6.99***
150	-3.70±5.63	76.85±9.80**
200	-42.59±30.0	88.89±5.56*

Data are presented as mean±SEM. Statistical significance was carried out between SLLE and EDTA at each concentration level using student's t test. (P<.05); ** (P<.01); *** (P<.001); n = 3*

3.4 Effect of SLLE on Hepatic Malondialdehyde Levels of Rats Subjected to Ischaemic / Reperfusion Injury

The result of the treatments on the hepatic MDA levels of ischemic / reperfusion-injured rats showed a reduction by Vit C although the reduction was not significant (Fig. 3). The lower dose of the SLLE (50 mg/kg) resulted in a slightly higher levels of MDA compared with saline-treated animals while the higher dose (100 mg/kg) resulted in a significant (P<.05) reduction of MDA levels. This reduction was also found to be much better than that provided by Vit C.

4. DISCUSSION

The safety potential of the methanol extract of *S. lehmbachii* had earlier been reported for *in-vivo* acute toxicity LD_{50} in rats [11]. While the acute toxicity evaluation in rats showed that the animals could tolerate a dose as high as 5,000 mg/kg, the in-vitro lethality assay in brine shrimps in the current study however resulted in a LC_{50} of 4.66 µg/ml in a 24-hour exposure model. The very low value of this toxicity parameter signifies a very potent activity of the leaves of the plant which could be of benefit in activity-guided fractionalization of the plant extract as well as potent and significant activity on biological systems; this may form the basis for biological screening in the search for potentially useful therapeutic agents from this plant. The relatively high dose tolerated in the whole animals when related to the high potency of *in-vitro* lethality suggested that a significant fraction of the orally administered dose may have been subjected to first-pass metabolism thereby affecting oral absorption and entry of significant amount as to constitute any potential for adverse effects to the animals so exposed.

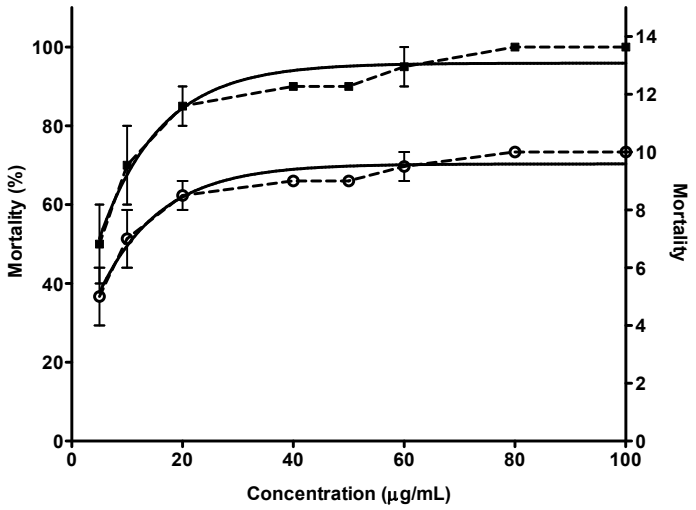

Fig. 1. Concentration vs mortality (right axis, ○) and % mortality (left axis, ■) data for SLLE in Brine Shrimp Assay

LC_{50} was calculated as 4.66 µg/ml. Data was fitted to an inverse exponent equation (Equation 2) with parameter values as follows: A = 69.99; B = 25.92; C = 0.091. Superimposed solid curves are nonlinear curves for the mortality plots. n = 15

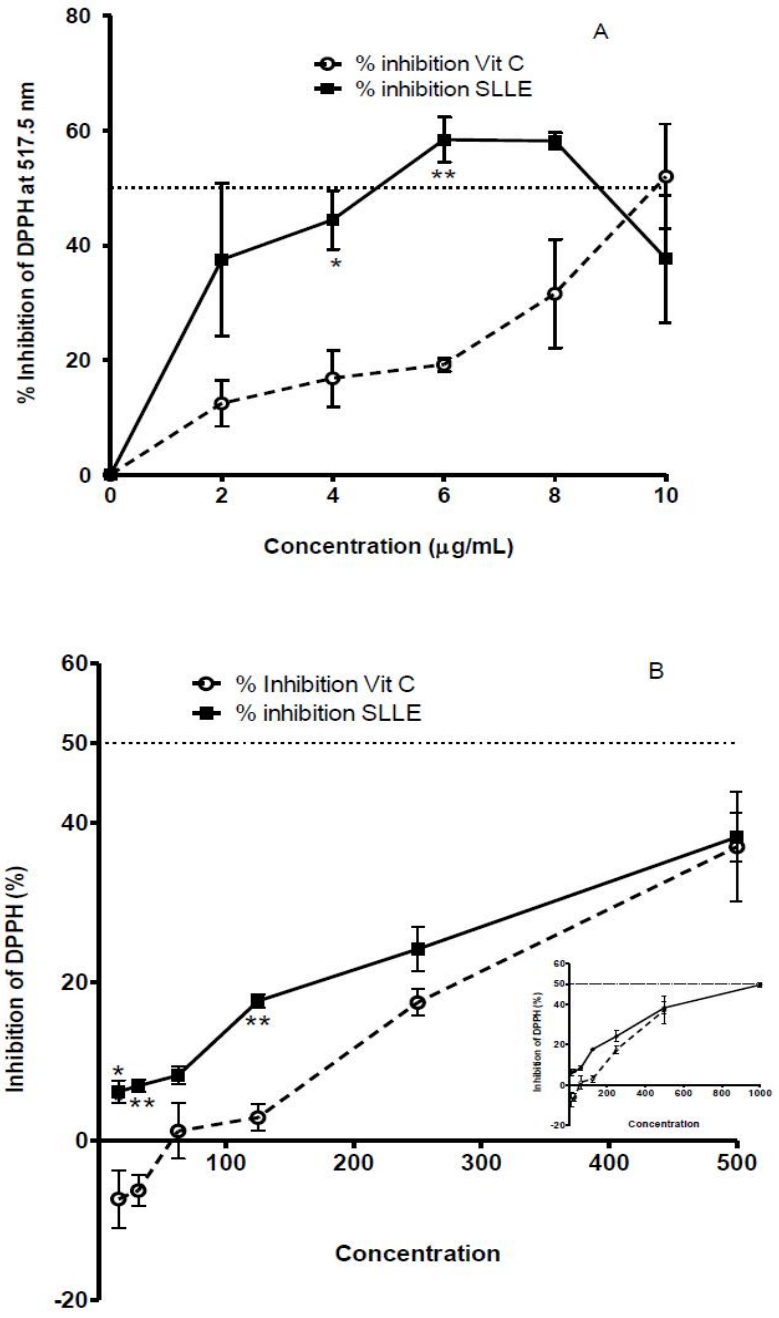

Fig. 2. DPPH radical scavenging activity of SLLE and Vit C at [A] low and [B] high concentrations

Low concentration range was 0-10 µg/mL while high concentration range was between 12.5-1000 µg/mL. Statistical significance was carried out between SLLE and Vit C at each concentration level using student's t test. (P<.05); ** (P<.01); *** (P<.001); n=3*

In the evaluation of antioxidant activity using the DPPH method, SLLE exhibited a much more superior antioxidant effect when compared with Vit C at both the low (0-10 µg/mL) and high (12.5-500 µg/mL) concentration ranges used in the study. When the two treatments were assessed by ANOVA over the entire concentrations in the two concentration ranges, the antioxidant effects of both Vit C and SLLE were found not to be significantly different though

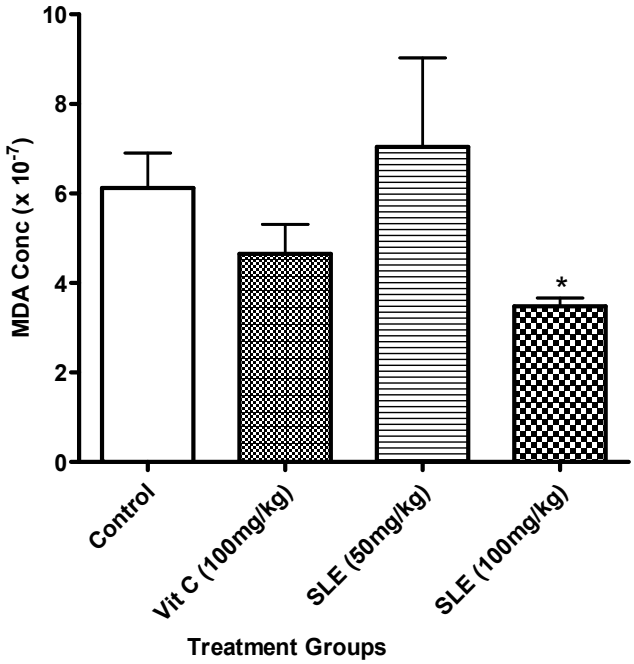

Fig. 3. Effects of SLLE and Vit C treatments on MDA levels in Ischaemic / reperfusion-injured hepatic tissue

*The concentration of the MDA was computed using the molar extinction coefficient of 1.56 x 10^5. Rats in the groups were subjected to ischemic/reperfusion injury of the liver and control rats were treated with normal saline while the other groups received either Vit C or two different doses of the SLLE. Each drug treated groups were compared with the control group using unpaired student's t-test. *. P<.05; n = 6*

when comparisons were made at individual concentrations using student's t-test, significant differences were found between the SLLE and Vit C. While the effects of the two treatments were easily observed and computed at the low concentration ranges, it was curiously observed that stable radical scavenging activity at higher doses were not as amenable and straightforward and in fact, radical scavenging activity at the higher concentrations were relatively minimal if not completely absent; this can be seen when cognizance is taken of the fact that IC50 of SLLE for DPPH radical scavenging will require a concentration above the maximum 1,000 µg/mL employed in the study (See insert of Fig. 2b).

In the FIC test, it was found that the standard compound, EDTA, was able to exhibit a concentration-dependent inhibition of the Fenton reaction of Fe^{2+} with 1, 10 phenanthroline; an action that is consistent with the known effect of EDTA. For the leaf extract however, there was a consistently decreasing effect of the extract on ion chelation after the increase elicited by 50 µg/ml. At the lower end of the concentration used in the study, both EDTA and SLLE showed positive chelating activity while at increasing concentrations, unlike EDTA, SLLE activity declined into the negative portion of the curve. Although comparison between the two agents revealed that SLLE was significantly (*P*<.05) inferior to EDTA, the reason for the decrease of the values of SLLE into the negative domain needs to be addressed. At the very least, total lack of chelating activity ought to limit the SLLE curve towards zero by approaching the x-axis. The fact that (50 ug/mL) of SLLE showed a positive chelating activity suggests that if the concentration range of the study had been extended below 50 ug/mL, we might probably have seen a positive, maybe concentration-dependent chelating activity. One possibility is that the extract may actually possess the ability to trigger a Fenton-type reaction that is capable of generating reactive oxidants. Given the fact that the plant is used indigenously for the treatment of malaria infection and bearing in mind the molecular activity of artemisinin antimalarials which involves oxidant effect on malaria parasites, the relationship between these effects of SLLE needs to be further explored and clarified.

It has been said that the main strategy in avoiding ROS generation that is associated with redox-active metal catalysis involves chelating of metal ions. The reducing power of polyphenols suggests that they will possess potential hydrogen donating abilities [12] which will be made available to the Fe^{2+}, thereby effectively removing them from solution and prevent the Fenton reaction that can generate ROS. Since SLLE was found to have relative abundance of polyphenolic compounds, it was expected to exhibit iron-chelating properties which should confer on it the ability to prevent Fenton-type reaction which is implicated in many diseases and neurodegenerative disorders like Parkinson's and Alzheimer's diseases [13]. The result of this study showed that SLLE lacks this activity and as such any antioxidant property exhibited must be due to other mechanisms. It is not yet known what nature of chemical species is contained in SLLE but phytochemical screening of the plant is ongoing. In terms of general group composition however, *S. lehmachii*, just as other *salacia* species is known to contain polyphenolics [11].

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is therefore used as an indicator of oxidative stress in cells and tissues [14]. Measurement of MDA is therefore widely used as an indicator of lipid peroxidation [15] and increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both human and animal models [16]. In the present study, a significant reduction (P<.05) in the production of MDA was observed in the group that was given SLLE 100 mg/kg. This implies the ability of the extract to protect membrane lipids from oxidation. This effect could be related to the presence of polyphenols as indicated in the preliminary phytochemical analysis [11]. Plant phenolic compounds trap chain-initiating radicals at the interface of the membrane, thus, preventing the progression of the radical chain reaction. The group that was given Vit C at a dose of 100 mg/kg showed slight decrease in MDA as compared to the group that was given 50 mg/kg of the extract and the control group that was given only vehicle. The effect of the extract on the MDA concentration, suggests a dose -dependent response, as a higher concentration decreased the MDA concentration significantly. This is also in agreement with similar effects reported for another *Salacia* species. Krishnakumar et al. [17] reported that *S. oblonga* root extracts possess anti-lipid peroxidative activity in the cardiac tissue of streptozotocin-diabetic rats. *S. oblonga* produced a significant decrease in peroxidation products viz., TBA-reactive substances, conjugated dienes and hydroperoxides. A study had shown that *S. reticulate* reduced the kidney, pancreatic and plasma peroxide levels as well as kidney aldose reductase activity [18] which suggests that it might equally have the same effect on other tissues such as the liver and this has been confirmed in the current study. In addition, Ismail and co-workers [19] reported that increased acid and alkaline phosphatase activity and decreased serum albumin in cotton pellet granulomatous rats were normalized after treatment with 1,000 mg/kg *S. oblonga* root bark powder in a study designed to evaluate the its anti-inflammatory activity. The activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were also increased in the heart tissue of diabetic animals treated with the extract suggesting its antioxidant activity. Also, nitric oxide production from lipopolysaccharide-activated mouse peritoneal macrophage and radical scavenging activities of the methanol extract of *S. chinensis* were reported in addition to potent antioxidant activity [20].

From this study, it can be seen that SLLE possessed robust antioxidant activity at low doses as well as the ability to prevent oxidative damage due to lipid peroxidation in the body; an ability that is shared with other species of the *salacia* genus. At higher concentrations however, its antioxidant activity is very weak or suspect and appears to be counteracted by other phenomena that has not been fully understood and therefore will require further studies to unravel.

5. CONCLUSION

In conclusion, the results showed that SLLE at low concentrations may have great relevance in the prevention and therapies of diseases in which oxidants or free radicals are implicated, and as such, SLLE could serve as an economic source of natural antioxidants. At higher doses or concentration however, the activity of the extract needs to be further clarified.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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